

Project report

Identification of site-related risk factors and organismic indicators of microbially influenced corrosion for sustainable repair management

Risk factors and indicators of microbially induced corrosion of hydraulic engineering systems

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Risk factors and indicators of microbially induced corrosion of hydraulic engineering systems

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Summary

Executive summary German

Bundeswasserstraßen werden als Schifffahrtswege genutzt; daher ist es unerlässlich, die Betriebssicherheit der darin befindlichen Wasserbauwerke zu gewährleisten. Die Instandhaltung und Sanierung dieser Bauwerke aufgrund von korrosionsbedingten Schäden kann jedoch hohe jährliche Ausgaben verursachen. Häufig ist Korrosion kein rein physikalisch-chemischer Prozess, sondern wird durch die Aktivität von Mikroorganismen beeinflusst (mikrobiell beeinflusste Korrosion, MIC). Mikroorganismen können korrosive Stoffwechselprodukte wie H_2S oder anorganische und organische Säuren ausscheiden, oder sie rufen die Korrosion direkt hervor, indem sie dem Stahl Elektronen entziehen. Das Wissen über die zugrundeliegenden mikrobiellen Stoffwechselvorgänge und molekularen Mechanismen ist jedoch noch sehr gering. Daher bestand das Ziel des Projekts darin, die mikrobiellen Gemeinschaften und Stoffwechselprozesse zu erforschen, die die Korrosion von Stahl in Bundeswasserstraßen fördern. Um geeignete Standorte zu identifizieren, entwickelten wir einen umfangreichen Fragebogen, der an die Wasserstraßen- und Schifffahrtsämter, Neubauämter und ggf. Außenbezirke verschickt wurde. Basierend auf den Ergebnissen des Fragebogens führten wir an einer ausgewählten Anzahl von Standorten Probenahmen durch. Wir konnten zeigen, dass die mikrobielle Gemeinschaft je nach Probentyp stark divergiert. Einige mikrobielle Taxa (z.B. *Desulfovibrio*, *Gallionella*, *Nitrospira*, *Geothrix* und *Holophaga*) wiesen in den Rosttuberkelproben eine signifikant höhere relative Häufigkeit auf als in Wasserproben. Diese mikrobiellen Taxa könnten als Indikatoren für die MIC geeignet sein. Da die Unterschiede zwischen den Standorten jedoch geringer waren als die Unterschiede zwischen den Probentypen, ist es (bisher) nicht möglich, standortbezogene Umweltrisikofaktoren für MIC zu ermitteln. Darüber hinaus untersuchten wir für einige Standorte die chemischen und taxonomischen Gradienten, die innerhalb der Rosttuberkel selbst auftreten. Mit Hilfe verschiedener Techniken konnten wir feststellen, dass sich das Milieu im Inneren eines Rosttuberkels stark von der umgebenden Luft- oder Wasserphase unterscheidet. Außerdem untersuchten wir die zeitliche Entwicklung potenziell korrosiver Biofilme mit Hilfe von Stahlcoupons, die parallel zu nicht korrosiven Biofilmen beprobt wurden. In nicht-korrosiven Biofilmen dominierten phototrophe Organismen und ein saisonaler Effekt. Die Zusammensetzung der mikrobiellen Gemeinschaft änderte sich im Laufe der Zeit so stark, dass einige Taxa zu einem bestimmten Zeitpunkt dominierten oder überhaupt nicht vorhanden waren. Zusätzlich zu den Feldstudien wurden verschiedene Laborstudien zur Anreicherung und Identifizierung von Schlüsseltaxa durchgeführt, die unter verschiedenen definierten Bedingungen zu MIC beitragen. Studien zur Anreicherung von Kulturen zeigten, dass Süßwasser-Rosttuberkel Prokaryoten beherbergen, die zur dissimilatorischen Reduktion von Nitrat, Sulfat und CO_2 mit $Fe(0)$ als einziger Elektronenquelle fähig sind. Im Falle der dissimilatorischen Nitrat- und Sulfatreduktion waren die Korrosionsraten im Vergleich zu abiotischen Kontrollen deutlich erhöht, was darauf hindeutet, dass diese Prozesse potenziell zu MIC in Süßwassersystemen beitragen können. Unter nitratreduzierenden Bedingungen waren Bakterien der Gattungen *Acidovorax* und *Thermomonas* dominant. Unter sulfatreduzierenden Bedingungen, mit Acetat als organischer C-Quelle, dominierten *Desulfovibrio* spp. Die Mitglieder dieser Gattung sind heterotroph und einige von ihnen sind dafür bekannt, dass sie stark korrosiv sind und eine Schlüsselrolle in marinen Lebensräumen und, wie hier bestätigt wurde, höchstwahrscheinlich auch in Süßwasserhabitaten spielen. Metaproteomanalysen deuten darauf hin, dass beide Mechanismen, die H_2 -vermittelte und die direkte Elektronenübertragung von der Stahloberfläche, eine Rolle spielen. Wir haben auch festgestellt, dass mit $Fe(0)$ als einziger Elektronenquelle, Methanogenese und Acetogenese stattfinden können. Um den molekularen Mechanismus der MIC zu entschlüsseln, wurden Rein-kulturstudien mit *D. vulgaris* und *D. ferrophilus* unter Verwendung metaproteomischer Ansätze

durchgeführt. Reinkulturversuche zeigten, dass die Stämme *D. vulgaris* und *D. ferrophilus* sowohl in Süßwasser- als auch in Salzwassermedium Stahl korrodieren. Die Optimierung der metaproteomischen Analyse von Fe-reichen Proben war eine Herausforderung. Wir fanden heraus, dass die SP3-Extraktionsmethode am besten für die Extraktion von Proben mit geringer Biomasse und starken Matrixeffekten geeignet war. Insgesamt hat die Kombination verschiedener Arten von Feld- und Laborstudien und die Anwendung interdisziplinärer Methoden definitiv dazu beigetragen, unser Verständnis von MIC in Bundeswasserstraßen zu vertiefen. Dennoch bleibt die Definition von Umweltrisikofaktoren eine Herausforderung. Die Ergebnisse des Rimic-Projekts sind ein hervorragender Ausgangspunkt für künftige Untersuchungen, von denen einige bereits angelaufen sind.

Full summary German

Bundeswasserstraßen werden als Schifffahrtswege genutzt, weshalb die Stabilität und Betriebssicherheit von Wasserbauwerken wie Spundwänden in Schleusen, Wehren und Uferbefestigungen unbedingt gewährleistet sein muss. Die Korrosion dieser (unlegierten) Stahlkonstruktionen birgt jedoch hohe Sicherheitsrisiken durch einen möglichen Funktionsverlust. Darüber hinaus verursachen die Instandhaltung und der Wiederaufbau dieser Bauwerke aufgrund korrosionsbedingter Schäden hohe jährliche Ausgaben.

Häufig ist Korrosion kein rein physikalisch-chemischer Prozess, sondern wird durch die Aktivität von Mikroorganismen beeinflusst (mikrobiell beeinflusste Korrosion, MIC). In den letzten Jahren wurden an mehreren Bundeswasserstraßen Korrosionsschäden an Stahlwasserbauwerken beobachtet, die durch eine erhöhte lokale Korrosion gekennzeichnet sind. Es ist wahrscheinlich, dass diese Korrosionsschäden durch die Anwesenheit von Mikroorganismen verstärkt werden. Unter oxidischen Bedingungen erfolgt die Korrosion des enthaltenen Eisens (Fe^0) hauptsächlich durch abiotische Oxidation zu Eisen(II) (Fe^{2+}), das weiter zu Eisen(III) (Fe^{3+}) oxidiert wird. Unter anoxischen Bedingungen wird Fe^0 durch die Reduktion von H^+ zu H_2 abiotisch zu Fe^{2+} oxidiert. Mikroorganismen können MIC auf viele verschiedene Arten verursachen. Sie können die Konzentrationen der Reaktanten verändern, z. B. sind unter mikrooxidischen oder anoxischen (z. B. nitratreduzierenden) Bedingungen hauptsächlich Bakterien für die Oxidation von Eisen(II) zu Eisen(III) verantwortlich. Sie können auch korrosive Metaboliten wie H_2S oder anorganische und organische Säuren ausscheiden. Außerdem können Mikroorganismen die Korrosion auch direkt beeinflussen, indem sie dem Stahl Elektronen entziehen. Hierfür nutzen sie Fe^0 als Elektronendonator zur Energiegewinnung und übertragen die Elektronen auf schwache Oxidationsmittel wie Sulfat oder CO_2 . Daher beschleunigen Mikroorganismen durch enzymatische Katalyse den gesamten Korrosionsprozess, insbesondere unter anoxischen Bedingungen, wo es an starken Oxidationsmitteln mangelt. Das Wissen über die zugrundeliegenden mikrobiellen Stoffwechselvorgänge und die molekularen Mechanismen ist jedoch noch sehr gering. Ziel des Rimic Projektes ist es daher, die mikrobiellen Gemeinschaften und Stoffwechselprozesse, die zur Korrosion von Stahl in Bundeswasserstraßen führen, besser zu verstehen. Unsere Ziele waren (i) der Vergleich von korrosiven Biofilmen mit nicht-korrosiven Biofilmen, um korrosive Mikroorganismen zu identifizieren, (ii) die Untersuchung der komplexen Ursache-Wirkungs-Beziehung zwischen MIC, Wasser- und Bodenchemie, sowie der mikrobiellen Diversität, um das Systemverständnis korrosiver mikrobieller Gemeinschaften zu verbessern, und (iii) die Identifizierung der Ursachen von MIC auf wasserbaulichen Materialien, um Risikofaktoren und potenzielle biologische Indikatoren für das Auftreten von MIC abzuleiten, die für eine frühzeitige Erkennung von biokorrosiven Prozessen geeignet sind. Im Rahmen des Rimic-Projekts haben wir verschiedene Feldstudien mit einer Reihe von Laborexperimenten kombiniert, um das Auftreten und die Mechanismen von MIC in Süßwassersystemen der Bundeswasserstraßen zu untersuchen. Zur Ermittlung geeigneter Standorte, haben wir einen umfassenden Fragebogen entwickelt, der an 43 Wasserstraßen- und Schifffahrtsämter, Neubauämter und ggf. deren Außenbezirke in Deutschland verschickt wurde. Von allen Rückmeldungen der Wasserstraßen und

Schiffahrtsämter (35) gaben 60% an, dass Korrosion auftritt. Auf der Grundlage der Ergebnisse des Fragebogens führten wir an ausgewählten Standorten Probenahmen durch, um die Zusammensetzung der mikrobiellen Gemeinschaft potenziell korrosiver Biofilme sowie andere Probenarten wie Wasser, Verfüllmaterial und Sedimentproben zu untersuchen. Parallel dazu wurden Umweltparameter wie Temperatur, pH-Wert, Nährstoff- und Sauerstoffkonzentration im Wasser der ausgewählten Standorte gemessen. Die Zusammensetzung der mikrobiellen Gemeinschaft wurde anhand von DNA-Extrakten durch 16S rRNA-Genamplikon-Sequenzierung und anschließende bioinformatische Verarbeitung analysiert. Wir konnten zeigen, dass die mikrobielle Gemeinschaft je nach Art der Probe stärker divergiert als nach Probenort, und dass sich die mikrobielle Gemeinschaft in Wasserproben deutlich von der in Rosttuberkelproben unterscheidet. Einige mikrobielle Taxa wiesen in den Tuberkelproben eine deutlich höhere relative Häufigkeit auf als in den Wasserproben. Dabei handelte es sich unter anderem um Sulfatreduzierer aus den *Desulfovibrionaceae* (insbesondere *Desulfovibrio*), Eisenoxidierer aus den *Gallionellaceae* (insbesondere *Gallionella*), Nitritoxidierer aus den *Nitrospiraceae* (*Nitrospira*) und Eisenreduzierer aus den *Holophagaceae* (insbesondere *Geothrix* und *Holophaga*). Diese mikrobiellen Taxa könnten geeignete Indikatoren für MIC sein. Darüber hinaus wurden statistische Tests durchgeführt, um die Umweltparameter des beprobten Wassers (z. B. Sauerstoff, pH-Wert, Temperatur) mit der Zusammensetzung der mikrobiellen Gemeinschaft zu korrelieren, um somit Umweltrisikofaktoren für das Auftreten von MIC zu identifizieren. Da die Unterschiede zwischen den Standorten jedoch geringer waren als zwischen den Probentypen (d. h. Rosttuberkel vs. Wasserproben) und sich die Umweltparameter in der Wasserphase stark von denen im Inneren der Rosttuberkel unterschieden, ist es (bisher) nicht möglich, aus Wasserproben standortspezifische Umweltrisikofaktoren für MIC zu ermitteln. Darüber hinaus wurden einige wenige Standorte für eine detailliertere Untersuchung von Rosttuberkel ausgewählt. Dabei lag der Schwerpunkt auf der Untersuchung der chemischen und taxonomischen Gradienten, die innerhalb der Rosttuberkel auftreten. Wir haben eine breite Palette von Analysetechniken wie Mikrosensor-Profilung, Rasterelektronenmikroskopie, Mössbauer-Spektroskopie, μ -Computertomographie und 16S rRNA-Genamplikon-Sequenzierung angewendet, um unser Verständnis der Prozesse und der Entwicklung korrosiver Biofilme zu vertiefen. Mithilfe dieser verschiedenen Techniken fanden wir heraus, dass Rosttuberkel eine komplexe Struktur aufweisen, die aus verschiedenen Schichten, Kammern und Poren besteht. Darüber hinaus beobachteten wir starke Gradienten innerhalb eines Rosttuberkels, mit anoxischen Bedingungen, niedrigem pH-Wert und negativem Redoxpotential im Inneren des Rosttuberkels. Es ist daher von entscheidender Bedeutung zu berücksichtigen, dass sich die Umgebung innerhalb eines Rosttuberkels stark von der umgebenden Luft oder Wasserphase unterscheiden kann. Darum ist es wichtig nicht nur Parameter im Wasser eines Standorts zu messen, um die potenzielle MIC-Belastung abzuschätzen, sondern sich auch der Tatsache bewusst zu sein, dass die Bedingungen innerhalb der Tuberkel und damit an der Stahloberfläche sehr unterschiedlich sein können. In einer dritten Feldstudie untersuchten wir die zeitliche Entwicklung korrosiver Biofilme mit Hilfe von Expositionsexperimenten mit Stahlcoupons direkt im Feld. Wenn die Coupons für kürzere Zeiträume exponiert wurden, waren die Korrosionsraten, hochgerechnet auf ein Jahr (mm/Jahr), deutlich höher als bei längeren Expositionszeiten. Obwohl dies bereits bekannt ist, ist es wichtig, diesen Unterschied zwischen den in kurzfristigen Laborstudien ermittelten Korrosionsraten, die oft nur einige Wochen dauern, und den in der Praxis beobachteten oder vorhergesagten Korrosionsraten zu berücksichtigen. Dennoch kam die Korrosion auch nach einem Jahr Inkubation im Feld nicht vollständig zum Stillstand. Die Analyse der mikrobiellen Gemeinschaft auf den Stahlcoupons ergab Unterschiede in der Zusammensetzung der mikrobiellen Gemeinschaft und ihrer zeitlichen Entwicklung im Vergleich zu nicht-korrosiven Biofilmen. In nicht-korrosiven Biofilmen dominieren phototrophe Organismen, und die zeitlichen Veränderungen sind viel stärker von den Jahreszeiten abhängig als in der mikrobiellen Gemeinschaft auf den Stahlcoupons. Außerdem ändert sich die Zusammensetzung der mikrobiellen Gemeinschaft stark mit dem Alter der Rosttuberkel, und zwar in einem solchen Maße, dass einige Taxa, die zu bestimmten Zeitpunkten dominieren, vorher und/oder nachher nicht mehr vorhanden sind. Aufgrund dieser "Alterung" von korrosiven Biofilmen

reichen Probenahmen direkt von Spundwänden im Feld, bei denen das Alter des korrosiven Biofilms oft nicht bekannt ist, möglicherweise nicht aus, um festzustellen, welche Mikroben bei der Korrosion eine wichtige Rolle spielen. Daher empfehlen wir, die mikrobiellen Taxa zu verschiedenen Zeitpunkten zu analysieren und Proben von Rosttuberkeln unterschiedlichen Alters (falls bekannt) am selben Ort zu nehmen, wo immer dies möglich ist. Insgesamt konnten wir zeigen, dass die dominanten mikrobiellen Taxa, die in den Feldproben von 13 verschiedenen Standorten gefunden wurden, auch in den Couponexpositionsversuchen als die dominanten Arten identifiziert wurden. Darüber hinaus konnten wir die wichtige Rolle der dissimilatorischen Sulfatreduktion bei MIC-Prozessen auch in Süßwassersystemen, trotz niedriger Sulfatkonzentrationen, nachweisen. Die dominierenden Sulfatreduzierer waren dabei Bakterien der Gattung *Desulfovibrio*. Dieses Taxon wurde auch unter sulfatreduzierenden Bedingungen aus Feldproben angereichert und diente als Modellorganismus für die Reinkulturstudien zur Entschlüsselung der MIC-Mechanismen (siehe unten). Unsere Ergebnisse deuten auf eine wichtige Rolle von (mikroaerophilen) Fe(II)-Oxidierern wie *Gallionella*, die neben der abiotischen Oxidation durch O₂, Fe(II) oxidieren. Darüber hinaus spielen Schwefeloxidierer eine wichtige Rolle bei der Auffüllung des Sulfatpools für die Sulfatreduzierer.

Zusätzlich zu den Feldstudien haben wir verschiedene Laborexperimente durchgeführt, um die wichtigsten Taxa zu identifizieren, die unter bestimmten Bedingungen für MIC verantwortlich sind. Dabei fokussierten wir uns insbesondere auf die anaeroben Atmungsprozesse, bei denen Nitrat, Sulfat oder CO₂ als Elektronenakzeptor dienen. Dazu wurden korrosive Biofilmproben von verschiedenen Feldstandorten als Inokulum für Anreicherungskulturen unter Sulfat-, Nitrat- oder CO₂-reduzierenden Bedingungen mit Stahl als einzigem Elektronendonator, d.h. Elektronenquelle, verwendet. Zusätzlich haben wir die Verfügbarkeit einer organischen C-Quelle auf das Wachstum getestet. Parallel dazu wurden Versuche mit H₂ als alternativem Elektronendonator sowie abiotische Kontrollen (ohne Inokulum) durchgeführt. Die Anreicherungskulturstudien haben gezeigt, dass Süßwasserrosttuberkel Prokaryoten beherbergen, die zur dissimilatorischen Reduktion von Nitrat, Sulfat und CO₂ mit Fe(0) als einziger Elektronenquelle fähig sind. Im Falle der dissimilatorischen Nitrat- und Sulfatreduktion waren die Korrosionsraten im Vergleich zu abiotischen Kontrollen deutlich erhöht, was auf ihren potenziellen Beitrag zu MIC in Süßwassersystemen hinweist. Unter nitratreduzierenden Bedingungen waren Taxa der Gattungen *Acidovorax* und *Thermomonas* dominant. Durch Nitritbildung und die Oxidation von Eisen(II) zu Eisen(III) trugen diese Organismen zur Korrosion bei. Unter sulfatreduzierenden Bedingungen, mit Acetat als organischer C-Quelle, dominierten *Desulfovibrio* spp.

Die Mitglieder dieser Gattung sind heterotroph und einige von ihnen sind dafür bekannt, dass sie hoch korrosiv sind und eine Schlüsselrolle in marinen Lebensräumen spielen, und, wie hier bestätigt wurde, höchstwahrscheinlich auch in Süßwasserhabitaten. Neben *Desulfovibrio* spp. waren autotrophe sulfatreduzierende Bakterien der Familien *Desulfocapsaceae/Desulfobulbaceae* und der Gattungen *Desulfomicrobium* und *Desulfosporosinus* in Anreicherungskulturen vorherrschend. Dies deutet darauf hin, dass die dissimilatorische Sulfatreduktion im Freiland auch in Abwesenheit von organischem C ablaufen könnte. Die Metaproteomanalyse zeigte, dass zwei am Elektronentransfer beteiligte Proteine, eine periplasmatische Hydrogenase und ein Porin der Außenmembran, in Anreicherungskulturen unter sulfatreduzierenden Bedingungen sehr häufig vorkamen. Dies deutet darauf hin, dass beide Mechanismen, die H₂ vermittelte, und die direkte Elektronenübertragung von der Stahloberfläche, eine Rolle spielen. Des Weiteren haben wir festgestellt, dass Methanogenese und Acetogenese auch mit Fe(0) als einzige Elektronenquelle stattfinden können. Um den molekularen Mechanismus der MIC zu entschlüsseln, führten wir Studien in Reinkultur mit *Desulfovibrio vulgaris* und *Desulfovibrio ferrophilus* unter Verwendung metaproteomischer Ansätze durch. Dabei wurden biotische und abiotische Behandlungen mit Stahl oder H₂ im Zeitverlauf verglichen, um zwischen den verschiedenen MIC-Mechanismen zu unterscheiden. Die Mikrokosmos-Experimente mit Reinkulturen gaben Aufschluss über den möglichen Energiemetabolismus der Sulfatreduzierer. Insbesondere wurde die Rolle von Fe(0) als potenzieller Elektronendonator untersucht. Die Reinkulturexperimente zeigten, dass die getesteten Stämme von *D. vulgaris* und *D. ferrophilus* sowohl in Süßwasser- als auch in Salzwasserkulturmedien

Stahl korrodieren. Dies deutet darauf hin, dass diese beiden Bakterienstämme erhebliche Schäden an Stahlkonstruktionen verursachen können. Die Optimierung der metaproteomischen Analysen der eisenreichen Proben war hierbei eine technische Herausforderung. Die SP3-Bead-Extraktionsmethode erwies sich als am besten geeignet für die Extraktion von Proben mit geringer Biomasse und hohem Matrixeffekt. Der klassische Ansatz, die Proteine anhand der Metagenomik-Ergebnisse zu analysieren, war aufgrund der geringen Qualität und Quantität der extrahierten DNA, insbesondere aus gemischten Anreicherungsproben, nicht möglich. Daher nutzen wir stattdessen die Uniprot-Datenbank der TOP10-Familien für die Auswertung von Mischkulturproben.

Insgesamt hat die Kombination verschiedener Feld- und Laborstudien sowie interdisziplinärer Methoden unser Verständnis von MIC in Bundeswasserstraßen vertieft. Dennoch bleibt die Identifizierung von standortbezogenen Umweltrisikofaktoren eine Herausforderung. Die Ergebnisse des Rimic-Projekts bieten eine hervorragende Grundlage für künftige Untersuchungen, von denen einige bereits angelaufen sind. So führt die BAW beispielsweise ein Experiment durch, bei dem Platten aus verschiedenen Materialien (Eisen, Aluminium, Zink und Kupfer) an verschiedenen Standorten ausgebracht wurden, um den Materialverlust, die Materialeigenschaften sowie die Zusammensetzung der mikrobiellen Gemeinschaft über längere Zeiträume zu untersuchen. In ähnlicher Weise wurde eine Studie an der Universität Erlangen-Nürnberg (auch hier in Zusammenarbeit mit der BfG zur Analyse der mikrobiellen Gemeinschaften) begonnen, um verschiedene Beschichtungen (z.B. Smart Repair) auf Metallplatten und ihre Resistenz gegenüber MIC zu untersuchen. Die Ergebnisse dieser Studien werden dazu beitragen, ein besseres Verständnis dafür zu entwickeln, welche Materialien am anfälligsten für Korrosion sind und wie der Korrosionsschutz in Zukunft verbessert werden kann.

Executive summary English

Federal waterways are used as shipping routes; thus, it is essential to ensure the stability and operational safety of hydraulic structures within. Maintenance and reconstruction of those structures due to corrosion-induced damage may account for high annual expenditure. Often, corrosion is not a purely physical-chemical process, but it is influenced by the activity of microorganisms (microbially influenced corrosion, MIC). Microorganisms may excrete corrosive metabolites such as H₂S or inorganic and organic acids, or they evoke corrosion directly by withdrawing electrons from the steel. However, knowledge on the underlying microbial metabolisms and molecular mechanisms is still very scarce. Therefore, the overall aim of the project was to shed light on the microbial communities and metabolic processes promoting corrosion of steel in federal waterways.

To identify suitable field sites, we developed an extensive questionnaire which was sent to the waterways and shipping offices within Germany. Based on the results of the questionnaire, we performed sampling at a selected number of sites. We could show that the microbial community is diverging strongly according to sample type, some microbial taxa (e.g. *Desulfovibrio*, *Gallionella*, *Nitrospira*, *Geothrix* and *Holophaga*) showed a significantly higher relative abundance in the tubercle samples compared to water samples. These microbial taxa might be suitable as indicators for MIC. However, as site difference were smaller than differences between sample type, it (so far) remains elusive to identify site based environmental risk factors for MIC. Furthermore, for a few sites we investigated the chemical and taxonomic gradients which occur within the rust tubercles itself. Using a set of different techniques, we found that the environment inside a rust tubercle differs strongly from the surrounding air or water phase. We also investigated the temporal development of potentially corrosive biofilms using steel coupon exposition in parallel measurements of non-corrosive biofilms. In non-corrosive biofilms, phototrophic organisms and a seasonal effect were dominant. The microbial community composition was strongly changing over time, to such an extent that some taxa dominate a certain time point or are not present at all. Overall, we verified the important role of dissimilatory sulfate reduction (especially by *Desulfovibrio*) in MIC also in freshwater systems, despite low sulfate concentrations.

In addition to the field studies, different sets of laboratory studies were performed to enrich and identify key taxa performing MIC under different defined conditions. Enrichment culture studies showed that freshwater corrosion scales harbor prokaryotes capable of the dissimilatory reduction of nitrate, sulfate, and CO₂ with Fe as sole electron source. In case of dissimilatory nitrate and sulfate reduction, corrosion rates were significantly enhanced compared to abiotic controls, suggesting that these processes have a potential for contributing to MIC in freshwater systems. Under nitrate reducing conditions, members of the genera *Acidovorax* and *Thermomonas* prevailed. Under sulfate reducing conditions, with acetate as organic C source, *Desulfovibrio* spp. dominated. Members of this genus are heterotrophic and some of them are known to be highly corrosive being key players in marine habitats and, as confirmed here, most likely also in freshwater habitats. Metaproteome analysis suggest that both mechanisms, H₂ mediated and direct electron transfer from the steel surface, play a role. We also found a potential of methanogenesis and acetogenesis to occur with Fe⁰ as sole electron source. To unravel the molecular mechanism of MIC, pure culture studies with *D. vulgaris* and *D. ferrophilus* were performed using metaproteomic approaches. Pure culture experiments showed that the strains *D. vulgaris* and *D. ferrophilus* were corrosive to steel in both freshwater and marine media. Optimizing metaproteomic analysis of Fe-rich samples was challenging, we found the SP3-bead extraction method was best suited for the extraction of low biomass samples with strong matrix effects. Overall, the combination of different types of field and laboratory studies and the application of interdisciplinary methods definitely helped to deepen our understanding of MIC in federal waterways. Nevertheless, defining environmental risk factors remains challenging. The results of the Rimic project are an excellent starting point for future investigations, of which some have already started.

Full summary English

Federal waterways are used as shipping routes, making it is essential to ensure the stability and operational safety of hydraulic structures, such as sheet pile walls in locks, weirs and bank enforcements. However, corrosion of those (unalloyed) steel structures bear high safety risks due to a potential loss of function. Additionally, maintenance and reconstruction of those structures due to corrosion-induced damage, but also preventive measures account for high annual expenditure.

Often, corrosion is not a purely physical-chemical process, but it is influenced by the activity of microorganisms (microbially influenced corrosion, MIC). In recent years, corrosion damage to hydraulic steel structures has been observed in several federal waterways, characterized by increased local corrosion, likely enhanced due to the presence of microorganisms. Under oxic conditions, corrosion of the contained iron (Fe⁰) occurs primarily via abiotic oxidation to ferrous iron (Fe²⁺), which is further oxidized to ferric iron (Fe³⁺). Under anoxic conditions, Fe⁰ is oxidized abiotically to Fe²⁺ by the reduction of H⁺ to form H₂. Microorganisms may mediate MIC in many different ways. They may change concentrations of the reactants, e.g. under microoxic or anoxic (e.g. nitrate reducing) conditions, mainly bacteria account for the oxidation of ferrous to ferric iron. They may excrete corrosive metabolites such as H₂S or inorganic and organic acids. Microorganisms may evoke corrosion directly by withdrawing electrons from the steel. They use Fe⁰ as electron donor for energy generation and transfer the electrons to weak oxidants such as sulfate or CO₂. Hence, by enzymatic catalysis, microorganisms accelerate the overall corrosion process, especially under anoxic conditions, where there is a lack of strong oxidants.

However, knowledge on the underlying microbial metabolisms and molecular mechanisms is still very scarce. Therefore, the overall aim of the project was to shed light on the microbial communities and metabolic processes that promote corrosion of steel in federal waterways. Our prime objectives were (i) the comparison of corrosive biofilms with non-corrosive biofilms to pinpoint corrosive microorganisms or even whole microbiocoenoses, (ii) the investigation of the complex cause-effect relationship between MIC, water and soil chemical properties, and microbial diversity to improve the system

understanding of corrosive microbial communities and (iii) the identification of the causes of MIC on hydraulic engineering materials to derive risk factors and potential biological indicators for the occurrence of MIC that might be suitable for an early detection of biocorrosive processes. Within the Rimic project, we combined different field studies with a set of laboratory experiments to investigate the occurrence and mechanisms of MIC in freshwater systems of the federal waterways.

To identify suitable field sites, we developed a comprehensive questionnaire which was sent to 43 waterways and shipping offices in Germany. Of all responding waterways and shipping offices (35), 60% indicated that corrosion is occurring. Based on the results of the questionnaire, we performed sampling at selected sites to investigate the microbial community composition of potentially corrosive biofilms as well as other sample types such as water, backfilling material and sediment samples. In parallel, we measured and collected environmental parameters such as temperature, pH, nutrient concentrations, and oxygen concentrations in the water at the chosen sites. The microbial community composition was analysed from DNA extracts through 16S rRNA gene amplicon sequencing and subsequent bioinformatic processing. We could show that the microbial community is diverging stronger according to the sample type than to sample site, and that the microbial community in water samples clearly differs from that in rust tubercle samples. Some microbial taxa showed a significantly higher relative abundance in the tubercle samples compared to the water samples. Those were, among others, sulfate reducers from the *Desulfovibrionaceae* (especially *Desulfovibrio*), iron oxidizers from the *Gallionellaceae* (especially *Gallionella*), nitrite oxidizers from the *Nitrospiraceae* (*Nitrospira*) and iron reducers from the *Holophagaceae* (especially *Geothrix* and *Holophaga*). These microbial taxa might be suitable indicators for MIC. Furthermore, using statistical tests, we correlated the environmental site parameters of the sampled water (e.g. oxygen, pH, temperature) to the detected microbial community composition to identify environmental risk factors for the occurrence of MIC. However, as differences between sites were smaller than between sample type (i.e. rust tubercle vs. water samples) and the environmental parameters in the water phase differed strongly from those within the rust tubercles, it (so far) remains elusive to identify site-specific environmental risk factors for MIC from water samples.

Furthermore, a few sites were selected for a more detailed study of rust tubercles. Hereby, the focus was to investigate the chemical and taxonomic gradients that occur within the rust tubercles. We applied a wide range of analytical techniques such as microsensor profiling, scanning electron microscopy, Mössbauer spectroscopy, μ -computer tomography, and 16S rRNA gene amplicon sequencing to deepen our understanding of the processes and the development of corrosive biofilms. Using this set of different techniques, we found that the rust tubercle is a complex structure consisting of distinct layers, chambers and pores. Furthermore, we observed strong gradients within one rust tubercle, with anoxic conditions, low pH and negative redox potential present in the inner core of the tubercle. Accordingly, the relative abundance of microbes performing sulfate reduction (e.g. *Desulfovibrionaceae*) and fermentation (*Geothrix*) increased towards the inside of the tubercles. Thus, it is crucial to consider that the environment inside a rust tubercle can differ strongly from the surrounding air or water phase. This highlights the importance to not only measure water parameters to estimate the potential risk of MIC at certain sites, but also to be aware of the fact that conditions can differ enormously inside the tubercle and thus at the steel surface.

A third field study investigated the temporal development of corrosive biofilms using steel coupon exposition experiments directly in the field. When coupons were exposed for shorter time periods, the corrosion rates, upscaled to one year (mm/year), were considerably higher than when coupons were incubated for longer periods. Even though this is consistent with previous results, it is important to consider the difference between corrosion rates determined in short-term laboratory studies, often lasting only a couple of weeks, with those observed or predicted in the field. Nevertheless, even after one year of incubation in the field, corrosion did not completely cease. The analysis of the microbial community on the steel coupons in parallel measurements of non-corrosive biofilms revealed differences in the community composition and its development over time. In non-corrosive biofilms,

phototrophic organisms are dominant and changes over time are much more dependent on seasonality than within the microbial community of steel coupons. Furthermore, the microbial community composition is strongly changing with tubercle age, to such an extent that some taxa which are dominant at certain time points are not present before and/or afterwards. Due to this “aging” of corrosive biofilms, sampling directly from steel piles in the field where the age of the corrosive biofilm is often unknown, may not be sufficient to determine which microbes play a major role in corrosion. Hence, we recommend analysing microbial taxa at different timepoints, and sampling rust tubercles of different age (if known) at the same site, wherever possible.

Overall, we showed that the dominant microbial taxa found in field samples collected from 13 different sites were also identified as the dominant species in the coupon exposition experiments. Furthermore, we verified the important role of dissimilatory sulfate reduction in MIC processes also in freshwater systems despite low sulfate concentrations, with members of the genus *Desulfovibrio* being the dominant sulfate reducers. This taxon was also enriched under sulfate reducing conditions from field samples and it was used as a model organism for the pure culture studies to unravel the MIC mechanisms (see below). Our results point to an important role of (microaerophilic) Fe(II) oxidizers such as *Gallionella* next to the abiotic oxidation by O₂. In addition, sulfur oxidizers play an important role in replenishing the sulfate pool for sulfate reducers.

In addition to the field studies, we conducted various sets of laboratory experiments to identify key taxa responsible for MIC under defined conditions. Here, we were specifically interested in the anaerobic respiration processes in which nitrate, sulfate or CO₂ serve as electron acceptor. For this, corrosive biofilm samples from different field sites were used as inoculum for enrichment cultures under sulfate, nitrate or CO₂ reducing conditions with steel as sole electron donor, i.e. electron source. In addition, we tested the availability of an organic C source on growth. Assays with H₂ as alternative electron donor as well as abiotic controls (without inoculum) were run in parallel. Enrichment culture studies have shown that freshwater corrosion scales harbor prokaryotes capable of the dissimilatory reduction of nitrate, sulfate, and CO₂ with Fe(0) as the sole electron source. In case of dissimilatory nitrate and sulfate reduction, corrosion rates were significantly enhanced compared to abiotic controls, indicating their potential contribution to MIC in freshwater systems. Under nitrate reducing conditions, members of the genera *Acidovorax* and *Thermomonas* prevailed. The production of the corrosive agent nitrite and the oxidation of ferrous to ferric iron were the means by which these organisms contributed to corrosion. Under sulfate reducing conditions, with acetate as organic C source, *Desulfovibrio* spp. dominated. Members of this genus are heterotrophic and some of them are known to be highly corrosive, being key players in marine habitats and, as confirmed here, most likely also in freshwater habitats. Besides *Desulfovibrio* spp., autotrophic sulfate reducing bacteria affiliated to the families *Desulfocapsaceae*/*Desulfobulbaceae* and the genera *Desulfomicrobium*, and *Desulfosporosinus* were predominant in enrichment cultures. This suggests that dissimilatory sulfate reduction in the field could also proceed even in the absence of organic C. Metaproteome analysis showed that two proteins involved in electron transfer, a periplasmic hydrogenase and an outer membrane porin were highly abundant in steel coupon assays under sulfate reducing conditions. This suggests that both mechanisms, H₂ mediated and direct electron transfer from the steel surface, play a role. We also found a potential of methanogenesis and acetogenesis to occur with Fe⁰ as the sole electron source.

To unravel the molecular mechanism of MIC, we performed pure culture studies using metaproteomic approaches with *Desulfovibrio vulgaris* and *Desulfovibrio ferrophilus*. Biotic and abiotic treatments with steel or H₂ were compared over time to distinguish between the different MIC mechanisms. The pure culture microcosm experiments provided insight into the possible energy gain mechanisms that sulfate reducers employ to grow. Specifically, the role of Fe⁰ as a potential electron donor was investigated. Pure culture experiments demonstrated that the tested strains of *D. vulgaris* and *D. ferrophilus* were corrosive to steel in both freshwater and marine culture media. This suggests that these two bacterial strains can cause significant damage to steel structures. Overall, it was a challenge for us to optimize metaproteomic analysis of the Fe-rich samples. The SP3-bead extraction method was found to be

best suited for the extraction of lower biomass and high matrix effected samples. The classical approach of performing protein analysis using metagenomics results of our samples, could have served as an ideal databank for proteomics, but the quality and quantity of the DNA extracted especially from mixed enrichment culture samples were not sufficient for a good analysis, due to lower biomass and the interference of Fe-minerals, which tend to bind to DNA, thereby rendering losses. Hence, the databank, Uniprot of the TOP10 families, for the evaluation of mixed culture samples was our next best solution.

Overall, the combination of various field and laboratory studies, along with interdisciplinary methods, has deepened our understanding of MIC in federal waterways. Nevertheless, defining environmental risk factors remains challenging. The results of the Rimic project provide an excellent foundation for future investigations, of which some have already started. For example, the BAW, conducted an experiment where plates of different materials (iron, aluminium, zinc and copper) were placed at different sites and to investigate the material loss, material properties, as well as the microbial community composition over longer timescales. Similarly, a study at the University Erlangen-Nürnberg (cooperation with BfG for microbial community analysis) started to investigate different coatings (e.g. smart repair) on metal plates and their resistance towards MIC. This will lead to a better understanding of which materials are most prone to corrosion and how corrosion protection can be improved in the future.

1 Introduction

For the federal waterways to function as shipping routes, it is essential to ensure the stability and operational safety of the many hydraulic structures, such as bare sheet pile walls in locks, in weirs or as bank enforcement. However, corrosion of those (unalloyed, not corrosion protected) steel structures by iron oxidation results usually in average corrosion rates below 0,03 mm/a in freshwater for longer exposition durations (Hein, 1990; Binder and Graff, 1995; Heeling, 2017; Arbeitsausschuss "Ufereinfassungen", 2013). Maintenance and reconstruction of those structures due to corrosion-induced damage, but also preventive measures account for high annual expenditure, which is valued at up to 4% of a nation's gross domestic product. Globally this corresponds to about 2.5 trillion USD (Koch, 2017).

Corrosion is often not a purely physical-chemical process, but in many cases microbially influenced. In recent years, a number of unusual cases of damage to hydraulic steel structures have also been observed in several federal waterways. These cases were caused by an increased local corrosive attack due to the presence of microorganisms as could be demonstrated, for example, on sheet pile walls along the Rhine-Herne Canal where accelerated corrosion with local rust perforation led to a partial collapse of the wall and made repair imperative (personal communication with Sven-Erik Wulf and Roland Baier, BAW). Cases of this so-called microbially induced corrosion (MIC) have been described in both freshwater (Rao et al., 2000; Odokuma and Ugboma, 2012) and seawater (Videla, 1994; Lee et al., 2004), and have even been detected in engineered systems (oil, gas, or sewer pipelines) or (nuclear waste storage) tanks (Vincke et al., 2001; Crolet, 2005). MIC is initiated by the metabolic activity of microorganisms that cover metal surfaces with biofilms, but can also affect organic materials, glass, ceramics, concrete and plastics (Heitz et al., 1996; Zuo, 2007). Biofilms, consisting of consortia of various microorganisms embedded in an extracellular matrix, bind water, can accumulate salts, locally change the pH, and excrete complexing substances, exoenzymes as well as other metabolic products (Flemming, 2016; Flemming and Wingender, 2010; Videla, 1994). Those microbial metabolic processes in the biofilm can significantly interfere with the kinetics of the cathodic and anodic reactions at the interface accelerating the physical-chemical corrosion processes. According to estimates, 20-50% of all corrosion damage to metals and other building materials is microbially influenced (Heitz et al., 1996; Beech and Gaylarde, 1999). Microorganisms thus contribute significantly to the weathering of hydraulic engineering materials.

Sheet pile steel, containing a maximum permissible carbon content of 0.2% (DIN EN 10248), is one of the most used construction materials in the federal waterways making it highly susceptible to MIC. Under oxic conditions, corrosion of the contained iron (Fe^0) occurs primarily via abiotic oxidation to ferrous iron (Fe^{2+}), which is further oxidized to ferric iron (Fe^{3+}) in an aqueous environment. Under microoxic conditions, oxidation of ferrous iron can, for example, be accelerated by Fe(II)-oxidizing bacteria (Bryce et al., 2018). Under anoxic conditions, microbially induced oxidation to ferric iron can additionally occur via dissimilatory nitrate reduction (Bryce et al., 2018). Accordingly, microbially induced iron corrosion is highly dependent on various physicochemical factors, such as the presence of oxygen, the type of electron acceptor, the pH or the redox potential, which all influence the composition of the microbial community as well as the corrosion mechanism (Heitz et al., 1996; Little et al., 2020). Within the corrosive biofilm, limited diffusion enables anoxic micro niches to evolve, in which different anaerobic microorganisms (e.g., sulfate-reducing bacteria, iron-reducing bacteria, iron-oxidizing bacteria, acetogenic bacteria, and methanogenic archaea) can exist in close proximity to each other as well as to aerobic organisms (Beech and Sunner, 2004; Bond and Lovley, 2005; Kip et al., 2017; Miller et al., 2018). Those microorganisms can evoke MIC directly by withdrawing electrons from the steel (Alabbas et al., 2013) as well as indirectly, for example, by the secretion of corrosive metabolites (CMIC) such as H_2S , inorganic acids (H_2SO_4 , HNO_3) or organic acids (e.g., acetic acid or propionic

acid) (Flemming, 1995). Moreover, indirect microbial destruction of building materials can also be caused upon consumption of O_2 resulting in the formation of corrosion-prone aeration cells or upon consumption of H^+ leading to depolarization and thus initiation of corrosion (Flemming, 1995). Most studies on MIC focus on MIC by sulfate-reducing bacteria (SRBs), which can reduce sulfate to sulfide (H_2S) and thus enable the dissolution of iron from steel and its conversion to iron sulfide (Stipaničev et al., 2013; Skovhus et al., 2017). The initially steel-protecting molecular hydrogen is consumed, the corrosion products iron sulfide and iron hydroxide are formed. At the same time, sulfur-oxidizing bacteria (SOBs) can produce sulfuric acid, which directly attacks the steel. Co-colonization with SOBs can therefore prevent a sulfate deficiency for the SRBs, giving an example for a symbiotic relationship within the consortia of a corrosive biofilm (Beech and Sunner, 2004). While all SRBs can initiate MIC to some extent by synthesis of the corrosive metabolite H_2S , some strains (e.g., *Desulfovibrio ferrophilus*, IS5) have been described to additionally use iron directly as electron donor and consequently severely enhance corrosion processes (Enning et al., 2012; Venzlaff et al., 2013).

Generally, microbially induced Fe oxidation by electron transfer under anaerobic conditions is thought to take place via three main mechanisms: (i) H_2 -mediated iron-to-microbe electron transfer (HIMET), (ii) shuttle mediated iron-to-microbe electron transfer (SIMET), and (iii) direct iron-to-microbe electron transfer (DIMET) (Lekbach et al., 2021). MIC through HIMET can accelerate Fe^0 corrosion by many different microorganisms through the consumption of H_2 formed abiotically by H^+ reduction coupled to Fe^0 oxidation (Deutzmann et al., 2015; Philips et al., 2019; Philips, 2020). The microbial anaerobic oxidation of H_2 can hereby be coupled to various electron acceptors (NO_3^- , SO_4^{2-} , CO_2) (Lekbach et al., 2021). Prominent examples for HIMET-performing microorganisms are, among others, acetogens, for example *Acetobacterium* (Philips et al., 2019; Philips, 2020). MIC through SIMET utilizes shuttle molecules (redox active organic molecules such as flavins and phenazines as well as humic acids), which could function similar to H_2 as electron shuttles between Fe^0 and the present electron acceptor (Lovley et al., 1999; Glasser et al., 2017; Little and Lee, 2014; Lekbach et al., 2021). A proposed microorganism performing SIMET is, for example, *Pseudomonas aeruginosa* via the production of the electron shuttle substance phenazine-1-carboxamide (PCN) (Huang et al., 2018). MIC through DIMET is increasingly mentioned as corrosion pathway, however difficult to verify in culture, but even more so in the environment (Lekbach et al., 2021). It was suggested that DIMET occurs among others within the archaeal species of the genus *Archaeoglobus* (Ali et al., 2020) and within the bacterial species of the genera *Bacillus* (Xu et al., 2013), *Desulfobacterium* and *Desulfovibrio* (Dinh et al., 2004; Enning et al., 2012), *Desulfopila* (Enning et al., 2012), *Prolixibacter* (Iino et al., 2015), *Shewanella* (Miller et al., 2018), and *Geobacter* (Tang et al., 2019). Mechanistically, DIMET is thought to occur via extracellular cytochromes and electrically conductive pili (Dinh et al., 2004; Venzlaff et al., 2013; Holmes et al., 2019; Lekbach et al., 2021; Chatterjee et al., 2021). A recently published study revealed overexpression of outer surface multiheme c-type cytochromes in *D. ferrophilus* IS5 upon cultivation with steel samples suggesting that this enzyme group may serve as a potential biomarker for MIC (Chatterjee et al., 2021). Beside cytochromes, also hydrogenases have been described to be mediators of corrosion in SRBs or methanogens, as they were found to be absent in non-corrosive strains (Deutzmann et al., 2015). In aerobic corrosion processes, extracellular catalases and peroxidases, catalyzing the transfer of electrons via their heme group, have been suggested to be responsible to MIC in marine biofilms. To conclude, all MIC-linked enzymes identified so far belong to the class of oxidoreductases with the ability to exchange electrons. Yet, full identification of the involved enzymes and understanding of the underlying mechanisms are still lacking due to the complexity of the microorganisms' metabolism.

2 Motivation and research needs

Corrosion damage, abiotic as well as microbially induced, jeopardizes the function of hydraulic structures, the safety of shipping traffic and local residents, and causes high economic costs every year. For example, at the Wilhelmshaven outer harbor, an unfavorable constellation of silt and microbial fouling led to severe corrosion of sheet pile walls, causing costs of ~100 million euros for the required replacement (D. Stammerjohann, BfG, pers. comm.). The degree of corrosion depends on various environmental factors, such as temperature, conductivity, pH or the backfilling of structures such as sheet piles. However, despite the obvious importance of microbial processes for the longevity of hydraulic engineering materials, knowledge on the underlying microbial processes and factors that promote the occurrence of MIC is still scarce. In particular, the role of individual bacterial species within the same functional groups, such as sulfate reducers (Enning et al., 2012; Venzlaff et al., 2013), iron reducers (AlAbbas et al., 2013; Lovley et al., 2011) and methanogens (Lahme et al., 2021), is increasingly discussed controversially with respect to MIC, whether they are able to promote as well as inhibit corrosion processes (Kip and van Veen, 2015). Most studies on MIC focus on SRBs (Skovhus et al., 2017; Stipanichev et al., 2013). However, recent studies show that other bacterial groups such as iron-oxidizing bacteria, manganese-oxidizing bacteria, acid-producing bacteria and methanogens are also important (Beech and Sunner, 2004; Jan-Roblero et al., 2008; Maruthamuthu et al., 2005; Rajasekar et al., 2010), but their role in corrosion processes is often underestimated (Javaherdashti 2008). This shows that the causative mechanisms are not yet sufficiently understood, as they can be attributed either to individual biochemical reactions or to specific microbial species or groups. Reliable organismic indicators for MIC, which allow a clear quantification and differentiation from non-microbially influenced corrosion, are therefore missing. Further complicating matters, the composition and activity of the biofilm microbial community is influenced by various environmental factors such as temperature, pH, and availability of electron donors/acceptors (Miller et al., 2018; Palacios et al., 2019; Schutz et al., 2014; Li et al., 2017; Phan et al., 2020; Jin et al., 2015). Studies that comprehensively illuminate these complex interactions are lacking.

A conspicuous structure commonly forming upon corrosion are tubercles. These mound-like accumulations of corrosion products may reach sizes of several millimeters to centimeters in diameter and in height and appear to be 'hot-spots' of corrosion. Corrosion tubercles are often associated with MIC and microbial activities appear to be the driving force of tubercle formation (Jin et al., 2015). Several studies have investigated the morphology and the mineral composition of these tubercles (Sarin et al., 2001; Ray et al., 2010b; Albahri et al., 2019) but only few combined these investigations with the molecular analysis of microbial community (Jin et al., 2015; Kip et al., 2017) and/or high resolution concentration profiles of solutes (Lee and de Beer, 1995; Jin et al., 2015). Furthermore, only few studies examined tubercles from a natural freshwater site (Ray et al., 2010b; Odokuma and Ugboma, 2012; Jin et al., 2015; Wakai et al., 2022).

Observations on the Rhine-Herne Canal, for example, show that the corrosion of sheet pile walls can vary in severity despite apparently identical boundary conditions and spatial proximity. However, the reasons for the different degrees of corrosion are unknown (R. Baier, BAW, pers. comm.). A better understanding of the microbial communities and their metabolic activities in corrosive biofilms and rust tubercles are seen as a crucial key for a better prediction of microbial corrosion and finally its consideration in the planning and maintenance of hydraulic engineering works. Thus our main objectives were (i) comparison of corrosive biofilms with non-corrosive biofilms to pinpoint corrosive microorganisms or even whole microbiocoenoses, (ii) Investigation of the complex cause-effect relationship between MIC, water and soil chemical properties, and microbial diversity to improve the systems understanding of corrosive microbial community mechanisms and (iii) identification of the causes of MIC on

hydraulic engineering materials to derive risk factors and potential biological indicators for the occurrence of MIC that might be suitable for an early detection of biocorrosive processes.

We aimed at combining field studies with laboratory experiments to pursue complementary approaches with the objective of ensuring a sustainable maintenance management of hydraulic engineering facilities. Hereby, field investigations were carried out in close coordination and cooperation with the Federal Waterways Engineering and Research Institute (Bundesanstalt für Wasserbau, BAW) and the Waterways and Shipping Office (Wasser- und Schifffahrtsamt, WSA) responsible on site. In parallel, defined studies under controlled laboratory conditions are conducted. For this, different sets of experiments using enrichment cultures from field samples, with and without steel, were conducted to enrich potentially corrosive microorganisms (cooperation with Dr. Jutta Meier, Institut für Integrierte Naturwissenschaften, Universität Koblenz). Additionally, pure culture experiments have been performed to analyse the enzymes involved in MIC and further understand the MIC mechanisms.

Through the targeted identification of indicators and risk factors for the occurrence of MIC, new methods were to be established to derive specific proposals for minimization and early detection of microbial corrosion, thus strengthening the BfG's advisory function in water chemistry issues. The results of this project contribute to a better understanding of MIC in federal waterways and will support the Waterways and Shipping Administration (Wasserstraßen- und Schifffahrtsverwaltung, WSV) in estimating the corrosion to be expected in the planning of hydraulic structures and to take them into account in order to minimize the expenses for the maintenance or reconstruction of hydraulic structures. This may ensure a sustainable repair management of hydraulic engineering facilities, maintaining their functionality and safety as long as possible.

The project Rimic aimed to close some of the described knowledge gaps in close cooperation of the BfG, with the University of Koblenz and the Federal Waterways Engineering and Research Institute (BAW; R&D topic). The BfG's work focused in particular on the targeted application of modern molecular biological methods (genomics, proteomics) for a comprehensive analysis of microbial communities and on investigating the environmental conditions under which these microorganisms are active. The knowledge gained should reveal new possibilities for detecting microbial corrosion at an early stage, increasing the service life and safety of hydraulic structures and reducing maintenance costs.

3 Methodology

3.1 Questionnaire and site selection

The formation of rust tubercles during microbially influenced corrosion (MIC) was observed in many natural marine but also industrial systems. However, their occurrence in freshwater systems caught more and more attention in recent years.

To investigate freshwater rust tubercles in German Waterways we conducted a questionnaire in 2020 (see S1 for specific questions) within the Rimic project. The questionnaire was developed together with the BAW and distributed through the GDWS (Günther Steil). The questionnaire was sent to 43 Waterways and Shipping offices in Germany. Of all Waterways and Shipping Offices (81% replies), 60% indicated that corrosion is occurring (Figure 1).

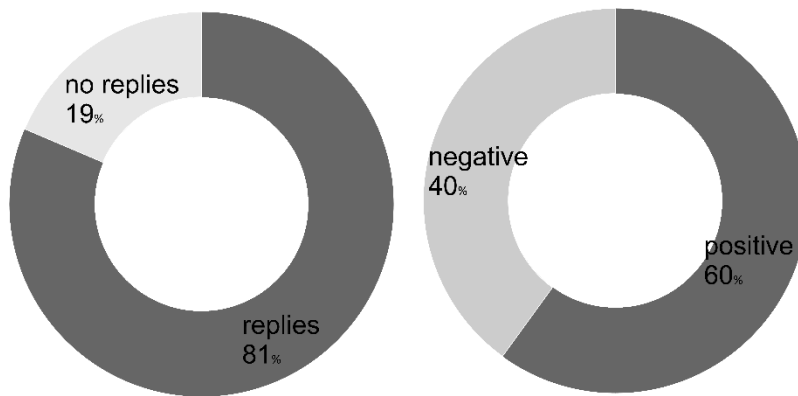


Figure 1: Results of the questionnaire, ratio of replies to no replies and positive findings of corrosion.

The first set of questions was targeted to corrosion on the water side, the side of the metal facing the water body. Of the water and shipping offices which found corrosion, 63% reported that they have water side corrosion on 1 to 15 locations and 8% reported that they find water side corrosion on more than 15 locations within their jurisdiction. They indicated that 45% of the corrosion was particularly localized and 37% reported a vertical zonation of the corrosion with water depth. Corrosion occurred on sheet piles i) in locks and outer ports (32%) and ii) on canal tracks (15%). Corrosion was also found on pile moorings and shore leaves (12%), on weirs (12%), other metal parts (10%), culverts (2%) and even galvanized metal parts (7%). No corrosion was reported on stainless steel (Figure 2).

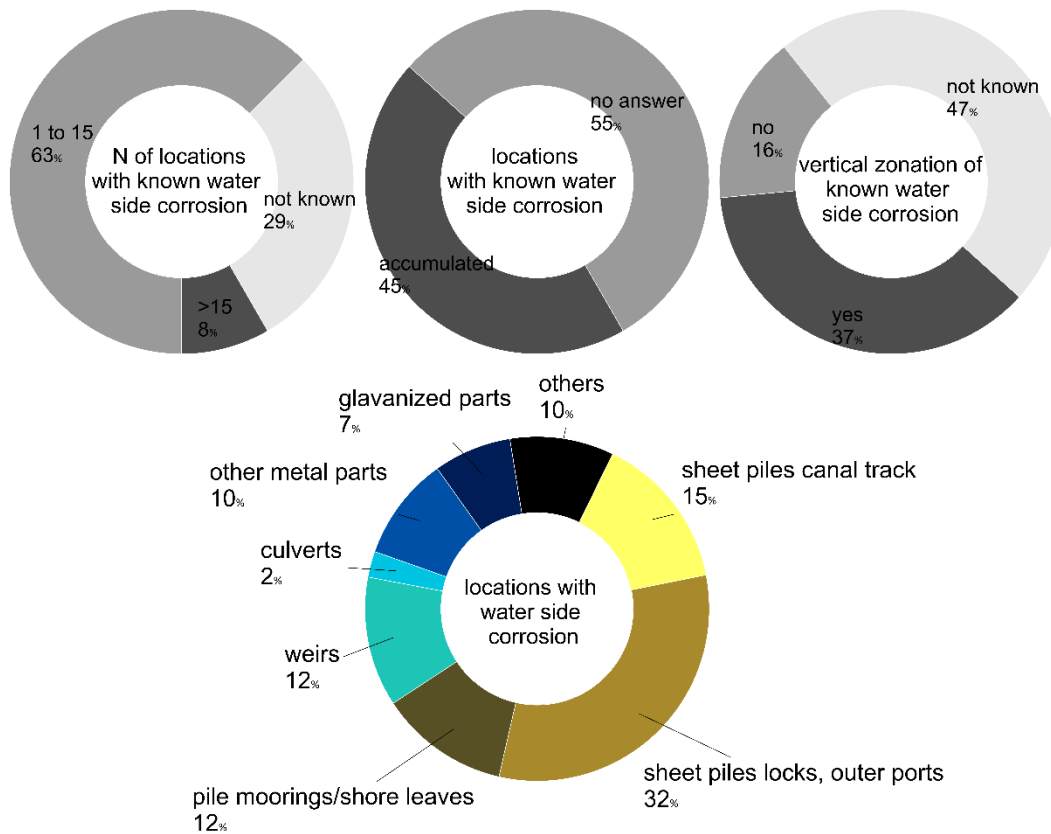


Figure 2: Results of the questionnaire to the questions regarding water side corrosion.

The second set of questions was targeted to corrosion from the land side e.g. the side facing the back-filling of a sheet pile (e.g. soil, cement etc.). In total 4% reported more than 15 location with land side corrosion and 25% with 1 to 15 locations of land side corrosion. Of the reported land side corrosion 22% reported that those occur accumulated. For the land side corrosion 31% reported that the material loss was particularly fast and verified by residual wall thickness analysis. Land side corrosion was reported only for sheet piles in locks, outer ports (73%) and sheet piles in the free canal track (27%).

In general, we concluded, that corrosion also is a severe problem occurring ubiquitous within inland waters. We want to note that the occurrence of corrosion as well as the identification of MIC as influencing the corrosion is likely an underestimation as i) not all responded ii) during site investigations some negative responses were found to actually have rust tubercle formation iii) MIC is not easily identified by visual inspection only.

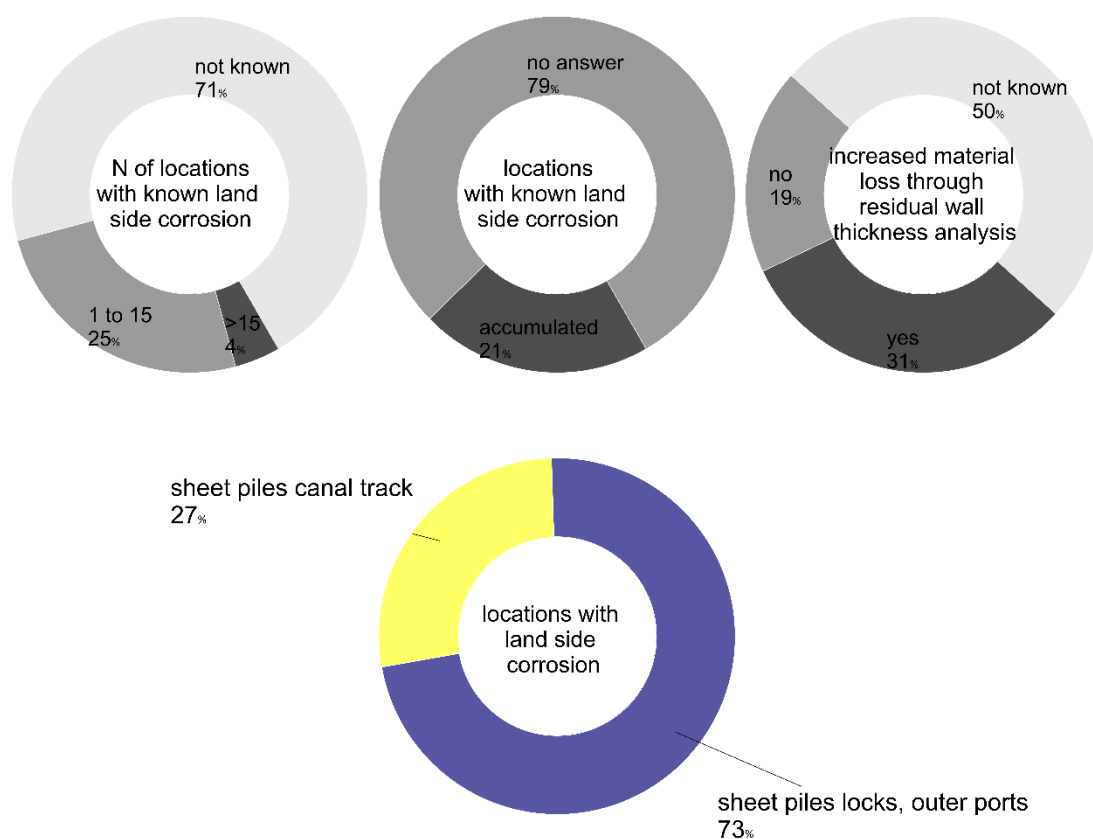


Figure 3: Results of the questionnaire to the questions regarding land side corrosion.

3.2 Sampling and sample preparation in the field

Our sampling sites, 12 in total, were located over Germany (Figure 5). Based on potential MIC cases (questionnaire) and observations from the BAW, we investigated different sites and sampled different sample types (e.g. sediment, water and tubercle samples) at different sites.

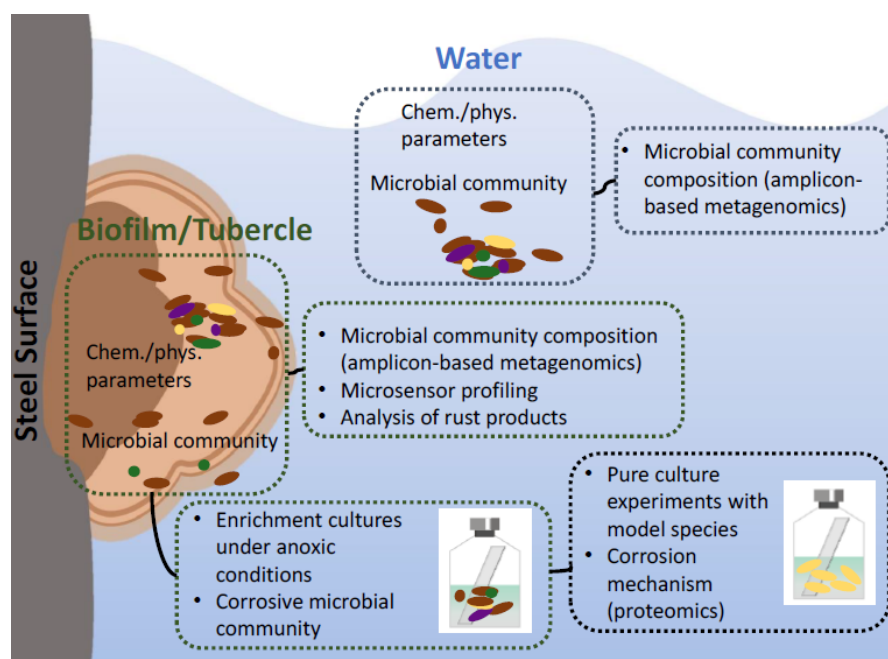


Figure 4: General sampling and downstream analysis scheme.

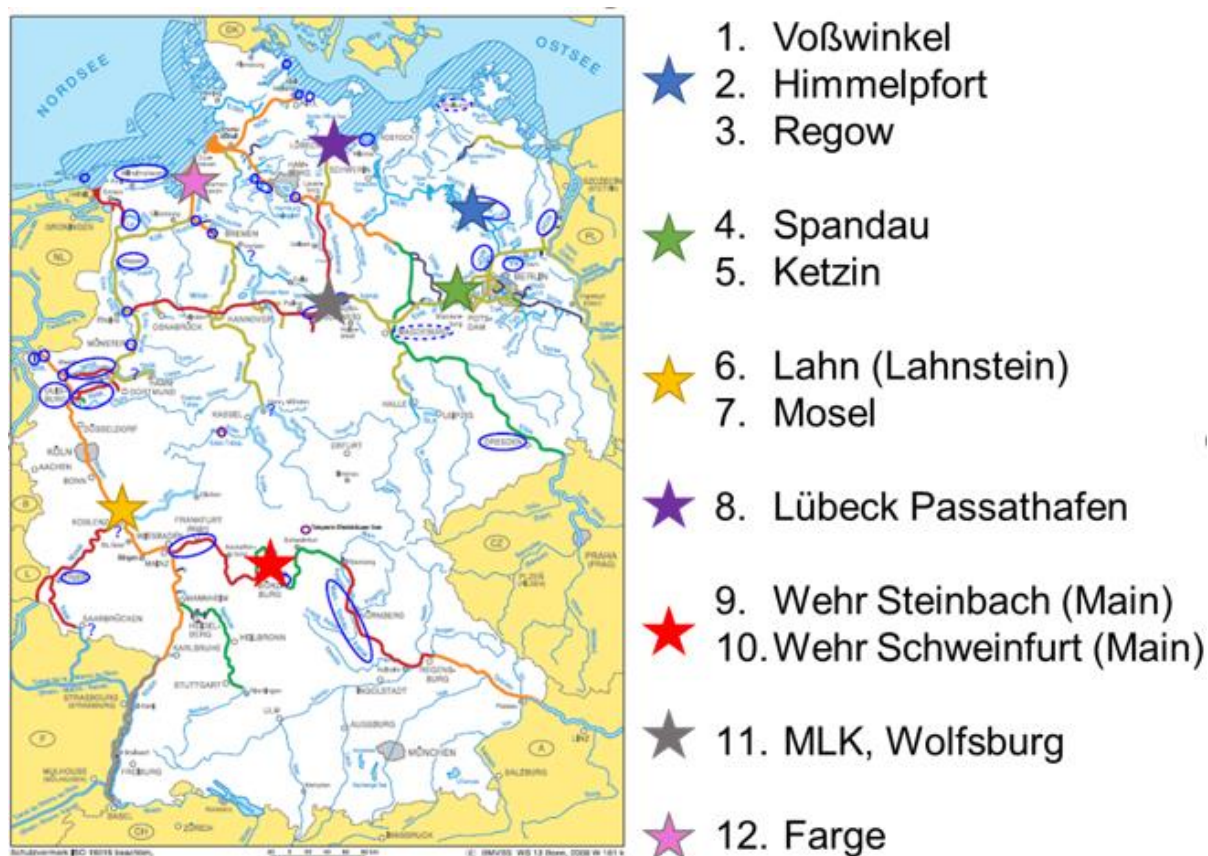


Figure 5: Map showing all sites where corrosion was reported by the water shipping administrations (blue circles) and site where we sampled at (stars), changed after a map created by G. Steil (GDWS).

One location (Figure 5, blue star), where most samples were acquired and experiments were conducted, was located within the Havel river system, Germany. This is a moderately eutrophic lowland river system containing sheet pile walls in ship locks and bank walls. In that area 3 sites were sampled. These sites were chosen because they contained corroded metal structures requiring maintenance or reconstruction. Safe access to the sites for sampling was made possible through the Waterways and Shipping Office (WSA Oder-Havel) in Eberswalde, Germany. We sampled tubercles from three locks in the Upper-Havel Waterway (Regow, RG and Himmelpfort, HO, Vosswinkel VW). Additionally, we sampled also from a bank wall of the Lower-Havel Waterway (Ketzin, Ke) and in Spandau (SP). For DNA extraction, Mössbauer spectroscopy, μ CT, and SEM, and enrichment cultures, tubercles were removed from the metal structures using either a sterile metal spatula (i.e., cleaned with 70% ethanol between sampling) or a single-use sterile plastic spatula. All samples were stored in sterile containers.

At some other sites, samples were taken and sent by the WSV employees (wehr Steinbach, Schweinfurt) and or during building inspections by BAW employees (Farge, MLK, RHK).

3.3 Steel exposure experiments in the field

Scale free steel coupons (10 mm x 30 mm x 2 mm, machining and delivery by Recknagel Präzisionsstahl GmbH), were assembled in arrays containing all timepoints for each site (Figure 6) using a nylon thread (7 mm). Prior to assembly all coupons were drilled (small hole, 2 mm in diameter, to attach the nylon thread), washed with EtOH, dried and weighted for later calculation of weight loss. Additionally, at site FBL, HO, VW and RG one plate of sheet pile steel (approximately 10 cm x 10 cm) was exposed together with the coupons. For elemental composition of each steel please see SI Table

1. The coupon arrays were either attached to sheet pile walls within locks (RG, VW, HO), at a measuring buoy (FBL) using neodymium magnets or free floating on a pond attached to a rope (with weights) for Rhein and Mosel. At sites HO, VW and RG, O₂ and temperature were recorded using MiniDots (Figure 7).

At site Lahn a YSI probe was attached to the same measuring ton, delivering O₂, pH, temperature and conductivity data, additionally bimonthly sampling of Chla and nutrients was also conducted. For Rhein and Mosel weekly samplings of water parameters and nutrients were performed.

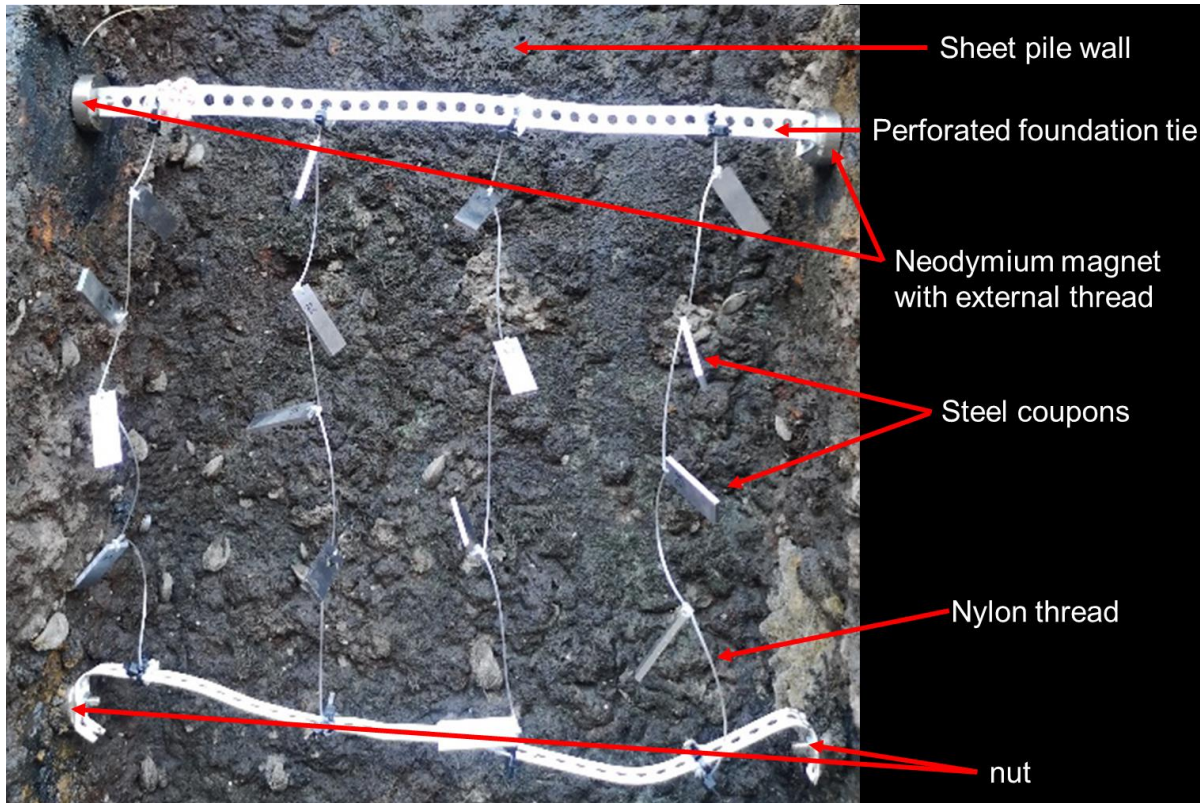


Figure 6: Example of a 4 x 4 coupon array (4 coupons each for 4 timepoints) used at sites HO, RG and VW. Arrays at site FBL contained 12 x 3 coupons (3 coupons each for 12 timepoints), and at site Mo and Rh 5 x 4 coupons (4 coupons each 5 timepoints).

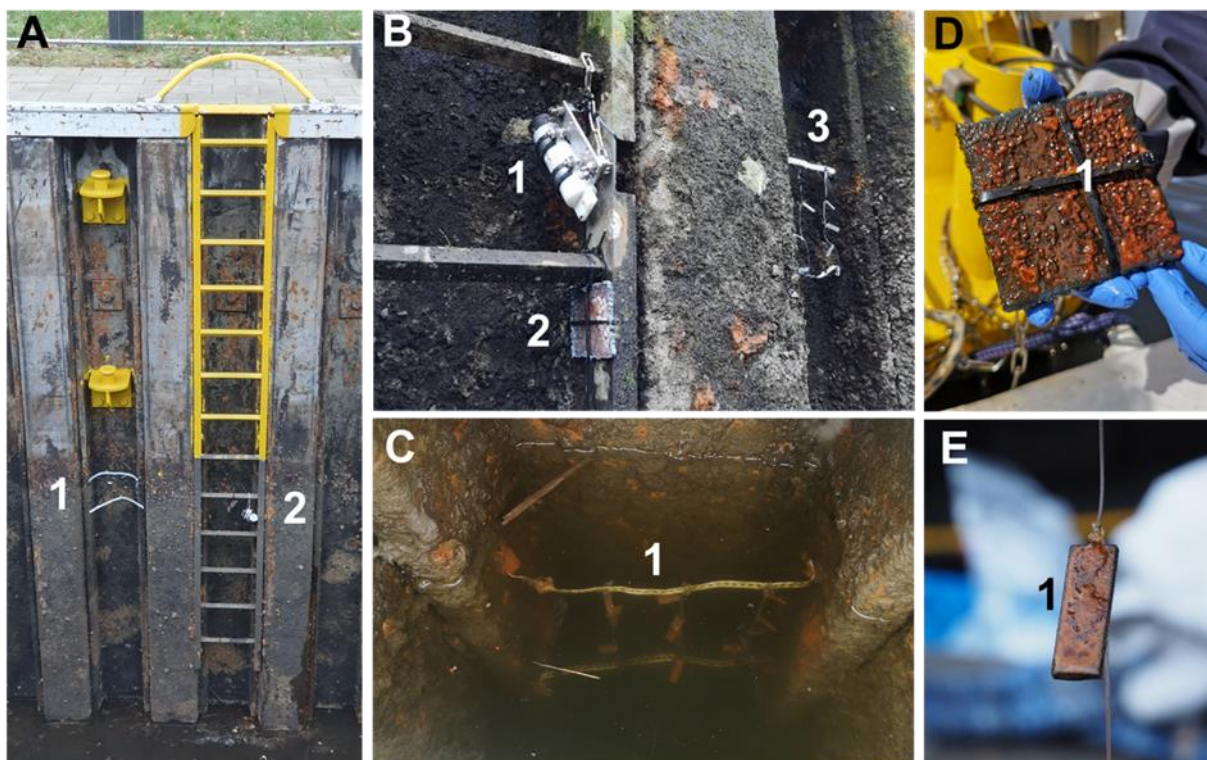


Figure 7: Impressions on the exposition experiments at the field sites. A: steel coupon array, mini Dot and steel metal plate attached to the steel pile wall and the leader, respectively, inside the lock (still drained) just below the low water level at site HO (T0). B: Mini Dot, steel metal plate and coupon array (3) at the site RG (still drained) just below the low water level (T0). C: sampling of the steel coupon array at site RG (T2). D: sampling of the steel plate at site FBL, in the background the yellow buoy can be seen where the plate and the coupons were attached to (T6). E: one steel coupon still attached to the nylon thread from site VW after retrieving from the coupon array (T1).

3.4 Corrosion studies with enrichment cultures

3.4.1 Experimental set-up

Environmental samples used as inoculum for primary enrichments came from the sites of Himmelpfort (2.), Regow (3.), MLK/Wolfsburg (11.), and Farge (12.) (Figure 5). Selected primary enrichments were then used as inoculum for corrosion studies. Primary enrichments and corrosion studies were performed under anoxic conditions with either nitrate, sulfate, and/or CO₂ as terminal electron acceptor. Elemental iron was provided as steel coupons (7.6 x 1.0 x 0.2 cm³; from Xometry for primary enrichments, from Recknagel for corrosion studies, for elemental composition of different steels used see S2) or iron beads (5 g ≈ 78 beads, Ø 2 – 2.4 mm; Metallpulver24; only for primary enrichments) (Figure 8). Molecular hydrogen served as alternative electron donor for comparison (Figure 8). Experiments were performed in 250-ml serum bottles containing 100 ml medium with the exception of the primary enrichments from Himmelpfort and Regow samples for which 120-ml serum bottles with 50 ml medium were used. The composition of the liquid medium was based on the freshwater medium by Widdel & Bak (1992) (Table 1). Solutions were filter-sterilized or autoclaved separately and stored anoxically.

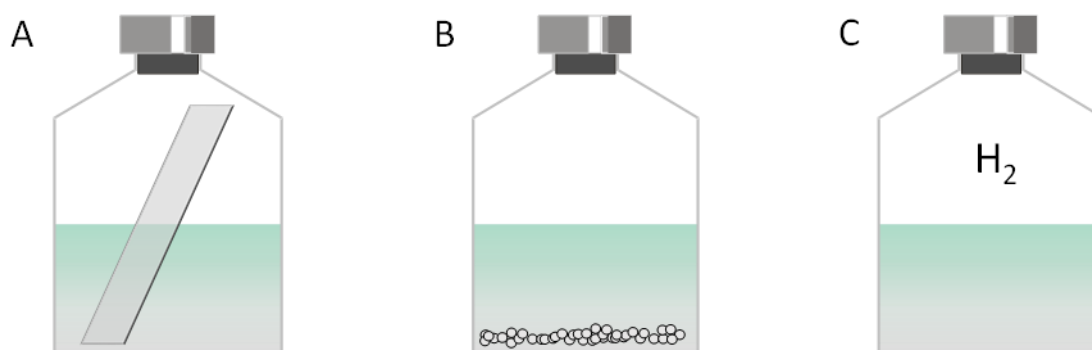


Figure 8: Experimental set-up under anoxic conditions: Serum bottles with medium and headspace using either steel coupons (A), elemental iron beads (B) or molecular hydrogen (C) as electron donor.

Table 1: Composition of the medium for primary enrichments and corrosion studies.

	MW	ml l ⁻¹	g l ⁻¹	mM
NH ₄ Cl	53.49		0.267	5
KH ₂ PO ₄	136.09		0.204	1.5
KCl	74.55		0.447	6
NaCl	58.44		0.234	4
CaCl ₂ ·2H ₂ O	147.02		0.147	1
MgCl ₂ ·6H ₂ O	203.3		0.305 (0.386) ²⁾	1.5 (1.9) ²⁾
MgSO ₄ ·7H ₂ O	246.48		0.123 (0.0246) ²⁾	0.5 (0.1) ²⁾
Vitamins ¹⁾		1		
Trace elements ¹⁾		1		
5x Se-Wo ¹⁾		0.2		
NaHCO ₃	84.01		2.52	30
NaNO ₃	84.99		1.70	20 ³⁾
Na ₂ SO ₄	142.04		2.84	20 ⁵⁾
Na-acetate	82.03		0.082	1 ⁵⁾

1) According to Widdel & Bak (1992).

2) Only in assays with CO₂ as only electron acceptor: sulfate as S-source for methanogens and acetogens.

3) Only in assays with nitrate as electron acceptor.

4) Only in assays with sulfate as electron acceptor.

5) Only in assays with acetate as organic C-source.

Iron beads were treated with acetone to remove rusty dust particles. Coupons from Xometry were treated with i) acetone to remove the oil and ii) 10% HCl for descaling. Coupons from Recknagel were treated with i) inhibitory HCl (45 g hexamethylenetetramine in 1 l 25% HCl) to remove rust and ii) 96 – 100% ethanol (after which coupons were blow-dried with N₂). For electron microscopy, small steel platelets were glued (metal glue) on polycarbonate (PC) coupons. Steel coupons, iron beads, and steel platelets mounted to PC coupons were heat sterilized together with the serum bottles at 120°C

two times for 5 h on two consecutive days. After addition of 100 ml medium, the biotic assays were inoculated with either material from environmental samples (primary enrichments) or with the material scraped off from the steel coupon mixed with culture liquid of the corresponding primary enrichment (corrosion studies). All experiments included an abiotic control, i.e. steel coupons incubated in non-inoculated medium. The bottles were sealed with butyl rubber stoppers and aluminium crimps, and the headspace was flushed with either 80% N₂/20% CO₂ (elemental iron serves as electron donor) or 80% H₂/20% CO₂ (molecular hydrogen serves as electron donor).

Medium preparation and inoculation took place within a vinyl anaerobic chamber (Coy Lab Products, Grass Lake, Michigan/USA). The bottles were placed on a shaker (150 rpm) and incubated at 20°C in the dark for four (primary enrichments) or six to nine weeks (depending on the experiment).

3.4.2 Sampling

All bottles were sampled on day 0. Primary enrichments from Farge and MLK/Wolfsburg were sampled twice a week. Primary enrichments from Himmelpfort and Regow were sampled only a second time at the end of incubation (i.e. after four weeks). For corrosion experiments, bottles were sampled once a week during the length of incubation. Sampling took place within an anaerobic chamber.

Samples were retrieved through the butyl rubber stopper using sterile syringes and needles. Samples from the headspace were analysed for H₂ and CH₄. The culture liquid was sampled for the analysis of pH, Fe compounds, N compounds, S compounds, acetate and total cell protein (liquid phase). On the first and last sampling day, additional samples for cell counts were taken. Furthermore, samples for 16S rRNA gene amplicon sequencing and metaproteome analysis were retrieved during the final sampling which also included the harvesting of the steel coupons. Before sampling, pressure compensation was performed with syringes – either empty or filled with N₂ – allowing the determination of either gas production or gas consumption. For H₂ and methane measurements, each 1 ml of the gas mixture was retrieved and injected into 3-ml (Exetainer standard cap; Labco Ltd., Lampeter, UK) or 4.9-ml vials (G4, DIN ISO 719; Klaus Ziemer GmbH, Langerwehe Germany). The sample volume was replaced by the same volume of N₂ in the headspace. For regular sampling, 4.5 – 5 ml were removed from the liquid phase and which were replaced by the same volume of corresponding medium. The measured concentrations of the different parameters were afterwards corrected for dilution or accumulation effects. Steel coupons were scraped off with a sterile high-grade steel spatula and the material was re-suspended in 2 ml mineral medium. From the measured immersion depth of the coupon the submerged area was calculated. Depending on the experiment, the material from the three replicate coupons had to be pooled.

3.5 Corrosion studies with pure cultures

3.5.1 Pre-experiment

Microcosm assays were set up with two model corrosive strains *Desulfovibrio vulgaris* (DSM 2119) and *Desulfovibrio ferrophilus* (DSM 15579) (Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) respectively (Table 1). Fe⁰ was provided as steel coupons in the size 7.6 x 1.0 x 0.2 cm³ (Gebr. Recknagel Präz. GmbH, Christes, Germany). Abiotic controls for each assay were also incubated along with the biotic assays. The biotic assays were set-up for each strain in triplicates. The abiotic assays were prepared as single assays. The respective media (80 ml per assay) were added to each assay and 5% v/v of a 4-day culture of each strain was inoculated in the biotic assays. The headspace of the H₂-biotic and -abiotic assays were flushed with 80% H₂/20% CO₂ gas mixture for 5 minutes and the outlet needle was removed first in order to have an overpressure in the bottles. The other assays were flushed in a similar manner with 80% N₂/20% CO₂ gas mixture in the headspace. The abiotic and biotic assays were incubated at 28°C

and 110 rpm for 63 days. The electron donors (lactate, H_2 and Fe^0) for the both the strains in each biotic assay were provided in excess (bold in Table 2) so that the chances for C-MIC (chemical MIC), H_2 -CMIC (H_2 -influenced CMIC), E-MIC (electrical MIC) in each of the biotic assays are maximized.

Table 2: Set-up of the microcosm assays for pre-experiment.

Assay abb.	Dsv-lac (<i>D. vulgaris</i>)	Dsv- H_2 (<i>D. vulgaris</i>)	Dsv- Fe^0 (<i>D. vulgaris</i>)	Dsf-lac (<i>D. ferrophilus</i>)	Dsf- H_2 (<i>D. ferrophilus</i>)	Dsf- Fe^0 (<i>D. ferrophilus</i>)
Electron donor	Lactate+ Fe^0	H_2 + Fe^0	Fe^0	Lactate+ Fe^0	H_2 + Fe^0	Fe^0
Medium	63.medium (DSMZ, 2017)	Widdel und Bak	Widdel und Bak	195c. medium (DSMZ, 2017)	Widdel und Bak	Widdel und Bak
C-source	Lactate & CO_2	CO_2	CO_2	Lactate & CO_2	CO_2	CO_2

Abiotic controls abb.	abio_sw	abio_ H_2	abio_ Fe^0	abio_mar	-	-
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3.5.2 Main experiment

The pre-experiment was repeated with a few modifications. Steel was not provided in the assays with lactate and H_2 (80% H_2 /20% CO_2). Fe^0 was provided as steel coupons in the size 7.6 x 1.0 x 0.2 cm³ (Gebr. Recknagel Präz. GmbH, Christes, Germany) and additional steel platelets 1.0 x 1.0 x 0.2 cm³ (Gebr. Recknagel Präz. GmbH, Christes, Germany) were also added for SEM analysis. Additionally, the H_2 and Fe^0 were spiked with 1 mM acetate as a C-source as *D. ferrophilus* strain is not a chemolithoautotrophic bacterium. *D. vulgaris* assays were incubated with freshwater media (Basal media for gram negative SRB, (Widdel and Bak, 1992)), whereas *D. ferrophilus* with saltwater media (~20 g l⁻¹ NaCl) (Basal media for gram negative SRB, (Widdel and Bak, 1992)). Abiotic controls for each assay were also incubated along with the biotic assays. All assays, were set up for each strain in triplicates. All assays, were set-up for each strain in triplicates. 100 ml of respective media were added to each assay, and for the biotic assays respective strains were inoculated (end concentration: 6 mg l⁻¹). The headspace of H_2 assays were flushed with 80% H_2 /20% CO_2 gas mixture for 5 minutes without overpressure, the outlet and inlet needle were removed at the same time, so no overpressure was maintained. Similarly, the other assays were flushed with 80% N_2 /20% CO_2 gas mixture in the headspace. The abiotic and biotic assays were incubated at 28°C and 110 rpm (optimal temperature for both strains to accelerate growth). Each assay, was incubated for different time periods till the bacterial growth curves were stabilized depending on the substrate concentrations (lactate, H_2 and sulfate) provided. This was done to ensure that the respective biomolecules were active during harvest. The lactate and hydrogen assays were incubated for 19 days and the steel assays for 34 days.

3.5.3 Sampling of pure cultures (pre and main experiments)

The first sampling took place on the same day (day 0) and the corresponding samplings were done on a weekly basis. The sampling process was the same for the pre- and main- experiment. However, the main experiment assays were incubated for differential time periods. The time period was decided during the weekly measurements, the assay was harvested when the sulfate concentrations were stabilized for the steel assays, and the substrates lactate and H₂ were below the limit of detection.

All sampling occurred in an anaerobic chamber (MEGA E-Line, M-Line, GS Glovebox Systemtechnik Malsch, Germany). Gas samples were collected for the measurement of H₂ concentration and liquid samples for pH, free sulfide, Fe (II) compounds, sulfate, qPCR (ddPCR for main experiment) and protein analyses. Furthermore, samples for metaproteomics were harvested on the final sampling day along with biofilm harvesting from the steel coupons (pre-experiment: replicates were pooled). Before sampling, the headspace pressure was brought to normal by compensation with Ar gas. For H₂ measurements 1 ml of the gas phase was aspirated and injected into 3-ml vials (Exetainer standard cap; Labco Ltd., Lampeter, UK) and for the main experiment 4-ml vials (Bottles G4, DIN ISO 719; Klaus Ziemer, Langerwehe, Germany) were used instead. The dsv-H₂ assay (pre-experiment), was flushed with H₂ at 4 intervals in the course of the incubation (3.5.1), the time stamps for H₂ refill was determined only when an under pressure was detected in the headspace during sampling. Additionally, 1ml of liquid was sampled in a separate vial for redox potential determination. Followed by, liquid sampling for the other analyses was done by aspirating 5ml of liquid phase and then replacing the same volume by the corresponding media. The measured concentrations of the different parameters were corrected for successive dilution or accumulative changes. Steel coupons were scraped off with a sterile high-grade steel spatula and the material was resuspended in ~2.5 ml mineral medium. The scraped steel coupons were weighed for weight loss.

3.6 Corrosion rates/weight loss

Corrosion rates were calculated based on the formula of An et al. (2021). First, rust products were scraped off for downstream analysis of DNA and mineral phases. Then coupons were placed in plastic petri dishes and cleaned using inhibited HCl (40 - 50 g/l hexamethylenetetramine in diluted HCl which is two parts HCl (3 %) and one-part water) for ca. 5 min. Afterwards coupons were cleaned and wiped using ethanol (96%) and dried. Clean and dry coupons were weighted. Corrosion rates were calculated using delta W (weight) (An et al., 2021) in g (coupons were pre-weighted before the start of the experiment). Averages and standard deviations were calculated from three coupons per timepoint and location (except for Lahn where only two coupons per timepoint could be analysed). Pictures were taken during preparation for SEM analysis (fourth coupon) and also after cleaning with inhibited HCl (not shown).

For enrichment cultures the amount of steel mass loss was determined for enrichment cultures by weighing the coupons before and after incubation subsequently to treatment with inhibitory HCl, rinsing in 96 – 100% ethanol and blow-dried with N₂. For pure cultures, coupons were rinsed in Milli-Q water and dried in the anaerobic chamber.

3.7 Microsensor measurements

3.7.1 Profiling

Measurements were performed using glass microelectrodes (500 µm tip size) attached to a micromanipulator and a field microsensor system (Unisense, Aarhus Denmark). The whole setup was mounted on a magnetic plate which was fixed by strong magnets and was perpendicular to the vertical sheet piles. This allowed the sensors to move horizontally to measure within different depths of the tubercle

(see also SI Figure 1 and SI Text S1 in Fiskal et al. (2023)). Sensors for O₂, E_h, pH, H₂ and H₂S were used, and calibrated in the field according to the instruction manual. Briefly, the O₂ sensor was calibrated with Na-L-ascorbate dissolved in 0.1 M NaOH (zero read) and with saturated river water (100% read). The H₂S sensor was calibrated using the Unisense calibration kit. The pH sensor was calibrated using pH 3.00, 4.01, 7.00, and 10.00 calibration buffers (WTW, Xylem Analytics Germany GmbH, Weilheim Germany). The H₂ sensor was calibrated using a serum flask containing a gas mixture of 80% H₂ and 20% CO₂. The sensor for redox potential was calibrated using a redox buffer at 124 mV. All calibration solutions were equilibrated to the ambient air temperature (RG) or water temperature (Ke). Profiling was performed starting from the outer surface of the tubercle and penetrating into the tubercle at 500 µm increments, at each depth triplicate measurements were performed. While in RG tubercles, sensors were not lowered all the way to the steel surface to prevent sensor damage, all sensors reached the steel surface in Ke tubercles (except for O₂ sensors, where the measurement was stopped after reaching zero concentrations). Detection dwell times were 20 s per measurement and depth correction was performed by setting the tubercle surface to zero. All measurements were plotted using Grapher (Golden Software, USA).

3.7.2 H₂ and redox potential measurements for culture studies

Measurements of H₂ in the gas phase were performed by Unisense NP sensor (H₂-X-NP-103803, Unisense, Aarhus, Denmark). The anode of the hydrogen sensor was initially polarized to +100 mV against the cathode by connecting it to the Unisense field multimeter. Two-point calibration was performed at 0 µmol l⁻¹ (distilled water) and with a saturation concentration of 644 µmol l⁻¹ of H₂. The saturation concentration was achieved by bubbling the water with 80% H₂/20% CO₂ mixture. Calibration values were then adjusted to standards consisting of 1 ml 80% H₂/20% CO₂ mixture injected into 3-ml vials containing N₂. All measurements were recorded by the Unisense trace suite software (v 3.3). The pure culture studies (main experiment) were performed by gas calibration. Two-point calibration was performed at 0% sat (ambient air) and 5% sat using 5% H₂/ 95% N₂ gas mixture, which was injected into a 3-ml exetainer. All values were recorded by the Unisense trace suite software (v 3.3). For pure cultures the redox potential (mV) was measured using a redox microsensor (RD-N-009205, Unisense Aarhus, Denmark) against reference electrode (SHE, Ref-N-009208, Unisense Aarhus, Denmark). One-point calibration was performed with 124 mV standard redox solution (Sigma Aldrich, Steinheim, Germany).

3.8 Biophysicochemical analysis

3.8.1 Methane

Methane was measured with a gas analyser by tunable diode laser absorption spectroscopy applying Off-Axis Integrated Cavity Output Spectroscopy (OA-ICOS) technology (LGR-ICOS™ GLA131 MGGA, ABB Inc., Quebec, Canada). A syringe was drawn up to 3 ml with room air and injected into the vial containing the gas sample. After mixing, 3 ml gas were again drawn up with the syringe and injected into the gas analyser equipped with a 3-ml sample loop. For calibration, standards of 0.34, 0.85, 2.50, and 4.10% CH₄ were prepared from aliquots of 100% methane gas injected into N₂-filled 120-ml serum bottles. From these, 1 ml was withdrawn and injected into 3-ml vials in order to have the same procedure as for the gas samples.

3.8.2 pH

Measurements of pH were performed directly after sampling with i) WTW-IDS pH-electrode SenTix 940-3 and a WTW-Multi 3430 meter (Xylem Analytics Germany Sales GmbH & Co. KG, Weilheim,

Germany) for enrichment cultures and ii) pH-meter and pH-electrode HA405-DPA-SC-S8/225 from Mettler-Toledo AG (Schwerzenbach, Switzerland) for pure cultures.

3.8.3 Fe(II)/Fe(III)

HCl-soluble Fe(II) and hydroxylamine-reducible Fe(III) were determined photometrically on fresh samples according to Lovley and Philipps (1987) using the ferrozine method (Stookey, 1970). For the solid-only fraction [Fe(II)s], 250 µl liquid sample were centrifuged at 17,500 x g for 5 min and the pellet was extracted with 500 µl 1 M HCl. Additionally, for the pure cultures, dissolved ferrous ion [Fe (II)_{aq}] was estimated from the supernatant, obtained from [Fe (II)s] estimation, approximately 230 µl of culture supernatant was fixed with 230 µl of 0.5M HCl and determined by the Ferrozine method (Stookey, 1970).

3.8.4 Sulfide

Free sulfide was determined on fresh samples using the photometric method of Cline (1969).

3.8.5 Sulfate, acetate, lactate and nitrogen compounds

Nitrogen compounds, sulfate, lactate and acetate were measured in the supernatant (centrifugation at 17,500 x g and 10°C for 10 min; stored at -20°C). Concentrations of nitrate, nitrite, and ammonium were determined by continuous flow analysis using a Bran+Lueb Autoanalyser-3-digital-colorimeter (Seal Analytical, Norderstedt, Germany) and standard protocols. Sulfate was determined by ion chromatography using an IC 690 (Metrohm, Herisau, Switzerland) equipped with a PRP-X100 column (Hamilton, Bonaduz, Switzerland) and 2 mM Na-benzoate as eluent. Acetate was measured using MAK086 Acetate Colorimetric Assay Kit (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and a 2300 EnSpire Multimode plate reader (PerkinElmer, Inc., Waltham, Massachusetts, USA). For pure cultures, acetate, lactate and sulfate ion concentrations of sample supernatants were measured by ion chromatography with a Metrosep A Supp 19 - 250/4.0 column (Metrohm, Herisau, Switzerland).

3.8.6 Total cell protein

Whole cell protein was determined for pellets obtained from 2 ml liquid sample after centrifugation (17,500 x g, 10°C, 10 min; stored at -20°C). Pellets were resuspended in 200 µl 0.5 M NaOH and lysed at 100°C for 5 min. Protein concentrations were measured photometrically using the Bio-Rad Protein Assay based on the Bradford method (Bio-Rad Laboratories, Inc., Herkules, California/USA) and a BSA standard (Uptima Interchim, Montluçon Cedex, France).

3.8.7 Cell counts

Samples for cell counting were fixed with formaldehyde (end concentration: 3.7%) and stored anoxically. In order to dissolve minerals and disrupt cell-mineral aggregates, aliquots (25 – 250 µl) of cell suspensions were treated with i) 0.5 M HCl, ii) 4% NaCl, and iii) 1 M HCl together with ultrasonication prior to filtering on black Isopore PC membrane filters of 0.2-µm pore size (Merck Millipore Ltd., Carrigtwohill, Ireland) and staining with 1:250 diluted nucleic acid stain SYBR Green I (Invitrogen by Thermo Fisher Scientific, Waltham, Massachusetts, USA). Cells were counted from approximately 30 images recorded per filter using an AxioImager.M2 epifluorescence microscope (Carl Zeiss Microscopy, Jena, Germany) with a 38HE filter set and camera.

3.9 Metal analysis (OES)

The chemical composition of the used steel sheet samples (delivered by Recknagel Präzisionsstahl) and one piece of a steel sheet pile (approx. 100x100x10 mm) was determined with a mobile spectrometer (PMI Master Pro, Oxford Instruments) by optical emission spectrometry (OES). This method allows to analyse the content of up to 22 elements usually found in steels in one measurement in a short time and with a high accuracy. At each sample three measurements were made. Before the measurement the surface was slightly grinded with coarse SiC abrasive paper and cleaned with acetone. The determined elemental composition of the sheet samples delivered by Recknagel lay within the specification of the ordered unalloyed S355J2 structural steel (material number 1.0577, see S2). The analysed piece of an (older) steel sheet pile showed a slightly different composition. Worth to be mentioned is here the relatively high copper content of 0.415 M.% (Recknagel sample 0.013 M.%), higher Sn content 0.025 vs. 0.004 M.%, higher content in chromium 0.117 M.% vs. 0.034 M.% and nickel 0.160 M.% vs. 0.017 M.% and the low Al content of 0.002 M.% vs. 0.022 M.%. The latter indicate that this steel is only semi-killed (partly deoxidized) in contrast to actual structural steels.

3.10 ATR-FTIR spectroscopy

Examination of corrosion products regarding mineral phases which could be associated with MIC was done by using Attenuated Total Reflection (ATR) Fourier Transform Infrared (FTIR) spectroscopy. The FT-IR spectrometer used was a Spectrum Two™ by Perkin Elmer. Prior the investigation the dried samples of corrosion products were pestled to obtain homogenized fine powders for analysis. The spectra were acquired in the spectral range between 4000 cm^{-1} – 600 cm^{-1} . The ATR-FTIR system was operated and the acquired spectra were analysed using the software provided by the manufacturer of the spectrometer. Spectra were also compared to references spectra (in house database) of probable minerals to identify occurring minerals in the corrosion products.

3.11 Elemental analysis

Accumulation of sulphur containing compounds such as metal sulfides, sulfates and elemental Sulphur are often used as one indicator during the evaluation of MIC. Therefore, solid material samples of rust tubercles, surface films, backfill material were analysed by a carbon/sulphur (C/S) elemental analyser (G4 ICARUS Series 2, Bruker) to determine the total amount of carbon and sulphur, respectively. During these measurements a distinct amount of material is combusted in a ceramic crucible in a high frequency induction furnace (temperature up to 1800°C) in a stream of oxygen (grade 5.0). The formed exhaust gases are analysed with a CO_2 and a SO_2 detector. From these values the total amount of carbon and sulphur could be calculated by the analyser. Prior to the measurements the samples were dried and powdered. For each single measurement approx. 100 mg of material were weighed out, depending on the amount of available material of the specific sample. For each sample it was envisaged to perform a three-fold determination, i.e. three times measuring each sample (3 x 100 mg).

3.12 Mössbauer spectroscopy

Mössbauer spectroscopy was used to determine the composition of Fe minerals from one RG and one HO tubercle. The RG tubercle was split roughly into three sections (outer, middle, and inner) to determine the spatial heterogeneity of the mineralogy whereas the HO tubercle was analysed as a bulk sample. All samples were prepared anoxically to preserve the redox speciation and mineralogy. Tubercles from both sites were placed into containers and immediately frozen in liquid N_2 . These containers

were opened inside a glovebox (Mbraun Unilab Workstation, 100% N₂ atmosphere, <20 ppm O₂) and samples were loaded into Plexiglas® holders (area 1 cm²) to form a disc. These samples were kept in airtight jars at -20°C until analysis. Sample holders were inserted into a closed-cycle exchange gas cryostat under a backflow of He to minimize exposure to air. Spectra were collected at 77 K and in some cases 5 K using a constant acceleration drive system (WissEL, Munich, Germany) in transmission mode with a ⁵⁷Co/Rh source. All spectra were calibrated against a 7 µm thick α-⁵⁷Fe foil that was measured at room temperature. Analyses were carried out using Recoil (Lagarec and Rancourt, 1998) and the Voigt Based Fitting (VBF) routine (Rancourt and Ping, 1991). The half width at half maximum (HWHM) was constrained to 0.123 mm s⁻¹ during fitting.

3.13 Scanning electron microscopy

The tubercle, previously analysed by µCT, was split along preferential plains of fracture using a sterile razor blade to receive distinct cross-sections for SEM analysis. By this means, we broadly identified 'inner', 'middle', and 'outer' section from the tubercle and were subsampled. These subsamples were then attached to aluminum stubs using either conductive carbon adhesive pads (G3347, Plano GmbH, Wetzlar, Germany) or Tempfix adhesive (G3305, Plano GmbH, Wetzlar, Germany). The samples were coated with a 10 nm thick deposition of platinum to reduce charging effects during analysis. The structure of the tubercle sections was characterized using a Zeiss Crossbeam 550L (Carl Zeiss Microscopy GmbH, Oberkochen, Germany) focused ion beam-scanning electron microscope (FIB-SEM). Images were taken in secondary electron mode using an acceleration voltage of 2 keV.

For pure culture and enrichment cultures the steel coupons for scanning electron microscopy (SEM) were sampled and one of the triplicates were considered for SEM. The coupons were transferred to Karnovsky fixans. The coupons were stored at 4°C overnight. The next day they were reintroduced into the anaerobic chamber, washed three times in 0.1 M cacodylate and through an ethanol series (20, 30, 50, 70, 90, and 2x 100% ethanol). Outside the anaerobic chamber, under an extraction hood, they were dipped through an ethanol:HMDS series (2:1, 2:2, 1:2 and 2x 100% HMDS), blown dry with N₂ and finally dry stored under N₂ atmosphere until microscopy. The coupons were mounted on stubs with double sided adhesive pads and coated with a thin layer (10 nm) of Au/Pt. The coupons were subjected to SEM imaging and Energy dispersive X-ray spectroscopy (Max-Planck-Institute für Neurobiologie des Verhaltens- Caesar, Bonn).

3.14 3D imaging via micro-computed tomography

A representative HO tubercle was selected for visualization of the morphology and its biological and mineral components by µCT and SEM. Upon sampling, the tubercle was placed into a sterile container with river water to keep the sample completely hydrated. To fix any bacteria occurring on or within the tubercle, 25% electron microscopy-grade glutaraldehyde was added to a final concentration of 2.5%. This fixed tubercle was stored at 4°C for at least 24 h. Dehydration of the sample was started by removing the fluid (river water and glutaraldehyde mixture) and replacing it by 25%_(aq) ethanol and left to incubate for 15 min. This dehydration process was continued in the same way by incubation the sample in 50%_(aq), 75%_(aq), and 100% ethanol (3x). After the final ethanol dehydration step, the tubercle was submersed for 15 min into a glass flask containing a mixture of 100% ethanol and hexamethyldisilazane (HMDS). The tubercle was then transferred to a container containing ~2 ml of fresh HMDS. The lid of the container was left partially open to allow the HMDS to gradually evaporate and the samples to air dry overnight. For imaging, the tubercle was attached to an aluminum SEM sample holder by plastic conductive carbon cement (Leit-C-Plast, G3302, Plano GmbH, Wetzlar, Germany) and was sputter coated with a 10 nm thick deposition of platinum. The latter was performed as surface information of the tubercle was first imaged by SEM (see also below), before performing the µCT imaging.

For the μ CT scan the sample was placed on a holder and scans were acquired using a Nikon XTH 320 μ CT scanner (Nikon Metrology, Leuven, Belgium) operated by the Visualization, Digitalization and Replication joint-lab at the Eberhard Karls University of Tübingen and Senckenberg Center for Human Evolution and Palaeoenvironment, Germany. An X-ray tube with a multi-metal reflection target with a maximum acceleration voltage of 225 kV was used and the sample was scanned with 200 kV and 35 μ A. A total of 4476 projections were acquired at 9.58532 μ m isometric voxel size, holding four frames per projection. During the scan a copper filter of 0.1 mm was used. Visualizations were created using AMIRA software (ThermoFisher Scientific, Waltham, MA, USA).

3.15 DNA analysis

3.15.1 DNA extraction and quantification

Tubercles for DNA extraction were immediately placed in sterile and sealable plastic contains and frozen in liquid nitrogen. Some tubercles were cleaved into outer, middle, and inner sections similarly to the previous analyses. However, tubercles were still frozen when subsamples were taken therefore separation was achieved by scraping off different sections from frozen tubercles using sterile spatulas. DNA was extracted from tubercles using the Fast DNA Spin Kit for Soil and FastPrep (MP Biomedicals, Irvine, CA USA). DNA was extracted from ~ 0.5 g tubercle material following the manufacturer's protocols with additional steps to optimize the yield and quality of the extracted DNA (for a detailed description see also SI Text 2 in Fiskal et al. (2023)).

For pure cultures DNA was extracted from 1 ml of sample by using the “DNeasy Blood and Tissue Kit” (Qiagen, Hilden, Germany). DNA was eluted in 100 μ l of DNase free buffer. For enrichment cultures DNA was extracted from pellets obtained by centrifuging 30 ml of culture liquid or from 2 ml biofilm suspension per steel coupon using “DNeasy Power Soil Pro Kit” (Qiagen, Hilden, Germany). For samples with low biomass, samples were also extracted using Fast DNA Spin Kit for Soil (see above).

3.15.2 qPCR

For pure cultures quantification of the *dsrAB* was done with the following set of primers, DSR1-F+ (5'-ACS CAC TGG AAG CAC GGC GG-3') and DSR-R (5'-GTG GMR CCG TGC AKR TTG G-3') (Biomers, Ulm, Germany). The absolute abundance of bacterial *dsrAB* genes were determined for all samples using both qPCR and ddPCR techniques. In the pre-experiment the *dsrAB* gene copies are depicted based on qPCR methods. However, a dd-PCR protocol was optimized using the DNA of the pre-experiment, because the need to prepare additional calibration standards was eliminated and the quantification of the gene is less laborious. The protocol was according to Leloup (2007). The reaction mix ($V_f = 25$ μ l) was composed of 12.5 μ l of MasterMix (SyBrGreen 2X, Eurogentec, Seraing, Belgium), 1 μ l of bovine Serum Albumin (Luyssaert et al.) (3 mg ml⁻¹), 1 μ l of MgCl₂ (50 mM), 1.25 μ l of each primer (1000 nM) and 2 μ l of template DNA. Quantitative PCR was carried out in Stratgene MX3005P (Agilent technologies, Santa Clara, USA) thermal cycler with temperature profile as follows, 2 min at 50°C, 10 min at 94°C for initial denaturation, 40 cycles of 15 s at 94°C, 1 min at 59°C, 45 s at 72°C. Each run included a non-template control (nuclease free water) and standard dilution series of *Desulfovibrio vulgaris*, DNA (10⁷-10² copies) (DNA extracted from pure culture stock). The unknown sample copies were estimated against the standard curve (C_i value vs copies) of the standard dilution series.

3.15.3 ddPCR

Absolute abundance of bacterial 16S rRNA genes were determined for all samples from which DNA was extracted by droplet digital PCR (ddPCR) using Bac908F_mod (5'-AACTCAAAGGAATTGAC-GGG-3')/Bac1075R (5'-CACGAGCTGACGACARCC-3') primers (biomers, Ulm, Germany). PCR

reactions were performed in 22 µl total volume (10 µl QX 200 ddPCR EvaGreen®Smx (Bio-Rad, Hercules, CA, USA) 8 µl nuclease-free water, 1 µl of forward and reverse primers (250 nM), and 2 µl of DNA template). The DNA template was diluted 1:100, 1:1000, 1:10,000, and 1:100,000 for 16S rRNA analysis. 20 µl of the reaction mix were used to generate droplets (Bio-Rad QX200™ Droplet Generator, Bio-Rad, Hercules, CA, USA). The resulting emulsion (40 µl) was transferred to a 96-well plate and sealed. PCR was run in a Bio-Rad C1000-Touch™ Thermal Cycler. The amplification conditions were as follows: 5 min at 95°C; 40 cycles of 10 s at 95°C, 30 s at 55°C, and 15 s at 72°C (ramp rate 2°C s⁻¹). Enzymes were deactivated by a 5 min hold at 4°C, followed by a 5 min hold at 90°C. Each run included a non-template control (nuclease free water) and a positive control (digested gDNA of *Shewanella baltica* DSMZ 9439). Droplets were read on a Bio-Rad QX200 Droplet Reader. Only samples with at least 11,000 accepted droplets were used for further analysis. The positive droplet threshold was set manually using the QuantaSoft™ Analysis Pro software (Bio-Rad, Hercules, CA). Gene abundance (copies per µl) was corrected for initial dilution of DNA template and dilution of the reaction volume during droplet generation. Gene copies per gram sample (tubercle material, wet weight) were calculated.

3.15.4 16S rRNA and 18S rRNA amplicon sequencing

16S rRNA amplicon sequencing was performed on all samples whereas 18S rRNA amplicon sequencing was only performed on the samples which were separated into different sections (outer, middle, inner). Sequencing was performed by Novogene Co. (Ltd, Beijing, China). DNA extracts were sent on dry ice to Novogene (UK) Company Limited (Cambridge, UK) where their standard workflow was performed (SI Text 3). 16S rRNA amplicons were generated using 16S rRNA gene (V3-V4) specific primers (341F: 5'-CCTAYGGGRBGCASCAG-3' and 806R: 5'-GGACTACNNGGGTATCTAAT-3') and 18S rRNA amplicons using 18S rRNA gene (V4) specific primers (528F: 5'-GCGG-TAATTCCAGCTCCAA-3' and 706R: 5'-AATCCRAGAATTTCACTCT-3'). The library was sequenced on an Illumina platform (NovaSeq 6000 System, Illumina, San Diego, CA, USA) and 250 bp paired-end reads were generated.

For the enrichment samples DNA extracts were sent on dry ice to Novogene (UK) Company Limited (Cambridge, UK) where their standard workflow was performed (see Fiskal et al. (2023)Text S3). To further explore 16S rRNA amplicon sequencing data (only for samples which were split into different sections), FAPROTAX (Functional Annotation of Prokaryotic Taxa, version 1.2.3. [Louca et al., 2016]) was used. This data base offers an option to automatically assign microbial processes/functional groups to the taxonomic information using the current literature on cultured strains. The output table was created using the provided collapse_table.py script on the OTU information (.biom files) through Python. The output table was shortened based on abundance of the assigned process and general classes of processes such as chemoheterotrophy, cellolysis and ureolysis were not considered. Bubble plots were created using R.

For field study 4.1 and 4.3. the raw data was processed using the nf-core/ampliseq Pipeline (version 2.3.2, Straub et al. (2020)). This pipeline utilizes the workflow tool Nextflow and combines several steps of data processing: Primers are trimmed using cutadapt (version 3.4, Martin (2011)), then DADA2 (version 1.22.0, Callahan et al. (2016)) is used to filter out sequences with low quality and infer Amplicon Sequence Variants (ASVs); for taxonomic classification the QIIME2 classifier (version 2021.8.0, Bokulich et al. (2018)) using SILVA database (version 138.1 Quast et al. (2013)) is run. The pipeline output was imported into (version 4.2.2, R Core Team (2022)) using the phyloseq package (version 1.42.0, McMurdie and Holmes (2013)).

Principal Coordinate Analysis (PCoA) and Constrained Analysis of Principal Components (CAP) was performed after aggregating ASVs on Genus level using the package microViz (version 0.11, Barnett et al. (2021)). The package microViz was also used to create heatmaps, correlation plots, and treeplots. Statistical differences between sample types were tested using linear models on centered log ratio transformed data.

To explore potential functional differences of microbial communities based on 16S rRNA amplicon sequencing data, FAPROTAX (Functional Annotation of Prokaryotic Taxa, version 1.2.3. (Louca et al., 2016)) was used. This data base offers an option to automatically assign microbial processes/functional groups to the taxonomic information using the current literature on cultured strains. The output table was created using the provided `collapse_table.py` script on the ASV information (.biom files) using Python. The output table was shortened based on abundance of the assigned process and general classes of processes such as chemoheterotrophy, cellolysis and ureolysis were not considered. Bubble plots were created using R.

All data processing steps demanding high compute power were run on a virtual machine on the BMBF-funded de.NBI Cloud within the German Network for Bioinformatics Infrastructure (de.NBI).

3.16 Proteome analysis via shotgun proteomics

3.16.1 Protein extraction of cell lysates

3.16.1.1 Pre-experiment

The cell lysate was prepared according to (Castronovo, 2023). Based on the whole cell protein concentrations from the final day of sampling the appropriate culture volumes was estimated (required protein range is 0.025-10 µg). Subsequently, 1ml of liquid culture was used for the dsf-lac assay and 45 ml for the dsf-H₂, dsf-Fe⁰ assays and abiotic controls. Additionally, the complete biofilm-suspensions were also extracted. The dsf assays were not extracted since the biomass for dsf-H₂ and dsf-Fe⁰ was not sufficient for proteomics. The given volumes of cultures were thawed and centrifuged at 4863 x g and 10°C for 10 min. The supernatant was discarded and the cell pellet was resuspended in 10 ml of Tris buffer A (50 mM TRIS, 50 mM NaCl, adjusted to a pH of 8.0 with 3 M HCl). This washing procedure was repeated a second time to get rid of adhering remains of the culture medium. Subsequently, the washed cell pellets were resuspended in 1.2 ml of buffer A and transferred to lysing matrix E tubes (2 ml, MP Biomedicals, Eschwege, Germany) for cell lysis via bead-beating. The tubes were pulsed four times for 20 seconds at an intensity of 6.0 m/s with a FastPrep-24™ 5G (MP Biomedicals, Eschwege, Germany), with intermittent cooling of the tubes on ice for 5 minutes between each round. In order to remove the cell debris and the remaining intact cells, the samples were then centrifuged for 15 minutes at 4863 RCF and 20°C (Hettich Rotina 380 R, Tuttlingen, Germany) and sterile-filtered (Acrodisc® syringe filters, 0.2 µm, PALL).

3.16.1.2 Enrichment cultures

The cell lysate preparation was done according to (Starke, 2017). 60 ml of liquid samples from the for each replicate was centrifuged (4,200 x g and 10°C for 20 min). The cell pellets of all samples were resuspended in 1.25% SDS Buffer and incubated for 1 hour at room temperature on a shaker (250 rpm). The cell resuspension was transferred to Lysing Matrix tubes E and subjected to bead beating at 20 s, 6.0 m/s for 4 cycles. The tubes were incubated on ice for 5 minutes between the cycles. Thereafter the cell sludge was ultrasonicated (Transonic Digital, Elma, Germany) for 3 minutes, power 8 for 2 cycles. The tubes were incubated on ice for 1-minute in between the cycles. The cell suspension was then centrifuged (18,000 x g and 4°C for 15 min) to separate the lysate phase from the solid material. The supernatant was filtered using a 1ml syringe with sterile 0,2-µm filter into fresh protein low binding 1.5 ml tubes. The extracted lysates are further stored at -20°C.

3.16.2 Measurement of protein concentrations and digestion of proteins

3.16.2.1 Pre-experiment (in-solution digestion method)

Protein concentrations of lysates were measured via bicinchoninic acid (BCA) method, using the Pierce BCA Protein-Assay kit (Thermo Fisher Scientific, Schwerte, Germany) according to the manufacturer's instructions and the concentrations were determined photometrically with the plate reader (TECAN Infinite M200 PRO Tecan, Männedorf, Switzerland). Pierce™ In-Solution Tryptic Digestion Kit (Thermo Fisher Scientific, Illkirch, France) was used to digest the proteins into peptides according to the manufacturer's instructions, except the guanidination step. The proteins were denatured and disulfide bonds were subjected to reduction by adding dithiothreitol (end concentration: 5.5 mM) and incubation at 95°C, followed by alkylation (end concentration: 10 mM) with iodoacetamide and, lastly digested with trypsin (trypsin ~0.2 µg).

3.16.2.2 Enrichment cultures

30 µl each of sample lysate were subjected to reduction with dithiothreitol (end concentration: 5 mM) at 60°C and alkylation with iodoacetamide (end concentration: 20 mM) at 37°C. The derivatized proteins were further immobilized on to SP3 magnetic beads (Cytiva Sera-mag SpeedBeads Carboxyl-Magnet-Beads- hydrophilic, Cytiva 45152105050250; -hydrophobic, Cytiva 65152105050250, Fischer scientific, Illkirch, France). The protocol for SP3 treatment was adapted from (Mikulášek, 2021). The purified proteins were then digested with sequencing grade modified Trypsin (Promega Corporation, Walldorf, Germany) at a concentration of one part of trypsin to 50 parts protein. On the next day, the digests are eluted into fresh tubes. The digests were vacuum dried (RVC 2-18 CDplus, Martin Christ, Germany) and stored in -80°C.

3.16.3 Shotgun proteomics measurements via UHPLC-HRMS/MS

3.16.3.1 Pre-experiment

An Ultimate 3000 RSLCnano UHPLC System (Thermo Fischer Scientific) was coupled to an Orbitrap Q Exactive HF HRMS (Thermo Fischer Scientific), using an EASY-Spray ion source for electrospray ionization in positive ionization mode. Peptide lysates were injected at 5 µl/min. For pre-concentration and online clean-up, a cartridge-based trap column was implemented (PepMap100, 300 µm x 5 mm, C18, 5 µm, 100 Å) before the chromatographic separation with a PepMap RSLC column (75 µm x 50 cm, C18, 2 µm, 100 Å; all instruments and columns from Thermo Fisher Scientific). A gradient of ultrapure water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B) was run with a flow of 0.3 µL/minutes for 100 minutes of chromatographic separation runtime, followed by 20 minutes column re-equilibration. Column temperature was set to 40°C. Full-scans (MS1) were acquired at a resolution of 120,000 with a range of 300 to 1550 m/z, an AGC (Automatic Gain Control) target value of 3×10⁶ and a maximum injection time of 50 ms. MS 2 spectra were acquired at a resolution of 15,000 in data-dependent acquisition mode, with the top 20 precursors chosen for fragmentation, an AGC target of 1×10⁵ and a maximum injection time of 65 ms.

3.16.3.2 Enrichment cultures

The lyophilised peptides were rehydrated with 0.1% LC/MS grade formic acid. Nanoliquid chromatography mass spectrometry (UltiMate 3000 RSLCnano, Dionex, Thermo Fisher Scientific) and Exactive HF mass spectrometer (Thermo Fisher Scientific) coupled with a TriVersa NanoMate (Advion, Ltd., Harlow, UK) was used for the LC/MS analyses of the digested peptides. The peptide lysates were injected at 5 µl/min flow rate onto a trapping column (Acclaim PepMap 100 C18, 3 µm, nanoViper, 75 µm x 2 cm, Thermo Fisher Scientific) by using 98% water/2% ACN 0.5% trifluoroacetic acid and separated on an analytical column (Acclaim PepMap 100 C18, 3 µm, nanoViper, 75 µm x 25 cm, Thermo Fisher Scientific) with a flow rate of 300 nL/min. Mobile phase was 0.1% formic acid in water (solvent

A) and 80% ACN / 0.1% formic acid in water (solvent B). Full MS spectra (350–1,550 m/z) were obtained in the Orbitrap at a resolution of 120,000 with automatic gain control (AGC) target value of 3×10^6 ions. The generated peptide ions were subjected to data dependent acquisition of N=TOP 20, where N represents the most intense ion signals to be selected for fragmentation in each cycle.

3.16.4 Data processing of shotgun proteomics measurements

The Proteome Discoverer v2.5 software from Thermo Fisher Scientific was used to process the MS-data resulting from the LC/MS measurements. The spectra were processed for the pre-experiment and enrichment experiment as two separate runs. The workflow settings were closely resembling the settings used by (Osório et al., 2021; Junior, 2021). In the processing step, Sequest-HT node was used as search engine, with a set precursor mass tolerance of 10 ppm and a fragment mass tolerance of 0.02 Da. A maximum of two missed cleavage sites was allowed. In accordance to the used digestion protocol, carbamidomethylation of cysteine was set as static modification. The dynamic modifications were oxidation of methionine, deamidation of asparagine and glutamine, pyroglutamate formation of peptide N-terminal glutamine as well as protein N-terminal acetylation, methionine loss and methionine loss with acetylation. For the enrichment experiment, INFERYS rescoring node was implemented to increase peptide identifications (Zolg et al., 2018) and Minora Feature Detector node was used for label-free quantification (LFQ). The maximum delta Cn in Percolator node was set to 0.05 and false discovery rate was set to 0.05%. In the consensus step, the maximum retention time shift was set to 10 minutes and mass tolerance to 10 ppm for chromatographic alignment at the Feature Mapper node. Unique plus razor peptides were used for precursor ions quantification, with precursor abundance based on intensity and summed abundances of all connected peptide groups used for protein quantification. For the pre-experiment a single run was performed with the protein databases as shown below without normalization. And the main experiment was normalized against the total peptide amount of the protein identifications. The following protein databases were used for protein identification

- Pre-experiment
 - Complete protein sequences of *Desulfovibrio vulgaris* from Uniprot/Swissprot database, Uniprot consortium (Boutet et al., 2016)
 - the list of common Repository of Adventitious Proteins (cRAP, 116 entries)
- Second sulfate enrichment experiment with environmental samples
 - Custom protein sequence created from the protein sequences of the TOP10 abundant families of the enrichment samples (16S rRNA results) from Uniref 90 cluster database (Suzek et al., 2014)
 - the list of common Repository of Adventitious Proteins (cRAP, 116 entries)

Further, analyses and categorization of proteins were performed by Uniprot mapping tool (Huang et al., 2011). BlastKOALA annotation tool was used to retrieve KEGG Orthology identifiers (K numbers) as functional orthologs for the identified proteins (Kanehisa M, 2016).

3.16.5 Proteomics optimization

We observed from the pre-experiment attempt that proteomics was unsuccessful and more investigation was required to optimize the proteomics method to our samples. The optimization experiments performed in this chapter are with the dsv-lac assay lysate (SRB lysate) and liquid cultures in order to evaluate the problematic hindering proteomics analysis. The dsv-lactate assays were chosen since it had the most biomass compared to the autotrophic assays, and more optimization experiments can be thereby performed.

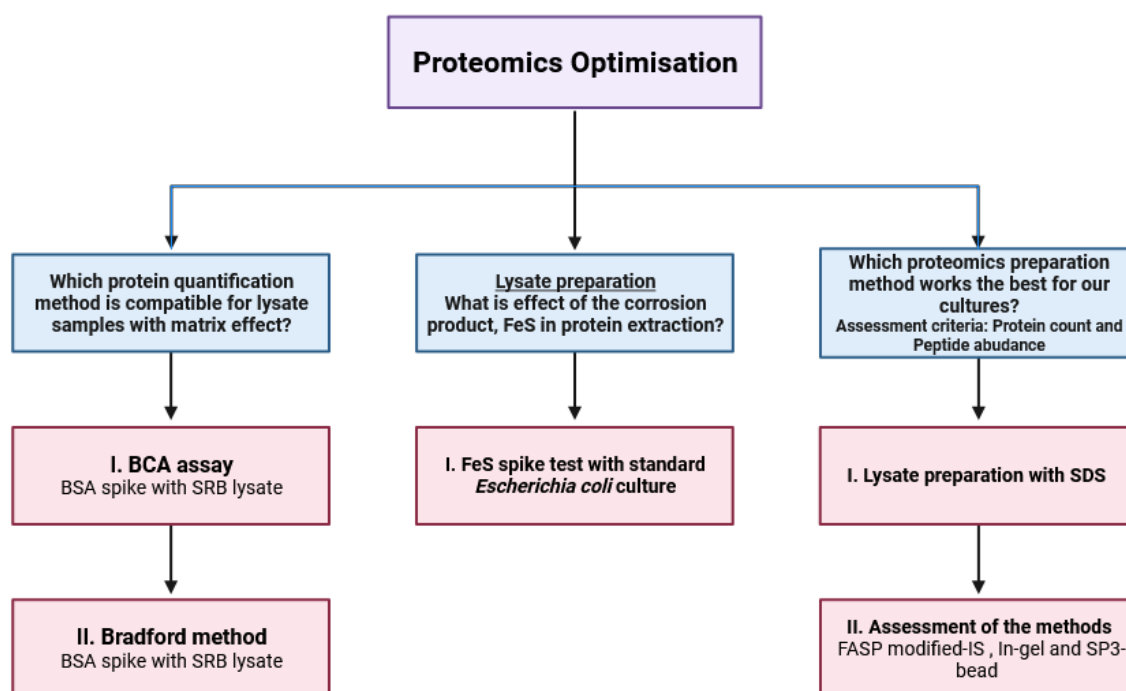


Figure 9: Schematic of the approach used for proteomic optimization of SRB lysate, created from Biorender.com.

3.16.5.1 BCA method assessment: BSA protein spike test with SRB lysate

This experiment was performed to validate the compatibility of the Bicinchoninic acid assay (BCA) protein determination kit with our sample. The experiment was set up as shown in Table 3. Calibration standards with BSA protein (Bovine Serum Albumin Standard, 2 mg/ml, Pierce, Thermo Fischer scientific, Germany) were prepared as given in the manual (working range 20-2000 µg/ml). 5 µl of each BSA standard was spiked with 20 µl of SRB lysate (dsv-lac). 25 µl of spiked calibration standards and pure standards were measured in a 96-well plate (Greiner bio-one, VWR, Darmstadt, Germany) with 200 µl of the BCA-reagent (50-parts Reagent B to 1-part Reagent A). The plate was incubated at 37°C for 30 minutes in an incubator (Orbital Incubator SI500, Stuart, UK). Thereafter, the samples were cooled to room temperature and the concentrations were photometrically determined at 562 nm (Infinite M200 PRO, Tecan, Switzerland). The measured O.D. of the spiked samples were estimated for protein concentration from the regression curve of the calibration standards. Thereafter, the measured BSA concentration spikes was calculated by subtracting the spiked sample concentrations with the unspiked SRB lysate ("0"). The percentage difference between the theoretical BSA concentration and calculated BSA concentration was determined.

Table 3: Spike experiment concentration table for BCA method assessment.

BSA Standard row (µg/ml)	2000	1000	500	250	125	62.5	31.25	15.625	0
SRB Lysate (µl)	20	20	20	20	20	20	20	20	20
BSA-Standard from Standard row (µl)	5	5	5	5	5	5	5	5	0
TRIS A (µl)	0	0	0	0	0	0	0	0	5
Total Volume (µl)	25	25	25	25	25	25	25	25	25
Concentration of BSA in spiked Lysate (µg/ml)	400	200	100	50	25	12.5	6.25	3.125	0

3.16.5.2 Bradford method assessment: BSA protein spike test with SRB lysate

This experiment was performed to validate the compatibility of the Bradford protein determination assay (Working range: 0.5-4 µg/ml) (similar to 3.16.5.1). The experiment was set up as shown in Table 4.5 µl of each BSA standard was spiked with 20 µl of SRB lysate (dsv-lac). Pure calibration standards were set-up (0.5 - 4 µg/ml). The charge of the proteins was altered by the addition of alkaline 0.5M NaOH (end vol. 200 µl). The total reaction volume was made up to 1000 µl with 800 µl of sterile MilliQ water. To which, 250 µl of Coomassie reagent was added to each sample. The samples were vortexed at room temperature for 5 minutes. Thereafter, the concentrations were estimated photometrically at 595 nm (UV-VIS Spektralphotometer DR5000, Hach Lange, Düsseldorf). The measured O.D. of the spiked samples were estimated for protein concentrations from the regression curve of the calibration standards. The measured BSA concentration spikes was calculated by subtracting the spiked sample concentrations with the un-spiked SRB lysate ("0"). The difference between the theoretical BSA concentration and calculated BSA concentration was determined.

Table 4: Spike experiment concentration table for Bradford method assessment.

BSA Standard row (µg/ml)	2000	1000	500	250	125	62.5	31.25	0
SRB Lysate (µl)	50	50	50	50	50	50	50	50
BSA-Standard from Standard row (µl)	2	2	2	2	2	2	2	
TRIS A (µl)	948	948	948	948	948	948	948	950
Total Volume (µl)	1000	1000	1000	1000	1000	1000	1000	1000
Concentration of BSA in spiked Lysate (µg/ml)	4	2	1	0.5	0.25	0.125	0.063	0

3.16.5.3 FeS spike test with *Escherichia coli*

In the biotic assays, greyish black precipitates were observed in the bulk solution and in the biofilm. And, it is known that sulfate reduction to sulfide is an important energy metabolism pathway. And sulfides can readily precipitate to Fe_xS_y in the presence of Fe^0 and Fe^{2+} (King and Wakerley, 1973). Sulfides can adsorb onto the protein surface by ionic interactions, hence making it difficult to isolate the proteins from the sulfide matrix (Benndorf et al., 2009). The aim here was to identify if iron sulfide influences lysate extraction and protein quantification using the standard *E. coli* culture. The salts $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ were used to generate 0-20mM of FeS reactions. 20mM was chosen to be the maximum concentration based on the maximum sulfate (20 mM) concentrations provided in our media of the microcosm experiments.

$\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ stock solutions (50 mM) were prepared. A pellet obtained from 10ml *E. coli* overnight culture (O.D₆₀₀ of 1.2) was resuspended in 3 ml of sterile MilliQ water (*E. coli* stock). The *E. coli* stock was quantified for whole cell protein concentration by Bradford assay. Thereafter, six sets from 0-2.49 mg/l concentrations were set-up from the *E. coli* stock. Each concentration set was spiked with corresponding volumes of $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ stocks to obtain FeS concentrations from 0.16 - 20mM, as shown in Figure 1, in an anaerobic chamber, because the reaction was sensitive to oxygen. A non-spiked set was also set-up as control. The total volume of the reactions was 1 mL and 0,01 g of SDS salt was added to each reaction for an end concentration of 1% SDS. Furthermore, lysate was extracted (details see 3.16.3.2) and the concentrations were determined by the BCA-assay detergent compatible kit (Thermo Fisher Scientific, Schwerte, Germany). Lastly, the influence of the FeS mineral on the *E. coli* protein concentration, were determined by dividing the difference of the concentration with and without FeS spike from the respective control concentration and then the percentage was calculated.

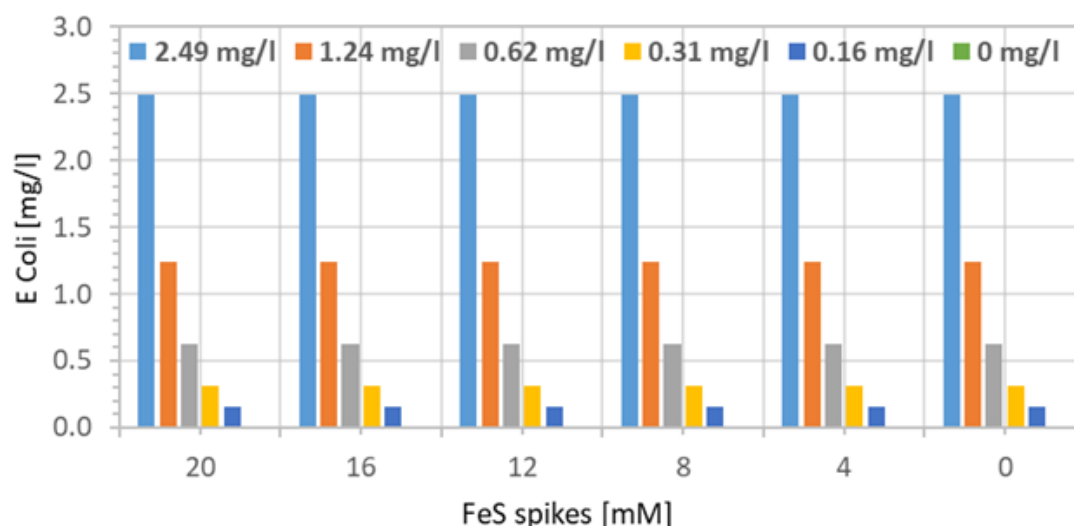


Figure 10: Experimental scheme showing the FeS spike concentrations and *E. coli* concentrations.

3.16.5.4 Optimisation of proteomic sample preparation methods

The lysate preparation as described in section (details see 3.16.1.1), was performed so far only with TRIS A buffer. In this section, the lysate preparation with SDS was tested, and compared with the proteomics results of TRIS A buffer (total number and abundance of identified proteins). Both the extraction buffers were compared by the in-gel and in-solution digestion methods. The dsv-lac was extracted with 50 mM TRIS A buffer and 0.2% SDT buffer. The SDT buffer is a combination of SDS detergent and dithiothreitol (5 mM). In-solution digestion was performed according to the Pierce in-solution tryptic digestion and guanidination kit manual (Thermo scientific, USA) and no subsequent peptide clean-up was done before injection in LC/MS (details see 3.16.2.1, 3.16.3.1). The second approach involved SDS-Page gel electrophoresis and a subsequent in-gel digestion (Starke, 2017) (Detailed protocol: see Bachelorarbeit_Seibicke). The LC/MS measurements were analysed by Proteome Discoverer 2.5 software against the protein database of *Desulfovibrio vulgaris* from Uniprot kb, the workflow settings were done as mentioned in (details see 3.16.4). Total protein counts and peptide abundances were estimated from the identified protein list and their corresponding peptide abundances.

3.16.5.5 Comprehensive analyses of proteomics preparation methods on sulfate-rich cultures

In this experiment, a new extraction method, called SP3 method was performed along with a modified In-solution method, with a pre-digestion clean-up step and also In-gel method. It was a detailed study performed as a part of a Bachelor thesis (Bachelor thesis-Seibicke). The aim of the experiment was the application of three proteomic preparation methods namely FASP in-solution, in-gel and SP3 solid phase extraction methods on sulphide rich pure cultures of *D. ferrophilus* and assessment of the peptide identification and biomass thresholds of each method. Firstly, in this study, 10 µg/µl (M100) of protein and 1 µg/µl (M10) were extracted by each method, a wide range was selected because typically anaerobic respiring bacteria yield variable biomasses depending on the nutrient conditions- heterotrophic or autotrophic. Secondly, the cultures were grown under high sulfide conditions that hinder with the protein preparation methods. Hence, based on these two parameters, biomass tolerance and matrix effect, the efficiency of the methods were analysed with proteomics. The best performing method would be thereafter, implemented for the analyses of the further corrosion experiments.

For the experimental approach, pure cultures of *D. ferrophilus* (DSMZ 15579) (DSMZ, Germany) were chosen for the study. The media, 195c was used as prescribed from DSMZ with lactate concentration of 22 mM (C-source) and sulfate as the electron acceptor was 21 mM. A mother culture was first prepared from -80°C glycerol stocks of *D. ferrophilus*. It was incubated at 25°C for 3 days. The cultures for the experiment were set-up in triplicates with 74 mL of media inoculated with 5% v/v of the mother culture. Thereafter, they were incubated at 25°C for two weeks. Furthermore, the distribution of the

biomass from the cell cultures for the proteomics preparation is described in the experimental design (Figure 11). The detailed steps of the lysate preparation and the three proteomics methods are explained in the bachelor thesis (Bachelor thesis- Seibicke). In brief, the lysate was extracted with 1.25% SDT buffer and was further processed by the proteomics methods. The in-solution digestion method, as mentioned before was done using the Pierce™ In-Solution Tryptic Digestion Kit (details see (3.16.2.1)). However, an additional lysate clean-up was performed before the lysate was subjected to post processing with the kit. Filter-aided sample preparation (30KDa MWCO filters, Merck, Molsheim, France) with 8M urea was used for lysate purification. The MWCO filters are included with a spin column that functions based on the size exclusion principle, hence retaining proteins larger than 30KDa in the column. The second proteomic approach, in-gel digestion was performed with a 1D-SDS Page (Bachelor thesis-Seibicke). The third method, SP3-extraction, using paramagnetic beads was a completely new method and was carried out for the first time. This method is based on the principle of solid-phase extraction, in which the surface of the bead is fabricated with carboxylate groups that allow proteins to immobilize on the bead surface. The protocol for SP3 treatment was adapted from (Mikulášek, 2021). Lastly, the peptides generated from each of the methods were subjected to LC/MS measurement (details see (3.17.3.1)). The resultant spectra were evaluated against the protein sequences of *Desulfovibrio ferrophilus* from Uniprot/TrEMBL database, Uniprot consortium (Boutet et al., 2016) with the Proteome Discoverer 2.5 software (details see (3.17.4)). The obtained protein list was evaluated from protein count and the peptide abundance data.

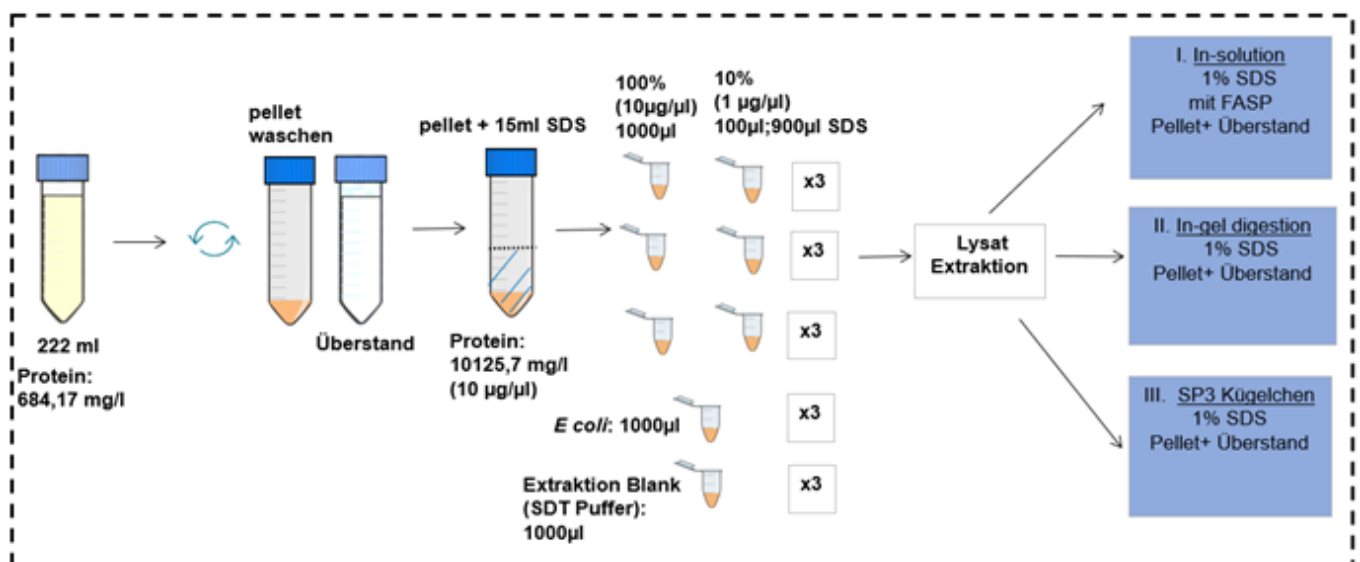


Figure 11: Experimental design of the proteomics method evaluation.

4 Project results

4.1 Field study: Rust tubercles in German Waterways – investigations on their abundance, microbial community composition and the influence of environmental parameters

The table below gives an overview about all samples collected, the location where they were collected as well as sampling date, river name, river km, GPS coordinates and the type of sample.

Table 5: ID, site, river, km GPS coordinates, sampling date and sampling type of all samples taken and analysed for field study 4.1: “Rust tubercles in German Waterways”, N = 76.

ID	site	river	km	GPSN	GPSE	samplingdate	type
Farge_Backfilling	Farge	Weser	27	53.205	8.510	23. Jun 20	backfill
Farge_constrpit_soil	Farge	Weser	27	53.205	8.510	23. Jun 20	soil
Farge_P_L1	Farge	Weser	27	53.205	8.510	23. Jun 20	tubercle
Farge_P_W1b	Farge	Weser	27	53.205	8.510	23. Jun 20	tubercle
Farge_rust_W2	Farge	Weser	27	53.205	8.510	23. Jun 20	tubercle
Farge_Water	Farge	Weser	27	53.205	8.510	23. Jun 20	water
HO_BF_algae0	Himmelpfort	Lychener Gewässer	0	53.177	13.230	01. Nov 20	biofilm
HO_BF_algae2	Himmelpfort	Lychener Gewässer	0	53.177	13.230	01. Nov 20	biofilm
HO_BFandP	Himmelpfort	Lychener Gewässer	0	53.177	13.230	01. Nov 20	tubercle
HO_P_Anker_1	Himmelpfort	Lychener Gewässer	0	53.177	13.230	01. Nov 20	tubercle
HO_P_Anker_2	Himmelpfort	Lychener Gewässer	0	53.177	13.230	01. Nov 20	tubercle
HO_P_SW_1	Himmelpfort	Lychener Gewässer	0	53.177	13.230	01. Nov 20	tubercle
HO_P_SW_2	Himmelpfort	Lychener Gewässer	0	53.177	13.230	01. Nov 20	tubercle
HO_P_SW_3	Himmelpfort	Lychener Gewässer	0	53.177	13.230	01. Nov 20	tubercle
HO_P_SW_sub	Himmelpfort	Lychener Gewässer	0	53.177	13.230	01. Nov 20	tubercle
HO_Sed	Himmelpfort	Lychener Gewässer	0	53.177	13.230	01. Nov 20	sediment
HO_W_IW_1m	Himmelpfort	Lychener Gewässer	0	53.177	13.230	01. Nov 20	water
HO_W_OW_1m	Himmelpfort	Lychener Gewässer	0	53.177	13.230	01. Nov 20	water
Ke_P_0	Ketzin	Untere Havel-Wasserstrasse	34	52.463	12.857	04. Jul 21	tubercle
Ke_P_1	Ketzin	Untere Havel-Wasserstrasse	34	52.463	12.857	04. Jul 21	tubercle
Ke_P_2	Ketzin	Untere Havel-Wasserstrasse	34	52.463	12.857	04. Jul 21	tubercle

Ke_P_3	Ketzin	Untere Havel-Wasserstrasse	34	52.463	12.857	04. Jul 21	tubercle
Ke_P_MS_a	Ketzin	Untere Havel-Wasserstrasse	34	52.463	12.857	04. Jul 21	tubercle
Ke_P_MS_b	Ketzin	Untere Havel-Wasserstrasse	34	52.463	12.857	04. Jul 21	tubercle
Ke_Sed	Ketzin	Untere Havel-Wasserstrasse	34	52.463	12.857	04. Jul 21	sediment
Ke_W	Ketzin	Untere Havel-Wasserstrasse	34	52.463	12.857	04. Jul 21	water
LP_Lubeck	Lubeck	Trave (Mündung)	26	53.957	10.879	21. Jun 22	tubercle
FBL_W_OW	Lahn	Lahn	129	50.336	7.674	24. Jan 21	water
LT_P1	Lahn	Lahn	136	50.308	7.617	04. Aug 21	tubercle
LT_P2	Lahn	Lahn	136	50.308	7.617	04. Aug 21	tubercle
LT_P3	Lahn	Lahn	136	50.308	7.617	04. Aug 21	tubercle
MLK_corrhole_N1	Mittellandkanal	Mittellandkanal	231	52.388	10.594	21. Jul 20	backfill
MLK_corrhole_N2	Mittellandkanal	Mittellandkanal	231	52.388	10.594	21. Jul 20	backfill
MLK_corrhole_S1	Mittellandkanal	Mittellandkanal	231	52.388	10.594	21. Jul 20	backfill
MLK_corrhole_S2	Mittellandkanal	Mittellandkanal	231	52.388	10.594	21. Jul 20	backfill
Mosel_Rust1	Mosel	Mosel	87	50.031	7.178	10. Jun 21	tubercle
Mosel_Rust2	Mosel	Mosel	87	50.031	7.178	10. Jun 21	tubercle
Mosel_Rust3	Mosel	Mosel	87	50.031	7.178	10. Jun 21	tubercle
Mosel_Sed	Mosel	Mosel	87	50.031	7.178	10. Jun 21	sediment
RG_P_140_3	Regow	Obere Havel-Wasserstrasse	42	52.717	13.238	03. Nov 20	tubercle
RG_P_150_a1	Regow	Obere Havel-Wasserstrasse	42	52.717	13.238	03. Nov 20	tubercle
RG_P_150_a2	Regow	Obere Havel-Wasserstrasse	42	52.717	13.238	03. Nov 20	tubercle
RG_P_158	Regow	Obere Havel-Wasserstrasse	42	52.717	13.238	03. Nov 20	tubercle
RG_P_26_6	Regow	Obere Havel-Wasserstrasse	42	52.717	13.238	03. Nov 20	tubercle
RG_P_34_4	Regow	Obere Havel-Wasserstrasse	42	52.717	13.238	03. Nov 20	tubercle
RG_P_43	Regow	Obere Havel-Wasserstrasse	42	52.717	13.238	03. Nov 20	tubercle

RG_P_5	Regow	Obere Havel-Wasserstrasse	42	52.717	13.238	03. Nov 20	tubercle
RG_P_C_w	Regow	Obere Havel-Wasserstrasse	42	52.717	13.238	03. Nov 20	tubercle
RG_P_C1150	Regow	Obere Havel-Wasserstrasse	42	52.717	13.238	03. Nov 20	tubercle
RG_P_C2	Regow	Obere Havel-Wasserstrasse	42	52.717	13.238	03. Nov 20	tubercle
RG_P_film_b	Regow	Obere Havel-Wasserstrasse	42	52.717	13.238	03. Nov 20	tubercle
RG_P_film_r	Regow	Obere Havel-Wasserstrasse	42	52.717	13.238	03. Nov 20	tubercle
RG_Sed_OS	Regow	Obere Havel-Wasserstrasse	42	52.717	13.238	03. Nov 20	sediment
RG_W_IW	Regow	Obere Havel-Wasserstrasse	42	52.717	13.238	03. Nov 20	water
RG_W_OW_1m	Regow	Obere Havel-Wasserstrasse	42	52.717	13.238	03. Nov 20	water
RG_W_OW_2m	Regow	Obere Havel-Wasserstrasse	42	52.717	13.238	03. Nov 20	water
RG_W_UW_1m	Regow	Obere Havel-Wasserstrasse	42	52.717	13.238	03. Nov 20	water
SB_WT	Wispertal	Sauerbrunnen Wispertal	0	50.105	7.879	13. Jun 21	water
SP_P_1	Spandau	Spree-Oder Wasserstrasse	0	52.537	13.211	27. Jul 21	tubercle
SP_P_2	Spandau	Spree-Oder Wasserstrasse	0	52.537	13.211	27. Jul 21	tubercle
SP_W	Spandau	Spree-Oder Wasserstrasse	0	52.537	13.211	27. Jul 21	water
ST1_Wehr	Schweinfurt	Main	332	50.040	10.229	01. Jul 21	tubercle
ST2_Wehr	Schweinfurt	Main	332	50.040	10.229	01. Jul 21	tubercle
VW_P_NL_UWZ	Vosswinkel	Obere Havel-Wasserstrasse	88	53.321	13.032	01. Dez 20	tubercle
VW_P_SPW_WWZ	Vosswinkel	Obere Havel-Wasserstrasse	88	53.321	13.032	01. Dez 20	tubercle
VW_P_UWZ_1	Vosswinkel	Obere Havel-Wasserstrasse	88	53.321	13.032	01. Dez 20	tubercle

VW_P_UWZ_2	Vosswinkel	Obere Havel-Wasserstrasse	88	53.321	13.032	01. Dez 20	tubercle
VW_P_UWZ_NL	Vosswinkel	Obere Havel-Wasserstrasse	88	53.321	13.032	01. Dez 20	tubercle
VW_P_WWZ_1	Vosswinkel	Obere Havel-Wasserstrasse	88	53.321	13.032	01. Dez 20	tubercle
VW_P_WWZ_2	Vosswinkel	Obere Havel-Wasserstrasse	88	53.321	13.032	01. Dez 20	tubercle
VW_W_OW	Vosswinkel	Obere Havel-Wasserstrasse	88	53.321	13.032	01. Dez 20	water
VW_W_UW	Vosswinkel	Obere Havel-Wasserstrasse	88	53.321	13.032	01. Dez 20	water
WSB_Pustel_3	Steinbach	Main	201	50.012	9.606	01. Mai 21	tubercle
WSB_Pustel_4	Steinbach	Main	201	50.012	9.606	01. Mai 21	tubercle
WSB_Pustel_6	Steinbach	Main	201	50.012	9.606	01. Mai 21	tubercle
WSB_Wasser	Steinbach	Main	201	50.012	9.606	01. Mai 21	water

All Metadata collected can be found in S3. An overview of environmental parameters (either measured on site or derived from various data bases) and the percentage of available data over the whole data set can be found in S4.

Microbial communities were profiled at 13 corrosion sites along German Waterways (Figure 5). At each site, corrosive biofilm was sampled together with other sample types (e.g. water, backfilling material, or sediment) to compare the microbial community composition among the different sample types. At some sites, only one or two samples were available for analysis. Thus, for some analysis only sites with sample size ($N \geq 3$) were included. Furthermore, the measurement of environmental parameters was not complete for all samples (S4). Since correlation of microbial abundance data with environmental factors is not feasible for sites, where those are missing, subsets of the whole data set are used in some cases (see below). Sequencing was performed in 4 separate runs, why artefacts due to the different sequencing runs cannot be excluded. DNA extraction and sequencing were performed as described in 3.15 and 3.15.4..

4.1.1 Microbial community composition between sample types and site

One project aim was to identify microbial indicators hinting towards MIC. By comparing the microbial community composition between different sample types, we tried to identify the microbes typically occurring within the rust tubercles.

The amplicon sequencing data show strong differences in the microbial community based on sample type. The bacterial community composition on the class level shows distinct differences based on sample type (Figure 12). Overall water samples (A) seem less diverse (on the class level) compared to tubercle samples. Sediment and backfilling material samples are more similar to tubercle samples than to water samples but still distinct from each other. Information on specific taxa will be given below.

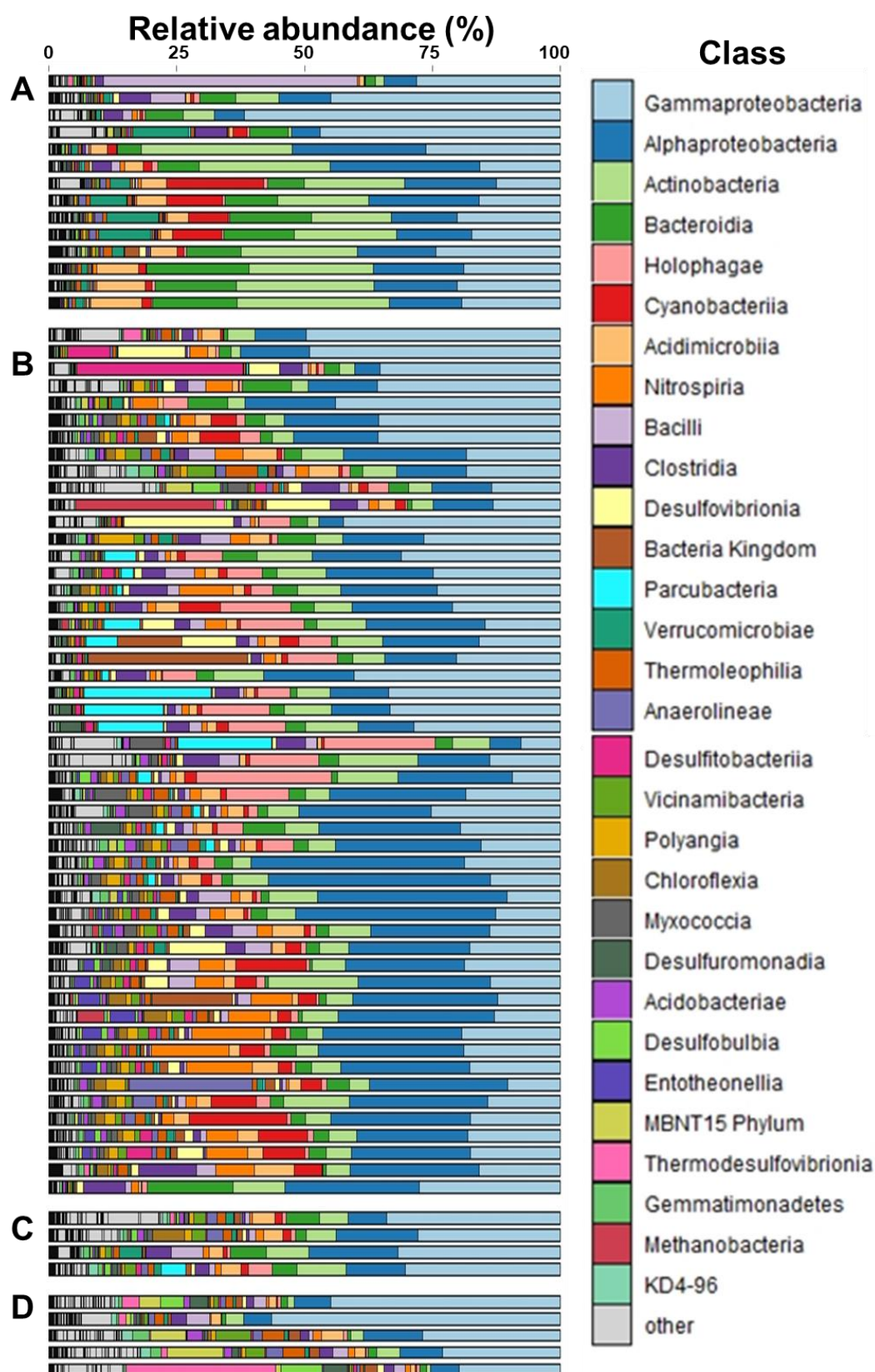


Figure 12: Bacterial community composition on the class level for different sample types (A=water, B=tubercle, C=sediment, D=backfilling material).

Based on the microbial community composition, samples were analysed using different statistical tests to infer whether we see trends based on sample site, sample type or with environmental parameters.

First, all sample types and sites were analysed by PCoA using bray curtis as distance metric. Here, clustering occurs by type and site, although type seems to be a stronger factor (Figure 13). This implies that the sample type, e.g. tubercle vs water, is a stronger factor in determining the microbial community composition than the environmental differences at different sites. The tubercle samples from different sites are more similar than a water and a tubercle sample from the same site. However, the spread within one site and sample type can be rather large (for example see Vosswinkel tubercle samples, Figure 13), implying that the difference of the microbial community composition of one type and site is sometimes larger than of the same type at different sites.

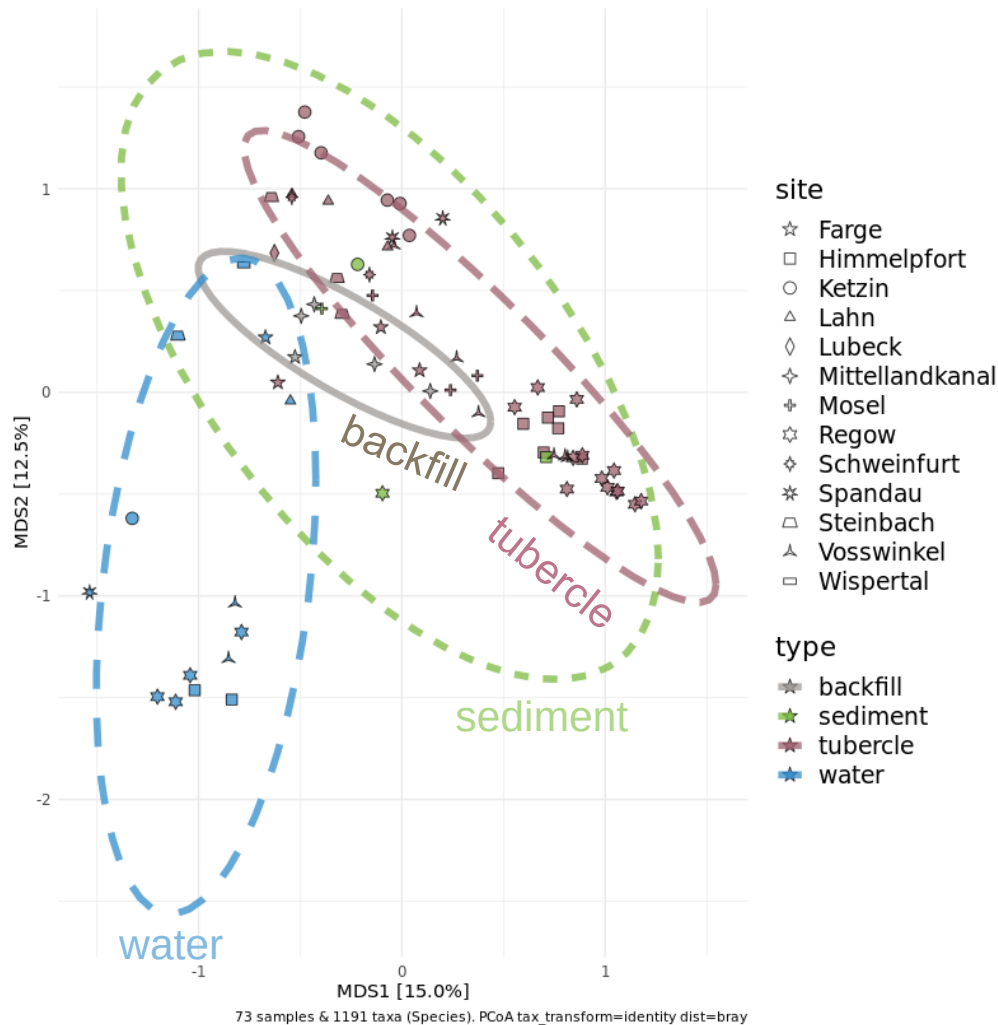


Figure 13: PCoA on bray curtis distance of all samples (all sites and all types), color depicts the different sites, shape the different types.

To further investigate the type difference, PCoAs were also performed for all sites individually. Here the type differences are very pronounced. For Vosswinkel, water and tubercle samples cluster opposite of each other on the first axes with an explanatory power of 35%, the difference between tubercle samples is shown along the second axes with 21.4% explanatory power. This is similar for site Regow, interestingly the sediment sample at site Regow clusters closer to the tubercle than to the water samples, likely reflecting the more reducing conditions in those environments. At site Himmelpfort biofilm samples (from a painted steel surface, not corrosive by visual investigation) are clustering closer to tubercle than to water samples but those samples still form their own cluster compared to the tubercles. One tubercle sample (HO_Anker_1) actually clusters together with the sediment sample, this is interesting as this sample is from a horizontal surface (anchor within the lock) which had visual sedimentation on top. Similar patterns (clustering of water and tubercle samples) also occur at site Ketzin and Spandau.

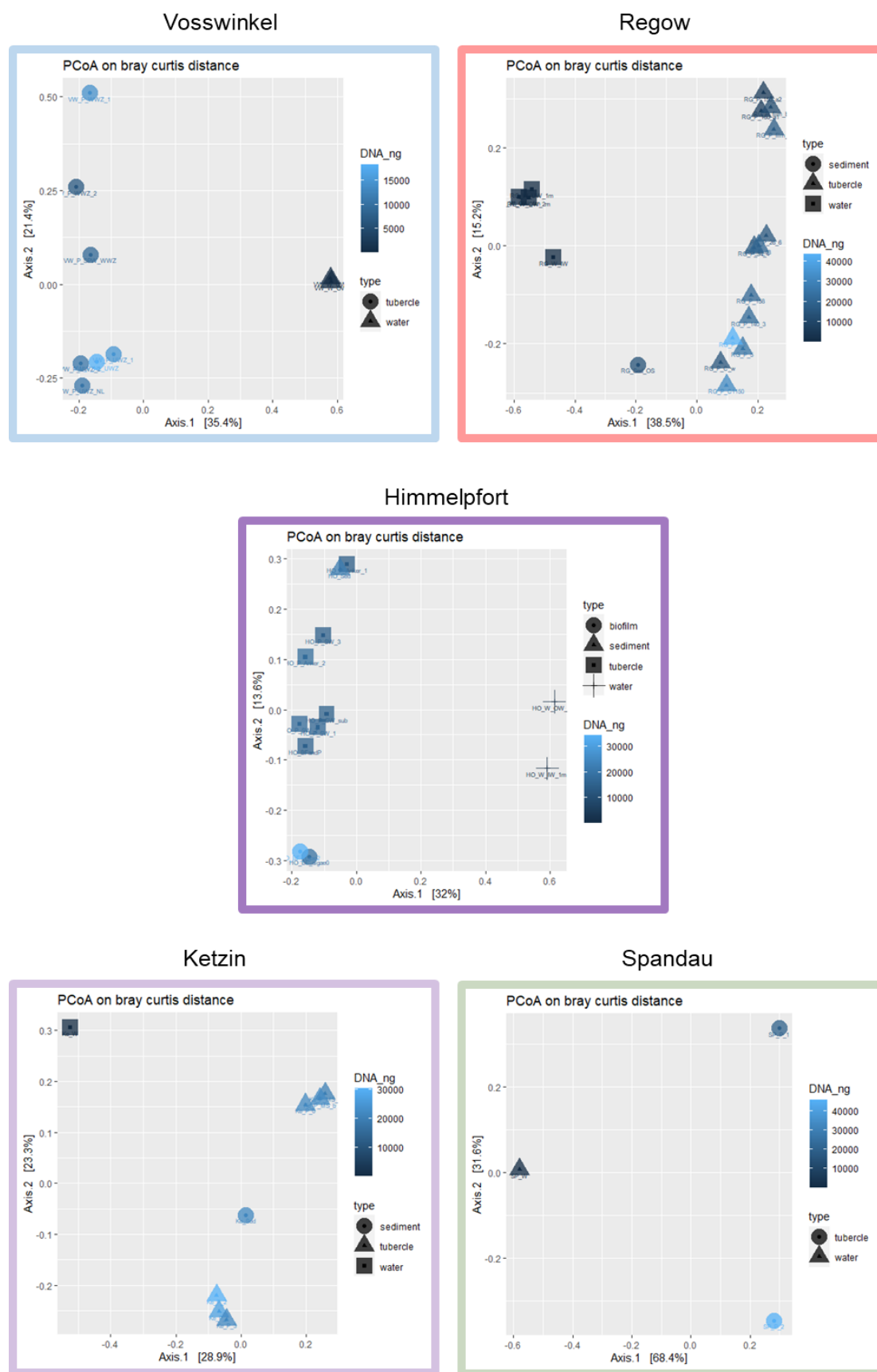


Figure 14: PCoA for Vosswinkel, Regow, Himmelfort, Ketzin and Spandau (sites with N≥4) for each site individually.

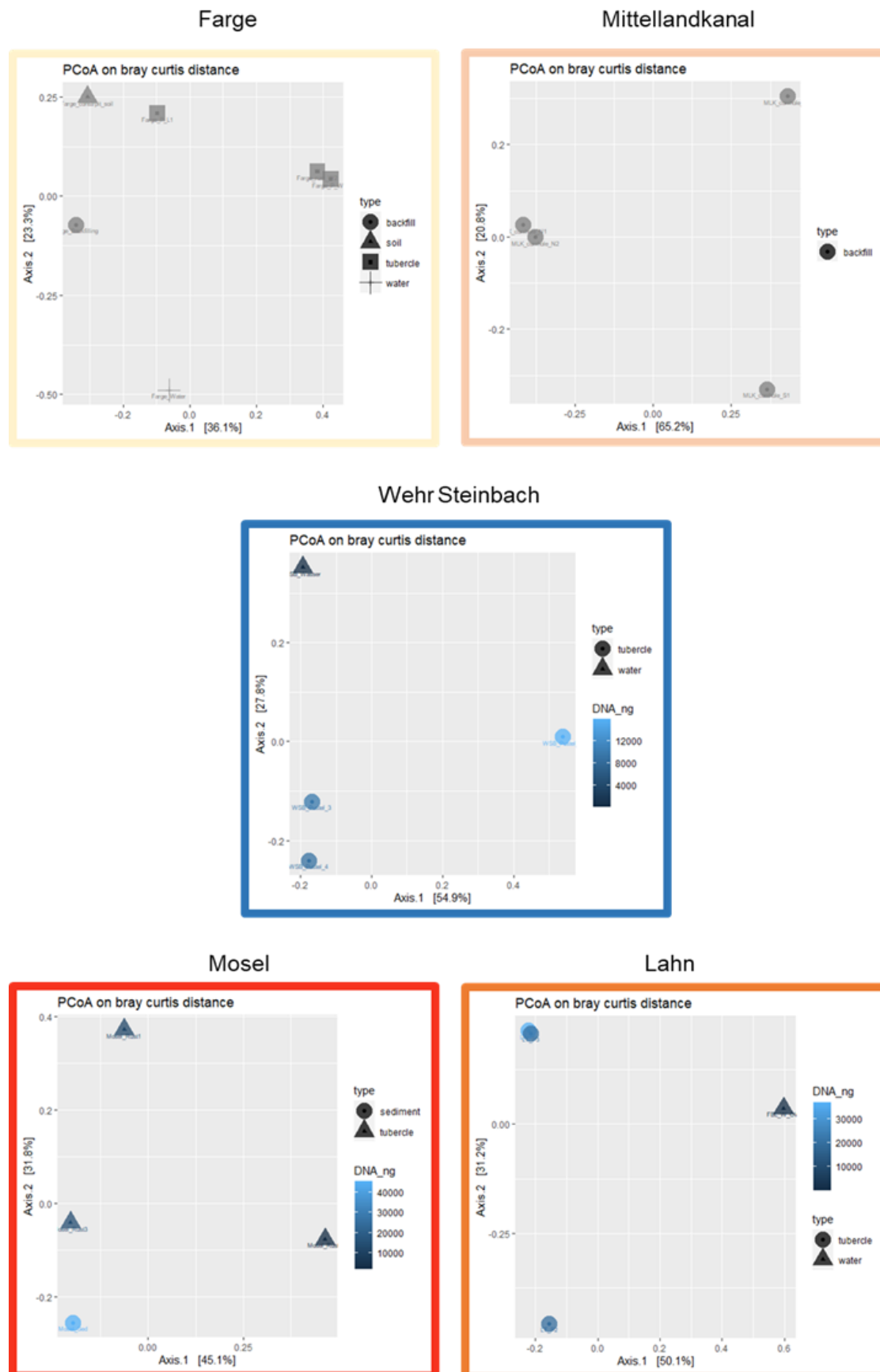


Figure 15: PCoA for Farge, Mittellandkanal, Wehr Steinbach, Mosel and Lahn (sites with $N \geq 4$) for each site individually.

For site Farge, material scraped from the land-facing side of a freshly cut-out piece of the sheet pile wall was also sampled. This sample clusters closer to the soil and backfilling material than to the tubercle samples facing the water side of the sheet pile wall. The microbial communities in the northwest samples of the MLK (Figure 15) form a different cluster from those samples taken at the southeastern side. All samples were a mix of backfilling material and corrosion products, because the sheet pile wall on this site showed various extends of corrosion and samples were taken at the corrosion holes. On the two sampling points on the northwestern site, the sheet pile steel was far more corroded (old corrosion holes of approximately 10 cm in diameter, backfilling material partly flushed away), than at the southeastern site (freshly opened corrosion hole, blackish backfilling material still present. At site Steinbach the samples cluster according to type (water vs tubercle) again. At site Mosel the rust samples show a rather large spread and one clusters closer to the sediment sample. At site Lahn the separation again occurs according to type water and tubercle. One tubercle also clusters away from the other two tubercle samples. Besides some specific features the main clustering effect is according to sample type (water vs. tubercle) e.g. at all sites where samples of both types are available.

When investigating only the type tubercle at all sites, 4 to 5 clusters according to site can be seen, the first distinct cluster is formed by all tubercle samples from site Regow (Figure 16, A), the other cluster is formed by Himmelpfort and Voßwinkel samples (Figure 16, C, which could be two separate clusters as well, B and C). These sites are located within the same river system; thus, the location/site plays a role in determining the microbial community as well. The tubercle samples from site Farge (3 samples only) seem to cluster separately from the other samples, likely because this site is a slightly brackish water site. There is another cluster with Ketzin (D) samples and part of Spandau and Lahn samples. The other samples are spread between those clusters or form smaller clusters within (Lübeck, Mosel, Schweinfurt, Steinbach).

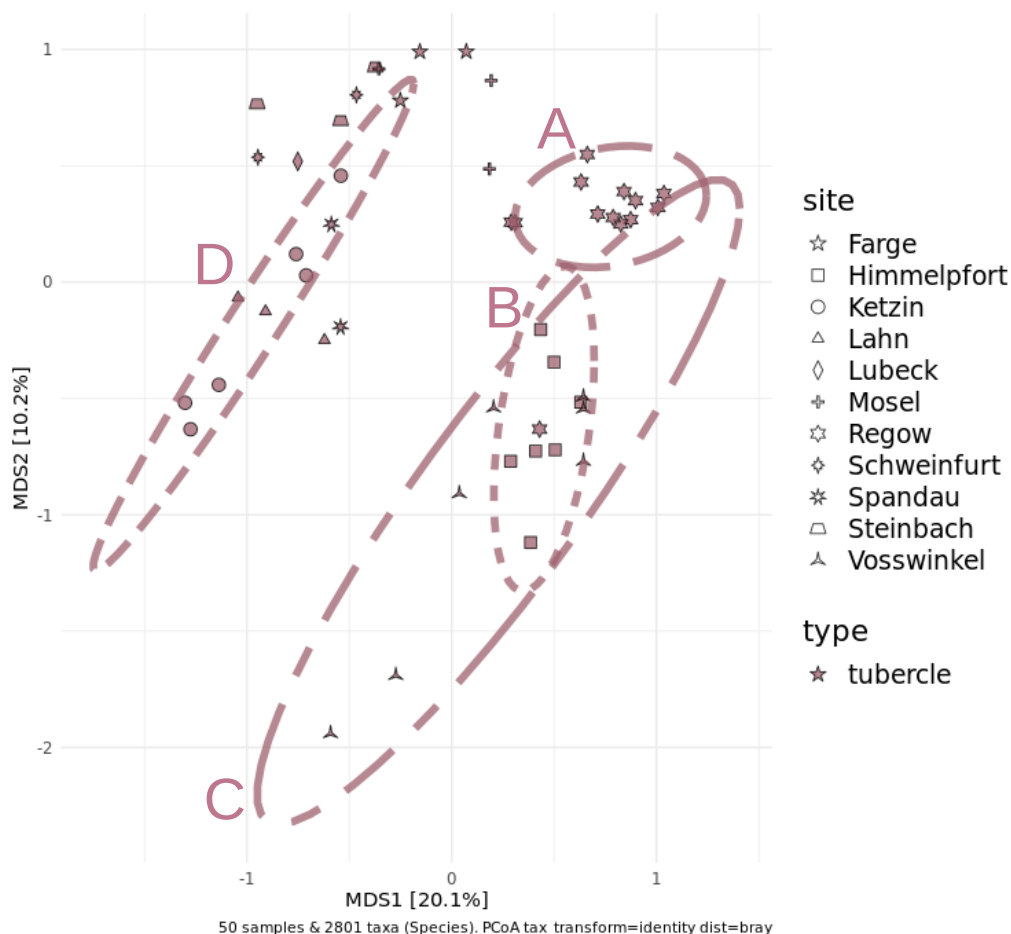


Figure 16: PCoA on bray curtis distance of only the type tubercle at all sites.

4.1.2 Correlation of environmental parameters to the microbial community composition

CAP analyses were performed to investigate the influence of the measured environmental parameters on the microbial community composition (Figure 17 and Figure 18). Please note that not all parameters were measured and or were available for all sites. Parameters used were the type of sample, the pH (as annual pH in the water), the annual temperature, the measured conductivity at the time of sampling, the oxygen concentrations (annual), and the GPS coordinates as these were the most complete in the dataset. Again, the strongest difference can be observed according to sample type (Figure 17). When only plotting the tubercle samples (Figure 18) some site-specific features can be observed.

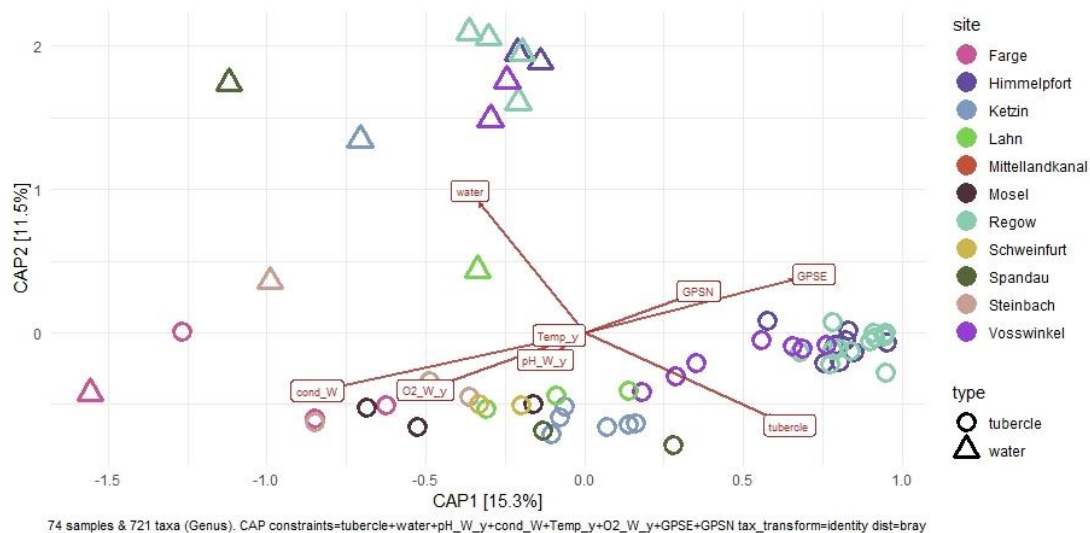


Figure 17: CAP analysis on sample type tubercle and water for all site (N=74). Only parameters which are available in at least 97% of the data set are plotted as arrows in the graph. The length of the arrow shows the importance of the environmental factor/parameter, while the direction shows whether it is positively or negatively correlated. Bray min prev 0.1.

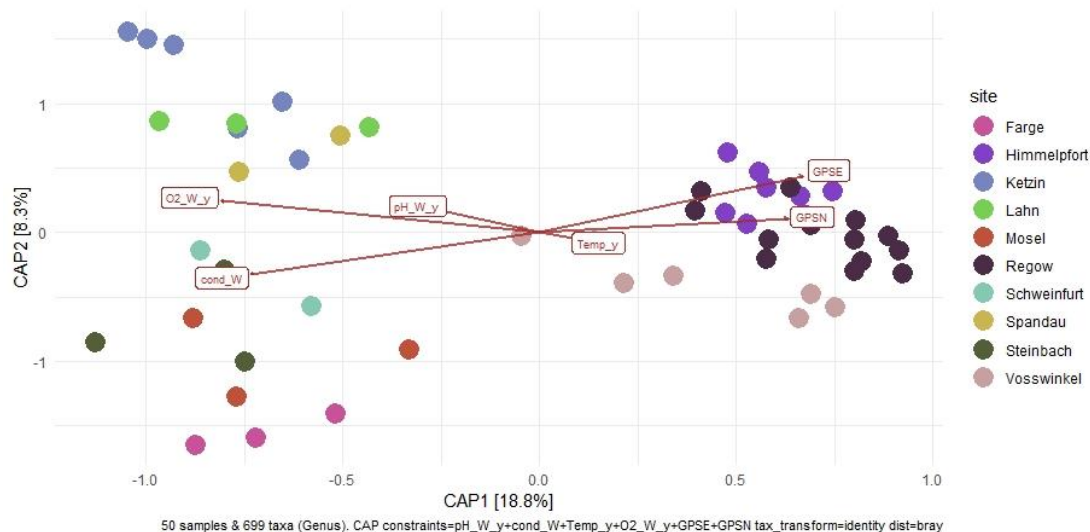


Figure 18: CAP analysis on sample type tubercle only (N=50), the length of the arrow shows the importance of the environmental factor/parameter, the direction shows whether it is positively or negatively correlated.

The conductivity points towards site Farge (also Steinbach and Mosel), Farge is the only brackish water site thus a shift of the microbial community towards more marine/brackish taxa might play a role here. However, another strong factor seems to be the coordinates (GPSE/GPSN) of the sampling location, thus site-specific factors not included in the analysis also shape the community. Site Lahn, Ketzin and Spandau seem to be positively correlated with the annual pH in the water and the annual

oxygen concentrations in the water. However, the explanatory power of the axis is not very strong. Overall with the environmental parameters tested we found no clear indication that environmental parameters strongly shape the community composition according to the site differences. This can have multiple reason, i) most importantly the environmental site-specific parameters were measured in the water and not inside the tubercles. Recent studies (Fiskal et al., 2023) have shown, however, that the environment inside the tubercle is extremely unique compared to the surrounding water. Therefore, it might just not be meaningful to compare the microbial community composition of the tubercles with the parameters measured in the water column. Especially parameters such as pH, redox conditions, and oxygen concentrations within the tubercle were shown to strongly deviate from the surrounding water environment. Thus, we cannot identify site specific risk factors/indicator parameters that promote or inhibit tubercle formation. ii) there is considerable diversity in the microbial community composition even within the tubercles of the same sites. Differences likely occur according to the stage/age of the tubercle, which tubercle layer was sampled (tubercle depth), the experienced difference in light exposure, different flow regimes, etc. Recent studies indeed show that the microbial community in freshwater tubercles changes over time (Wakai et al., 2022). iii) The taxa occurring within the tubercles are extremely diverse and even though sulfate reducers seem to play a role in most tubercles, it is not always the same taxa performing the metabolic process. Thus, the identification of the “MIC microbes” seems elusive. There is always a set of taxa performing the different metabolisms (Fiskal et al., 2023) and even this diverse set is changing from one tubercle to another regardless of site.

4.1.3 Analysis of the core microbiome

To further investigate the differences between water and tubercle samples we took a subset of the whole data set by choosing sites where we have a reasonable amount of water and tubercle samples from the same site. Thus, we choose only samples from site Himmelpfort (HO), Vosswinkel (VW) and Regow (RG) as subset data set. An overview of the bacterial families is shown in Figure 19. Clearly a different microbial community composition can be observed between type water and type tubercle samples. Water samples have on average higher relative abundance of *Comamonadaceae*, *Sporichthyaceae*, *Ilumatobacteraceae*, *Sphingomonadaceae*, *Chitinophagaceae*, and *Clade III*. In contrast tubercle samples have higher relative abundance of *Hyphomicrobiaceae*, *Xanthobacteraceae*, *Rhizobiales*, *Desulfovibrionaceae*, *Gallionellaceae*, *Holophagaceae* and *Nitrospiraceae*. *Comamonadaceae* are on average twice as abundant in water than in tubercle samples (in percent relative abundance). On the genus level it becomes clear that some taxa are similarly abundant (e.g. *Rhodospirillum rubrum*, *Hydrogenophaga*), whereas others are significantly more abundant (~ 25 times) in water samples (e.g. *Limnochlamydomonas*). Other genera such as *Leptothrix* and *Acidovorax* were significantly less abundant in water samples (3 times) compared to tubercles samples. As *Limnochlamydomonas* is described as planktonic aerobic freshwater organisms (Hahn et al., 2010) its higher abundance in the water samples is expected. *Leptothrix* is microaerophilic and oxidizes Fe(II), its mixotrophic or chemoorganoheterotrophic and depends on organic carbon (Emerson et al., 2010). *Leptothrix* spp. produces sheaths (Suzuki et al., 2011). The occurrence of *Leptothrix* (and other microaerophilic Fe(II) oxidizers (e.g. *Gallionella*)) at corrosion sites and their contribution to tubercle formation have been reported for many different marine and freshwater systems (Emerson, 2018; Emde et al., 1992; Ray et al., 2010a). Also some *Acidovorax* species can oxidize Fe(II), often coupled to nitrate reduction, some species live lithoautotrophic on hydrogen (Willems, 2013). *Sporichthyaceae* are on average 10 times more abundant in water than in tubercle samples. Here differences depending on the genus can be observed. All but one genera found in this family were significantly more abundant in water samples: *Candidatus Planktophilum* (3 times), *hgcl clade* (24 times), and *Sporichthya* (5 times). Only *Longivirga* was 7 times more abundant in tubercles compared to water samples. *Ca. Planktophilum* is a known planktonic freshwater bacterium (Jezbera et al., 2009), similarly the *hgcl* clade is known from freshwater systems (Zhang et al., 2023). *Sporichthya* are aerobic or facultatively anaerobic and chemoorganotrophic (Trujillo and Normand). Within the *Ilumatobacteraceae*, *CL500-29 marine group* was ~11 times more abundant in

water than in sediment samples and the genus *Ilumatobacter* was about the same in relative abundance. Within the *Sphingomonadaceae*, the genera *Sphingorhabdus* (4 times), *Novosphingobium* (6 times), *Rhizorhapis* (2 times), *Sandarakinorhabdus* (4 times), *Parablastomonas* (2 times), *Altererythrobacter* (10 times) showed a higher relative abundance in the water samples. The genus *Sphingomonas* was two times more abundant in the tubercle samples. Species of the genera *Sphingorhabdus*, *Sandarakinorhabdus* and *Sphingomonas* are known aerobic aquatic taxa (Gich and Overmann, 2006; Jogler et al., 2013). Some genera (e.g. *Parablastomonas*, *Sandarakinorhabdus*) are pigmented (Ren et al., 2015; Gich and Overmann, 2006). *Chitinophagaceae* are on average four times as abundant in water compared to tubercle samples. Especially the genus *Sediminibacterium* was the most abundant genus in water samples (~ 8 times more compared to tubercle samples). The relative abundance of the Clade III (*Alphaproteobacteria*) is 10 to 100 times higher water samples compared to tubercle samples.

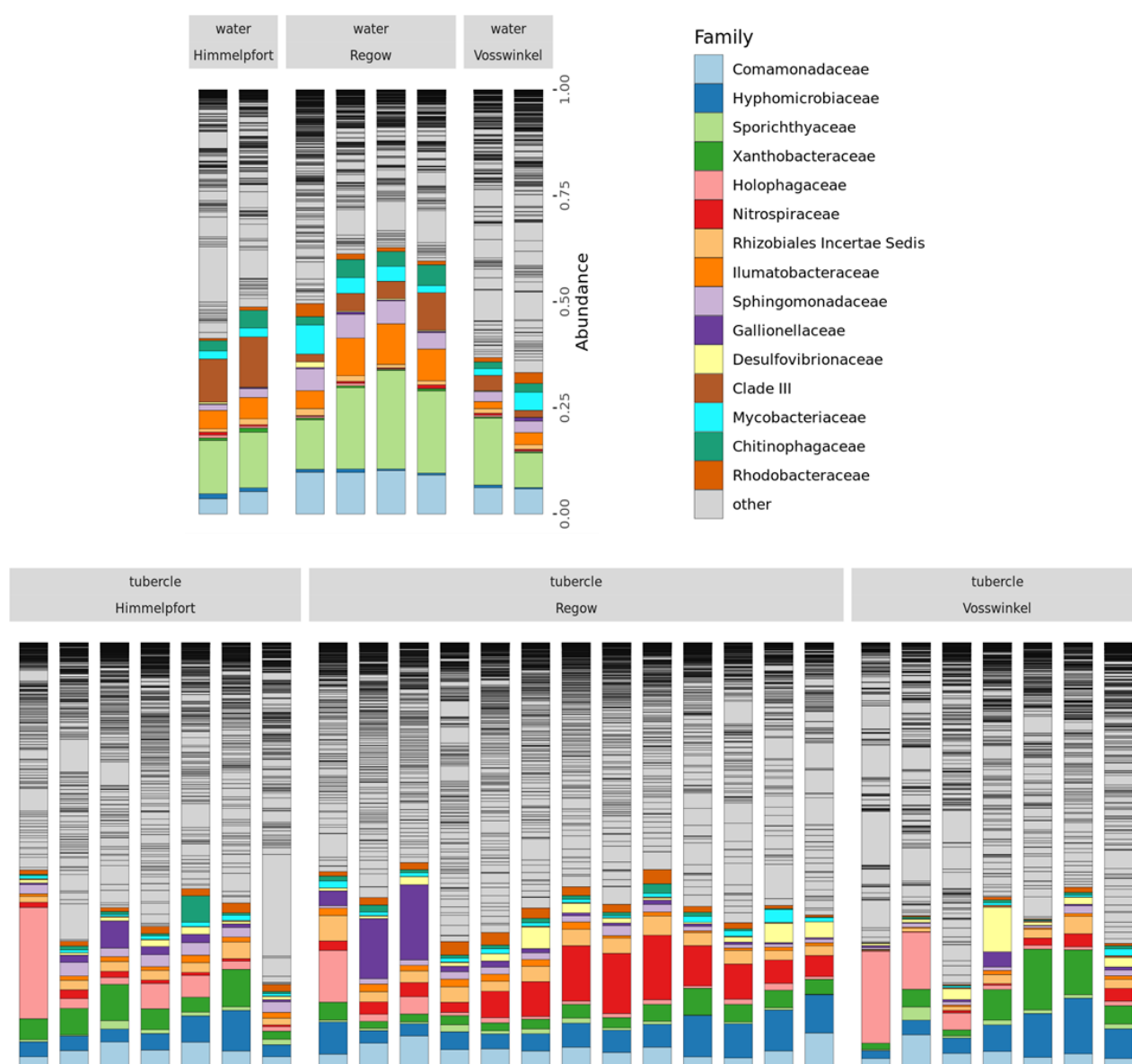


Figure 19: Bacterial community composition on the family level for site Himmelpfort, Regow, and Vosswinkel, showing water (first row) and tubercle (second row) samples in comparison.

The *Hyphomicrobiaceae* are on average 8 times more abundant in tubercles. Within this family, the genera *Pedomicrobium* was the most abundant followed by *Hyphomicrobium*. Both were on average 8 times more abundant. For the tubercle samples, some counts were annotated to *Filomicrobium*, which was not found in water samples. The higher occurrence of *Pedomicrobium* within the tubercles is interesting as it is known as Fe and Mn oxidizing and accumulating bacterium which can play a role in clogging of water systems and plays a general role in biofilm formation through hyphenation (Cox and Sly, 1997; Braun et al., 2009). The *Xanthobacteraceae* are on average 9 times more abundant in tubercles. The genera with the highest relative abundances were *Bradyrhizobium* (7 times), *Afipia* (8 times), *Pseudolabrys* (22 times), *Pseudorhodoplanes* (5 times), *Rhodopseudomonas* (7 times), *Rhodoplanes* (13 times) and *Tardiphaga* (8 times). All members of the *Xanthobacteraceae* are grow aerobic and chemoheterotroph, but facultatively chemolithoautotrophic on hydrogen and/or reduced S compounds (Oren, 2014). Genera of the *Rhizobiales* are on average 2 times more abundant in tubercles. Most abundant were *Alsobacter* (2 times), *Bauldia* (7 times), *Phreatobacter* (3 times) and *Nordella* (4 times). Some *Alsobacter* species (*A. ponti*) were found to perform denitrification and sulfate reduction (Deng et al., 2023).

Within the *Desulfovibrionaceae* (5 times more abundant in tubercle samples (even 8 times, if one water sample is excluded which was pumped water from inside the lock which was rich in particles and sediments)), the genus *Desulfovibrio* is almost the only annotated taxon (> 95% of all counts). Only very few counts were assigned to other genera such as *Bilophila*, *Desulfobaculum*, or *Mailhella*. *Desulfovibrio vulgaris* (species found in this study if annotated were *D. vulgaris*, *D. africanus* and *D. desulfuricans*) is a well-studied sulfate reducer and known from microbially induced corrosion sites. Generally, if *D. vulgaris* is incubated on carbon steel EET (extracellular electron transport) - MIC is expected to occur. However, the production of H₂S itself can cause indirect corrosion processes. Magnetite nanoparticles can increase weight loss by *D. vulgaris* by over 60% though the promotion of EET-MIC (Wang et al., 2022). The *Gallionellaceae* are on average six times more abundant in tubercle samples compared to water samples, yet their abundance between samples is heterogeneous. Within the *Gallionellaceae*, the genera *Gallionella* and *Sideroxydans* were by far the most abundant genera, 8 and 6 times more abundant in tubercles than in water samples, respectively. Other genera such as *Ferriphaselus* and *Ca. Nitrotoga* were significantly less abundant. *Gallionella* and *Sideroxydans* are both chemolithoautotrophic Fe(II) oxidizers. *Gallionella* produces twisted stalks (Suzuki et al., 2011). The occurrence of *Gallionella* at corrosion sites has been reported before (Emerson, 2018; Emde et al., 1992; Ray et al., 2010a). Within the *Holophagaceae*, *Geothrix* (10 times more abundant in tubercle samples) was the dominant genus (>95% of all counts), a few counts were also assigned to *Holophaga* (2 times more abundant). *Geothrix* is a known Fe(III) reducer however some species couple the Fe(III) reduction to fermentation (Coates et al., 1999). *Geothrix fermentans* was found to secrete electron shuttle compounds (Bond and Lovley, 2005) and was reported before to occur in corrosion tubercles (Jin et al., 2015; Fiskal et al., 2023). *Nitrospira* is the only assigned genus within the *Nitrospiraceae*, the genus is on average 9 times more abundant in tubercle samples. Also, the abundance of *Nitrospira* is patchy between tubercle samples, in RG tubercles *Nitrospira* seem more abundant in general in comparison to VW and HO. *Nitrospira* are lithoautotrophs and are known to perform nitrification (Spieck and Bock, 2015).

To further investigate differences between water and tubercle samples, we analysed the core microbiome here defined as those families present in at least 85% of all samples of the same type and with a relative abundance of >1%. As there too many families in the dataset we will describe the core microbiome at the order level here. Orders in the core microbiome occurring in both types (water and tubercle) were *Rhizobiales*, *Microtrichales*, *Burkholderiales*, *Rhodobacterales*, *Sphingomonadales*, *Caulobacterales*, *Methylococcales*, and *Micrococcales*. Orders occurring only in the core microbiome of type water were *Chitinophagales*, *SAR11 clade*, *Methylacidiphilales*, *Corynebacteriales*, *Frankiales*, *Synechococcales*, *Pseudanabaenales*, *Sphingobacteriales*, *Cytophagales*, *Flavobacteriales*, *Verrucomicrobiales*, and *Chthoniobacteriales*. Orders occurring only in the type tubercle were *Holophagales*,

Gaiellales, *Alphaproteobacteria* Class, *Nitrospirales*, *Desulfovirbionales*, *Lactobacillales* and *Myxococcales*. Using a heatmap it becomes clear that there is a correlation between specific taxa and the sample type, this correlation is stronger than to site.

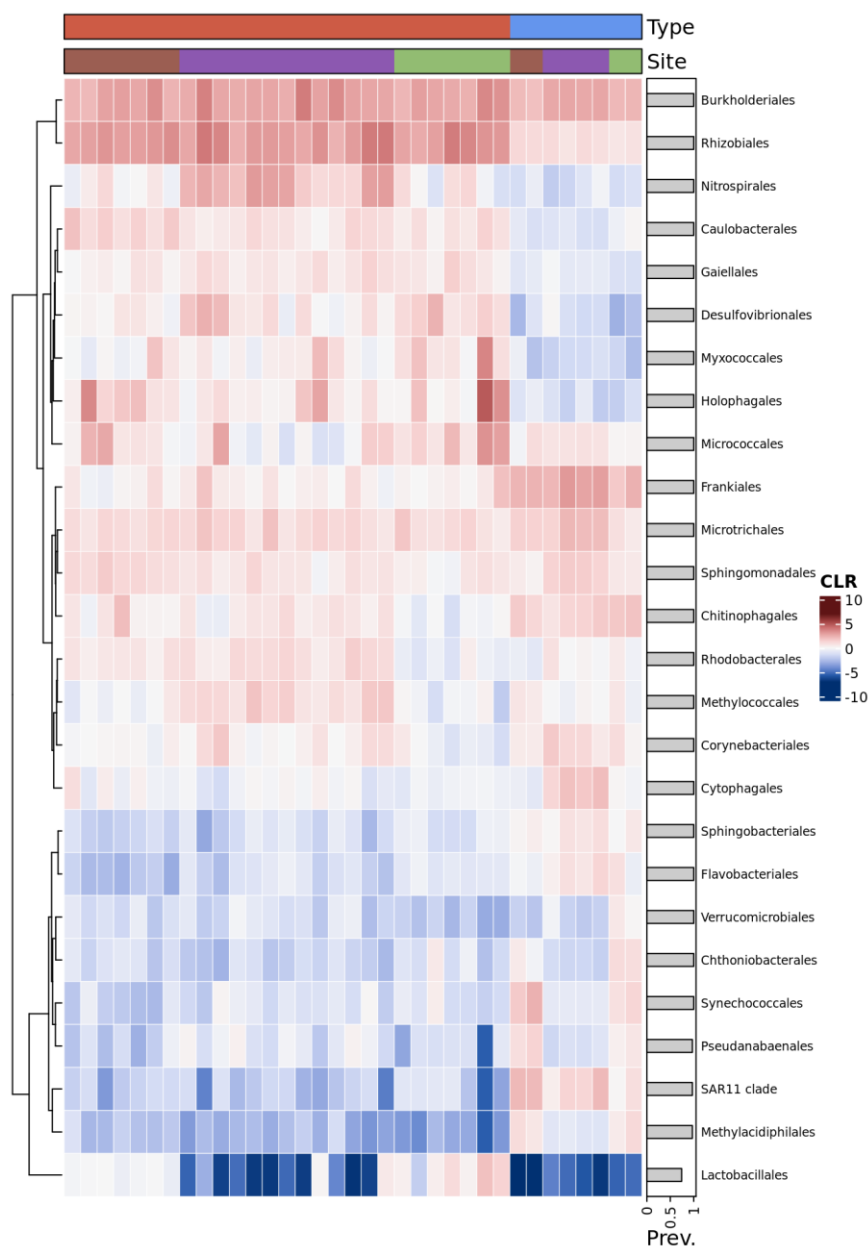


Figure 20: Heat map of the core microbiome for water and tubercle samples from site RG, HO and VW sorted according to sample type and site.

Additionally, we performed a statistical test to see which taxa occur significantly more often in sample type tubercle or water. For that only the core microbiome was tested (see above). The result is shown using a treeplot (Figure 21). Using the treeplot also the phylogenetic belonging of the taxa is shown. Interestingly there are whole phyla which occur significantly more often in type tubercle than type water for example the Firmicutes. On the other hand, some taxa only significantly differ on the class level (*Alphaproteobacteria* occur significantly more often within the tubercles) and/or on the family level (or below, not shown). Other orders occurring significantly more often in type tubercle are: *Desulfovirbionales*, *Micrococcales*, *Rhizobiales*, *Gaiellales*, *Nitrospirales*, *Holophagales*, *Rhodobacteriales*, *Xanthomonadales* and *Caulobacteriales*.

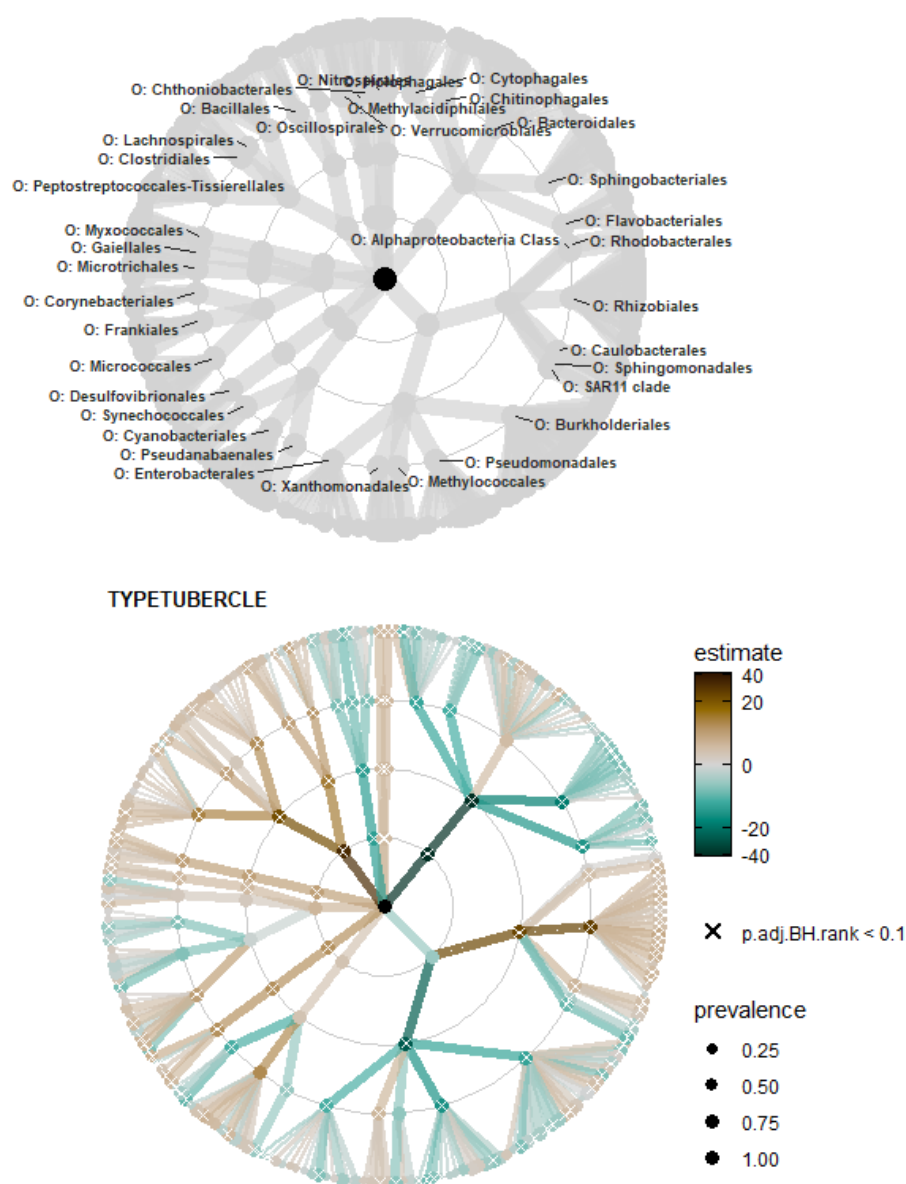


Figure 21: Tree plot showing the occurring core microbiome, all taxa occurring more often in type tubercle are in brown, all taxa occurring more often in type water are in blue. If this difference is significant the branch is marked (x). The key shows the most abundant orders exemplarily.

4.1.4 Correlation of the core microbiome with environmental parameters

To elucidate the influence of environmental parameters we correlated the most abundant taxa in tubercle and separately in water samples to environmental parameters. To do so, we only used the environmental parameters which were mostly complete in the dataset and chose the 25 most abundant taxa in each sample type on the genus level as we expect that the different species within one genus show similar correlations to environmental parameters. By comparing the two heat maps Figure 22 and Figure 23 it appears that the correlation is overall stronger to the “water taxa” than to the “tubercle taxa”. This supports the idea that the tubercle environment strongly differs from the water and so environmental parameters measured in the water column do not correlate to the tubercle microbial community. Nevertheless, for both sample types some correlations of taxa with specific environmental parameters can be observed. The tubercles show a block of genera (e.g. *Cellulomonas*, *Afipia*, *Geothrix*, *Rhodoferrax*, *Sideroxydans*) which correlate negatively with the conductivity and the pH on the sampling day and positively with the annual oxygen concentrations and the temperature. In contrast,

another block of genera (e.g. *Nitrospira*, *Sulfuricella*, (*Thiobacillus*, *Acidovorax*), *Gallionella*, *Desulfovibrio*) is positively correlated to the pH and the conductivity on the day of sampling and negatively correlated to the annual oxygen concentrations and the temperature on the day of sampling.

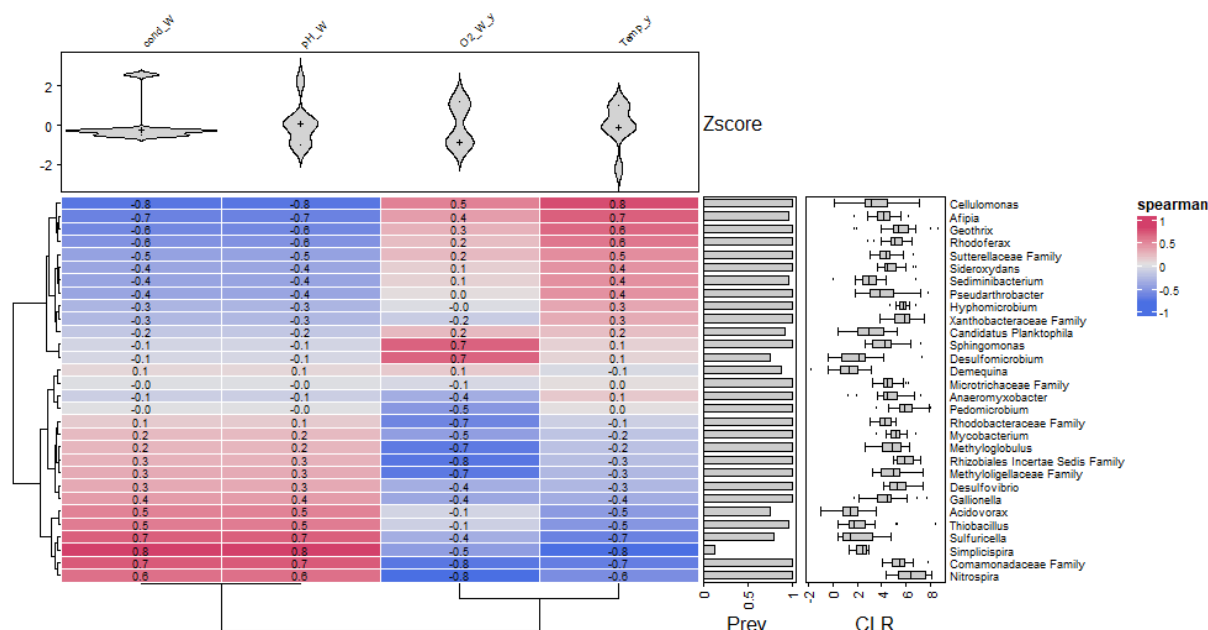


Figure 22: Heatmap showing the top 30 genera of tubercles in the Vosswinkel, Himmelpfort and Regow dataset.

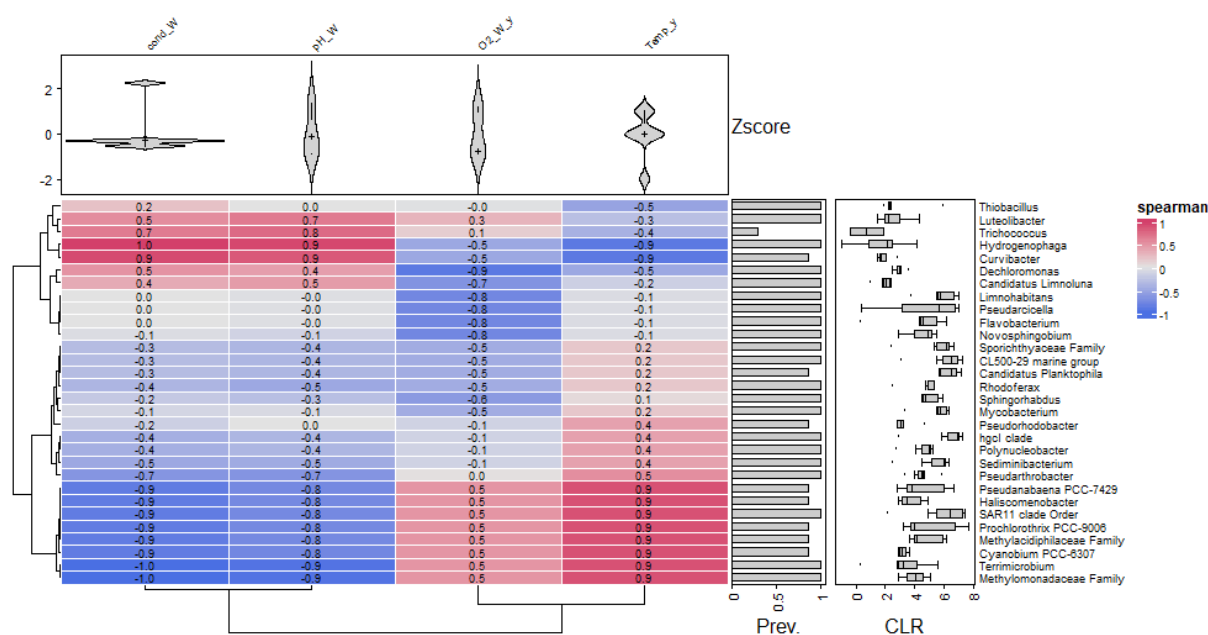


Figure 23: Heatmap showing the top 30 genera of water in Vosswinkel, Himmelpfort and Regow dataset.

Within the water samples, multiple blocks of genera can be observed. Some genera only correlate to the annual oxygen concentrations (e.g. *Ca. Limnoluna*, *Limnohabitans*, *Flavobacterium*). Another block correlates strongly (negatively) with the conductivity and pH on the day of sampling and positively with the annual oxygen and the temperature on the sampling day (e.g. *Methylomondaceae*, *Terrimicrobium*, *Pseudoanabaena*, *Prochlorothrix*, *Cyanobium*). Some genera (e.g. *Hydrogenophaga*, *Curvibacter*, *Dechloromonas*) correlate positively with the pH and conductivity on the sampling day and negatively with annual oxygen and temperature (sampling day).

To further investigate the influence of environmental parameters we correlated more measured environmental parameters with the core community of both types (water and tubercle). However, the dataset is not complete thus only a limited number of samples is included in this analysis. Interestingly, there are taxa correlating positively to the annual temperature and negatively to the temperature on the day of sampling (e.g. *Cellulomonas*, *Geothrix*, *Rhodoferrax*) or the other way around (e.g. *Acidovorax*, *Thiobacillus*, *Aeromonas*, *Sulfuricella*, *Shewanella*) similarly for pH. Other correlations appear more intuitive e.g. the strong positive correlation of taxa such as *Nitrospira* with the nitrate concentrations in the water.

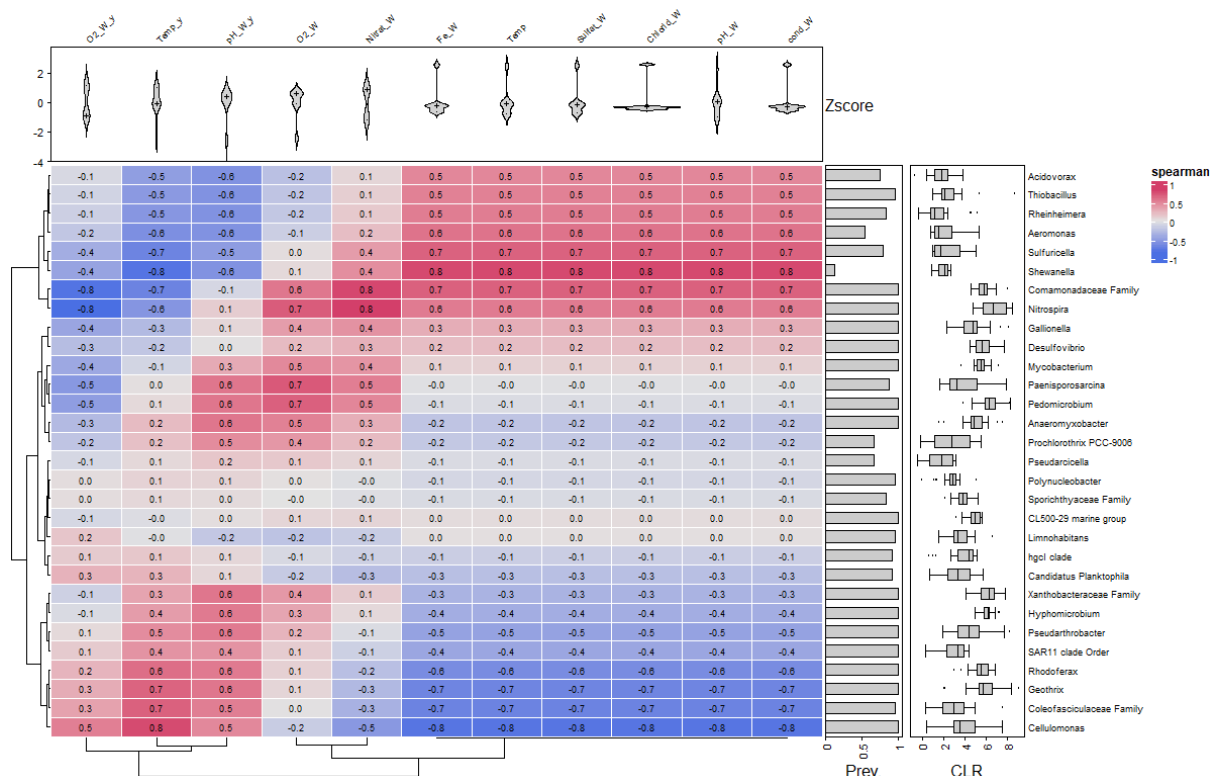


Figure 24: Heatmap showing the correlation between environmental parameters and the core community of type water and type tubercle samples.

4.1.5 Summary of the core microbiome in the sample types water and tubercle

As a summary the microbial orders only occurring in sample type water or tubercle as well as the taxa occurring in both sample types Figure 25 is shown below. Orders only occurring in type tubercle were *Gaiellales*, *Lactobacillales*, *Alphaproteobacteria Class*, *Nitrospirales*, *Myxococcales*, *Holophagales* and *Desulfovibrionales*.

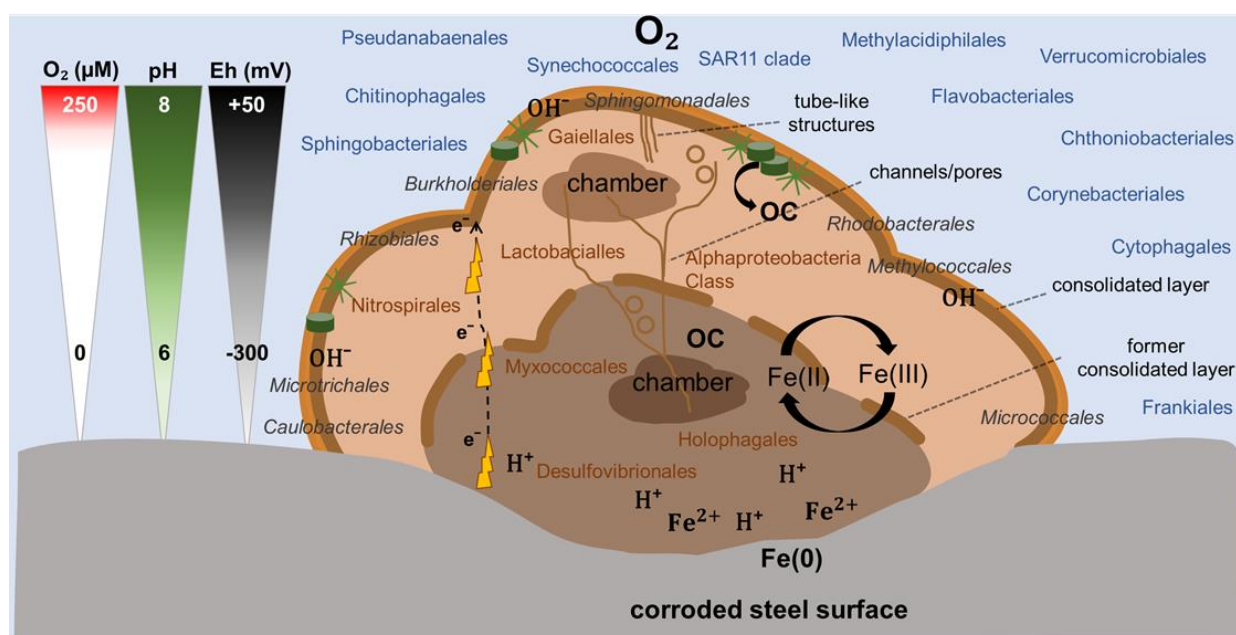


Figure 25: Summary scheme of a tubercle and its surrounding water phase, modified after Fiskal et al. (2023). Taxa occurring only within the tubercle are in brown, taxa occurring in water and tubercle are in grey and taxa occurring only in the water are in blue.

4.2 Field study: Microbially influenced corrosion and rust tubercle formation on sheet piles in freshwater systems

This field study is published open access, please see (Fiskal et al., 2023) for the results and discussion sections. Please find the abstract and a summary figure (Figure 26) from the published article below.

The extent of how complex natural microbial communities contribute to metal corrosion is still not fully resolved, especially not for freshwater environments. In order to elucidate the key processes, we investigated rust tubercles forming massively on sheet piles along the river Havel (Germany) applying a complementary set of techniques. In-situ microsensor profiling revealed steep gradients of O_2 , redox potential and pH within the tubercle. Micro-computed tomography and scanning electron microscopy showed a multi-layered inner structure with chambers and channels and various organisms embedded in the mineral matrix. Using Mössbauer spectroscopy we identified typical corrosion products including electrically conductive iron (Fe) minerals. Determination of bacterial gene copy numbers and sequencing of 16S rRNA and 18S rRNA amplicons supported a densely populated tubercle matrix with a phylogenetically and metabolically diverse microbial community. Based on our results and previous models of physicochemical reactions, we propose here a comprehensive concept of tubercle formation highlighting the crucial reactions and microorganisms involved (such as phototrophs, fermenting bacteria, dissimilatory sulphate and Fe(III) reducers) in metal corrosion in freshwaters.

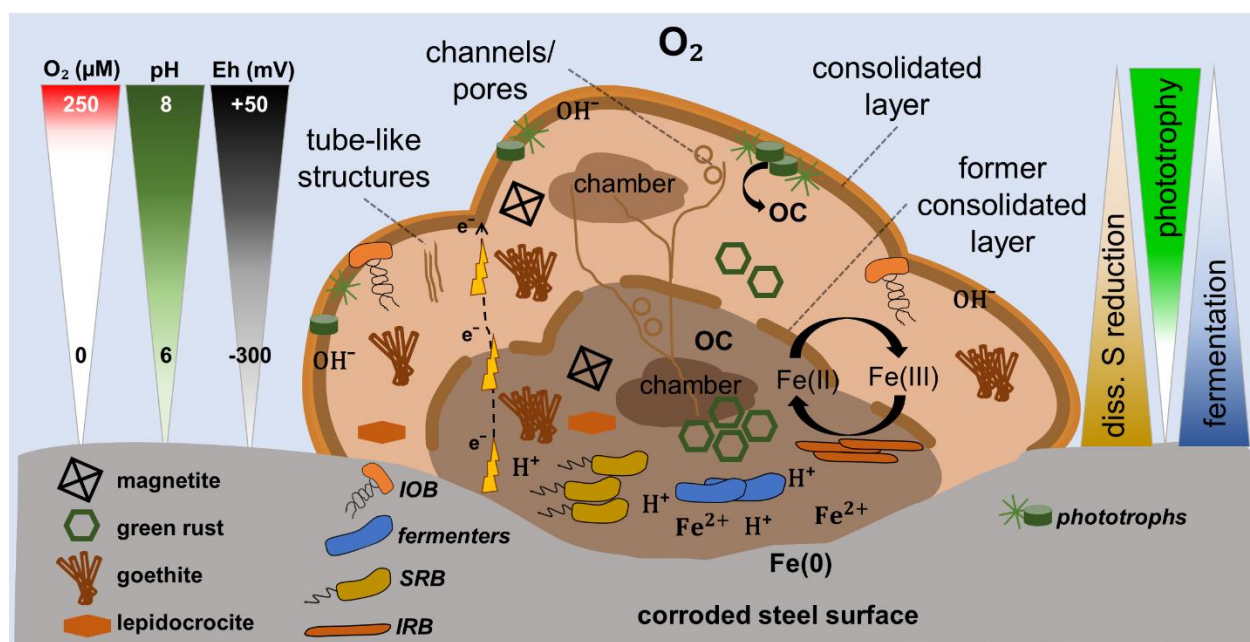


Figure 26: Conceptual model of a freshwater tubercle based on previously proposed schemes by Ray et al. (2010b) and Sarin et al. (2004b). Major features are the highly complex architecture with different compartments (in the scheme only two are shown for simplicity) containing chamber-like cavities, tube-like structures and highly consolidated layers. Strong gradients of pH, O_2 and redox potential occur. The relative importance of microbial metabolisms changes with penetration depth: fermentation and dissimilatory reduction of S compounds increase towards the inside, whereas phototrophy increases towards the outside of the tubercle. Labile organic carbon (OC) is provided by phototrophs supporting chemoorganoheterotrophy and mixotrophy. EPS stabilize tubercle matrix. Internal "cycling of Fe and S compounds accelerates and prolongs the overall corrosion process. A mixed mineral matrix develops over time, undergoing precipitation and re-dissolutions, some minerals are electrically conductive thus conducting electrons from anodic to cathodic sites and supporting microbes capable of extra cellular electron transport.

4.3 Field study: Temporal development of microbially influenced corrosion and rust tubercle formation on steel coupons in freshwater systems

For the experimental set up please see the Methodology parts 3.3 and 3.4. An overview of all site information and analysed parameters can be found in S5 A and B.

4.3.1 Visual observations of steel coupons during exposition experiments

Visual observations of the steel coupons over time show the progression of the biofilm formation (Figure 27 and Figure 28). Figure 28 shows exemplary images of coupons from Mosel before and after cleaning. In contrast to other sampling sites, the incubation time for coupons in Mosel and Rhein was rather short (5 weeks instead of over a year). Furthermore, sampling on these two sites was performed once a week instead of every three months. However, even in this short time period, the visual appearance of the tubercles differs between the two sites. At site Mosel the whole coupon has a thin rusty to whitish film (after drying) and some hot spots with actual tubercles. At T4 (4 weeks old coupon) other plant material can also be observed, indicating the formation of a more advanced biofilm. In contrast, coupons from the river Rhein do not show much growth at T1 (1-week-old coupon). Only at T2 (2 weeks old coupon) a rusty layer can be seen, although it is less homogenous and has small nub like mounds and stripes. The mounds seem to grow bigger over time and also grow together.

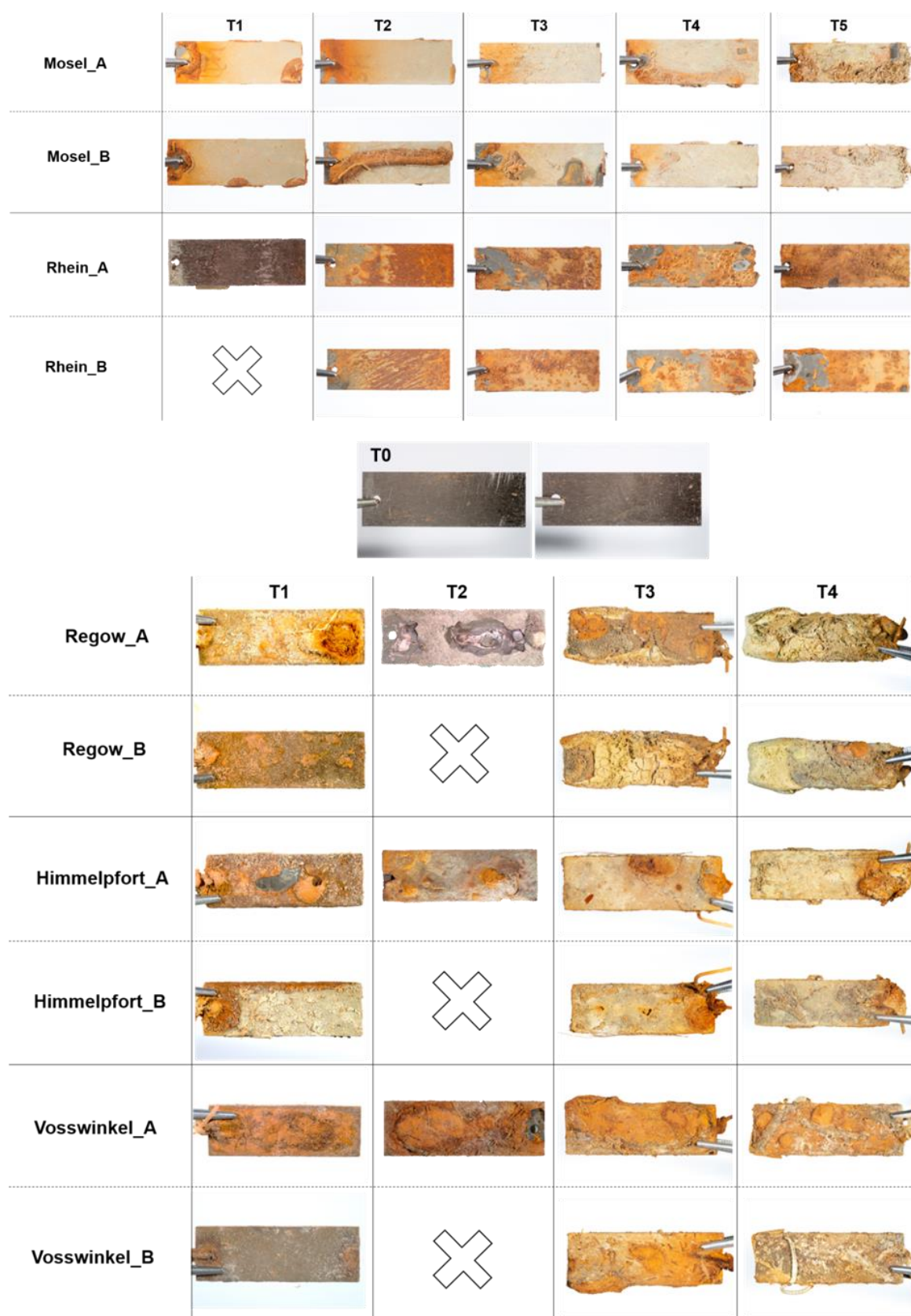


Figure 27: Photographs of the steel coupons after drying and fixation for SEM for top: Rhein and Mosel for T1-T5 (5 weeks in total); bottom: Regow, Himmelpfort and Voßwinkel (bottom) for T1-T4 (every three months).

At sites Regow, Himmelpfort and Vosswinkel, exposition was performed for over one year and coupons were sampled every three months (at 4 timepoints in total). T1 (~3 months old coupon) showed a

mature biofilm and rust tubercles. After 6 months the shape of the coupon was changing through increased growth of algae and other organisms even some chironomid larvae, their tube structures and sponges could be observed. After cleaning, it was recognized that below the rusty tubercles the steel surface was degraded whereas the surface looked visibly intact under other areas of the biofilm (exemplarily shown for three Mosel coupons, all T5, Figure 28).

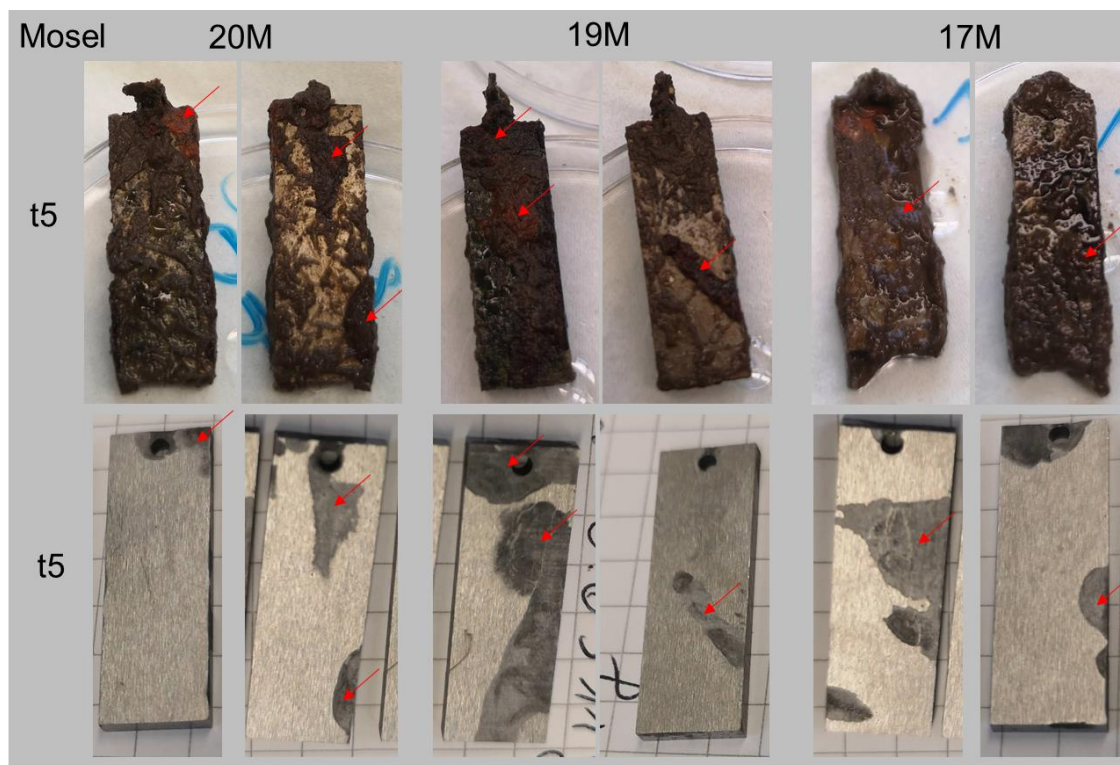


Figure 28: Photographs of the freshly sampled/unfrozen steel coupons after 5 weeks (t5) of exposure in the river Mosel before (top) and after cleaning with inhibited HCl. The red arrows indicate visible rust tubercle formation on the coupons and the corrosion/material loss visible on the cleaned coupon surfaces.

4.3.2 Corrosion rates over time at different sites

After cleaning of the coupons, corrosion rates were determined (Figure 29). At all sites corrosion was fastest at the beginning of exposure and slowed down over time (except for station Rhein where corrosion rates were relatively stable and showed a slight increase towards week 5). The calculated corrosion rates (extrapolated to 1 year for each timepoint) differed by one order of magnitude from a maximum of 0.32 mm/yr (calculated from 1 week of exposure, site Rhein) to a minimum of 0.02 mm/yr (calculated from 489 days of exposure, site Himmelpfort). Even though these extremes occurred at different sites, similar differences were also observed at the same site, emphasizing that exposure time strongly affects the calculated corrosion rates. Calculated corrosion rates showed a best fit using a power function (Figure 29). However just from the behavior of corrosion rates over time it is not possible to elucidate the influence of abiotic vs. biotic corrosion. One could argue that the “nice” fit of all sites and timepoints speaks for a more uniform abiotic process, however also biotic degradation processes are known to follow a similar shape overall. The different reasons for the slowdown of the corrosion rates will be discussed in the following including abiotic and biotic corrosion reactions as a separation is not possible with the current experimental data. At the beginning corrosion was very fast reaching values reported from highly corrosive sites in literature (Wakai et al., 2022). This could be due to i) the presence of oxygen and thus fast oxidation of Fe(II) (Fortin and Langley, 2005), ii) easy access to the surface through microbes (e.g. IOBs, hydrogen-using MOs), iii) reaction products that are still easily removed by the flow (no stagnant conditions), which makes the reaction still favorable

as no products are building up (Deutzmann et al., 2015). After some time, corrosion starts to slow down likely through the buildup of corrosion products (e.g. iron oxides, carbonates) and the formation of a biofilm which will then decrease O₂ concentrations at the metal surface as well as access to the surface by other microbes (Fiskal et al., 2023). After anoxic conditions are established, corrosion continues without direct contact of oxygen to the surface, and biotic corrosion might become more important. This could explain why corrosion is slowing down but does not stop even at 500 days exposition. If corrosion products are broken off or removed (e.g. cleaning of sheet pile walls in locks and wires for maintenance) corrosion rates could increase again. This is indeed also consistent with the observations in the Rhine river (much higher flow rates) and thus less/slower build-up of corrosion products and/or removal of corrosion products over time (Figure 27).

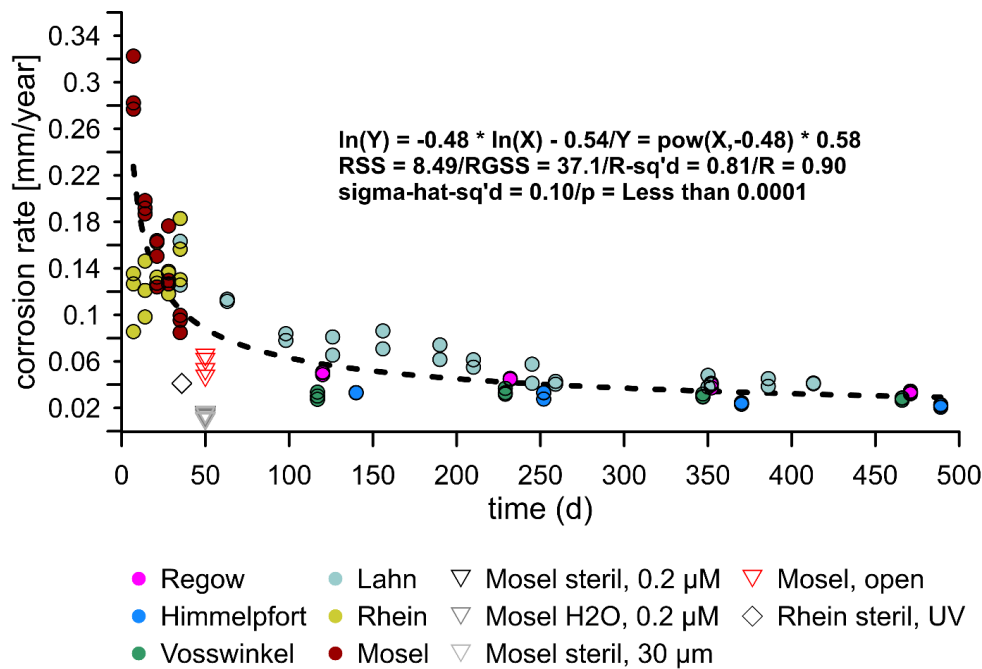


Figure 29: Calculated corrosion rates at all sites and timepoints (circles). Data points are fitted using a power function (formula see plot, $N=90$). Additionally, corrosion rates from an incubation test with sterile filtered Rhein water and UV light and from an exposition test in the Mosel using falcon tubes and different treatments are shown. These tests are not used to fit the power function.

4.3.3 Bacterial abundance in relation to corrosion rates at each site

At the stations Rhein and Mosel (5 weeks exposure time, 5 timepoints sampled), bacterial copy numbers increased over time. Bacterial copy numbers started with 5.79×10^8 nr/g (Rhein) and 1.95×10^8 (Mosel) and reached the highest values at week 5 with 3.03×10^9 (Rhein) and 1.52×10^9 (Mosel). These values are comparable to bacterial copies in tubercles from sheet piles in German water ways (Fiskal et al., 2023). Similarly, weight loss (%) increased over time. However, as discussed above corrosion rates itself decreased (Mosel) and were relatively stable or even increased towards week 5 at station Rhein (Figure 30). This could be due to the higher flow rates and shear forces at the station Rhein leading to a slower buildup of corrosion products/biofilm and keeping the system longer in the “starting” phase. Highest corrosion rates (average of three replicates was ~ 0.30 mm/year) was detected after 1-week exposure in the Mosel, with a decrease towards week 5 to ~ 0.10 mm/year.

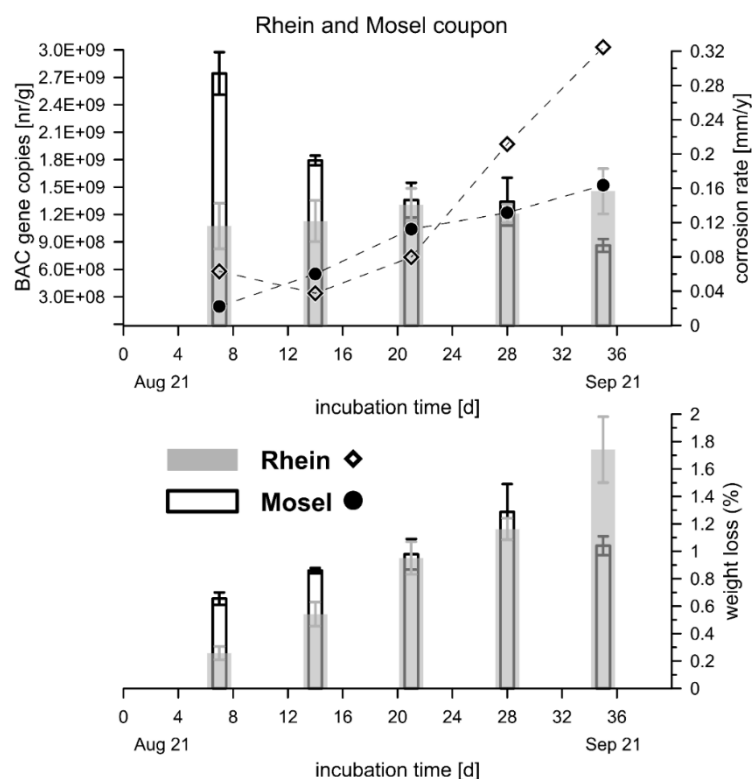


Figure 30: Bacterial copy numbers (lines), corrosion rates and weight losses (bars with SD from triplicate coupons) for Rhein and Mosel.

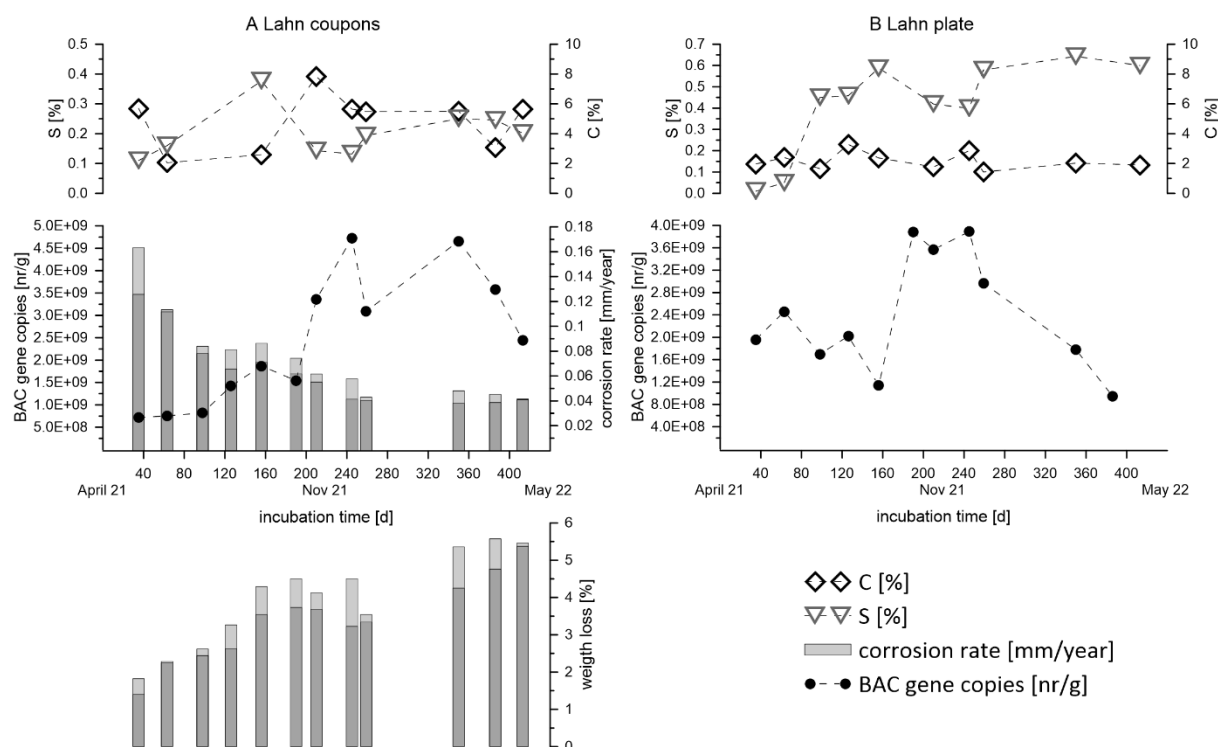


Figure 31: S and C content, bacterial copy numbers, corrosion rates and weight losses (bars for the 2 coupons per timepoint) for A the Lahn coupons and B the Lahn plate, please note corrosion rates could not be calculated for the plate.

At the site Lahn (413 days exposure time, 12 timepoints sampled), corrosion rates also decreased over time from about 0.15 mm/year (t1, 35 days) to 0.04 mm/year after 413 days of exposure. Bacterial copy numbers increased over time from 7.18×10^8 to 4.73×10^9 and then seemed to level off and or decreased again towards the end with values of 3.09 to 4.66×10^9 copies per g sample.

Carbon and sulfur contents were distinct from “non-corrosive” biofilm samples of the same site. Whereas in the non-corrosive biofilm carbon content reached between 16 to 66% C (for data see S5), in the biofilm on the coupons or steel pile plate a maximum of 10% was observed. Interestingly, S content varied less strongly, in all biofilms/tubercles a maximum of about 0.75% was reached (average coupon samples is 0.2%, N=9, of all plate samples 0.4%, N= 10, and of all non-corrosive biofilm samples 0.59%, N=4)).

A qualitative analysis (FTIR) of the mineral phases showed presence of calcite, goethite, lepidocrocite, feldspar and less often quartz (S5).

Corrosion rates at sites RG, VW and HO also decreased over time (less pronounced for VW). Highest corrosion rates were determined at site RG with 0.05 mm/year after 120 d of exposure (t1). Lowest rates were reached at site HO after 489 days with 0.02 mm/year. Similar to sites Lahn, Rhein and Mosel bacterial copy numbers generally increased over time from 6.71×10^8 (VW), 5.72×10^8 (HO) and 5.76×10^8 (RG) to 2.46×10^9 (VW), 1.86×10^9 (HO) and 1.88×10^9 (RG). These copy numbers are similar to copy numbers reported in field studies of corrosion tubercles (Fiskal et al., 2023).

The S and C contents of biofilms at RG, VW and HO were comparable to those of the site Lahn, and as before, samples from non-corrosive biofilms showed much higher C contents (~20%) compared to tubercles from coupons and or plates (S5).

Identified minerals (FTIR) were also similar to site Lahn with calcite, goethite and lepidocrocite occurring most often, and feldspar and quartz less often. In some samples ferrihydrite was additionally detected. In one sample (tubercle from the sheet pile in the lock) Schwertmannite was fitted.

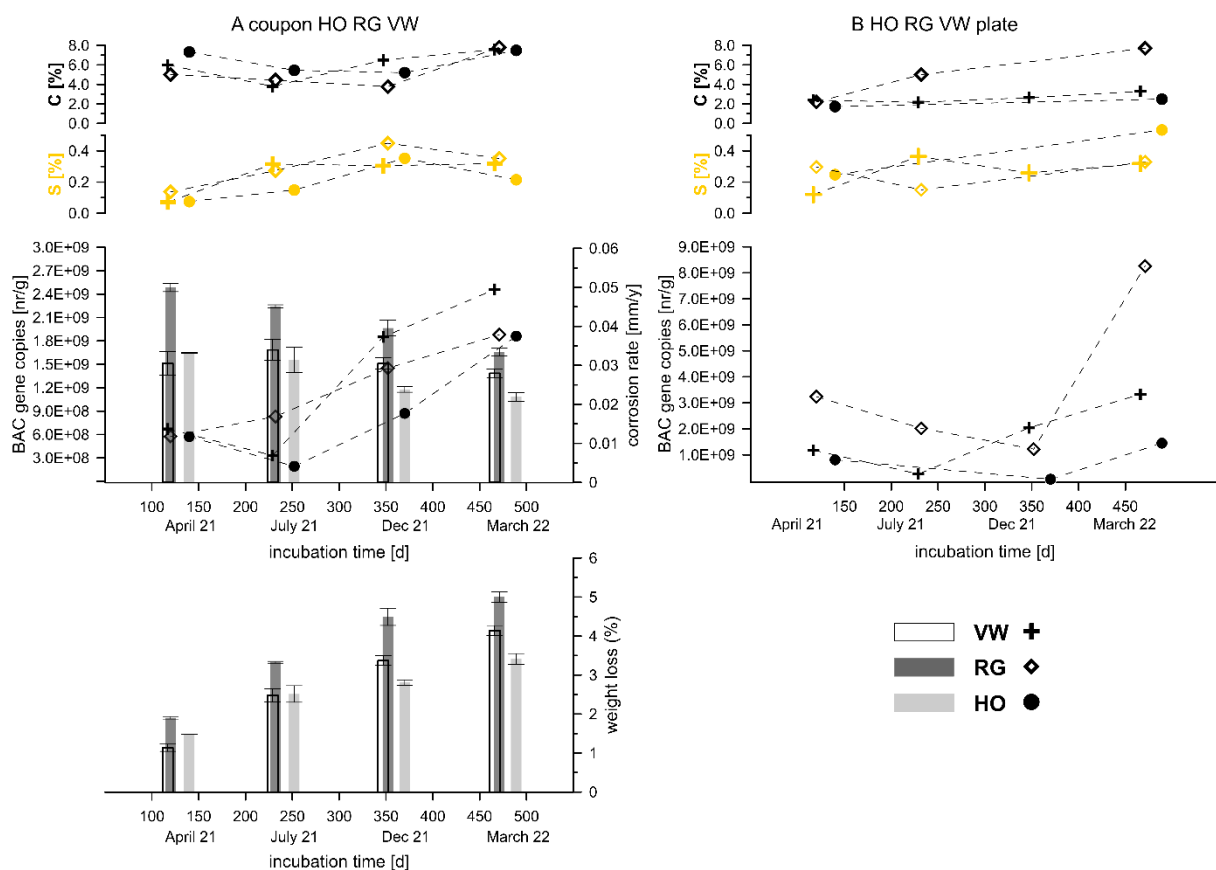


Figure 32: S and C content, bacterial copy numbers and the corrosion rate (as well as weight loss percent) for A the coupons and B the plate of HO, RG and VW, please note corrosion rate could not be calculated for the plate.

4.3.4 SEM analysis of the corrosion coupons at site Mosel and Rhein

Specific timepoints of each site (except site Lahn) were sampled for SEM analysis (see Methods section 3.13). Only site Mosel and Rhein are shown here due to the high-resolution sampling.

For site Mosel 3 timepoints were chosen for SEM analysis (T1, T3 and T5; six images per timepoint; *Figure 33*). At T1 (after 1 week) the coupon was already heavily encrusted by various particles (minerals and presumably organic matter; A, D, E; for T0 images see S6). However, the surface of the coupon is still at least partly visible (B, F) and showed the onset of corrosion pits (B, C). Below the original surface (characterized by parallel stripes/lines) the metal was already degraded (F). After 3 weeks (T3), the coupon was heavily inhabited by cells (diatoms and microbial cells; A-D). Below the diatom dominated biofilm, roundish pits of various size could be observed (E, F) and different mineral precipitates could be observed (B, C). At T5 the diatom dominated biofilm was even more dense and more prokaryotic cells can be observed within the diatoms.

For site Rhein, 3 timepoints were chosen for SEM analysis (T1, T2 and T4, six images per timepoint; *Figure 34*). At T1 (after 1 week) the coupon was nearly covered by mineral deposits (A-D), presumably Fe oxides (C) and carbonates (B). The original surface seemed to be mostly intact (E). However, small bumpy structures (F; presumably the start of a corrosion tubercle) could be found. At T2, mineral deposits still dominated the surface of the coupon (A, E, F). However, the surface looked more degraded (A-C). At T4, diatoms cells (F) could be found on the coupon surface, but their density was much lower than on the Mosel coupons at T3 or T5. Similar to Mosel on T3, corrosion pits could be observed (A, C) together with a number of different mineral deposits, presumably carbonates (D) and Fe oxides (E, F).

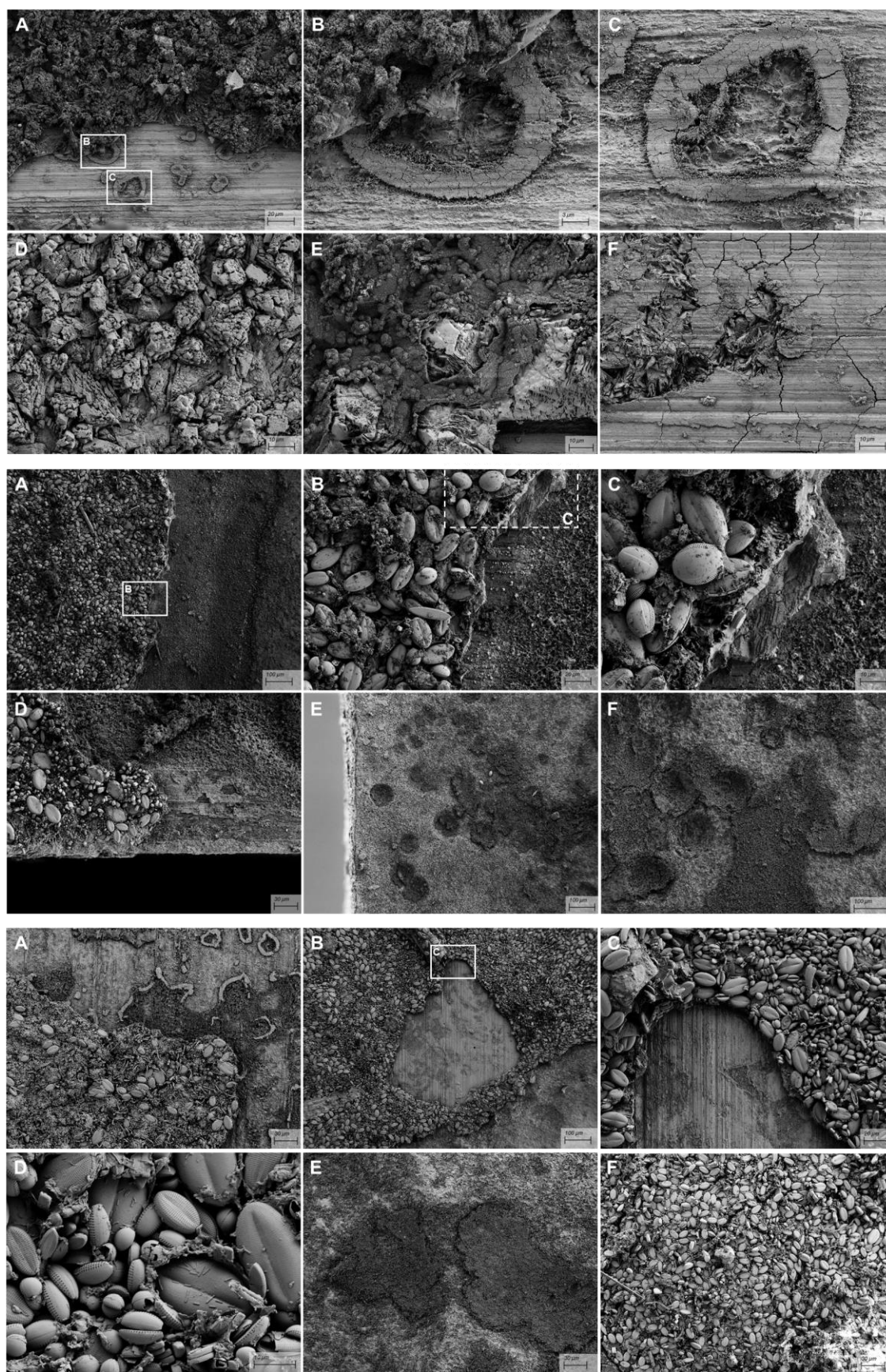


Figure 33: SEM micrographs of Mosel coupons T1, T3 and T5.

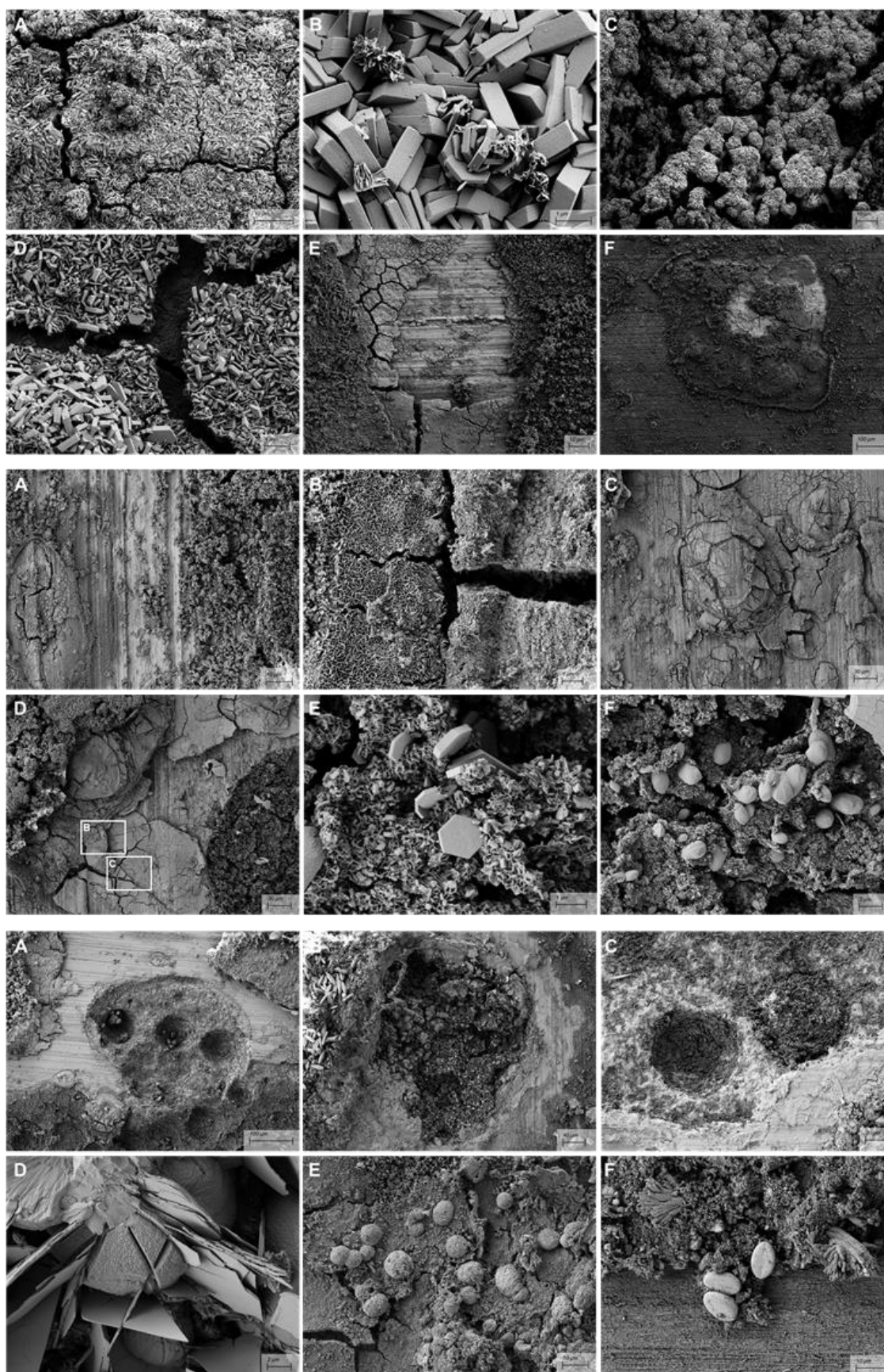


Figure 34: SEM micrographs of Rhein coupons T1, T2 and T4.

4.3.5 Changes of the microbial community over time

NMDS plots (Figure 35) revealed that composition of the microbial community of the buoy samples followed a circular change nearly following a yearly cycle. Thus, microbial community composition for T11 was very similar to those for T1 (both spring season of two following years). In contrast, the microbial communities in coupon and plate samples shifted more strongly and were not similar between the beginning and the end of the incubation. It seems that the community composition of the buoy samples was driven mostly by seasonal patterns, whereas the microbial communities in tubercles on coupon or plate samples were more influenced by other factors (i.e., the metabolic succession during corrosion). Furthermore, it seems likely, that coupon and plate samples were less dominated by phototrophic organisms (see also chapter 4.1.3) and are thus less dependent on seasonally changing light intensities. However, biofilm T1 of the buoy was not directly comparable to T1 of coupon and plate samples (see also Methods and above), because the surface of the buoy biofilm samples was not cleaned before the experiment was started.

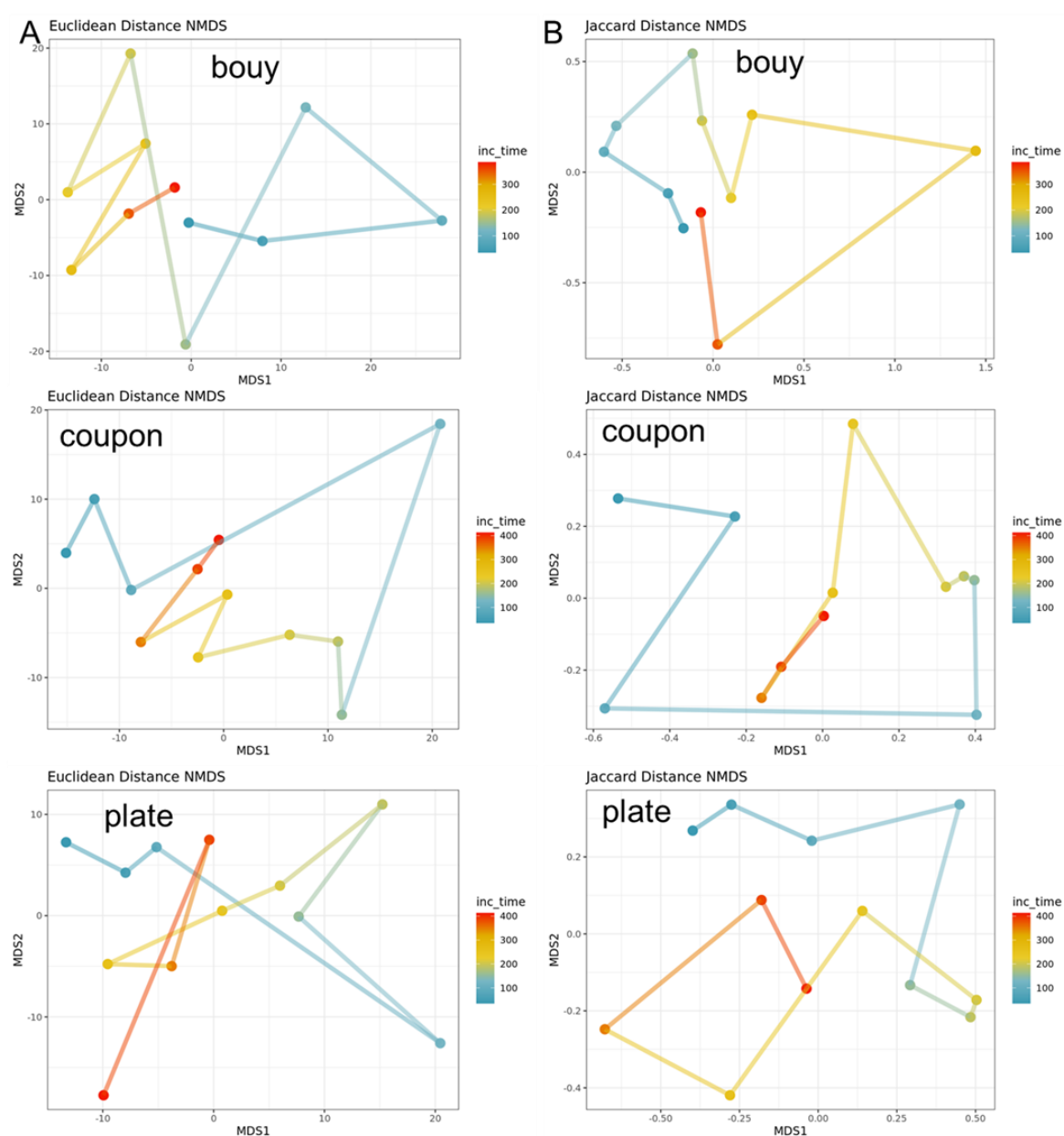


Figure 35: NMDS plot for the buoy (first row), coupon (second row) and plate (third row) at site Lahn for the microbial community (16S rRNA gene analysis) over time (days) using two different distance calculations (A: Euclidean, B: Jaccard).

4.3.5.1 Bacterial and archaeal taxa in relation to environmental/water parameters

For Lahn samples, 10-12 timepoints were available for over one year of exposition time, with a nearly complete set of environmental parameters. Hence, the following correlations were performed on the Lahn dataset only.

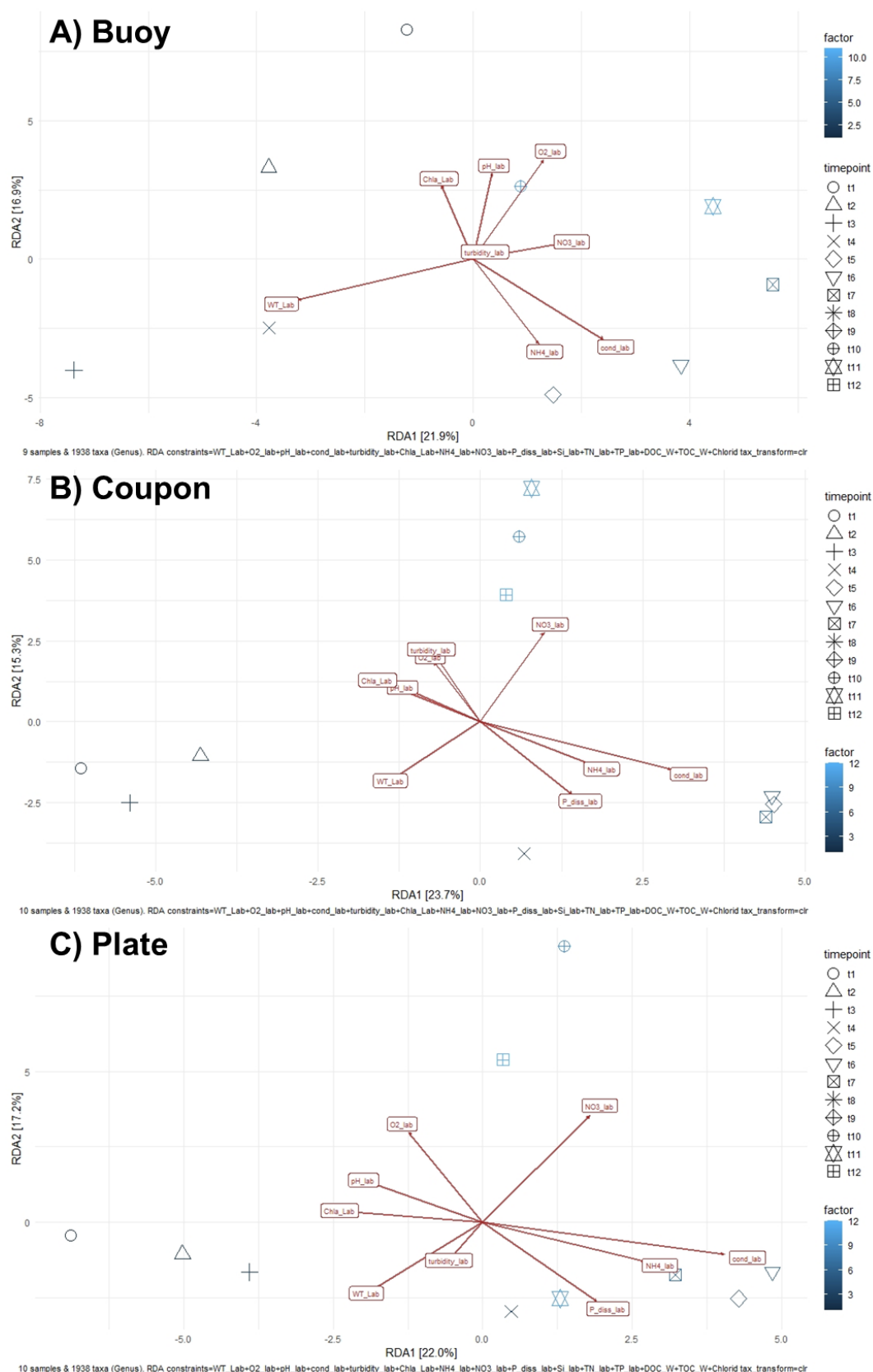


Figure 36: RDA analysis (on the genus level) using only environmental parameters as predictors for the microbial community composition for A) the buoy, B) coupon and C) the plate samples.

The RDA analysis in Figure 36 shows the influence of environmental parameters on the microbial community composition. It can give hints which environmental factors drive the observed changes within the microbial community composition and its change over time. Similarly, to the NMDS used in Figure 35 one can see that the buoy shows a more seasonal cycle of the microbial community compared to the coupon and plate. On average the arrows of the measured water parameters were longer which means the correlation were stronger with the buoy samples. The highest explanatory power had the water temperature pointing to timepoints T3 (07.07.2021) and T4 (04.08.2021). The microbial community composition at T11 was negatively correlated with temperature (21.04.2022). Other parameters which might explain the observed changes in the microbial community composition were the conductivity and ammonia concentrations (higher towards T5, 03.09.2021 and T6, 07.10.2021). Additionally, oxygen (and pH) as well as nitrate concentrations seemed to explain some of the variation (pointing towards T10, 16.03.2021, and T11, respectively). Chla concentrations point towards the microbial community composition of T1 (05.05.2021). Turbidity had nearly no explanatory power for the change in the buoy microbial community composition. The RDA of the coupon and the plate were comparable to each other, but different to that of the buoy sample. As shown before (NMDS analysis in Figure 35) the microbial community was less influenced by season compared to the non-corrosive biofilm on the buoy. Here the t12 samples were more different from t1 compared to the non-corrosive biofilm even though the same season of two consecutive years was sampled. For the RDA of the coupon and plate microbial community composition, the conductivity had the highest explanatory power pointing towards T5, T6, T7 (September to October), together with ammonia and phosphate concentrations. Also nitrate concentrations seemed to influence the distribution of the microbial community composition (positively correlated towards T10, T11 and T12 (March to May, 2022). Another cluster of samples formed, here no environmental parameter directly points towards those samples, namely T1 to T3 (May to July, 2021). Those samples lay in between the arrows of turbidity, oxygen, pH, Chla (opposite to the arrows of ammonium, phosphate and conductivity) and the water temperature.

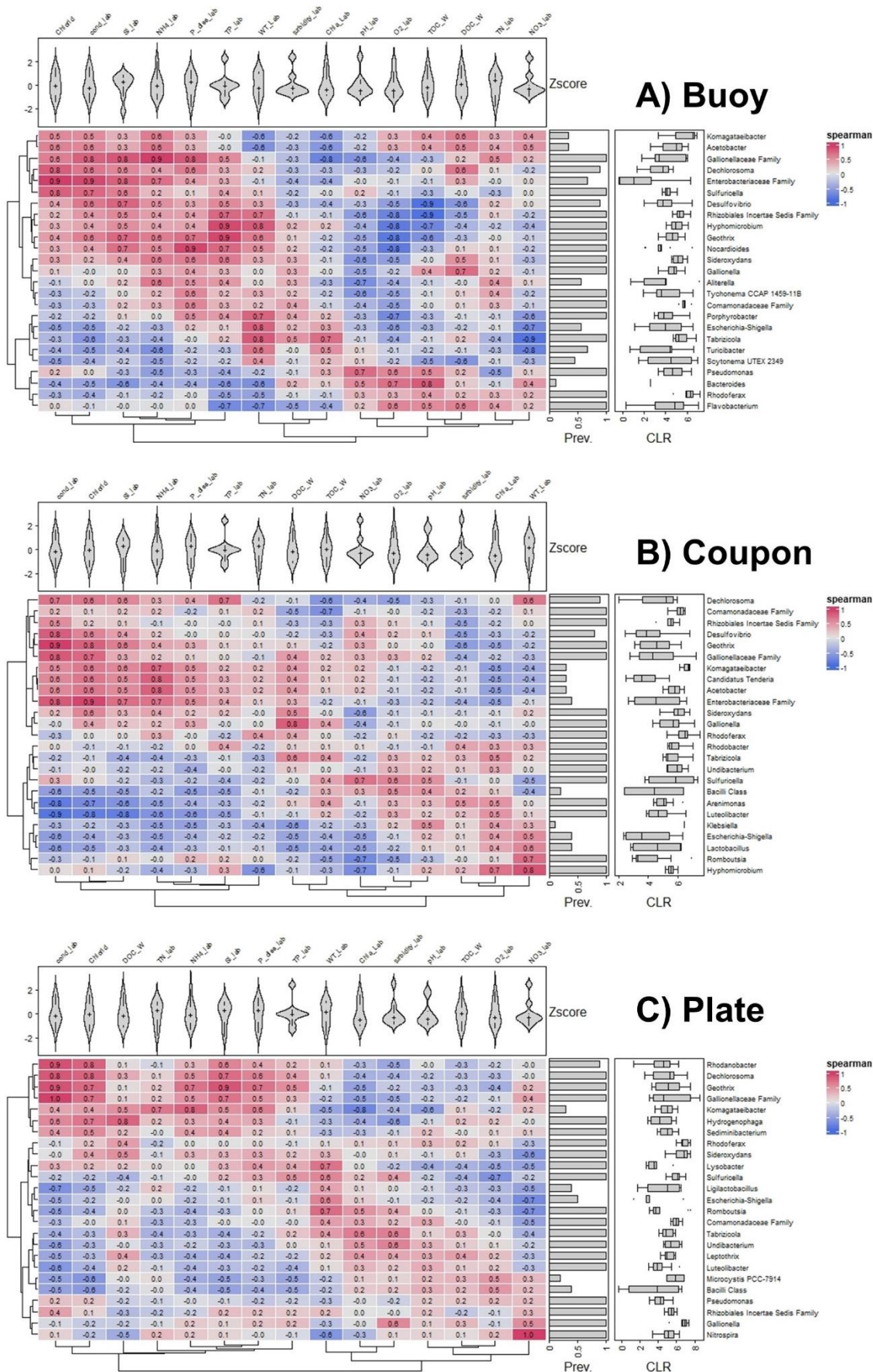


Figure 37: Spearman correlations of the most abundant taxa for A) the buoy, B) the coupon and C) the plate samples. Please note the most abundant taxa are different in A), B) and C).

When correlating the most abundant genera with environmental parameter one can see which genera were driven by which measured water parameter. Also, here one can observe that overall the most abundant taxa in the non-corrosive biofilm were more strongly correlated with the measured water parameters than the most abundant taxa (which differ with sample type) of the corrosive biofilm on the plate and the coupon. As shown also in the other field studies of this project (please see also 4.1 and 4.2), the tubercle environment is very different from the surrounding water phase, thus the measured environmental parameters in the water fail to explain the microbial community of the plate and coupon biofilm. The microbes change their environment through their metabolism, thus measuring water parameters in the field is not a good indicator for MIC in general.

Nevertheless, some correlations could be determined. For the non-corrosive biofilm on the buoy samples there was a cluster of taxa which correlated positively with chloride, conductivity, silicate, ammonium and phosphate concentrations, namely *Komgataibacter*, *Acetobacter*, *Gallionellaceae Family*, *Dechlorosoma*, *Enterobacteriaceae Family*, *Sulfuricella*, *Desulfovibrio*, *Rhizobiales*, *Hyphomicrobium*, *Geothrix Nocardioles*, *Sideroxydans* and *Gallionella*. Some taxa of this cluster were negatively correlated with oxygen concentrations, pH and TOC/DOC content, which are *Desulfovibrio*, *Rhizobiales Incertae Sedis*, *Hyphomicrobium*, *Geothrix* and *Nocardioles*. Others such as *Pseudomonas*, *Bacteroides*, *Rhodospirillum* and *Flavobacterium* were positively correlated with pH, oxygen TOC and DOC concentrations. Some taxa were positively correlated with water temperature (e.g. *Desulfovibrio*, *Rhizobiales*, *Hyphomicrobium*, *Escherichia-Shigella*, *Tabrizicola*, *Porphyrobacter*).

For the coupon (and plate) microbial community, the taxa show the same general correlation towards the environmental variables. For example, the cluster of taxa positively correlated to conductivity (*Dechlorosoma*, *Rhizobiales*, *Desulfovibrio*, *Geothrix*, *Gallionellaceae Family* and others). However, often the correlation was strong and the most abundant taxa were not the same.

4.3.5.2 Temporal development of eukaryotic taxa over time

On average *Bacillariophyceae* (genera with the highest relative abundance: *Stephanodiscus*, *Gomphonema*, *Nitzschia*, *Surirella*, *Cocconeis*, *Thalassiosira*, *Navicula*) are the most abundant group (relative abundance) in the buoy samples, especially from October to December their relative share is high. Another group found in relatively high abundance are the *Ulvophyceae* (genera with the highest relative abundance: *Cladophora*, *Basycladia*, *Trochosarcina*, *Pseudendoclonium*). Also, *Chlorophyceae* (*Chlamydomonium*, *Stegoclonium*, *Chlamydomonas*, *Paulschulzia*, *Chlorochytrium*) and *Trebouxioophyceae* (*Micractinium*, *Choricystis*, *Picochlorum*, *Chlorella*, *Microthamnion*) are abundant at nearly all timepoints. Some samples show high relative abundances in *Annelida* (many unassigned genera; *Aelosoma*, *Nais*, *Tubifex*, *Lumbricillus*, *Pristina*) *Bryozoa* (*Plumatella*, *Paludicella*), *Arthropoda* (*Crustacea*: *Eurytemora*, *Nitokra*, *Cypridopsis* (*Ostracoda*); *Hexapoda*: *Orthocladus*, *Simulium*, *Hydropsyche*, *Cricotopus*, *Tanytarsus*; *Arachnida*: *Hydrozetes*) and/or *Chytridiomycota* (*Rhizophidiales*, *Chytridiomycotina*, *Chytridiomycetes*, *LKM11-Rozella*, *Rhizophyidium*, *Mesochytrium*) however their distribution is patchier. This is similar for the coupon and plate samples, however on the coupon biofilm the *Ulvophyceae*, *Nematoda* (*Chromadorina*, *Punctodora*, *Monhystera*, *Enoplea*, *Rhabdolaimus*) and *Arthropoda* are more abundant on average. On the plate biofilm some samples show higher relative abundances of *Cnidaria* (*Cordylophora*, *Craspedacusta*, *Hydra*).

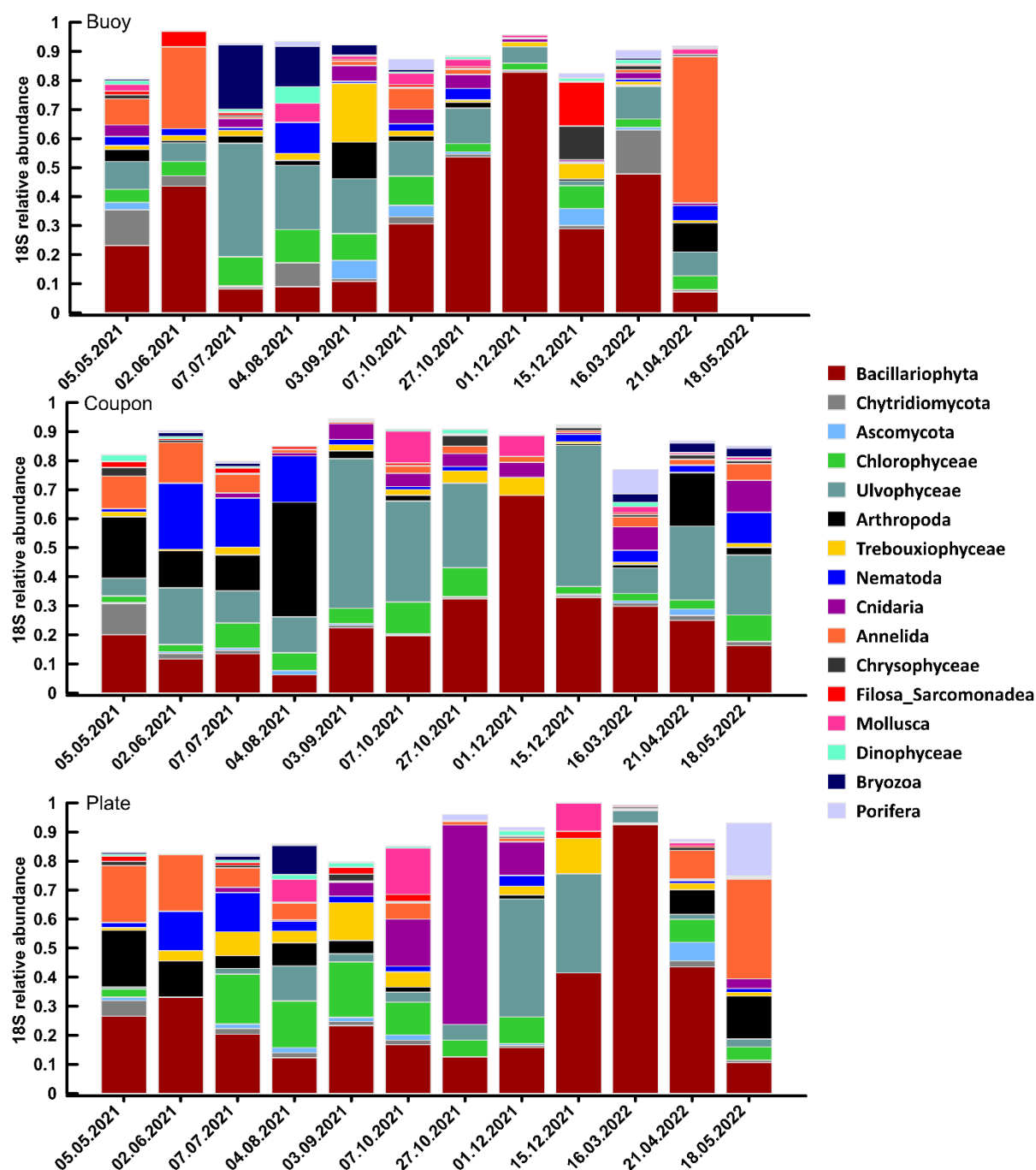


Figure 38: Eucaryotic community composition (based on amplicon sequencing) at site lahn for the bouy, coupon and plate samples over time.

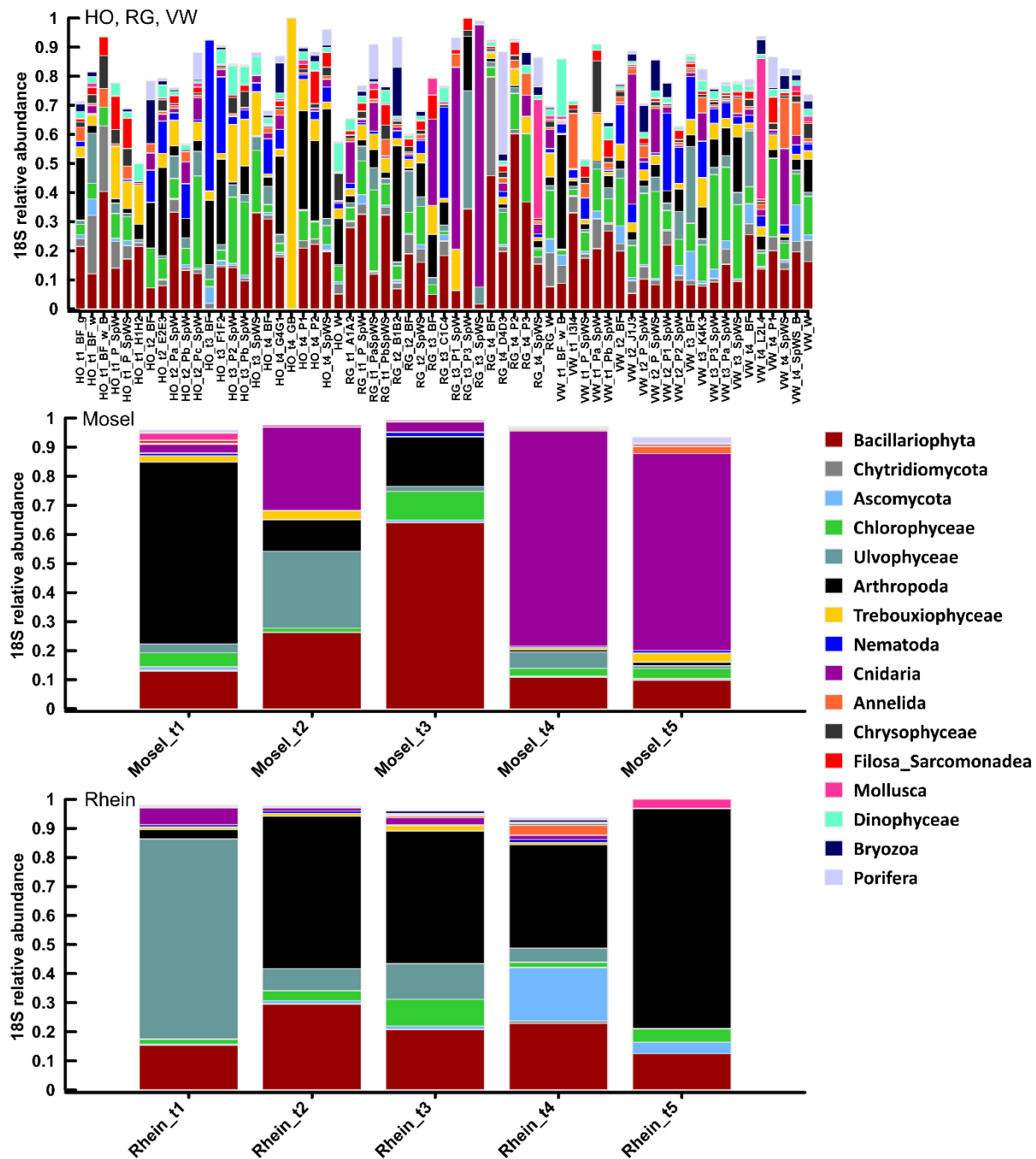


Figure 39: Eucaryotic community composition (based on amplicon sequencing) at sites Regow, Himmelpfort, Vosswinkel and site Mosel and site Rhein.

For sites RG, HO and VW, *Bacillariophyceae* (genera with the highest relative abundance: *Stephanodiscus*, *Navicula*, *Staurosira*, *Fragilaria*, *Pseudostaurosira*, *Gomphonema*) and *Chlorophyceae* (*Scenedesmus*, *Desmodesmus*, *Chlamydomonas*, *Stigeoclonium*, *Monoraphidium*) as well as *Trebouxiophyceae* (*Chlorella*, *Choricystis*, *Chloridium*, *Meyerella*, *Displosphaera*) are abundant at nearly all timepoints and sample types. Other groups found in relatively high abundance are the *Ulvophyceae* (*Trichosarcina*, *Cladophora*, *Dilabifilum*, *Choroithrix*, *Pseudendoclonium*, *Basicladia*) and *Arthropoda* Hexapoda: *Hydropsyche*, *Cardiocladius*, *Orthocladius*; Crustacea: *Nitokra*, *Itunella*, *Chydorus* (*Branchiopoda*), *Cypridopsis* and *Candona* (*Ostracoda*), *Arachnida*: *Afronothrus*). Some samples show high relative abundances in *Annelida* (many unassigned genera; *Aelosoma*, *Epirodrilus*, *Nais*, *Tubifex*, *Lumbricillus*, *Pristina*), *Bryozoa* (*Plumatella*, *Paludicella*), *Chytridiomycota* (*Rhizophidiales*,

Chytridiomycotina, *LKM11-Rozella*, *Rhizophyidium*, *Mesochytrium*), *Nematoda* (*Punctodora*, *Chromadorina*, *Tobrilus*, *Limnomermis*) and *Cnidaria* (*Cordylophora*, *Hydra*, *Craspedacusta*).

At site Mosel *Bacillariophyceae* (mostly *Diatoma* followed by *Navicula* and *Gomphonema* as well as *Thalassiosira*) increase in relative abundance until T3, and then decrease again. At the Rhein, *Bacillariophyceae* (mostly *Diatoma*, some *Navicula* and *Gomphonema* as well as *Thalassiosira*) increase from T1 to T2 and then decrease slightly again, no clear trend can be observed. At site Mosel a high relative share of *Cnidaria* (mainly *Cordylophora*) was found (especially T4 and T5) compared to site Rhein. *Arthropoda* (for Mosel: mainly *Endotribelos*, followed by *Simulium* and *Cricotopus*; for Rhein: mainly *Simulium* and *Cardiocladius*, some *Endotribelos*) show a relatively high share at both sites (T1 to T3 at site Mosel and T2 to T5 at site Rhein). Additionally, *Ulvophyceae* (mainly *Cladophora* at both sites) are found in relative high share at both sites (especially Rhein T1 and Mosel T2). *Chlorophyceae* (mainly *Stigeoclonium*) are found at all timepoints of both sites. At site Rhein also *Ascomycota* (*Leptosphaeria*, *Dothideomycetes*) are found (T4 and T5).

4.3.5.3 Temporal development of prokaryotic taxa over time

At site Lahn buoy, coupon and plate samples are clearly dominated by *Gammaproteobacteria* and *Alphaproteobacteria* at all timepoints followed by *Bacteroidia*, *Cyanobacteriia*, *Actinobacteria*, *Bacillilli*, *Clostridia* and others. At the buoy samples a strong shift in relative abundance with time can be observed especially within the *Gammaproteobacteria*.

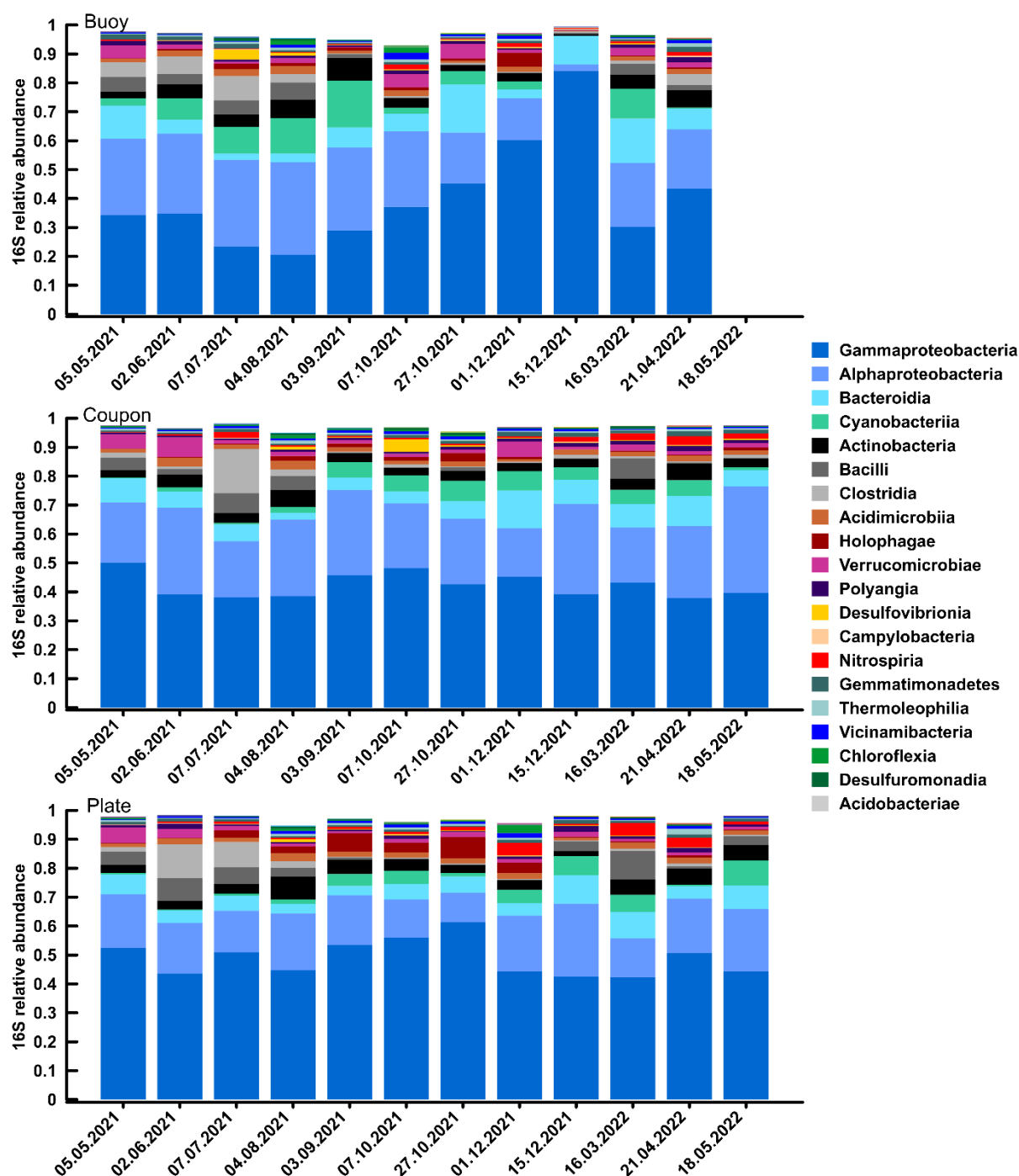


Figure 40: Prokaryotic community composition on the class level based in 16S amplicon sequencing over time for site Lahn (Buoy, Coupon and Plate samples).

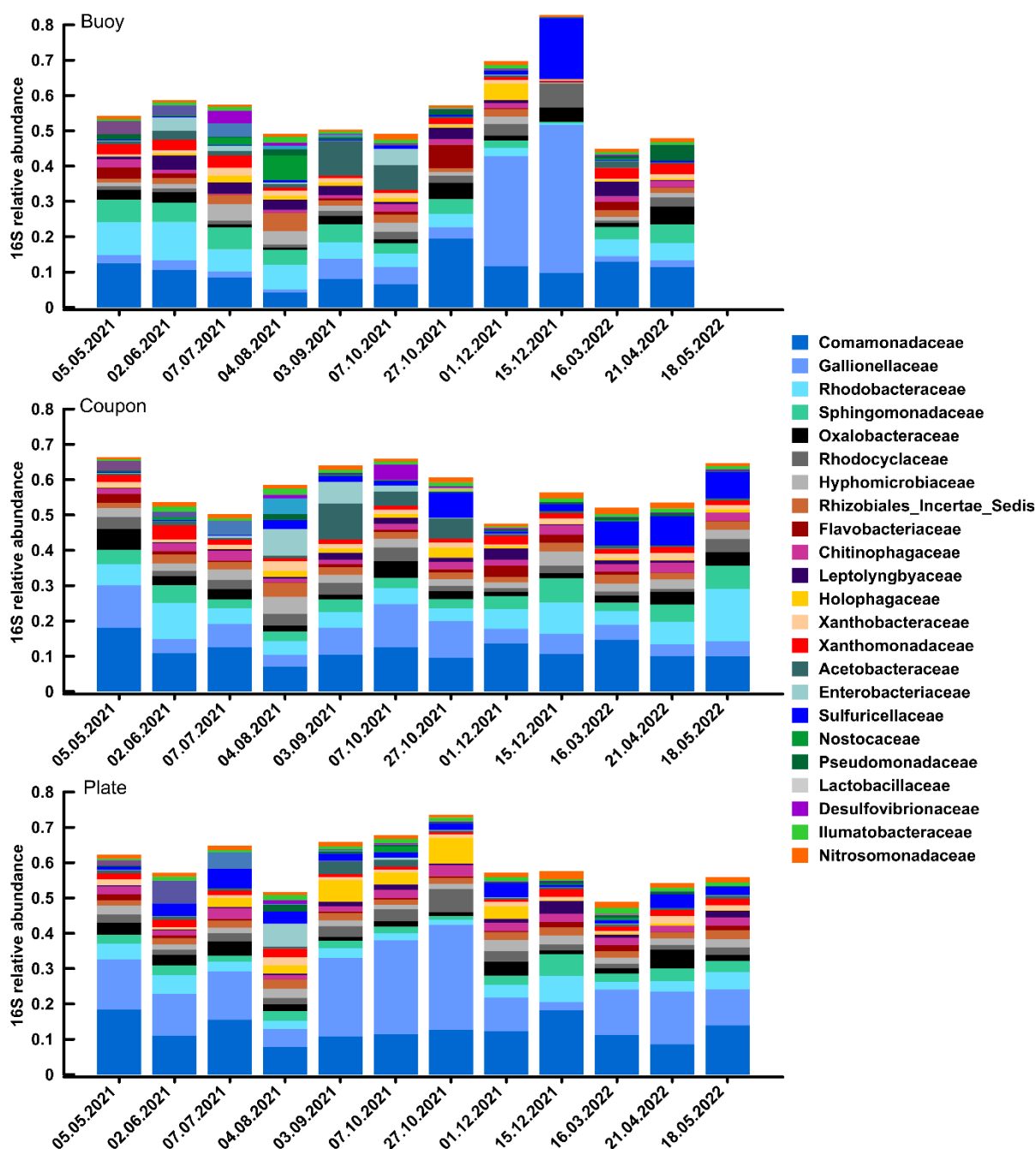
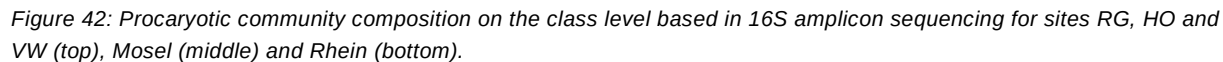


Figure 41: Prokaryotic community composition on the family level based in 16S amplicon sequencing over time for site Lahn (Buoy, Coupon and Plate samples).

On the family and genus level differences in time and between sample types (buoy, coupon and plate) becomes clearer. Most samples and timepoints are dominated by *Comamonadaceae* and *Gallionellaceae*. In buoy samples the *Comamonadaceae* are dominated by the genera *Rhodoferrax* and *Leptothrix* (Fe(II) oxidizer). The *Gallionellaceae* were dominated by *Gallionella* and *Sideroxydans* (Fe (II) oxidizers) and showed the highest relative abundance at T8 (01.12.2021) and T9 (15.12.2021). Other abundant taxa were *Sulfuricella* (*Sulfuricellaceae*), especially at T9, *Tabrizicola* and *Rhodobacter* and *Pseudorhodobacter* (*Rhodobacteraceae*), *Flavobacterium* (*Flavobacteriaceae*), especially at T7 (27.10.2021), *Rhizobiales*, *Undibacterium* (*Oxalobacteraceae*), *Hyphomicrobium* (*Hyphomicrobiaceae*). Also, *Geothrix* (*Holophagaceae*), *Pseudomonas* (*Pseudomonadaceae*), *Arenimonas* (*Xanthomonadaceae*), *Sphingorhabdus* (*Sphingomonadaceae*) and *Dechlorosoma* (*Rhodocyclaceae*), especially at T9, were abundant.

In the coupon samples *Comamonadaceae* are dominated by *Rhodoferax* with the highest relative abundance at T1 (05.05.2021) followed by *Leptothrix*. In comparison to the buoy samples *Sulfuricella* was found in higher relative abundance overall and also on more timepoints. Furthermore, their relative abundance increases over time. The *Gallionellaceae* are dominated by *Sideroxydans* and to a lesser extent by *Gallionella* compared to the buoy samples. *Rhodobacter* (and *Tabrizicola*, both *Rhodobacteraceae*) and *Undibacterium* show overall a higher relative abundance at the coupons compared to the non-corrosive biofilm at the buoy. Within the *Hyphomicrobiaceae* and the Rhizobiales also *Hyphomicrobium* and *Rhizobiales*, respectively, are dominating the coupon samples. Similarly, *Sphingorhabdus* dominates the coupon samples within the *Sphingomonadaceae*. Furthermore *Nitrospira* (*Nitrospiraceae*, not included in the graph above, as overall *Nitrospiraceae* are not in the top 23 families) are more abundant within the coupon samples compared to the buoy samples. Other abundant genera are *Pseudorhodobacter*, *Arenimonas*, and the *Komagataeibacter* (*Acetobacteraceae*, not included in the graph above, as overall *Acetobacteraceae* are not in the top 23 families). Interestingly, *Komagataeibacter* only occurs from T5 to T9 and was not detected at all other times at the coupon samples and similarly only occurs from T5 to T8 within the biofilm, which may indicate a specific response to seasonal changes within that species. Furthermore *Flavobacterium* (less abundant within coupon samples compared to biofilm samples) and *Romboutsia* (*Peptostreptococcaceae*, not included in the graph above) occurred.

The plate samples are also dominated by *Comamonadaceae* and *Gallionellaceae*, the dominant genera of both of these families, *Rhodoferax* (and *Leptothrix*), *Gallionella* (and *Sideroxydans*), respectively, all show a higher relative abundance on average compared to both the coupon and the biofilm samples. Furthermore, the relative abundance of *Gallionellaceae* seems to first increase and decrease again over time, with the highest relative abundance from T5 to T7. *Sulfuricella* and *Undibacterium* show overall a lower relative abundance in comparison to the coupon but higher in comparison to the biofilm samples. *Geothrix* shows the highest relative abundance on the plate in comparison to both coupon and plate samples. However, *Geothrix* increases and decreases again over time in relative abundance on all sample types. *Romboutsia* and *Nitrospira* also show highest relative abundance within the plate samples in comparison to coupon and buoy. Other abundant taxa are also *Rhizobiales Incertae Sedis*, *Dechlorosoma*, *Hyphomicrobium*, *Rhodobacter* and *Tabrizicola*. Less abundant were SC-I-84 (*Burkholderiales*), *Arenimonas*, and *Sediminibacterium* (*Chitinophagaceae*).



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Sideroxydans, its decrease however is more gradual. In contrast to other samples (e.g. all Lahn samples), *Comamonadaceae* of the Mosel coupons are dominated by *Hydrogenophaga* (and less by *Rhodoferrax* and even less by *Curvibacter* and *Acidovorax* as well as *Ideonella*), *Hydrogenophaga* (and *Ideonella*) show the highest relative abundance right at T1 and then gradually decreases over time. This is also the case for *Acidovorax*. Also abundant was *Dechlorosoma* (*Rhodocyclaceae*), with the highest relative abundance at T4 and *Rhodobacter* (*Rhodobacteraceae*) as well as *Rhizobiales Incertae Sedis*, both with higher relative abundance from T3 to T5. As for the other samples *Holophagaceae* are dominated by the genus *Geothrix*. The *Cyanobacteriia* class in the Mosel coupon samples is dominated by the family *Leptolyngbyaceae* and *Synechococcales* (*Schizothrix*). Other abundant genera include *Hyphomicrobium*, *Undibacterium* (*Oxalobacteraceae*), *Dechloromonas* (highest relative abundance at T2, *Rhodocyclaceae*), *Ca. Tenderia* (*Tenderiaceae*), and *Novosphingobium* (*Sphingomonadaceae*).

Site Rhein is less dominated by *Comamonadaceae* and especially *Gallionellaceae* compared to site Mosel. Instead *Leptolyngbyaceae* (*Cyanobacteriia*) show a higher relative abundance, especially towards the later timepoints (T3 to T5). Also, relatively more abundant in comparison to site Mosel is *Ca. Tenderia*, which strongly increases in relative abundance to T5 (at T5 it reaches 20% of the whole community on genus level). Similarly, at site Rhein *Pseudomonas* was relatively more abundant (highest at T2). The *Comamonadaceae* are dominated by *Rhodoferrax* (and less by *Acidovorax* and *Hydrogenophaga*) at site Rhein, *Rhodoferrax* and *Acidovorax* both increase in relative abundance at T3 and T4, whereas *Hydrogenophaga* increases towards T3 and then decreases again. *Escherichia Shigella* and *Klebsiella* (*Enterobacteriaceae*) were only present at T1 and T2 and absent at all other timepoints. In contrast *Beggiatoaceae* were present at site Rhine in high relative abundance compared to all other sites, *Beggiatoaceae* were absent at T1 and T2 and showed highest relative abundance at T5. *Flavobacterium* and the *Rhizobiaceae* were also abundant. The *Gallionellaceae* were less abundant at site Rhein overall and dominated by *Sideroxydans* (and less by *Gallionella*), both increasing in relative abundance until T3 and then decreasing again. Other abundant taxa were *Sphingorhabdus* and *Desulfovibrio*. *Desulfovibrio* increases from T1 to T3 and is then nearly absent after that. Other (less) abundant taxa include *Saprospiraceae* (*Chitinophagales*), *Lysobacter* (*Xanthomonadaceae*), *Rhizobiales* and *Incertae Sedis*.

4.3.5.4 Temporal development of potential functions and the corresponding bacterial and archaeal taxa (FAPROTAX)

Overall the potential functions found are similar between different sample types, but their relative contribution differs. The difference between the non-corrosive biofilm samples from the buoy in comparison to coupon and plate samples is the strongest. This difference is primarily driven by a higher relative contribution of phototrophy in the buoy biofilm samples. Especially photoautotrophy and photosynthetic cyanobacteria are of greater relative importance here. Furthermore, a strong seasonality of the phototrophic processes can be seen solely within the biofilm samples from the buoy. All photosynthetic processes increase in importance from the beginning of June until beginning of September and decline afterwards. This seasonal shift is missing in the plate and coupon samples.

In contrast, processes such as fermentation seem to show a higher relative importance within the corrosive biofilms of the coupon and plate material. In both sample types (coupon and plate) fermentation processes increase in relative importance, reach a plateau and then decline towards the beginning of October. Some processes, such as respiration of S compounds, only occur at single timepoints and are seemingly independent of sample type. Other processes (e.g., methylotrophy) seem to be present at nearly all timepoints, independent of sample type. Additionally, dark oxidation of Fe compounds occurs at nearly all timepoints for buoy and plate samples, whereas for the buoy samples it was assigned only for December and in low relative abundance in September and October.

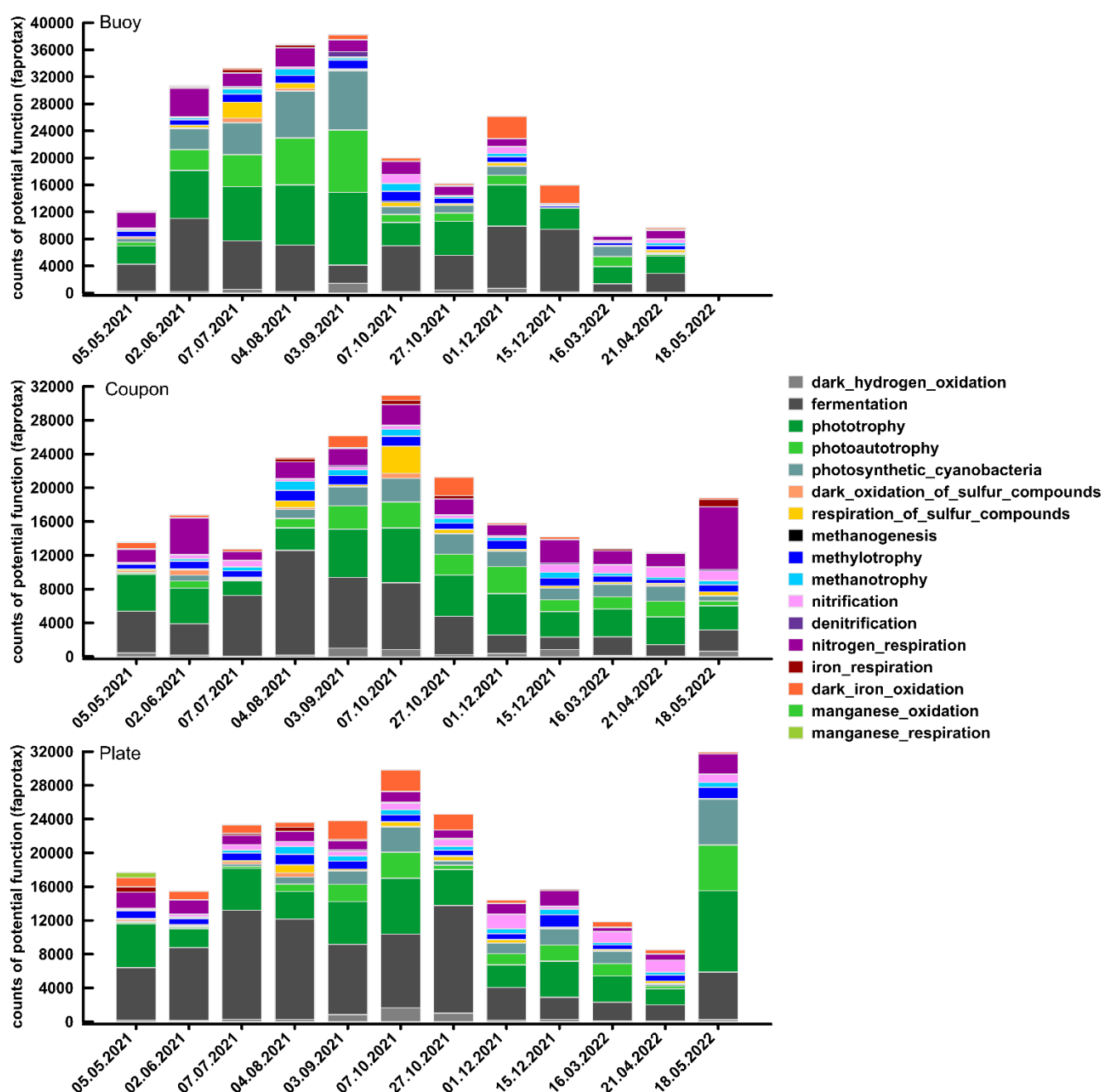


Figure 44: Most abundant potential metabolisms/functions annotated by FAPROTAX for site Lahn along time and for the biofilm, coupon and plate samples.

Overall at site RG, HO and VW similar main potential functions were assigned with fermentation and phototrophy dominating. Some samples however show a relatively higher contribution of respiration of S compounds and or additional processes (respiration of Fe compounds).

For site Mosel, already at T1 (1 week) most processes occur. Dark hydrogen oxidation is dominating and then decreasing in relative importance towards T5. Fermentation stays more or less stable over time. Phototrophy goes up and down and no actual trend can be seen over time. The dark oxidation of Fe compounds decreases over time. At Site Rhein this is different, fermentation and dark hydrogen oxidation first increase and then decrease again in relative importance and the dark oxidation of Fe is only assigned from T3 onwards. At site Rhein, the respiration of S compounds increases in relative abundance towards T3 and is then decreasing again.

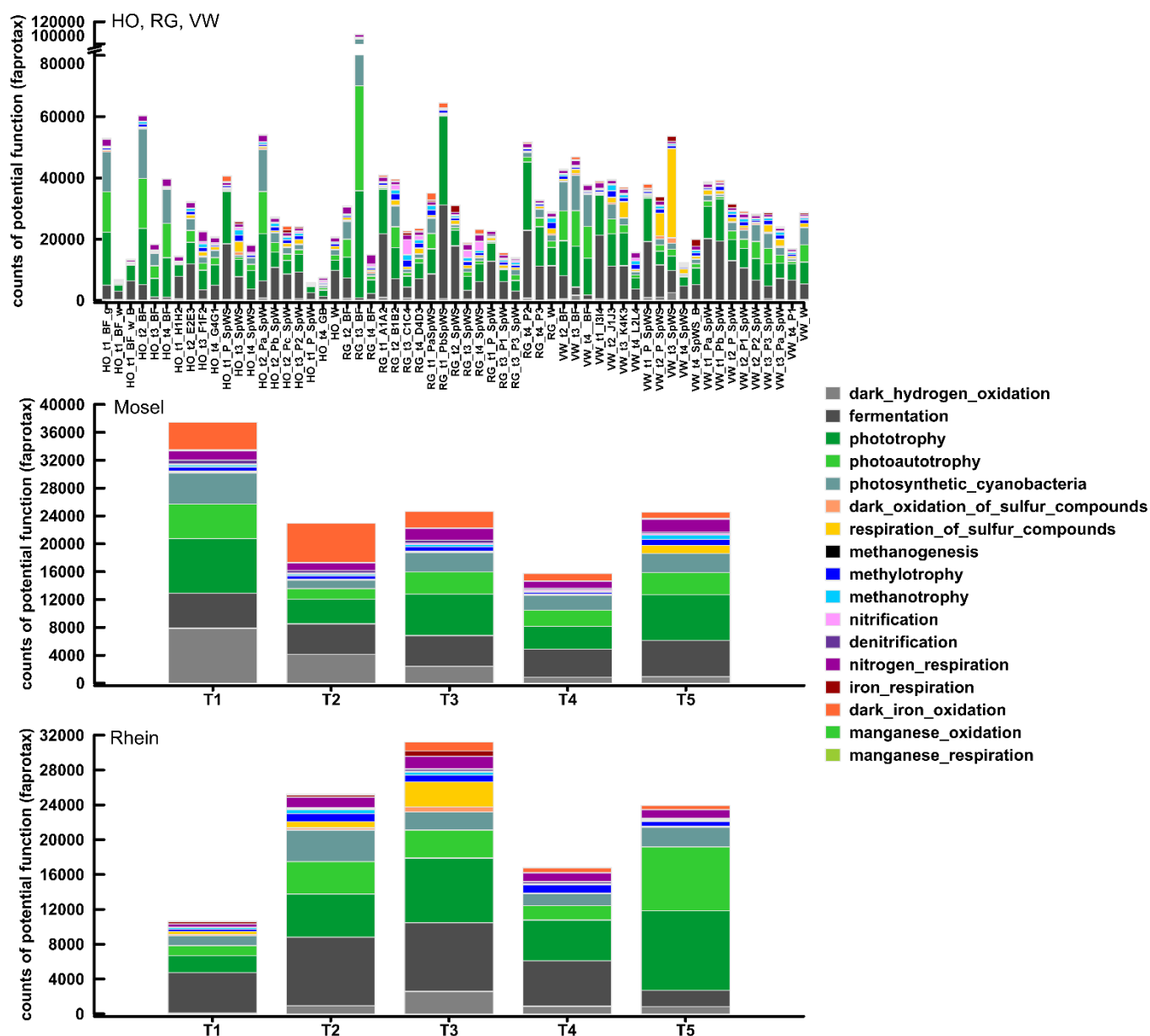


Figure 45: Most abundant potential metabolisms/functions annotated by FAPROTAX for site Himmelpfort, Regow and Voßwinkel for all samples and site Mosel and Rhein. Please not y-axis is broken.

In order to compare the different sites and sample types in more detail the most dominant and diverse potential functions were plotted individually and the taxa assigned to the process are shown accordingly (Figure 46, Figure 47, Figure 48, Figure 49, Figure 50).

For site Lahn, fermentation was assigned to be performed by *Erysipelotrichaceae*, *Rhodoferrax*, *Geothrix*, *Enterobacterales*, *Clostridiales*, *Propionivibrio*, *Bacilli*, *Desulfurococcales*, and *Actinobacteria*. Some trends over time are found in all sample types for example *Bacilli* only occurs from May to August. Similarly, *Geothrix* occurs from June to December for the buoy samples but only starts to be present in higher abundance from August onwards for the coupon and from July for the plate samples. The presence of *Geothrix* is interesting as it is also an Fe reducing bacterium. It has to be noted that the genus *Rhodoferrax* also includes Fe reducing species, although the species level could not be assigned here. The presence of *Desulfurococcales* (*Archaea*) is also interesting in terms of corrosion as they include taxa capable of reducing sulfur compounds for oxidation of hydrogen (under autotrophic growth).

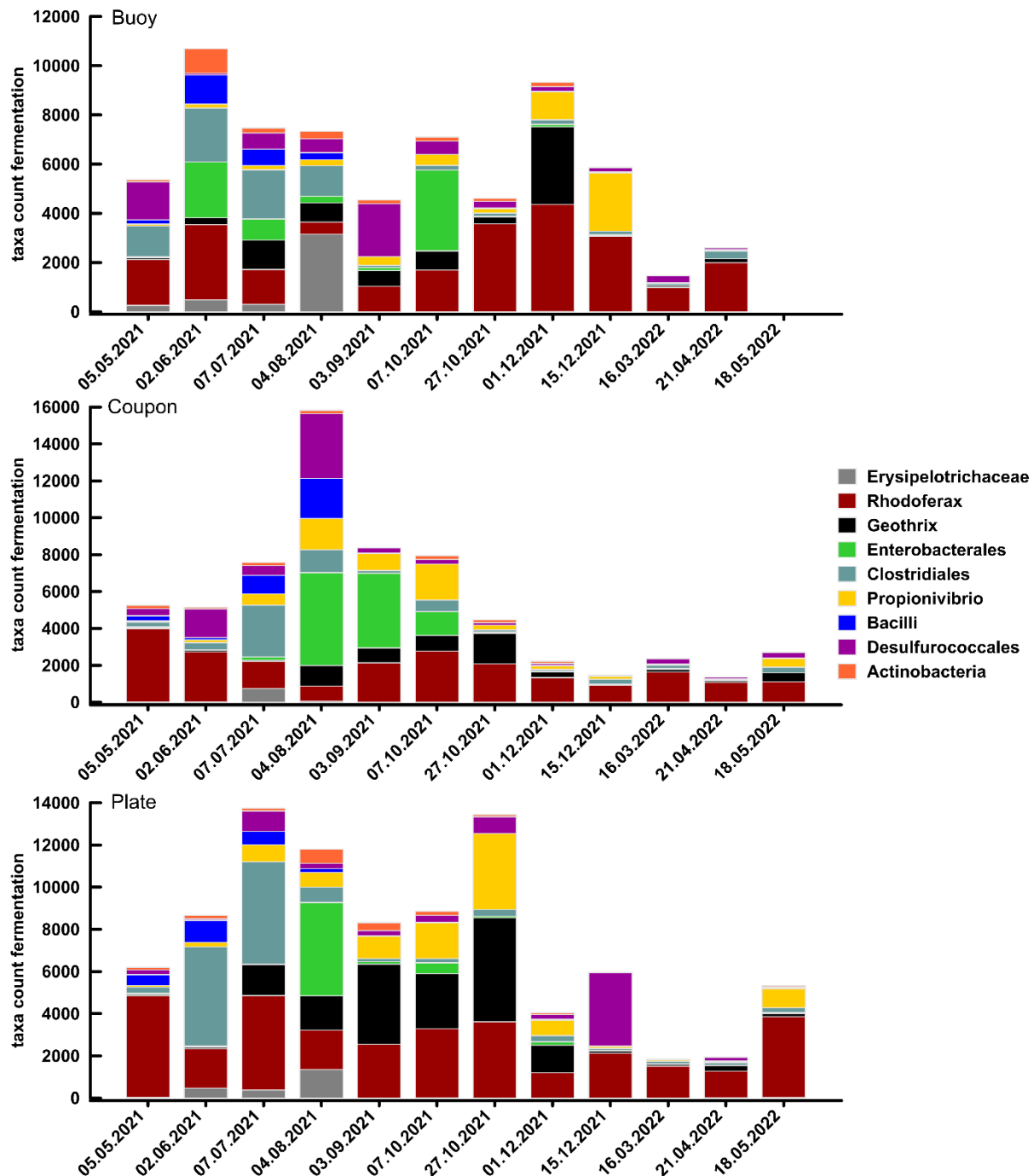


Figure 46: Taxa count for fermentation for samples of the buoy biofilm (top), the coupon biofilm (middle) and the plate biofilm (bottom) over time. Please note y-axes differ.

For phototrophy, the differences in the taxonomic composition between sample types and over time is stronger compared to fermentation. Especially in the buoy samples a strong seasonal signal can be observed. *Rhodoferrax* was assigned as the dominant phototrophic organism of the 16S community and is present at all timepoints and sample types. Within the buoy samples however between June and September the community changes drastically in relative abundance, *Oscillatoriales* increase in relative abundance during that time as well as *Rhodobacteraceae*. In July and August also *Nostocales* show a high relative abundance. In September the community is predominated by *Chroococcidiopsidaceae*. This seasonal pattern is missing in the plate and coupon biofilm samples. Within the coupon samples *Synechococcales* show a high relative abundance from August onwards, similar to the plate

samples. Additionally, *Chromatiales* have a higher relative abundance (from September to December only) within the coupon samples.

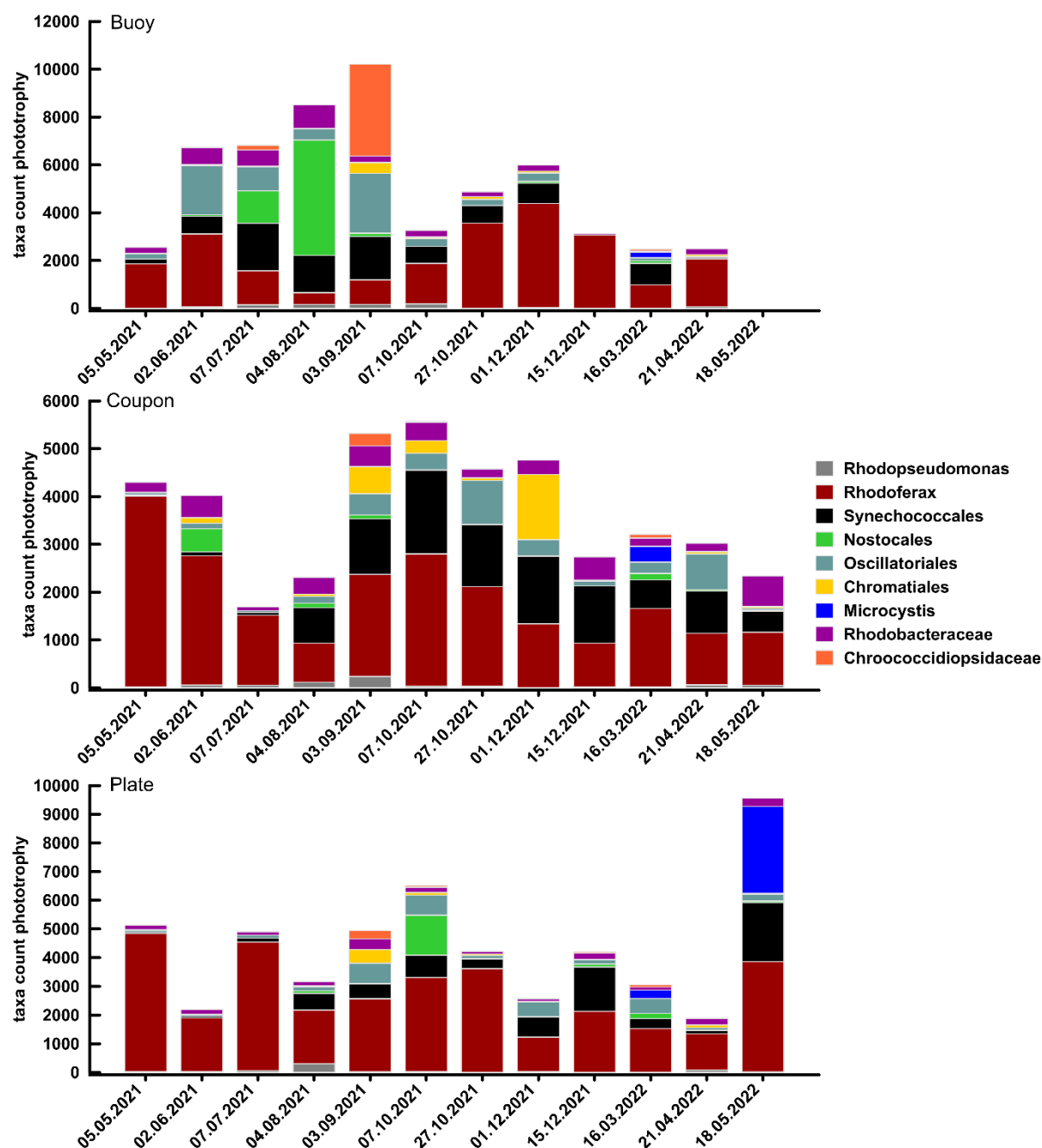


Figure 47: Taxa count for phototrophy for samples of the buoy biofilm (top), the coupon biofilm (middle) and the plate biofilm (bottom) over time. Please note y-axes differ.

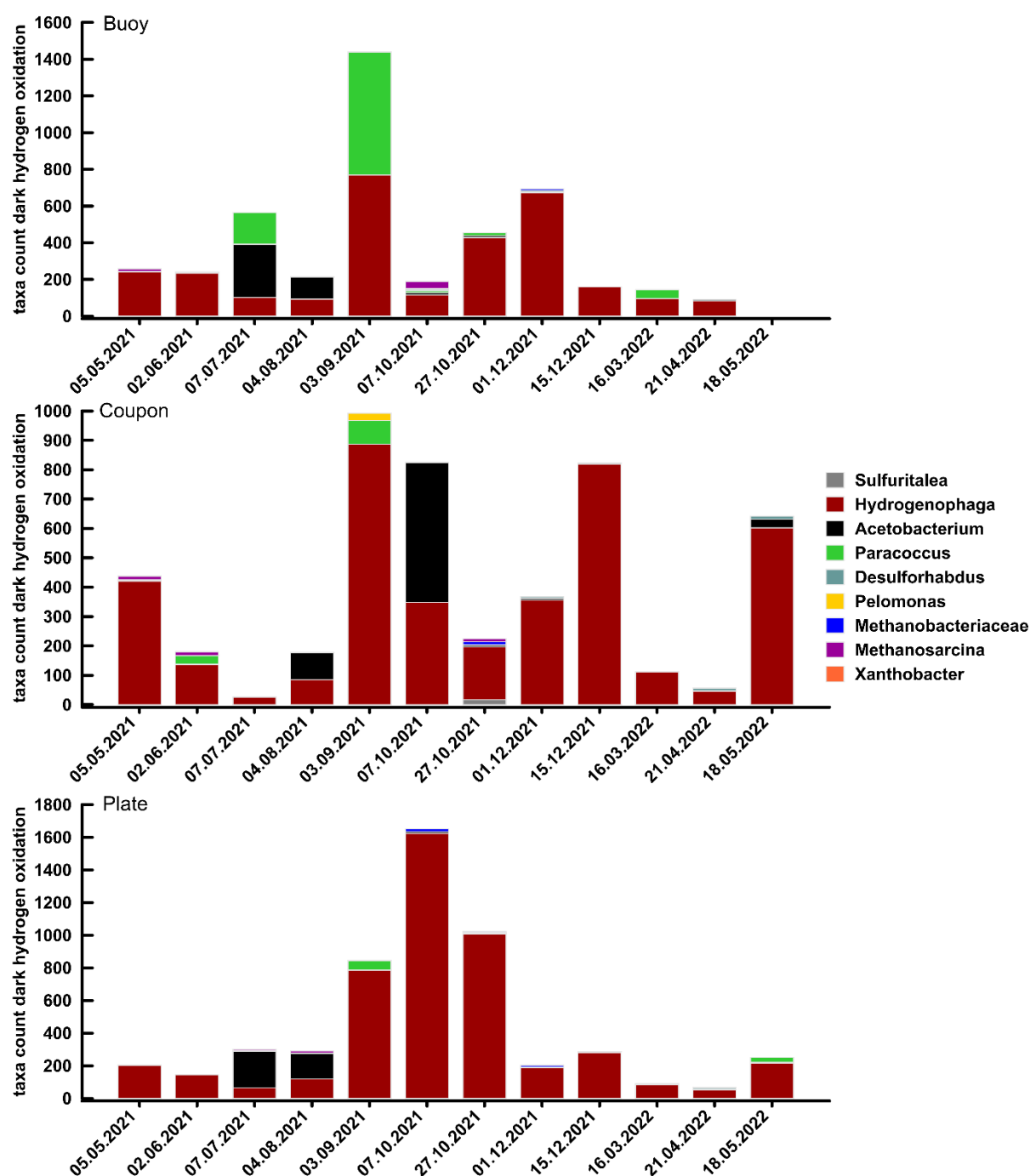


Figure 48: Taxa count for dark hydrogen oxidation for samples of the buoy biofilm (top), the coupon biofilm (middle) and the plate biofilm (bottom) over time. Please note y-axes differ.

The dark hydrogen oxidation at site Lahn was assigned mostly to *Hydrogenophaga*. Some timepoints also show high taxa count of others such as *Acetobacterium* or *Paracoccus*.

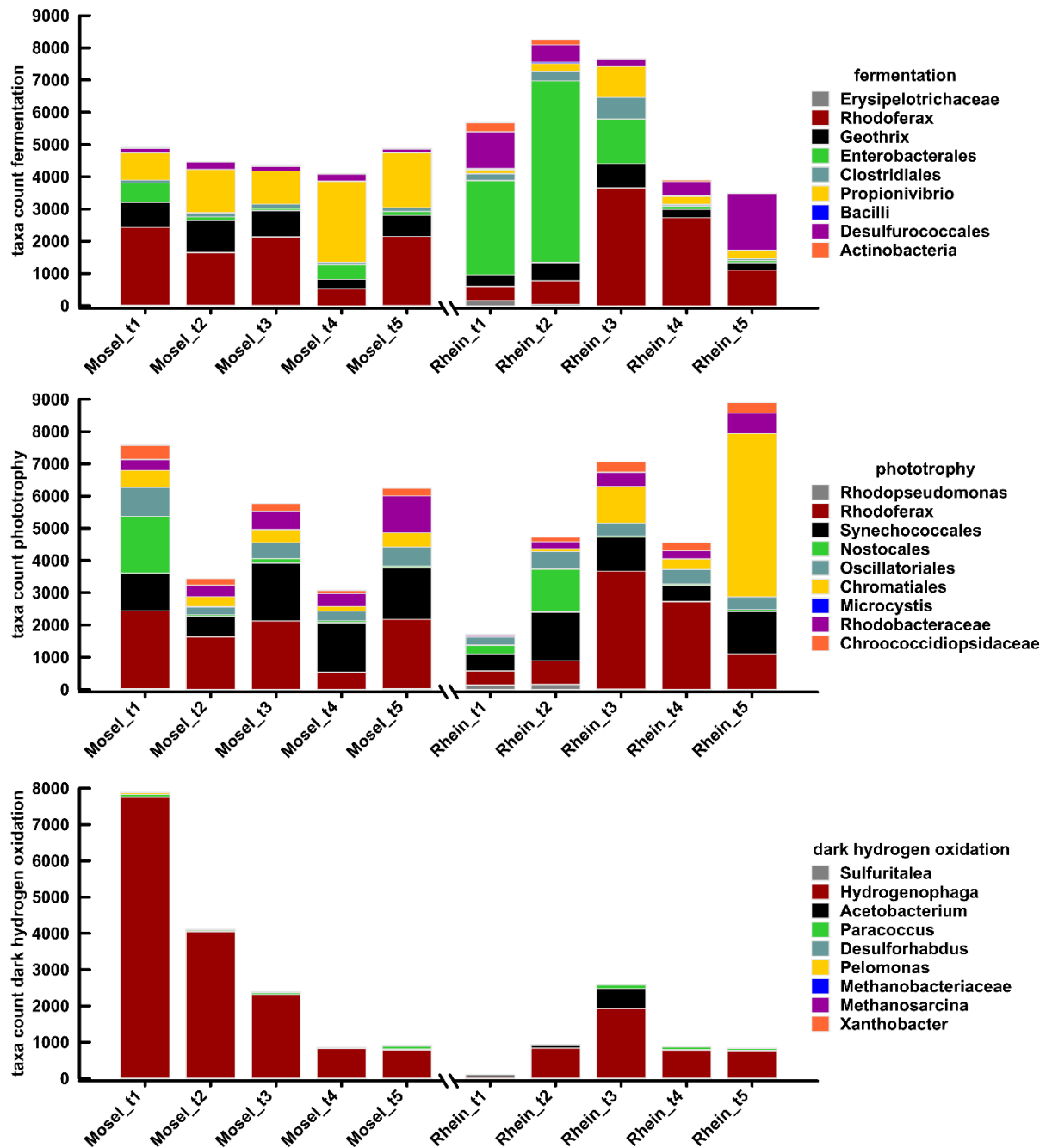


Figure 49: Taxa count for fermentation (top), phototrophy (middle), and dark hydrogen oxidation (bottom) for sites Mosel (left) and Rhein (right).

Similar to our findings at site Lahn, fermentation at sites Rhein and Mosel is performed dominantly by *Rhodoferrax*. However, at site Mosel *Propionivibrio* was also abundant whereas at site Rhein *Enterobacterales* was more abundant especially from T1 to T3. *Geothrix* is also abundant at all timepoints and at both sites. At site Rhein *Desulfurococcales* was also abundant.

As for site Lahn, phototrophy in Mosel and Rhein is dominated by *Rhodoferrax* and *Synechococcales*. At site Mosel *Rhodobacteraceae* were counted more often, whereas at site Rhein *Chromatiales* were more counted more often especially at later timepoints.

The dark hydrogen oxidation is dominated at both sites by *Hydrogenophaga*.

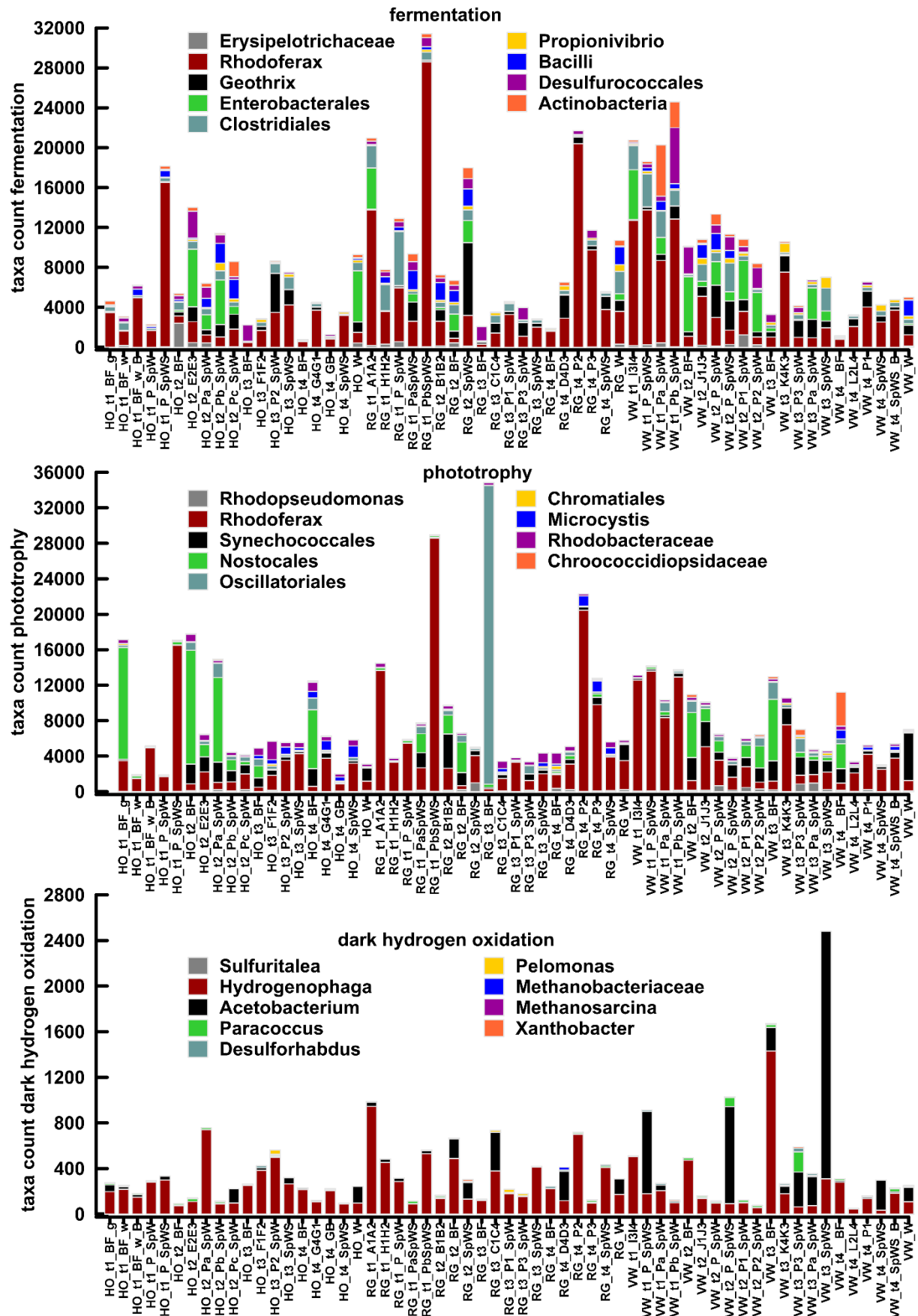


Figure 50: Taxa count for fermentation (top), phototrophy (middle), and dark hydrogen oxidation (bottom) for sites HO, RG and VW.

Respiration of S compounds is dominated by *Desulfovibrio* at all sites and sample types. Other taxa which occasionally occur are: the family *Desulfobacteraceae* and the genus *Desulfosporosinus*. Additionally, very few counts were assigned to *Fusibacter*, *Geoalkalibacter*, *Desulfocapsa*, *Desulfobulbaceae*, *Desulfatiglans*, *Desulfotobacterium*, *Desulfuromonas*, *Desulfobacca*, *Desulforhabdus*, *Desulfomicrobium*, and *Desulfohalobiaceae*.

Nitrogen respiration is dominated by one taxon: *Gemmobacter*, in some samples however also *Magnetospirillum*, *Comamonadaceae*, *Shewanella*, *Desulfovibrionaceae*, *Paracoccus*, *Burkholderiales*, *Rhodocyclales*, and *Nitrobacter* were assigned. Fewer counts were also assigned to *Rhodoplanes*, *Pseudomonas*, *Clostridium*, *Stenotrophomonas*, *Nitrosomonadales*, and *Vogesella*.

For nitrification *Nitrospiraceae* (mostly *Nitrospira*) were dominantly assigned in most samples and timepoints. In some samples *Nitrosomonadaceae* (*Nitrosomonas*, *Nitrosospora*, *Nitrosovibrio*) were assigned followed by *Nitrobacter*.

The dark oxidation of S compounds was assigned nearly exclusively to *Bosea*, with a few assigned other taxa such as *Thiothrix*, *Sulfuricellaceae*, and *Thioalkalivibrio*.

With time, diversity of the taxa assigned to perform specific pathways seems to increase, for example iron respiration taxa in Rhein and Mosel (5 weeks incubation time) are dominated by *Geobacter* with very few counts of other taxa such as *Desulfuromonas*, *Ferribacterium*, *Desulfotobacterium* and *Shewanella*. Even though the dominant taxa (*Geobacter*) is the same in samples with longer incubation times (Lahn, Regow, HO, VW) the relative share of other taxa (e.g. *Shewanella*, *Ferribacterium*) is increasing and other additional taxa are also assigned such as *Desulfovibrio* or *Geoalkalibacter*.

Similar to the dark oxidation of S compounds, dark iron oxidation was nearly exclusively assigned to one taxon, *Gallionella*, a few other counts were assigned to *Sphaerotilus*.

Methanogenesis was rarely counted, taxa found to perform methanogenesis in the samples were *Methanotrix*, *Methanoregula*, *Methanolinea*, *Methanomassiliicoccus*, and *Methanobacterium*.

4.4 Laboratory study: Results from corrosion studies with enrichment cultures

4.4.1 Overview of environmental samples used for enrichment cultures and corrosion experiments

In total, 81 primary enrichment cultures were established. For the sites Farge and MLK/Wolfsburg, with two samples each (Table 6; Figure 51), in total 36 cultures were established with either nitrate, sulfate or CO₂ as electron acceptor and with either steel coupon, iron beads or molecular hydrogen as electron donor (=2x2x3x3). These enrichments were part of the BSc thesis by Plochocki (2021), Wiegert (2021), and Köhler (2022). Köhler (2022) focused hereby on DNA extraction and cell counting by epifluorescence microscopy. For the sites Himmelpfort with one sample and Regow with six samples (Figure 51, Table 6), in total 45 cultures were established, again with either nitrate, sulfate or CO₂ as electron acceptor but with only steel coupon or iron beads as electron donor for six samples (=6x3x2+1x3x3). These enrichments were part of the BSc thesis by Steinhorst (2021). Steinhorst (2021) and Köhler (2022) also investigated the original prokaryotic community composition of the environmental samples.

In total, four corrosion experiments were performed (Table 6). Each corrosion experiment included five treatments which were performed in triplicate. One corrosion experiment was conducted with nitrate (BSc thesis by Raab 2021), two with sulfate (BSc thesis by Egerter 2022 and Rahm 2023), and one with CO₂ as electron acceptor (BSc thesis by Hürten 2023).

Table 6: Environmental samples used for enrichment cultures and corrosion experiments.

Environmental sample name (DNA extraction)	Sample name used for primary enrichments	Electron acceptors used in corrosion studies
FargePL1	#3	-
FargePW1b	#8	CO ₂
MLKcorholN2	#15	NO ₃ ⁻
MLKcorholS1	#16	SO ₄ ²⁻ (2x)
RG.Sed.OS	R-Sed	-
RG.P.150.a2	R-a2	-
RG.P.film.b	R-b1	CO ₂
RG.P.C1150	R-c1	-
RG.P.C1125.imo	R-e1	-
RG.P.MS1.omi	R-Tu	SO ₄ ²⁻
HO.P.SW.123	Hi-Tu	-

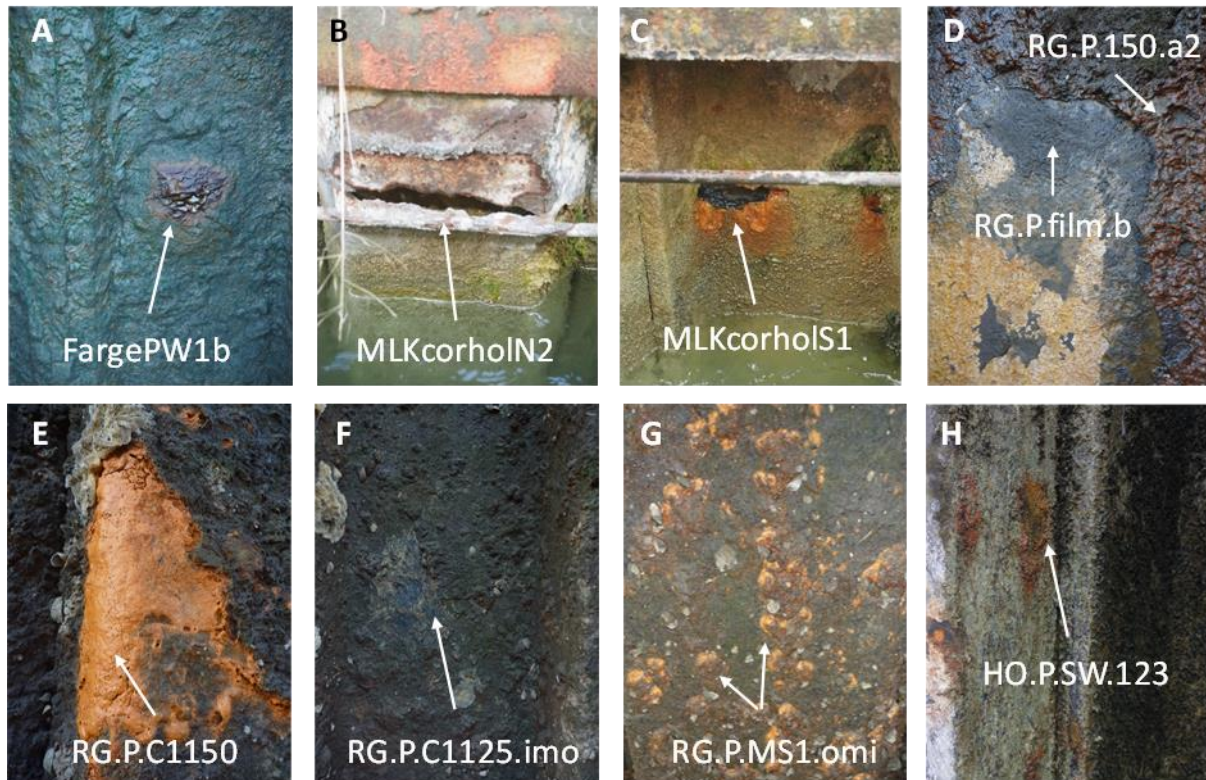


Figure 51: Environmental samples: corrosion tubercle within corrosion crust from sheet pile at Farge harbor (FargePW1b) (A), corrosion hole northern sheet pile MLK with backfill extruding (MLKcorholN2) (B), corrosion hole southern sheet pile MLK with backfill extruding (MLKcorholS1) (C), hard corrosion crust (RG.P.150.a2) with black film underneath (RG.P.film.b) in Regow lock (D), thick reddish soft corrosion material partially covered with algae in Regow lock (E), had dark corrosion crust in Regow lock (F), rust tubercles on sheet pile in Regow lock (G), and rust tubercles on sheet pile in Himmelpfort lock (H).

Primary enrichments were used to investigate whether steel corrosion would proceed under the test conditions applied (nitrate, sulfate, and CO₂ reducing conditions), what kind of prokaryotes were present in the different environmental samples and which might be responsible for MIC. Furthermore, they served for optimizing the experimental set-up and analytical tools. In the following we present the main results of the corrosion experiments.

4.4.2 Corrosion experiment under nitrate reducing conditions

For the corrosion experiment with nitrate as electron acceptor, the primary enrichment of environmental sample #15 (MLKcorhoIN2) was used. It showed the highest consumption of nitrate in comparison to all other environmental samples. The following five treatments were included (in triplicate): i) steel with nitrate and microorganisms (Steel_Nit_#15; I – III), ii) steel with nitrate, acetate, and microorganisms (Steel_Nit_Ac_#15; IV – VI), iii) steel with nitrate but without microorganisms (Steel_Nit_abiot; VII – IX), iv) steel only (Steel_abiot; X – XII), and v) H₂ with nitrate and microorganisms (H₂_Nit_#15; XIII – XIV) (Figure 52). Assays were incubated for six weeks.

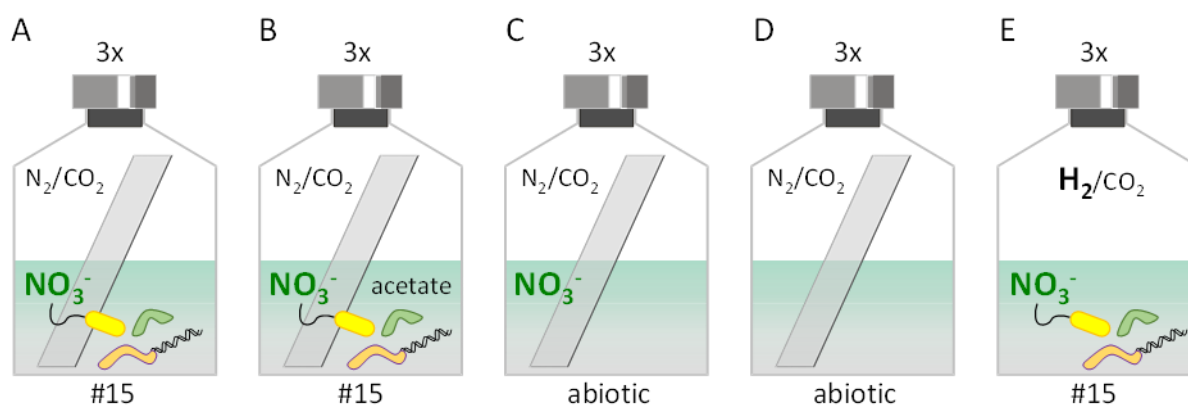


Figure 52: Experimental set-up for corrosion experiment under nitrate reducing conditions for six weeks: steel coupon with primary enrichment from sample #15 without acetate (Steel_Nit_#15; I – III) (A) and with acetate (Steel_Nit_Ac_#15; IV – VI) (B), abiotic control with nitrate (Steel_Nit_abiot; VII – IX) (C) and without nitrate (Steel_Ac_abiot; X – XII) (D), and primary enrichment from sample #15 with H₂ as electron donor (H₂_Nit_#15; XIII – XIV) (E). Each assay was run in triplicate.

Iron loss from the steel coupons was highest in the assays with microorganisms and with acetate (Figure 53). In assays with microorganisms but without acetate the iron loss was similar to the iron loss in the abiotic control with nitrate. The lowest corrosion rate was observed in sterile medium without nitrate.

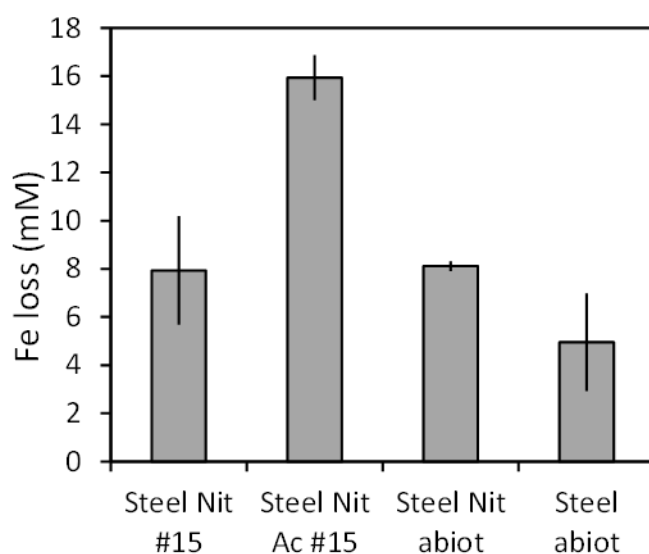


Figure 53: Iron loss from steel coupons in assays with nitrate after six weeks of incubation at 20°C. Arithmetic mean with standard deviation of triplicates.

In all assays the pH increased (Figure 54A) indicating the reduction of nitrate [either by the microorganisms using nitrate as terminal electron acceptor or abiotically by Fe(II)] and/or the production of H₂ [presumably abiotically by the reduction of H⁺ with Fe(II)]. In the assays with steel coupons, the highest nitrate removal rate (corresponding to nitrate reduction rate) was indeed found in those with microorganisms and acetate (Figure 54B). Microbial growth was demonstrated by protein and cell number increase (data not shown). Acetate was completely consumed (Figure 54E). These observations show, that dissimilatory nitrate reduction increased corrosion rates. However, an organic C source had to be present otherwise the microorganisms would not grow (or grow very slowly). As end products of nitrate reduction, nitrite only appeared transiently in elevated amounts (Figure 54C) whereas ammonium formed the largest fraction and accounted for 49 – 62% of N-NO₃⁻ reduced in the assays with microorganisms and acetate (Figure 54D). The amount of the end product N₂ was estimated from the N balance and accounted for 30 – 42% of N-NO₃⁻ reduced. Molecular hydrogen was only detected transiently in small amounts in assays containing steel coupons and microorganisms in contrast to the abiotic controls where H₂ accumulated over time (Figure 54F). This indicated that the microorganisms most likely consumed the abiotically formed H₂. In assays with steel coupons and microorganism, Fe(II) was for a great part further oxidized to Fe(III) (both fractions include dissolved species as well as resuspended solids; Figure 54G, H). Nitrate itself acted as a corrosive agent and was mainly reduced to ammonium (61 – 86%) and to a lesser extent to nitrite (14 – 18%). Fe(II) was only further oxidized to Fe(III) in the abiotic control with nitrate but at a smaller scale than in assays with microorganisms. In the assays amended with H₂ as electron donor, much higher nitrate reduction rates were achieved compared to the assays with steel coupons. A large proportion was reduced to nitrite (37 – 58%). The remaining part was most likely reduced to N₂ as the concentration of ammonium decreased. The main ammonium decrease coincided with the main decrease of both H₂ and nitrate as well as the main increase in protein (data not shown). This points to the uptake of ammonium as N source for biosynthesis. Final protein concentrations in assays with microorganisms were 1.2 ± 0.7, 5.8 ± 0.3, and 19.7 ± 1.5 mg l⁻¹ for those with steel coupons, with steel coupons and acetate, and with H₂, respectively.

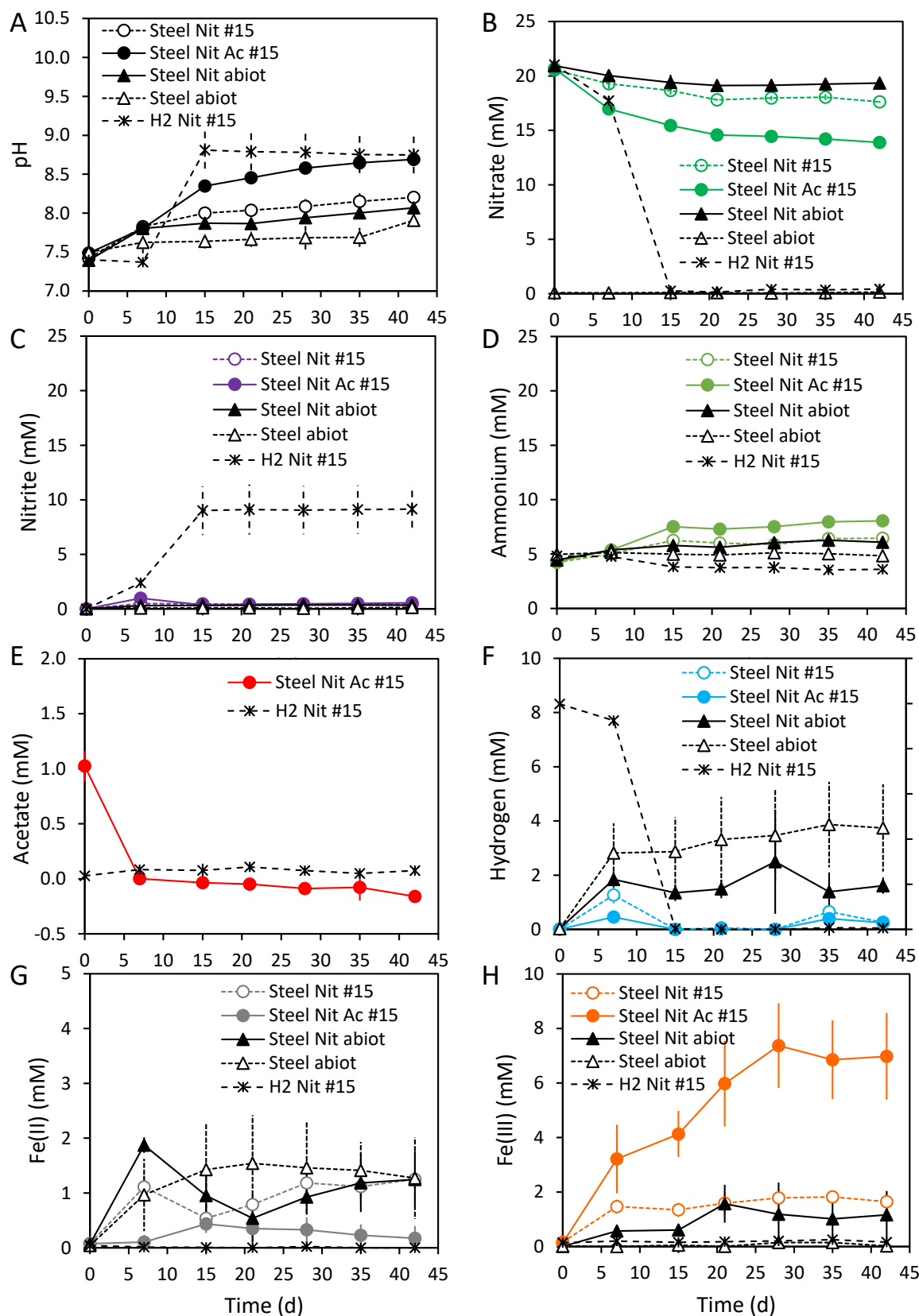


Figure 54: Time course of the different measurement parameters in assays with nitrate during six weeks of incubation at 20°C. Concentrations of H₂ (F) for assays H2_Nit_#15 are indicated on the secondary y-axis. Arithmetic means with standard deviation of triplicates.

The enriched prokaryotic communities were dominated by two OTUs (Figure 55). The one with the highest relative abundance was affiliated to the genus *Acidovorax* with 83% in the assays amended with H₂ and with 54 – 55% in those with steel coupons. The lowest proportion was found in the prokaryotic biofilm community scraped off from the steel surface (46%). The second most abundant was affiliated to the genus *Thermomonas* and was prominent in assays with steel coupons especially within the biofilm (43%).

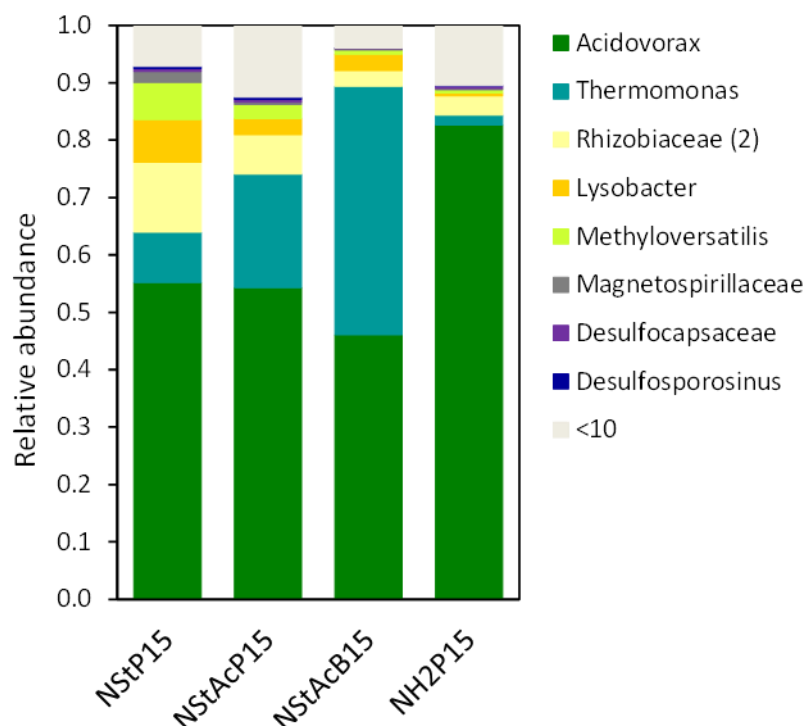


Figure 55: Prokaryotic communities enriched under nitrate reducing conditions using environmental sample #15 for six weeks. Composition is based on 16S-rRNA amplicon sequencing. Relative abundances of the top 10 OTUs with values >0.01. Mean of triplicates except NStAcB15 (biofilm samples of triplicates were pooled). N: nitrate, St: steel, Ac: acetate, P: planktonic, B: biofilm.

4.4.3 Corrosion experiments under sulfate reducing conditions

Two corrosion experiments were performed with sulfate as electron acceptor. In the first experiment, the primary enrichment of environmental sample #16 (MLKcorhoIS1) was used. It showed the highest consumption of sulfate in comparison to all other environmental samples. The following five treatments were included (in triplicate): i) steel with sulfate and microorganisms (Steel_Sul_#16; I – III), ii) steel with sulfate, acetate, and microorganisms (Steel_Sul_Ac_#15; IV – VI), iii) steel with sulfate but without microorganisms (Steel_Sul_abiot; VII – IX), iv) steel with acetate but without microorganisms (Steel_Ac_abiot; X – XII), and v) H₂ with sulfate and microorganisms (H₂_Sul_#16; XIII – XIV) (Figure 56). Assays were incubated for six weeks. In the second experiment, additionally the primary enrichment of environmental sample R-Tu (RG.P.MS1.omi) was used because the relative abundance of sequences affiliated to sulfate reducing prokaryotes was high. The following five treatments were included (in triplicate): i) steel with sulfate and microorganisms from #16 (Steel_Sul_#16; I – III), ii) steel with sulfate, acetate, and microorganisms from #16 (Steel_Sul_Ac_#15; IV – VI), iii) steel and microorganisms from R-Tu (Steel_Sul_R-Tu; VII – IX), iv) steel with sulfate, acetate, and microorganisms from R-Tu (Steel_Sul_Ac_R-Tu; X – XII), and v) steel with sulfate and acetate but without microorganisms (Steel_Sul_Ac_abiot; XIII – XIV) (Figure 57). Assays were incubated for eight weeks.

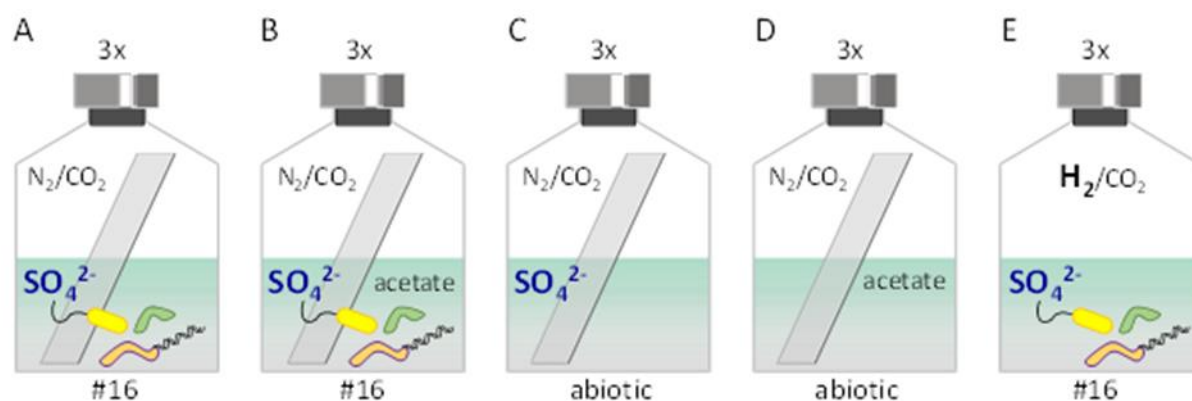


Figure 56: Experimental set-up for the first corrosion experiment under sulfate reducing conditions for six weeks: steel coupon with primary enrichment from sample #16 without acetate (Steel_Sul_#16; I – III) (A) and with acetate (Steel_Sul_Ac_#16; IV – VI) (B), abiotic control with sulfate (Steel_Sul_abiot; VII – IX) (C) or acetate (Steel_Ac_abiot; X – XII) (D), and primary enrichment from sample #16 with H_2 as electron donor (H2_Sul_#16; XIII – XIV) (E). Each assay was run in triplicate.

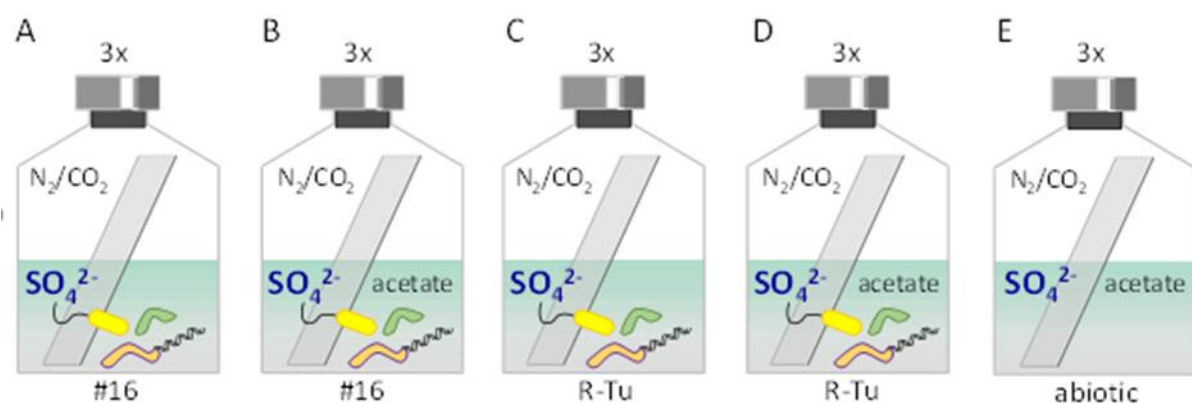


Figure 57: Experimental set-up for the second corrosion experiment under sulfate reducing conditions for eight weeks: steel coupon with primary enrichment from sample #16 without acetate (Steel_Sul_#16; I – III) (A) and with acetate (Steel_Sul_Ac_#16; IV – VI) (B), steel coupon with primary enrichment from sample R-Tu without acetate (Steel_Sul_R-Tu; VII – IX) (C) and with acetate (Steel_Sul_Ac_R-Tu; X – XII) (D), and abiotic control with sulfate and acetate (Steel_Sul_Ac_abiot; XIII – XIV) (E). Each assay was run in triplicate.

Iron loss after six weeks was only marginally higher in assays with microorganisms from environmental sample #16 and with acetate in comparison to abiotic assays (Figure 58). Iron loss in assays with microorganisms but without acetate was even lower. After eight weeks, iron loss was clearly greater in assays with microorganisms from environmental sample #16 than in the abiotic control. Here, the presence of acetate made no difference. However, assays with microorganisms from the environmental sample R-Tu showed lower corrosion rates than the abiotic assays even after an incubation time of eight weeks (Figure 58).

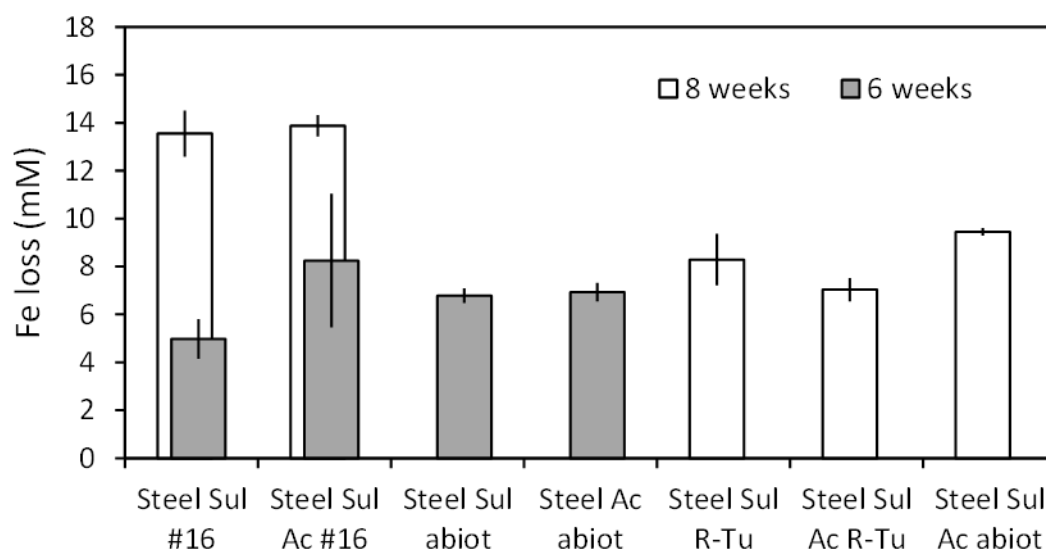
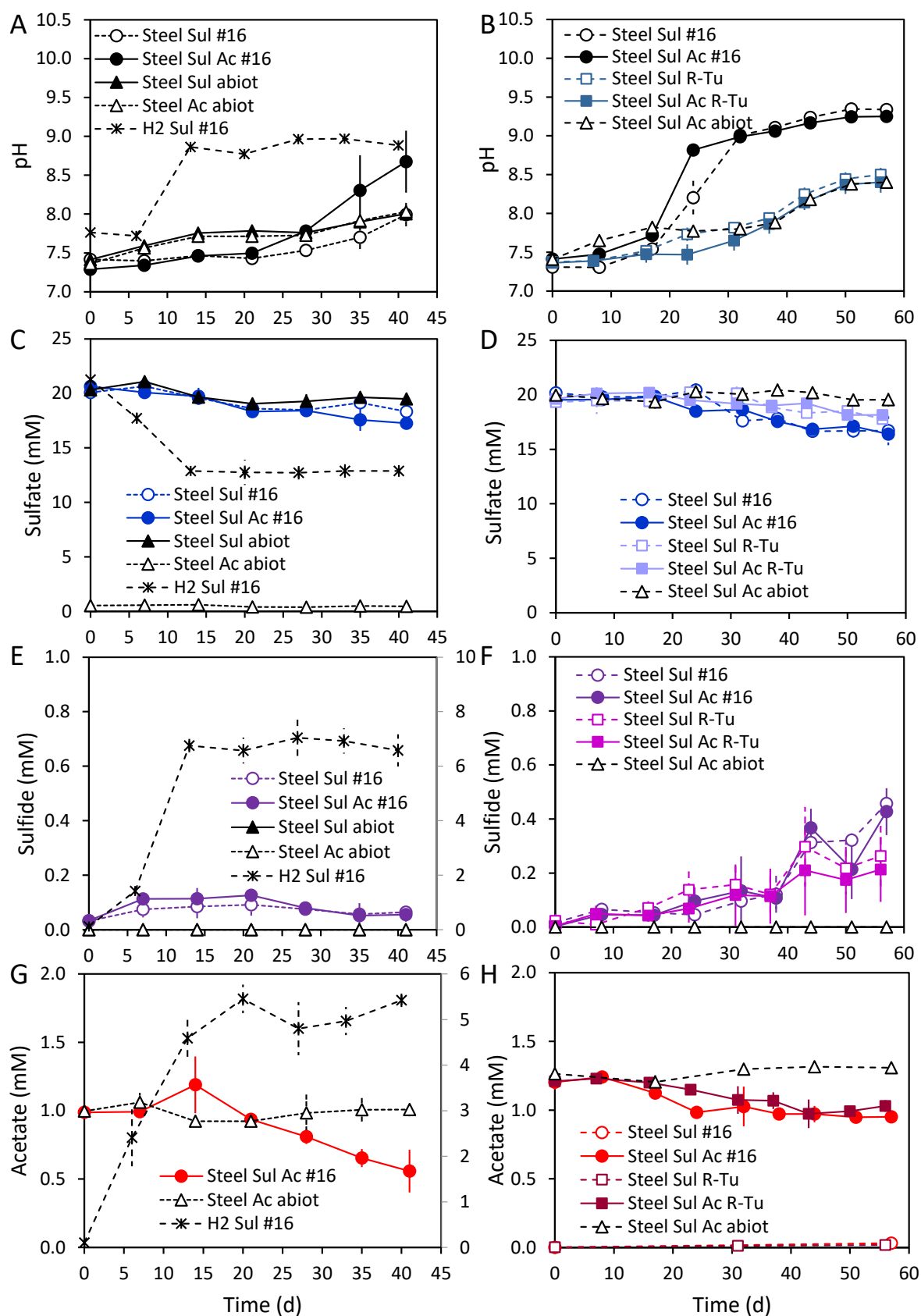


Figure 58: Iron loss from steel coupons in assays with sulfate after six weeks (grey bars) and eight weeks of incubation (white bars) at 20°C. Arithmetic mean with standard deviation of triplicates.

The pH values increased in all assays (Figure 59A, B). In assays with steel coupons, high iron losses corresponded to high final pH values and to a high sulfate removal in the assays with microorganisms (Figure 59C, D). An increase of pH was supposed to result from the abiotic reduction of H^+ by $Fe(II)$ and/or the dissimilatory reduction of sulfate. Protein content and cell numbers increased concomitant to sulfate removal (data not shown). A slight decrease of sulfate was also observed in the abiotic assays which was most likely due to co-precipitation with Ca/Fe carbonates as sulfate reduction does not occur abiotically under ambient conditions. Only small amounts of free sulfide were detected in the assays with microorganisms and steel coupons (Figure 59E, F) due to the immediate formation and precipitation of iron sulfides which was also evidenced by the blackening of the cultures. Acetate amended to the assays with microorganisms and steel coupons only decreased slightly indicating that it served as C source but not as electron donor (Figure 59G, H). While H_2 accumulated in the abiotic assays as a result of abiotic corrosion only small amounts were observed transiently in the assays with microorganisms (Figure 59I, J). Most likely H_2 was consumed by the microorganism and served as electron donor. In the, abiotic assays ferrous iron was released from the steel coupons early on and consisted for a great part of the soluble form Fe^{2+} whereas in assays with microorganisms an increase in ferrous iron occurred later and to a lesser extent (Figure 59K, L, M, N). No ferric iron was detected (data not shown).

In the assays amended with H_2 as alternative electron donor, sulfate reduction occurred much faster than in assays with steel coupons. Due to a lack of iron, large amounts of free sulfide accumulated. Parallel to dissimilatory sulfate reduction, acetogenesis occurred as shown by acetate production. The protein content was clearly higher with H_2 as electron donor yielding $5.2 \pm 0.7 \text{ mg l}^{-1}$ in comparison to $0.67 \pm 0.37 \text{ mg l}^{-1}$ (without acetate) and $1.02 \pm 0.20 \text{ mg l}^{-1}$ (with acetate) in assays with steel coupons after six weeks. After eight weeks, similar yields were determined for assays with microorganisms of either environmental sample with or without acetate (min. $1.16 \pm 0.29 \text{ mg l}^{-1}$ and max. $1.47 \pm 0.14 \text{ mg l}^{-1}$).



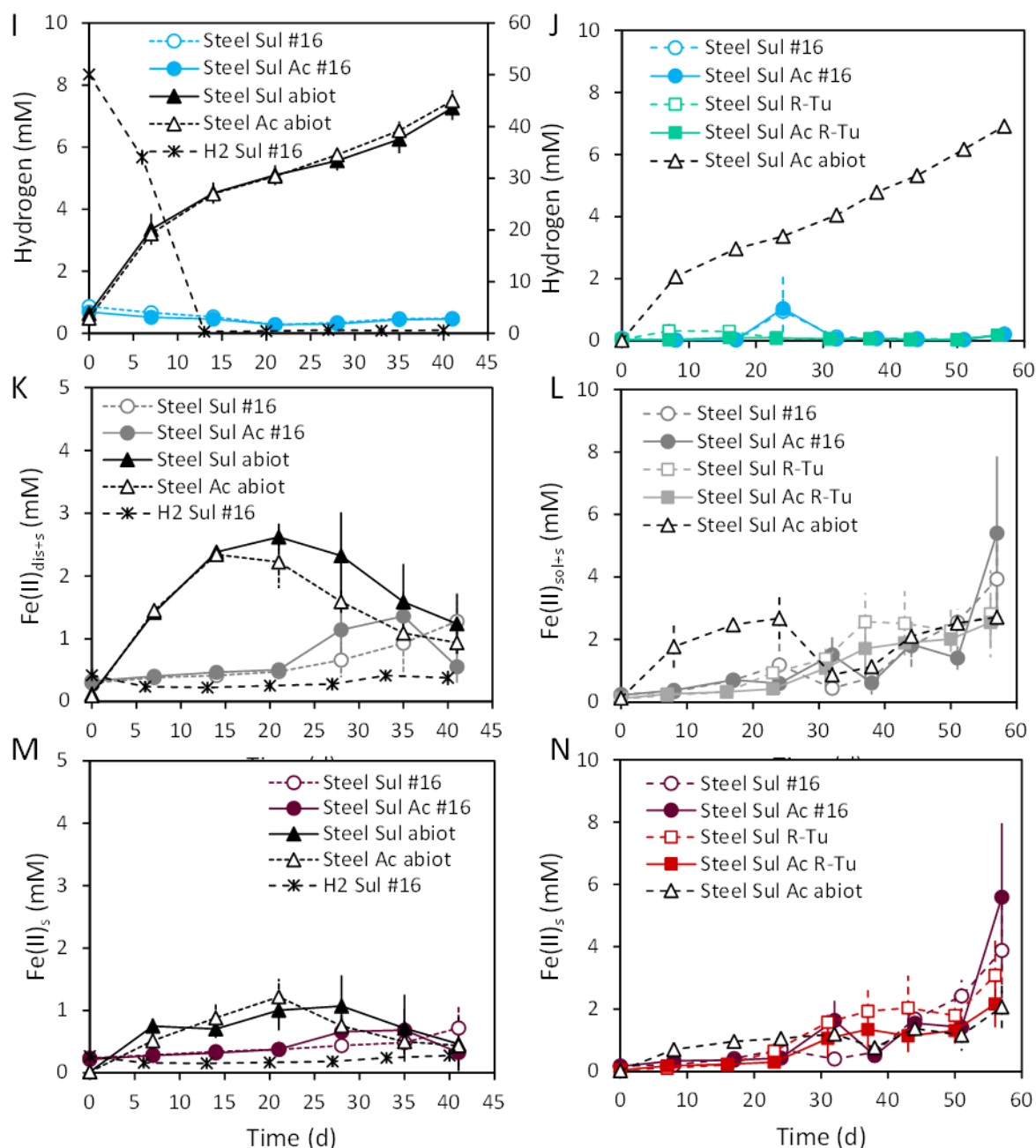


Figure 59: Time course of the different measurement parameters in assays with sulfate during six weeks (A, C, E, G, I, K, M) and eight weeks of incubation (B, D, F, H, J, L, N) at 20°C. Environmental sample #16 was used as inoculum for both incubations, environmental sample R-Tu was only used for the eight-week incubation. Concentrations of sulfide (E), acetate (G) and H₂ (F) for assays H2_Sul_#16 are indicated on the secondary y-axis. Arithmetic means with standard deviation of triplicates.

In assays with steel coupons and microorganisms of environmental sample #16 incubated for six weeks, the prokaryotic community was dominated by OTUs affiliated to genera and/ or families that comprise sulfate reducing bacteria (Figure 60). In assays without acetate, members of the family Desulfocapsaceae and the genus *Desulfosporosinus* (one OTU each) showed highest relative abundances. In the assays with acetate, OTUs affiliated to the genus *Desulfovibrio* dominated. In addition, members of the genera *Desulfomicrobium* and *Desulfosporosinus* were present in high relative abundances. In assays amended with H₂, the genus *Acetobacterium* showed highest relative abundances in accordance with the high production of acetate. Here, the most abundant sulfate reducers were

affiliated to *Desulfomicrobium* followed by *Desulfovibrio* and Desulfocapsaceae. No sequencing could be performed on biofilm samples as too little DNA was extracted.

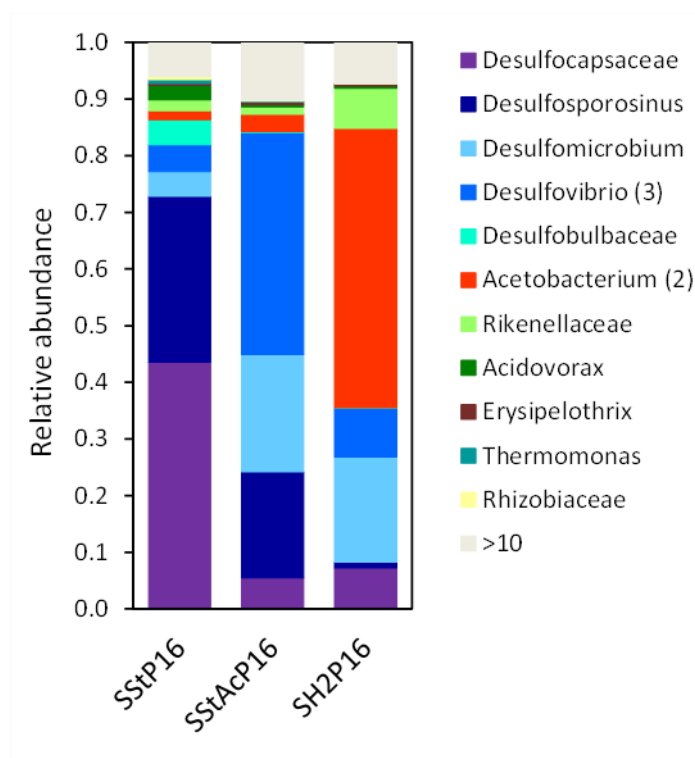


Figure 60: Prokaryotic communities enriched under sulfate reducing conditions using environmental samples #16 for six weeks. Composition is based on 16S-rRNA amplicon sequencing. Relative abundances of the top 10 OTUs with values >0.01. Mean of triplicates. S: sulfate, St: steel, Ac: acetate, P: planktonic, B: biofilm.

During the second corrosion experiment (incubated for eight weeks), sequences affiliated to the genus *Acetobacterium* became more prominent in assays with steel coupons without acetate (Figure 61). Apparently, growth of sulfate reducing bacteria was slowed down due to the lack of an organic C source which might be then supplied by co-cultured acetogens. In assays inoculated with the primary enrichments from #16, OTUs affiliated to the genus *Desulfomicrobium* dominated in the liquid phase, whereas OTUs affiliated to the genus *Desulfosporosinus* dominated within the biofilm. In assays inoculated with the primary enrichments from R-Tu, OTUs affiliated to the genus *Desulfovibrio* showed the highest relative abundances in both planktonic and biofilm communities in the presence of acetate, meanwhile OTUs affiliated to the genus *Desulfosporosinus* dominated in the absence of acetate.

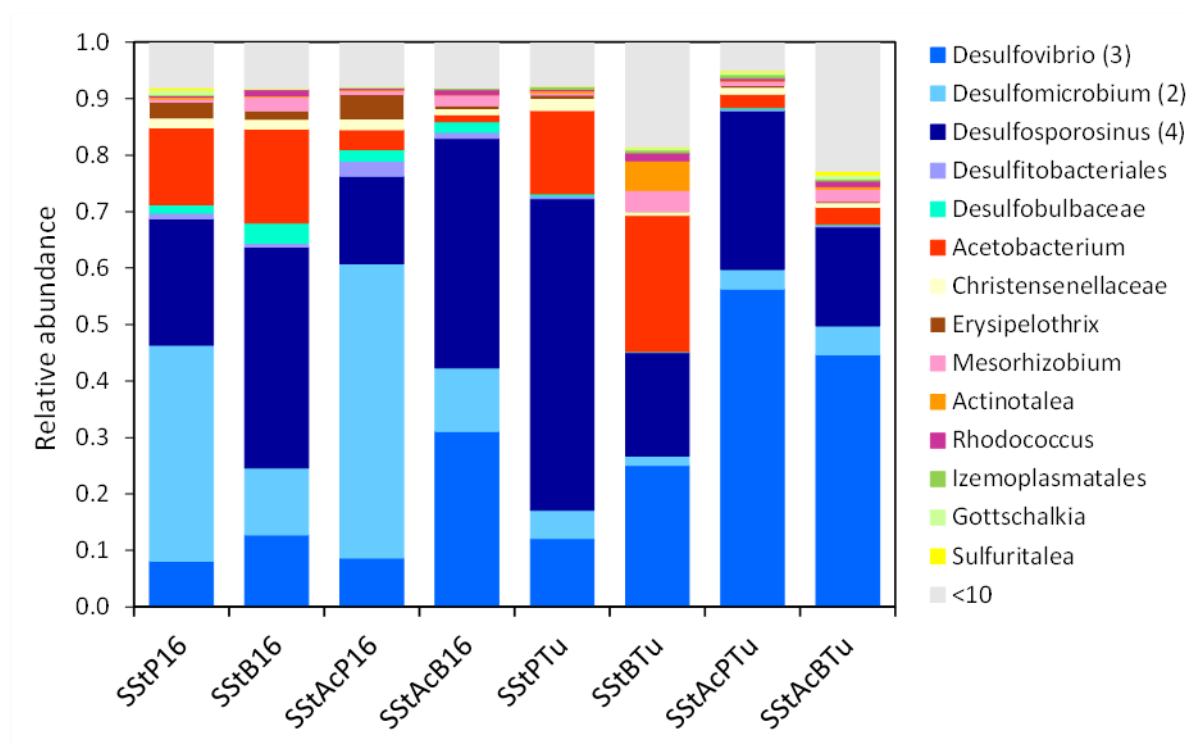


Figure 61: Prokaryotic communities enriched under sulfate reducing conditions using environmental samples #16 and R-Tu for eight weeks. Composition is based on 16S-rRNA amplicon sequencing. Relative abundances of the top 10 OTUs with values >0.01. Mean of triplicates; in case of biofilm samples mean of duplicates. S: sulfate, St: steel, Ac: acetate, P: planktonic, B: biofilm.

4.4.4 Proteomics of first corrosion experiment under sulfate reducing conditions

The proteomics results are mentioned and discussed in detail in the manuscript under preparation (Provisional title: “Exploration of the metabolic functions from enriched freshwater sulfate reducing communities under corrosive and non-corrosive environments”). The aim of this paper was to study the community shifts and elucidate the electron transport pathways of corrosive freshwater samples in the presence and absence of Fe^0 . Thereby, an enrichment of corrosive strains with Fe^0 can be achieved, which are capable of specifically utilizing steel for growth, either directly or mediated through H_2 . For which, the protein expression is adapted towards the synthesis of specific proteins that facilitate the transport of electrons from steel, e.g., outer membrane proteins and periplasmic hydrogenases. The community were analysed by 16S rRNA sequencing and the enzymatic/protein patterns by metaproteomic analyses. An enrichment of sulfate reducing bacteria was observed in the 16S rRNA composition (Figure 60) and concomitant sulfate consumption trend in all the assays. In the steel assays, the families *Desulfocapsaceae* (alternatively *Desulfobulbaceae*-100% sequence similarity with BLAST tool (Boratyn GM, 2013)), *Desulfobulbaceae*, *Desulfitobacteriaceae*, *Desulfomicrobiaceae* and *Desulfovibrionaceae* were among the TOP10 identified families. In the H_2 -amended assay, apart from sulfate reducing families, acetogens were found to be the second most abundant community. Subsequently, enzymes catalyzing the acetogenesis pathway (also Wood-Ljungdahl) assigned to acetogenic species were identified, and the key enzymes were CODH/ACS, acetate kinase and phosphotrans-acetylase. Acetogens in the H_2 -amended assays, exist mutualistically with sulfate reducers, and mainly play a role as the C-source, hence a concomitant increase in biomass was observed compared to the steel assays. Additionally, enzymes pertaining to the sulfate respiration (Figure 62), were found among all assays which is the primary energy metabolic pathway among sulfate reducers, the corresponding enzymes ATP sulfurylase (sat), APS reductase (aprAB) and dissimilatory sulfite reductase (dsrAB) and membrane complexes Qmo, Qrc were identified. The identification of SRB enzymes

concur with the 16S rRNA results, whereby an enrichment of sulfate reducing communities were observed. Another major metabolic pathway, is the C-metabolism, and autotrophic bacteria that with CO₂, would have to fix CO₂ to acetyl-CoA for biomass by the reductive acetyl-CoA or Wood-Ljungdahl pathway (Figure 62). Therefore, the corresponding key enzymes CODH/ACS complex, methyltransferase and corrinoid protein (Vit B12) were found to be abundant in the steel assay with CO₂. Further on, to more specific findings of corrosive bacteria and the energy transport pathways. In the steel assays, an abundance of a cable bacteria family, *Desulfobulbaceae* was identified, and a previous study showed that it was able to grow with Fe⁰ as the sole electron donor, and the probable mechanisms of electron transfer were H₂-mediated transfer (HIMET) and direct electron transfer (DIMET) (Dinh et al., 2004). Thereby, in order to find potential outer membrane electron transport proteins /complexes that play a role in electron transfer, by DIMET or HIMET mechanisms. The metaproteomic results were filtered only for the significantly abundant proteins ($p \leq 0.05$ and $-2 \geq FC \geq 2$) in the steel assays compared to the H₂-amended assay. Therefore, outer membrane porin proteins were identified explicitly in the steel assays that could potentially be involved in the transport of electrons via the DIMET mechanism. There have been studies associating the role of porins and multiheme cytochromes as conduits or channels for electron transport, in *Shewanella*, *Desulfopila corrodens* (Beese-Vasbender et al., 2015; David J. Richardson, 2012). However, multiheme cytochromes and also the associated organisms were not found. Nevertheless, periplasmic Ni-Fe hydrogenases, which metabolize H₂ oxidation in the periplasmic space and, cytochrome-c3 pools, that play a role in the transfer electrons from the periplasm to the cytoplasmic membrane complexes were also identified in the steel assays. Hence, these enzymes show that the HIMET mechanism was preferred in the steel assays, mediated through H₂, formed by the oxidation of Fe⁰, thereby causing iron loss. In both HIMET and DIMET, the bacteria would need to be in contact with the steel for the transfer of electrons. Hence, the identification of these proteins supports the fact that the observed loss of steel in these assays can be linked to the utilization of Fe⁰ as an electron source. Overall, the proteomic analysis of the sulfate enrichment showed us that the TOP10 families enriched with steel could be potential corrosive bacterial strains, and the overexpression of periplasmic Ni-Fe hydrogenases, and c3-cytochromes are potential indicators for MIC mediated through H₂ in sulfate reducers, and ideally an identification of porins associated with multiheme cytochromes are a probable indicator for DIMET mechanism, which is a more aggressive form of corrosion. In mixed communities, we observed that acetogens can enhance biomass of sulfate reducers, by providing acetate as the C-source: Subsequently, in nature, corrosive strains can thereby profit from acetogens and build higher biomass which would lead to more corrosion.

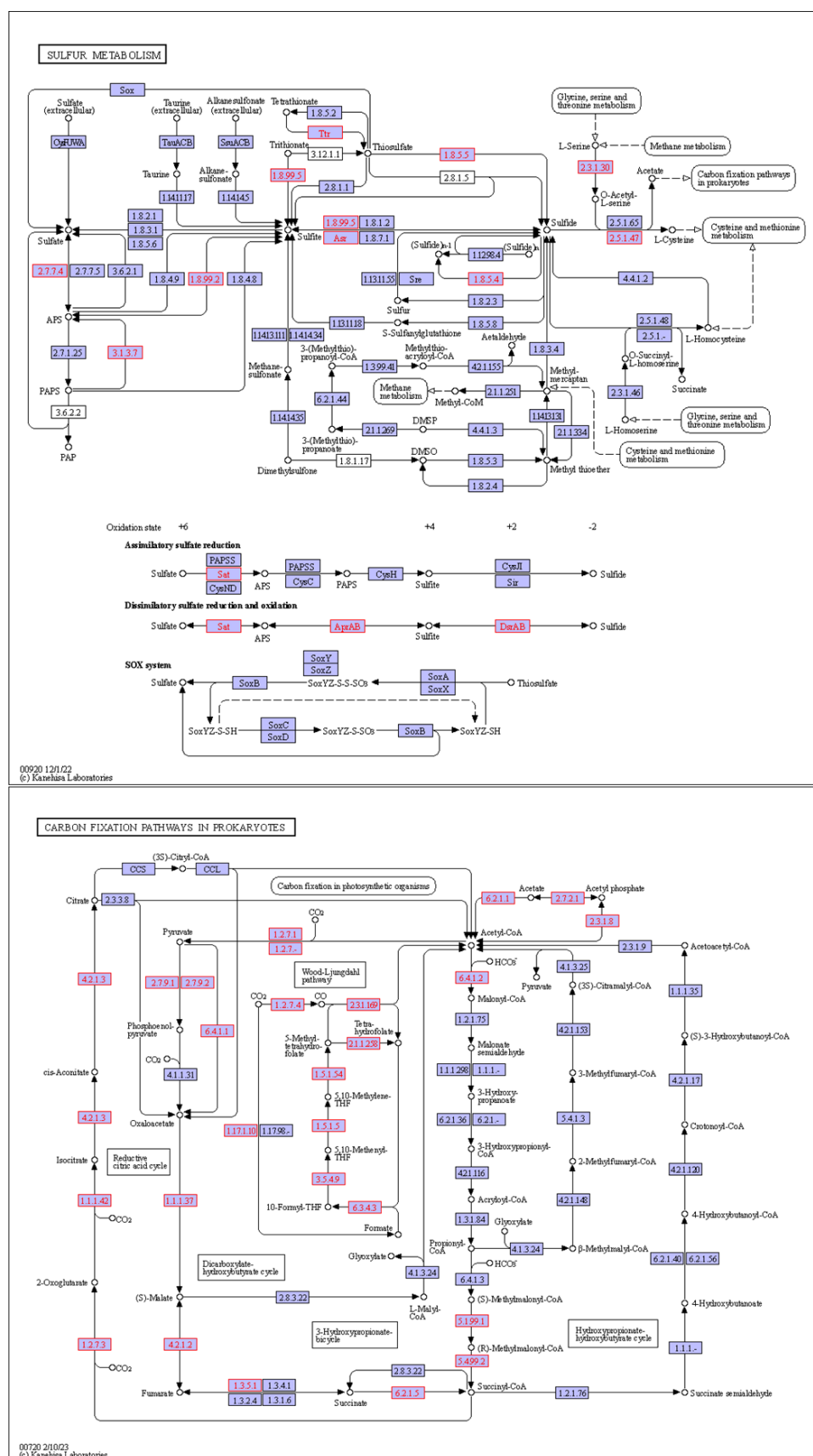


Figure 62: The highlighted KEGG pathways for dissimilatory sulfate reduction (top) and CO₂ fixation (bottom) are the depicted associated proteins identified in the steel-only assays, this plot was generated from KEGG (Kanehisa M, S. Y., Kawashima M, Furumichi M, Tanabe M (2016). "KEGG as a reference resource for gene and protein annotation." *Nucleic Acids Research* 44: D457-D462.).

4.4.5 Corrosion experiment under CO₂ reducing conditions

For the corrosion experiment with CO₂ as electron acceptor, the primary enrichments of the environmental samples #8 (FargePW1b) and R-b1 (RG.P.film.b) were used. These samples showed the highest relative abundances of sequences affiliated to methanogenic prokaryotes compared to the other samples used for primary enrichments. The following five treatments were included (in triplicate): i) steel with microorganisms from #8 (Steel_CO₂_#8; I – III), ii) H₂ with microorganisms from #8 (H₂_CO₂_#8; IV – VI), iii) steel with microorganisms from R-b1 (Steel_CO₂_R-b1; VII – IX), iv) H₂ with microorganisms from R-b1 (H₂_CO₂_R-b1; X – XII), and v) steel without microorganisms (Steel_CO₂_abiot; XIII – XIV) (Figure 63). Assays were incubated for nine weeks.

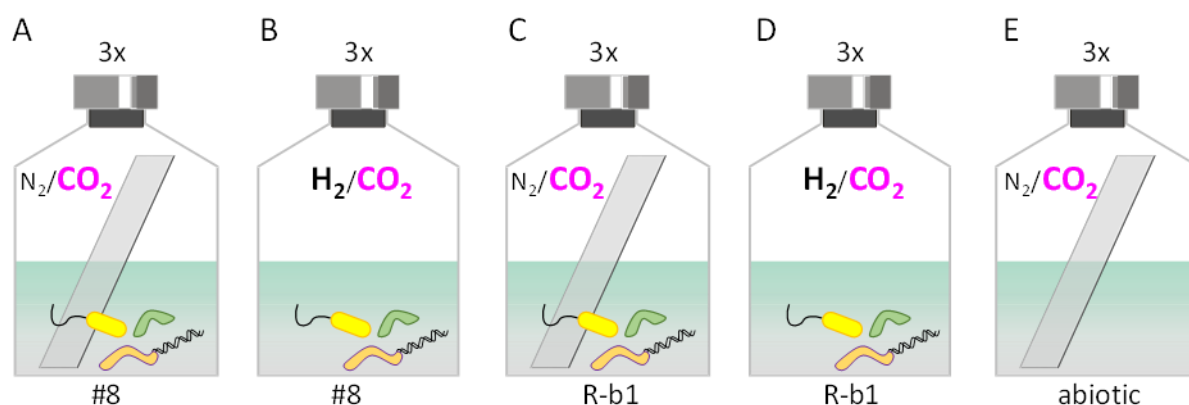


Figure 63: Experimental set-up for corrosion experiment under CO₂ reducing conditions for nine weeks: primary enrichment from sample #8 with steel coupon (Steel_CO₂_#8; I – III) (A) or H₂ as electron donor (H₂_CO₂_#8; IV – VI) (B), primary enrichment from sample R-b1 with steel coupon (Steel_CO₂_R-b1; VII – IX) (C) or H₂ as electron donor (H₂_CO₂_R-b1; X – XII) (D), and abiotic control (Steel_CO₂_abiot; XIII – XIV) (E). Each assay was run in triplicate.

Iron loss in the assays with microorganisms was less than in the abiotic assay (Figure 64). In assays containing microorganisms from sample #8 the iron loss accounted for only 36% of the iron loss determined for the abiotic control.

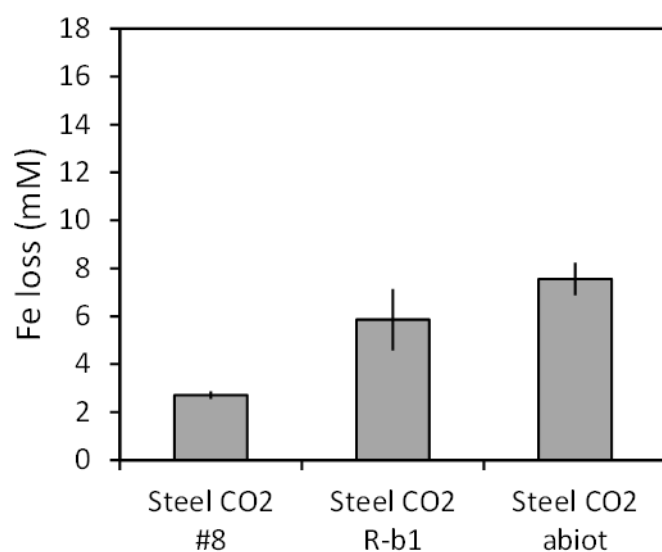


Figure 64: Iron loss from steel coupons in assays with CO₂ as sole electron acceptor after nine weeks of incubation at 20°C. Arithmetic mean with standard deviation of triplicates.

Accordingly, the pH increase was less in the assays with microorganisms and steel coupons in comparison to the abiotic control (Figure 65A). This is especially true for the assays with microorganisms from environmental sample #8. Here less methane was produced than in assays with microorganisms from environmental sample R-b1 (Figure 65B). Acetate production was similar for assays with steel coupons of either environmental sample (Figure 65C). Molecular hydrogen reached high concentrations in abiotic controls, whereas in the assays with microorganisms H_2 was kept at a low level after an initial increase (Figure 65D). Most likely the microorganisms present consumed the H_2 produced by the abiotic corrosion of Fe(II). Furthermore, higher corrosion rates in the absence of microorganisms were also corroborated by the higher release of ferrous iron from the steel coupons in the abiotic assay (Figure 65E, F).

In the assays amended with H_2 as electron donor, higher pH values were observed indicating higher microbial activities than in assays with steel coupons. In assays with microorganisms from #8 a high production of acetate was observed, meanwhile in assays with microorganisms from R-b1 a high production of methane was observed. Protein contents reached $3.5 \pm 0.5 \text{ mg l}^{-1}$ and $2.0 \pm 0.3 \text{ mg l}^{-1}$ in assays with microorganisms from #8 and R-b1, respectively. For steel coupons, only protein contents of $0.26 \pm 0.10 \text{ mg l}^{-1}$ and $0.26 \pm 0.10 \text{ mg l}^{-1}$ were detected for assays with microorganisms from #8 and R-b1, respectively.

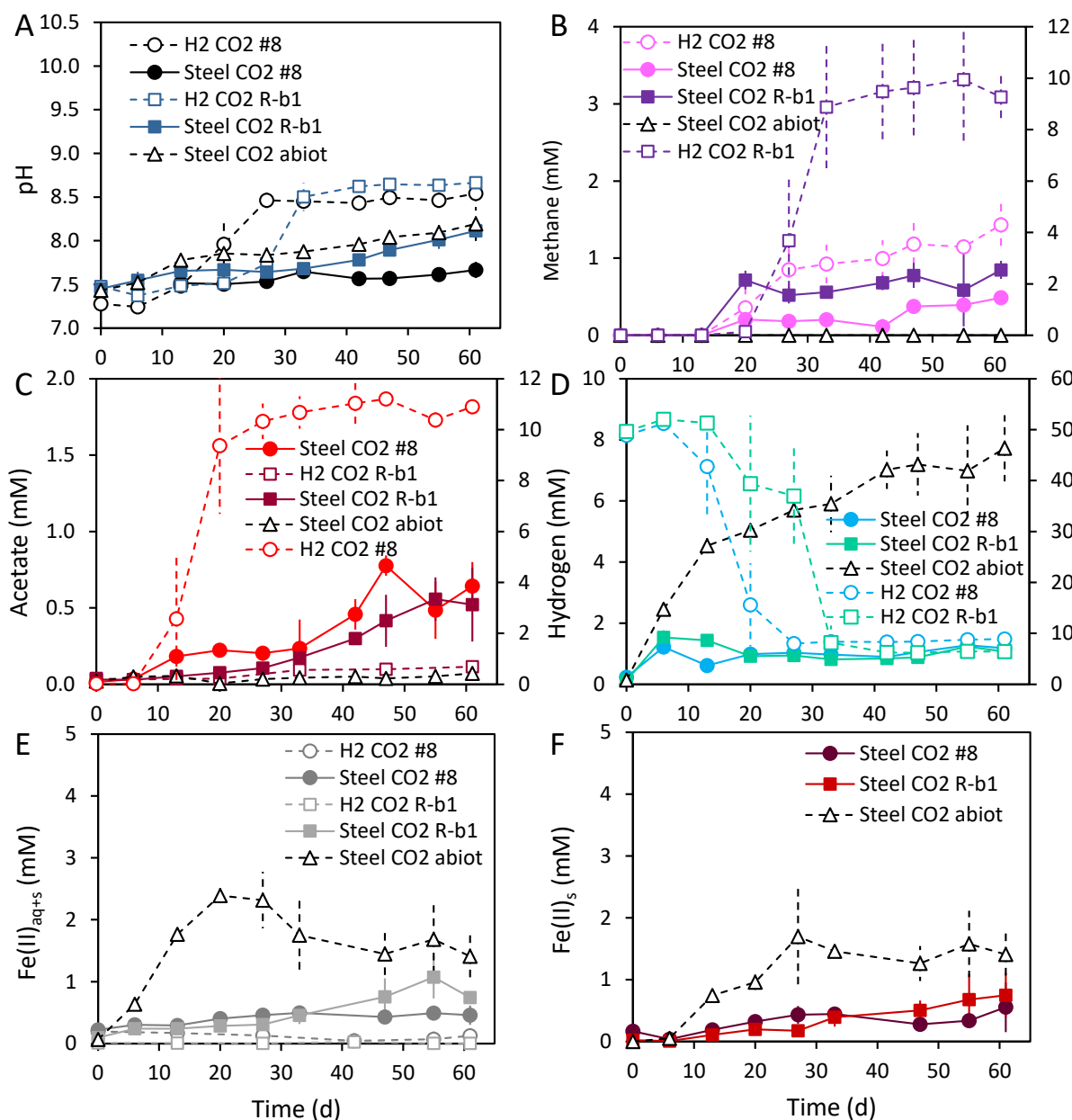


Figure 65: Time course of the different measurement parameters in assays with CO₂ as sole electron acceptor during nine weeks of incubation at 20°C. Concentrations of acetate (C) and H₂ (D) for assays H2_CO2_#8 as well as concentrations of methane (B) and H₂ (D) for assays H2_CO2_R-b1 are indicated on the secondary y-axis. Arithmetic means with standard deviation of triplicates; for assays H2_CO2_#8 only two replicates were included as one (XI) was most likely contaminated with O₂.

As expected, based on the high acetate production, the prokaryotic communities in enrichments from #8 were dominated by one OTU affiliated to the genus *Acetobacterium*, a well-known acetogen (Figure 66). Only the biofilm community contained a higher relative abundance of another OTU which was affiliated to a methanogen of the genus *Methanobacterium*. A large fraction in the assays with steel coupons was constituted by sequences affiliated to the genus *Desulfovibrio*. Assays with microorganisms from R-b1 were dominated by *Methanobacterium* when H₂ was used as electron donor. This was also to be expected as a high methane production was observed in these assays. However, when steel coupons served as sole electron source, sequences affiliated to the genus *Desulfosporosinus* dominated.

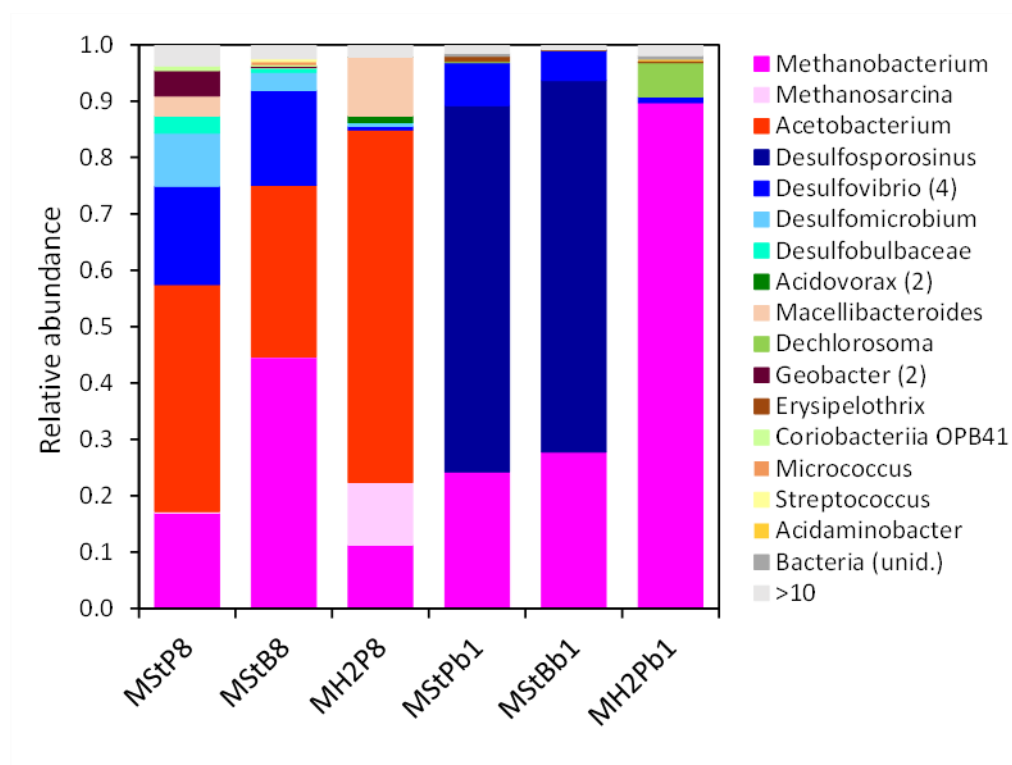


Figure 66: Prokaryotic communities enriched under CO_2 reducing conditions using environmental samples from #8 and R-b1 for nine weeks. Composition is based on 16S-rRNA amplicon sequencing. Relative abundances of the top 10 OTUs with values >0.01 . Mean of triplicates. M: methanogenic enrichment, St: steel, Ac: acetate, P: planktonic, B: biofilm.

4.5 Laboratory study: Microcosm experiments with pure cultures

4.5.1 Pre-experiment

The experimental set-up is mentioned in detail in section 3.5.1. The first objective of this study was to identify a difference in the protein expression patterns of model freshwater and marine water sulfate reducing bacterial strains (*D. vulgaris* and *D. ferrophilus*) under different nutritional conditions (lactate, H_2 and Fe^0). Secondly, a dominance of CMIC, H_2 -CMIC and EMIC (for theoretical background see Introduction) corrosion mechanisms on steel was simulated, with lactate (+steel), H_2 (+steel) and Fe^0 as nutritional substrates. These objectives were laid, in order to understand the metabolisms of the strain for nutritional condition and the extent of corrosion with CMIC, H_2 -CMIC and EMIC mechanisms. Also, with each mechanism signature proteins are expected for identification that are characteristic to each mechanism, such as outer membrane multiheme cytochromes, Ni-Fe hydrogenases and cytoplasmic/periplasmic cytochromes in the Fe^0 -only assay. Therefore overall, by investigating model organisms under controlled conditions, it is possible to understand the extent of corrosion (visual and quantitative) influenced by each strain, the associated biophysiochemical changes and, importantly, the functional adaptation of the bacteria, the expression of specific proteins to aid electron transfer when steel is the only electron donor. Thereby, the characteristic results (biophysiochemical and metaproteomic patterns) from this study can be extrapolated in MIC studies and diagnostics for corrosion damage in freshwater and also marine hydraulic steel structures.

4.5.1.1 Biophysiochemical parameters

The time course trends of the biophysiochemical parameters of the assays were studied and these parameters contributed in understanding the energy metabolism (SO_4^{2-} reduction) and C-metabolism, that were adapted according the nutritional conditions. Thereby, supporting our investigation on

studying the indicators of biotic corrosion. The samples were measured weekly (details see 3.5.1), the respective measurements are represented as time-trends (Figure 67). The nomenclature of abbreviations for the two strains are dsv and dsf, and the nutritional substrates provided for each strain are represented as lac, H₂ and Fe⁰ respectively. One of the triplicates from dsf-lac-assay was lost after day-3, due to an accident, hence only individual data points for the remaining two duplicates were plotted in the graphs for dsf-lac assay. Moving on to the individual results, the pH trend of the bulk solution was alkaline in all the assays due to H⁺ consumption as a result of sulfate reduction (Figure 67a). Also, lower redox potentials validate reducing anaerobic conditions in biotic assays compared to the abiotic assays (Figure 67c). In the lactate assays (dsv-lac and dsf-lac), lactate (Figure 67m) and sulfate was consumed steadily (Figure 67e). In the dsv-lac assay, sulfide had an increasing trend, as a result of sulfate reduction (Figure 67g). Also, in the presence of steel, the precipitation rate of sulfides in dsv-lac is slower than its production rate, showing that lactate oxidation was prevalent rather than Fe⁰ oxidation. On the other hand, in the dsf-lac assay sulfide concentrations were lower, mainly due to the precipitation of the released sulfide ions with the ferrous ions to iron sulfide, indicating that the oxidation rates of Fe⁰ in *D. ferrophilus* are higher compared to *D. vulgaris*. Correspondingly, ferrous ion concentration was almost undetectable in the dsf-lac assay compared to the dsv-lac (Figure 67i). And, the steel weight loss, (Figure 67k) and the electron budgets (Figure 67l) confirm that, in the dsf-lac assay, Fe⁰ was used as an electron source in addition to lactate for sulfate reduction.

In the dsv-H₂ assay, the sulfate and H₂ consumption trends were rapid (Figure 67a and o). Correspondingly, the sulfide concentration has an increasing trend (Figure 67g). H₂ is a bio accessible molecule, which easily diffuses into the cell, thereby metabolized for energy by sulfate reducers, so the steel that was provided, was otherwise not utilized as an electron source, therefore low concentration of ferrous ions were detected (Figure 67i), the steel weight loss (Figure 67k) may be as a result of anoxic Fe⁰ loss, and on an average it was lesser than the dsv-lactate assay, may be due to more biomass in the lactate assays (Figure 67q). The electron budgets (Figure 67l) of the dsv-H₂ assay, show that more H₂ was oxidized than the sulfate reduced, hence an overestimation of the H₂-concentration was found, probably resulting from the measurement method. Nevertheless, the overall H₂ trend shows a decrease and the excess of sulfide ion confirms that H₂ was utilized as the preferential electron donor. On the other hand, the dsf-H₂ assay showed only minimal activity due to the absence of an organic C-source (Figure 67a and o)

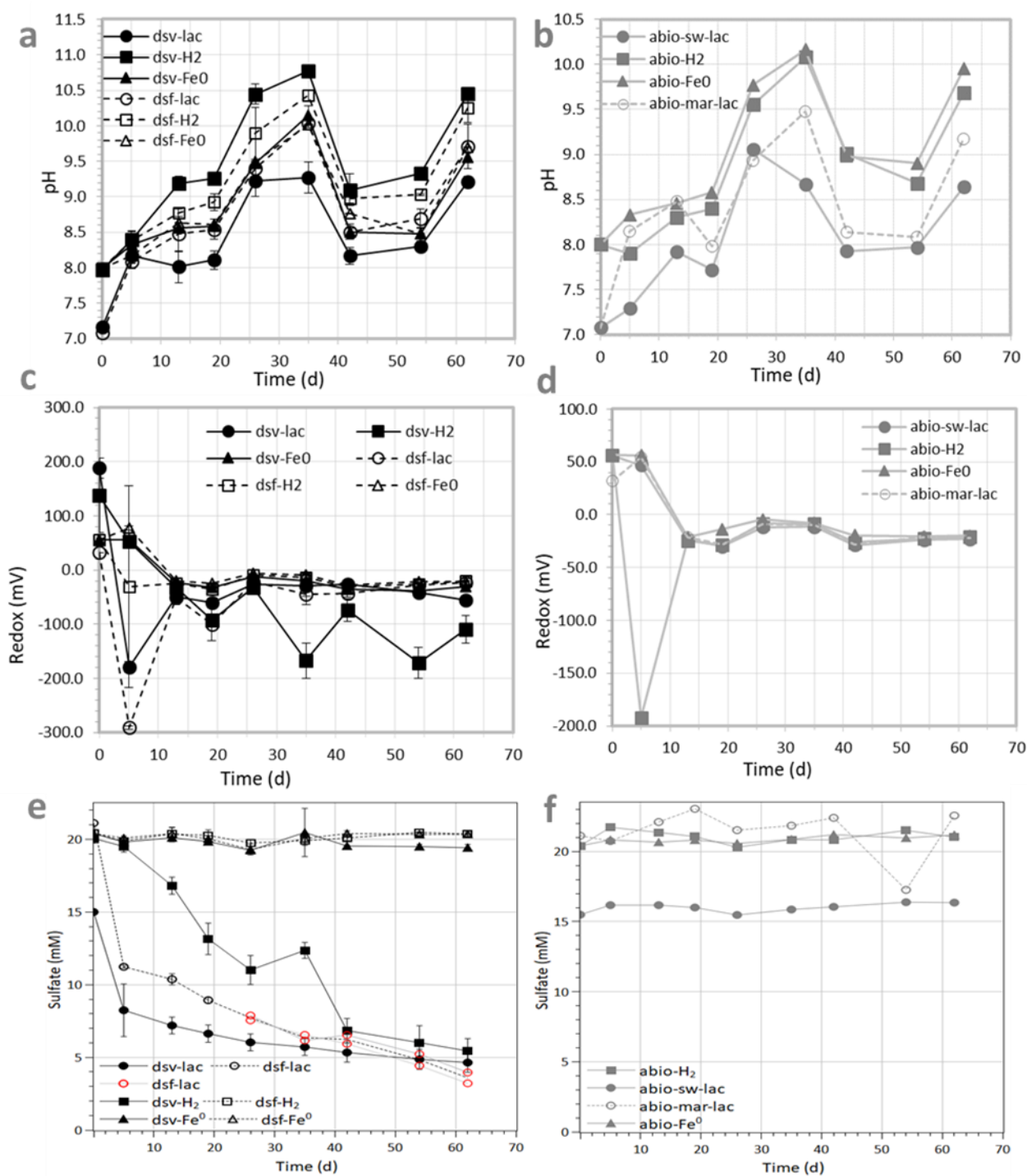
In the dsv-Fe⁰ a comparatively slower sulfate trend was observed than the corresponding H₂ and lactate assays (Figure 67e). Lactate and H₂, as electron donors are easily metabolized by sulfate reducers, and lactate also serves as a C-source, thereby facilitating biomass increase (Figure 67q).

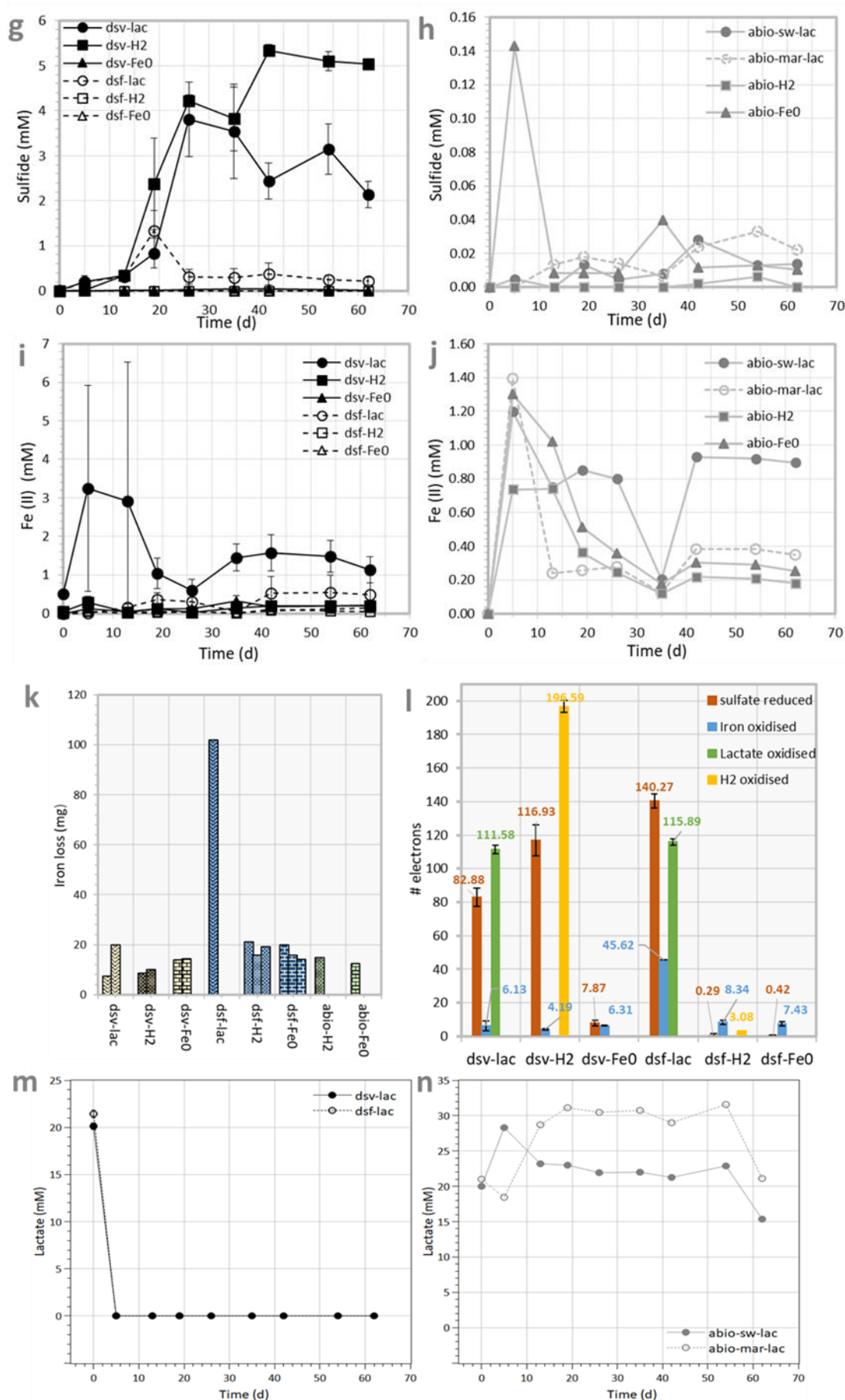
Whereas, Fe⁰ as a solid substrate would require the expenditure of extra energy to mediate ways of electron extraction. Hence, in Fe⁰ assay, the bacteria adapt themselves to facilitate ways to gain more energy rather than biomass increase, especially under autotrophic conditions (without organic C-source) which, is also evident by the lower gene copy numbers (Figure 67q). Nevertheless, steel weight loss and sulfate reduction show that the dsv strain could survive with Fe⁰ under autotrophic conditions. Hence, in order to extract electrons from metal, as per literature three main modes of microbial electron transfer from steel are possible (details see Introduction), direct electron uptake (DIMET), H₂-mediated electron uptake (HIMET) and shuttle-mediated electron uptake (SIMET). The evidence of shuttle molecules by *D. vulgaris* is not evident (Woodard et al., 2023). Hence, DIMET and HIMET would be considered as two possibilities of electron transfer in the Fe⁰ only assays. In a study, rapid sulfate reduction with steel was considered to be an indication for the aggressive DIMET mechanism (Dinh et al., 2004). However, in our study based on the weight loss and sulfate reduction in the Fe⁰ assay, it seems that HIMET mechanism was employed for electron transfer. In order to substantiate this assumption, the proteomics results need to be further studied. The dsf-Fe⁰ assay indicates marginal activity due to the lack of an organic C-source, yet more average weight loss was observed than the abiotic assay, also the same was observed in the dsf-H₂ assay, this shows that *D. ferrophilus*

inoculum were not able to increase in growth, but probably survived utilizing Fe^0 until starvation, thereby causing iron loss.

Apart from the biotic assays, abiotic assays also showed anoxic corrosion of steel (Figure 67k) (Figure 67b), and the abiotic assays had a higher weight loss than the biotic *D. vulgaris* assays, as a result of uniform abiotic corrosion. But, upon steel surface visualization, the biotic corrosion showed more surface damage, cracks were observed, whereas the abiotic corrosion did not indicate any such signs of damage (data not shown).

The biophysiochemical trends of the electron donors lactate, H_2 , Fe^0 (weight loss) and sulfate reduction indicate that H_2 and lactate seem to be the most preferred electron sources for *D. vulgaris*, and *D. ferrophilus* can also utilize electrons from Fe^0 . Additionally, *D. vulgaris* was able to survive with CO_2 and H_2 as an electron source, and H_2 seems to be more favorable as an energy source, followed by lactate and steel. Hence, according to our second objective (see 4.5.1), we observed a prevalence of CMIC, H_2 -CMIC and EMIC mechanisms in the dsv-lac, dsv- H_2 and dsv- Fe^0 assays, respectively, as well as a combination of CMIC and EMIC in the dsf-lac assay. Moreover, lactate is mainly used for biomass increase, as a favorable electron donor and C-source, thereby inducing more corrosion of steel by sulfide attack. This phenomenon was also validated by the average steel weight loss in the lactate assays, which was almost similar to the weight loss occurring in the steel only assays which are maintained under autotrophic conditions. Corrosion due to sulfide attack can also be attributed to H_2 , which serves only as an electron donor to sulfate reducers (in this case). However, the increase in biomass and weight loss were less pronounced compared to the lactate assays. Overall, if all the substrates were to be supplied in the anoxic zones of steel biofilms, it would then be a conducive environment for the occurrence of MIC. Hence, in conclusion, some MIC indicators that might be extrapolated from this study to the environment are rising pH trends towards the anoxic zones of steel biofilms, decreasing redox values (< -100 mV), and a higher abundance of sulfate reducers compared to other microorganisms, can be an indicator for prevalent MIC processes. The biophysiochemical trends from the environmental samples are shown in section 4.4.





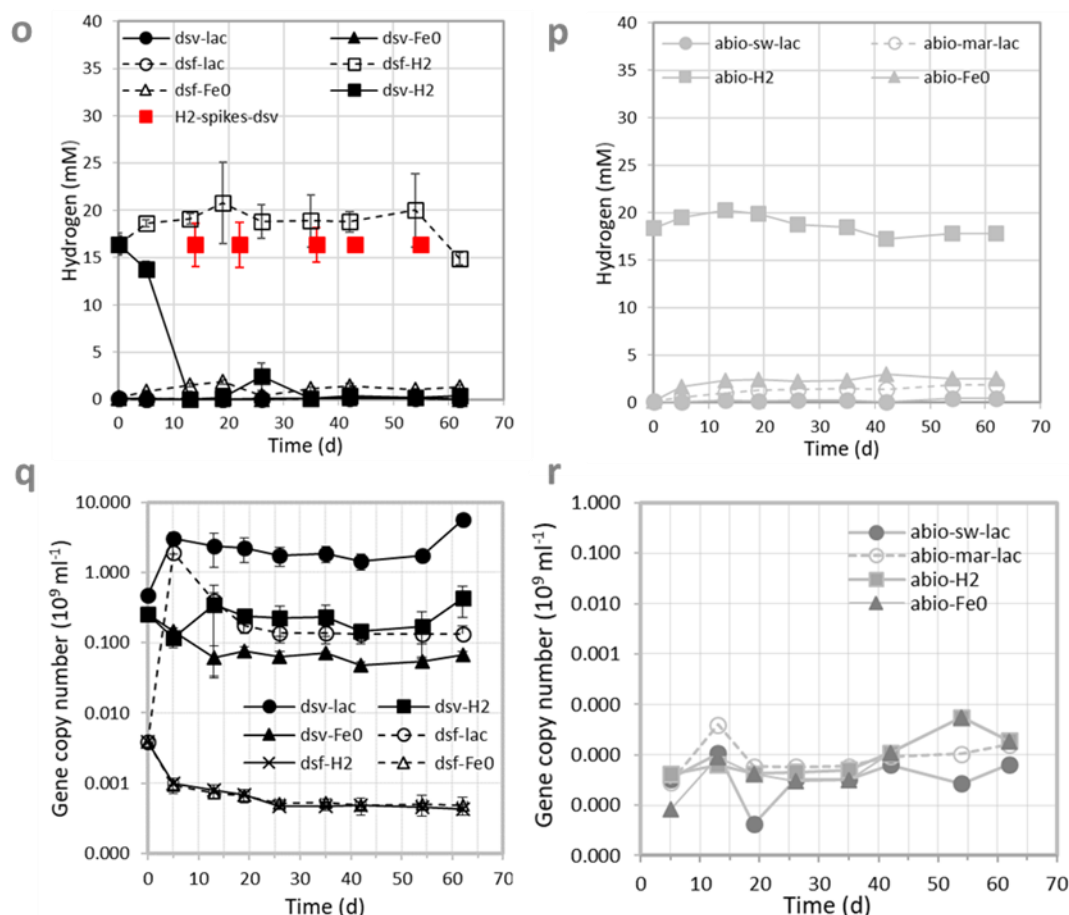


Figure 67: Time course measurements of the biochemical parameters in the biotic (left) and abiotic (right) assays. (a, b) pH, (c, d) redox, (e, f) sulfate, (g, h) sulfide, (i, j) ferrous ion, (k) iron loss, (l) electron budget, (m, n) lactate, measured in the liquid samples and (o, p) hydrogen, in the headspace (q, r) copy numbers of *dsr* gene from the liquid culture. All the data points represent the arithmetic mean and the corresponding standard deviations as error bars for triplicates.

4.5.1.2 SEM images

A steel coupon, from the triplicates per assay were analysed by SEM/EDX. We observed that all the coupons were rich with minerals and cells. And, we found that C and O was consistent in the element analysis for all the biotic assays, this could be to presence of bacterial cells and extracellular polysaccharides in a biofilm. The cells can be seen to be closely-knit within the polysaccharide webs and minerals. Additionally, evidence of cracks was observed in the dsv-H₂ assay coupon, and not in the abiotic assay, which is an indication that biotic corrosion causes damage to the lattice of steel which leads to structural stability, which would have more impact than uniform corrosion which is characteristic to abiotic corrosion. SEM images and EDX data not shown due to the use in publication that is under preparation.

4.5.1.3 Shortcoming of pre-experiment

The proteomics analyses were not successful because of insufficient biomass in the H₂ and Fe⁰ assays, and probably the interference of minerals (Fe_xS_y). Additionally, the H₂- measurement was over-estimated, so the method required optimization and lack of growth of *D. ferrophilus* was observed with CO₂. Hence, the experiment was repeated with certain modifications (main experiment, see 3.5.2). The proteomics analyses were also subjected to optimization, pertaining to lower biomass yields and higher mineral matrix (methodology and results, see 3.17.5 and 4.6).

4.5.2 Main experiment

The details of the experimental set-up are explained in detail in section 3.5.2. The aim for this experiment was to understand the protein expression patterns with Fe^0 as the sole electron donor, when compared to the electron donors, lactate and H_2 . The subjects for the study were again, *D. vulgaris* incubated in freshwater media and *D. ferrophilus* incubated in marine water media. Hence, in contrast to the pre-experiment, steel was not provided in the assays with lactate and H_2 . Originally, the pre-experiment, was designed to understand the differential protein expression patterns for the three conditions (lactate, H_2 and Fe^0) but also to study the influence of lactate and H_2 as substrates on Fe^0 corrosion by sulfate reducing bacteria. In the main experiment, individual assays with only one available electron donor (lactate, H_2 or Fe^0) were set-up to promote a stronger differentiation between the metabolic processes, thereby supporting the pre-experiment. Additionally, some modifications were implemented in the main experiment to overcome the shortcomings of the pre-experiment (details see 4.5.1.3). The following modifications were implemented, firstly 1mM acetate was provided as a C-source in the H_2 and Fe^0 assays for both the strains in order to ensure increase in biomass, since we observed in the pre-experiment that *D. ferrophilus* did not grow under chemolithoautotrophic conditions. And, 1mM acetate would be sufficient as a C-source for both strains. Secondly, an overestimation of H_2 concentration was observed in the pre-experiment (details see 4.5.1) which was tackled by changing the calibration method (details see 3.7.2)

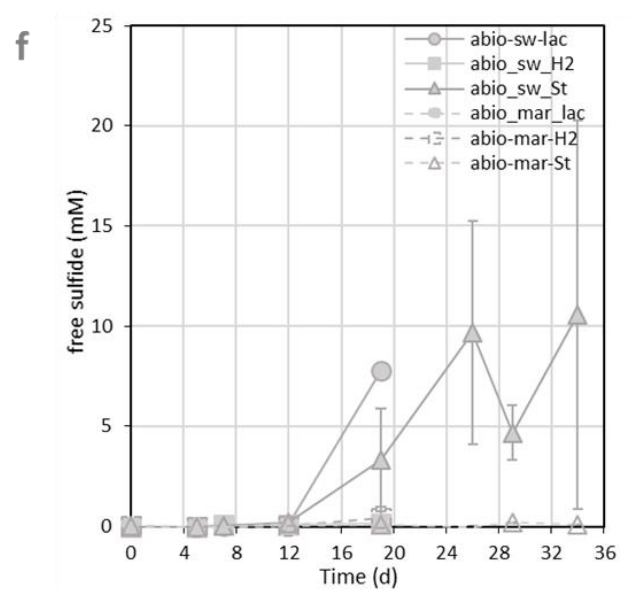
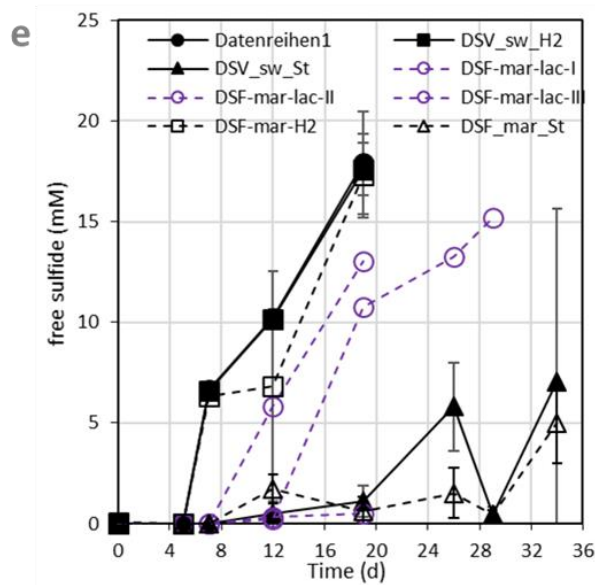
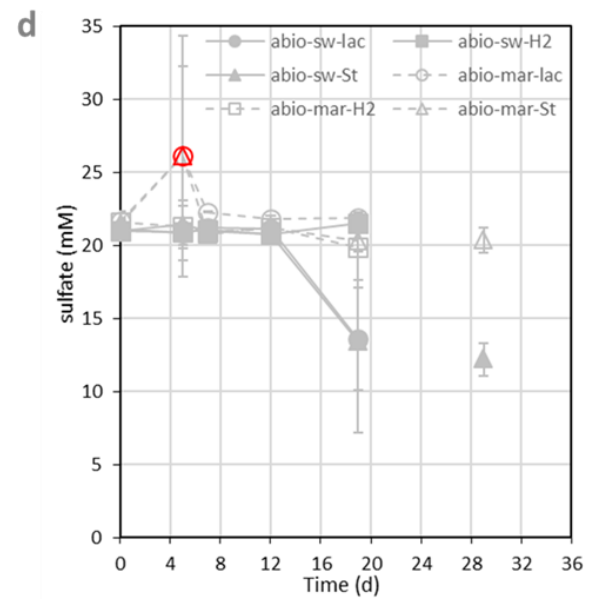
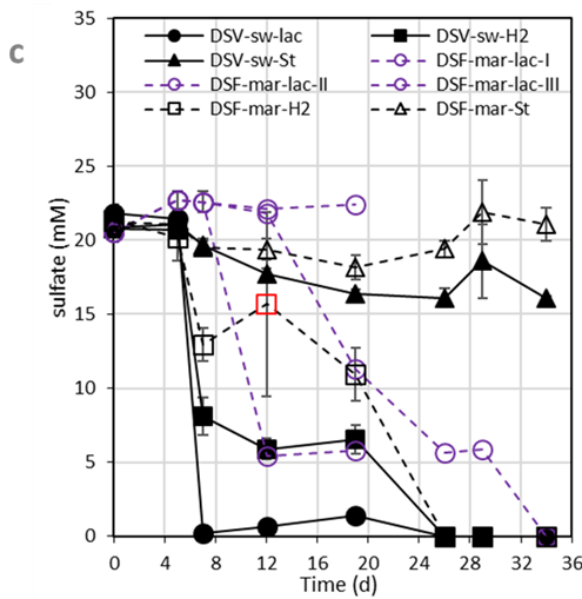
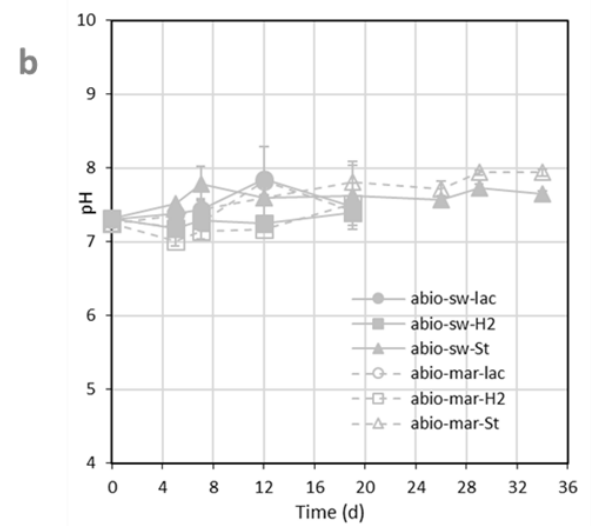
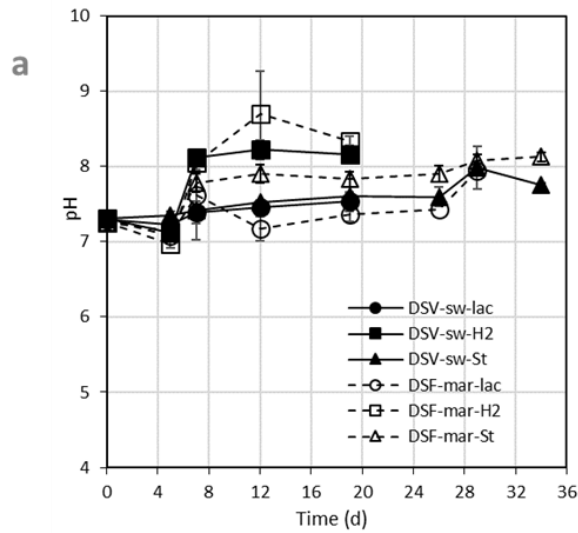
4.5.2.1 Biophysicochemical parameters

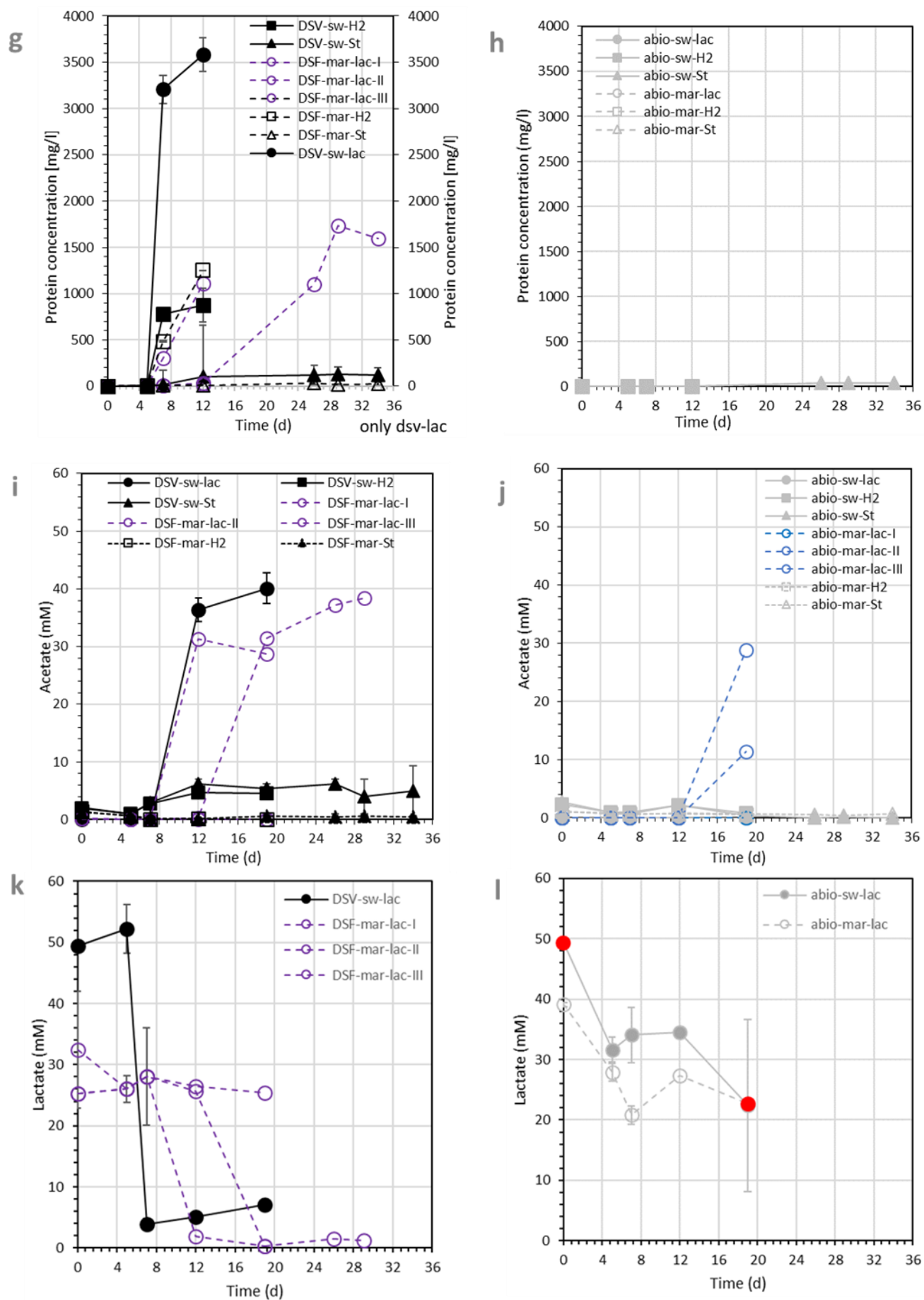
The results of the biophysicochemical trends of the biotic assays will be explained for this experiment in comparison to the pre-experiment. In order to make it comprehensible, the key results of the electron substrates (lactate, H_2 and Fe^0) and the associated metabolites (acetate, Fe (II)) will be discussed first followed by sulfate (electron acceptor) and sulfide trends. In the main experiment, the consumption of the substrates lactate (Figure 68k) and H_2 (Figure 68m) were similar to the pre-experiment. Whereby, lactate was rapidly consumed as a preferential electron donor in the lactate assays and H_2 in the H_2 assays of both the strains. Explicitly, we found a consumption of H_2 by *D. ferrophilus*, because of 1mM acetate, provided as C-source unlike the pre-experiment (grown with CO_2). However, an exception was observed in the triplicates of dsf-lac assays, lactate and also other parameters were inconsistent between the triplicates, may be due to handling errors during inoculation or due to growth delays. Therefore, the trends of individual replicates are represented in purple. But overall, from both the experiments that lactate and H_2 were easily available as electron donors for both the strains, which thereby promoted an increase in biomass (Figure 68g). Additionally, pertaining to the lactate utilization, the detection of the metabolite acetate, confirms lactate metabolises (Figure 68i). Furthermore, in the steel assays where Fe^0 is the main electron donor, weight loss results were particularly more pronounced compared to the pre-experiment. Whereby, a higher weight loss at the end of the incubation was observed by *D. ferrophilus* (48 ± 7) mg compared to *D. vulgaris* (28 ± 1) mg. Subsequently, indicating *ferrophilus* might be employing DIMET mechanisms to accelerate the Fe oxidation. Hence, the proteomics analyses are expected to show the expression of specific outer membrane multi-heme c-type cytochromes, or outer membrane electron transfer complexes that transport electrons into the periplasm (Proteomics: in preparation). As an outcome of Fe^0 oxidation, the total Fe (II) concentrations (Figure 68o) increases initially, followed by a decrease, as a consequence of precipitation as FeS, on the steel surface. Hence, an increase in solid Fe (II) would be expected when a decrease in total Fe (II) was observed, but we observed a decrease in the solid Fe (II). Because, the solid Fe (II) concentration (Figure 68q) was estimated from the liquid phase, and the precipitation of FeS mostly happens on the steel surface. Overall, similar to the pre-experiment, anaerobic respiration using biotic lactate, H_2 and Fe^0 assays in each assay was conducted as expected. Furthermore, the biomass (Figure 68 g) results, as in the pre-experiment indicate that strains with Fe^0 seem to investing most of their energy, for survival and synthesis of enzymes that aid respiration with Fe^0 , when compared to lactate and H_2 .

Further on, to the electron acceptor, sulfate consumption (Figure 68c) was higher in all the assays compared to the pre-experiment, except the dsv-H₂, that had almost the same amount (~14mM) of sulfate consumption. Higher sulfate consumption in the main experiment could be as a result of the increase in incubation temperature, 28°C compared to the pre-experiment, 20°C. The temperature was increased to 28°C, since it is the optimal growth temperature for both the strains, which can thereby lead to an increase in biomass (requirement for proteomics). Furthermore, similar to the pre-experiment highest sulfate consumption was observed in the lactate assay and H₂- assay and least in the steel only assay, in both strains. Thereby, indicating yet again, that lactate and H₂, if available can be easily be utilized by sulfate reducers for growth. Additionally, the sulfate consumption in the steel assays (dsv-st ~5mM and dsf-st~2mM) was not as high as the lactate and H₂ assays, hence in order to utilize Fe⁰, lower sulfate concentrations would suffice for the survival of *D. vulgaris* and *D. ferrophilus*, because reduction of one mole of sulfate, requires 4 moles of Fe⁰, when the bacteria is capable of utilizing steel directly or indirectly (via 4H₂) (Enning and Garrelfs, 2014). Furthermore, due to sulfate reduction, the released metabolite sulfite was also detected in all the assays (Figure 68e). Similar to the pre-experiment, lesser sulfide was detected in the steel assays, due to low sulfate consumption and sulfide precipitation as FeS.

Further onto the abiotic assays, another key difference compared to the pre-experiment was that lower abiotic corrosion was observed compared to the biotic assays (Figure 68n), probably because the growth of the bacteria in the steel only assays were more pronounced due to the added acetate as C-source (1 mM) compared to the autotrophic conditions of pre-experiment (CO₂). Moreover, more abiotic corrosion was observed in the freshwater samples than marine water, which is contrary to the fact that high chloride concentrations can enhance corrosion (Ukpaka, 2011) (Figure 68n), but this is true under oxic conditions, hence under anoxic conditions, freshwater corrosion could be as impactful as marine corrosion. Furthermore, total Fe (II) concentration was also measured in both the abiotic steel assays (Figure 68p) and the ferrous ions measured from abiotic Fe⁰ oxidation, can precipitate with carbonate ions abiotically to solid iron carbonates (Figure 68r). Additionally, in the abiotic assays some contamination was observed which was supported by the consumption of lactate, hydrogen and Fe⁰ (based on protein detection) and sulfate concentrations (Figure 68 d, l, j, h).

Overall, compared to the pre-experiment, here we achieved growth of bacteria in all the assays, especially the steel assays. And, *D. ferrophilus* strain was proved to be more corrosive than *D. vulgaris*.





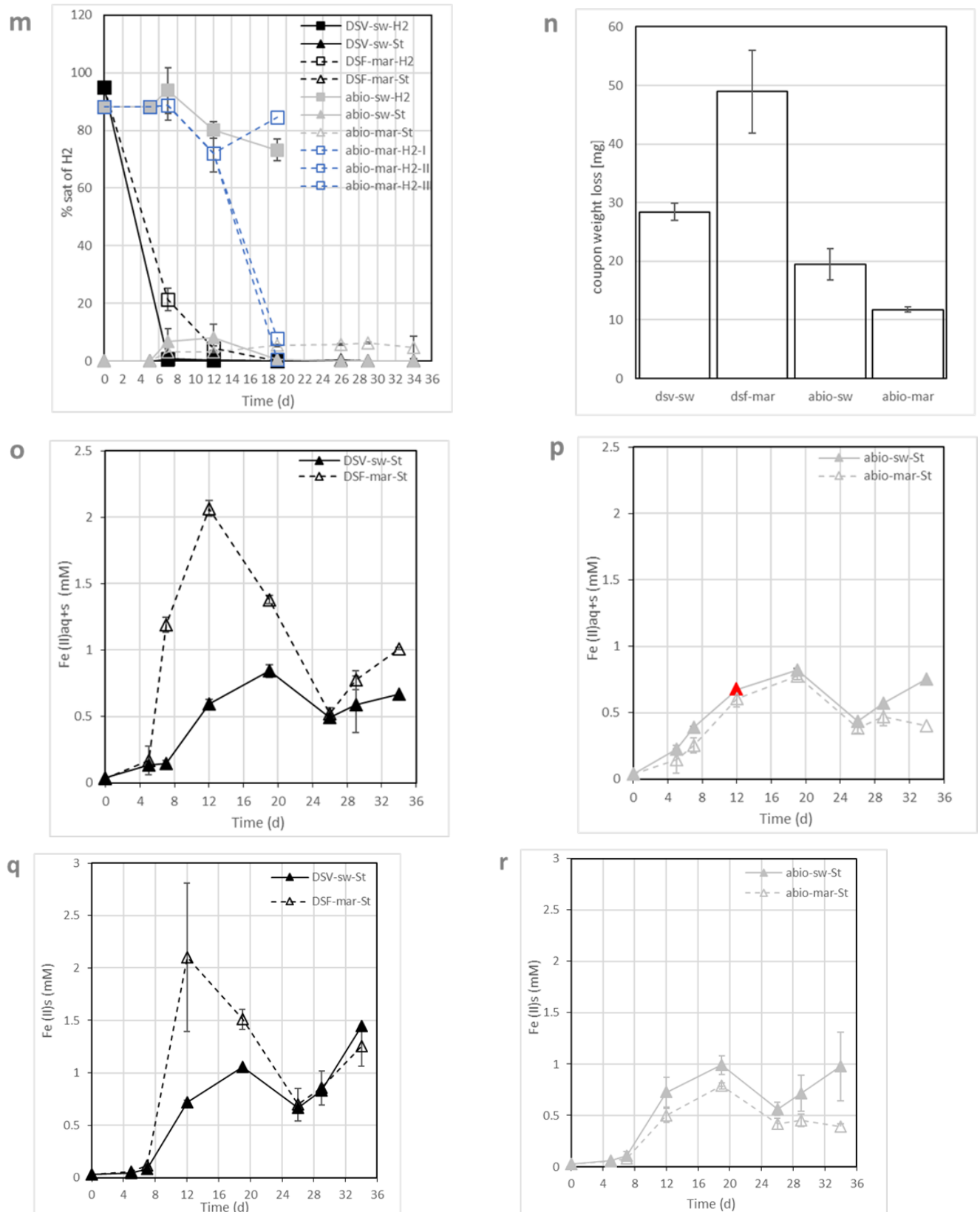


Figure 68: Time course measurements of the biochemical parameters in the biotic (left) and abiotic (right) assays. (a, b) pH, (c, d) sulfate, (e, f) sulfide, (g, h) protein, (i, j) acetate, (k, l) lactate, (o, p) total ferrous ions, (q, r) solid ferrous, measured in the liquid samples and (m, n) hydrogen, in the headspace. All the data points represent the arithmetic mean and the corresponding standard deviations as error bars for triplicates, except the dsf-lac assays which are represented as purple lines for each replicate. Some datapoints are indicated in red, which are either single value or means of duplicates due inconsistencies between the triplicates.

4.6 Proteomics optimization

The optimization experiments in sections 4.6.1, 4.6.2 and 4.6.4 with the dsv-lac assay lysate (SRB lysate with Tris buffer) and liquid cultures in order to evaluate the problematic hindering proteomics analysis. Hence, the dsv-lactate assays were chosen because it had the most biomass compared to the autotrophic assays. Thereby, multiple optimization experiments could be conducted with the same sample. A quick overview of the methods is shown in Figure 9.

4.6.1 BCA method assessment: BSA protein spike test with SRB lysate

The quantification of the protein content in the lysate was essential in order to determine trypsin concentration for successful digestion proteins. Hence, two assays, BCA assay and Bradford assay (4.6.1, 4.6.2) were assessed for compatibility with sulphide and Fe rich samples. BCA assay was commonly used as a standard assay for proteomics so far, and Bradford for the whole cell protein quantification of our anaerobic cultures. Thereby, these two methods were chosen for the assessment. The principle for this experiment is based on the classical spike test, in our case with BSA standard protein (methodology see 3.16.5.1). The BSA-spiked SRB lysate concentration was measured photometrically using the BCA kit (working range: 20-2000 µg/ml). The results are shown in Table 7, which is the measured concentrations of BSA-spiked SRB lysate. The recovered concentrations of the BSA spike were from 70-90% for concentrations above 25 µg/ml (LOQ). Thereby, the difference is not very extreme when estimating concentrations in the working range, since accurate quantification of the protein in the lysate was not absolutely necessary, because our aim for quantification of lysates was to estimate the trypsin concentration and to ensure that approximately the same amount of peptides are injected into LC/MS measurement.

Table 7: Measured BSA spiked lysate concentrations using BCA assay

BSA spike (Theoretical) (µg/ml)	Spiked lysate concentration (µg/ml)	Spiked sample concentration- unspiked SRB lysate (µg/ml)	% Recovery BSA
400	727	326	81
200	547	146	73
100	485	83	83
50	434	32	65
25	426	24	97
12.5	391	-11	-86
6.25	389	-12	-200
3.125	402	0.32	10.2
0	401	0	

4.6.2 Bradford method assessment: BSA protein spike test with SRB lysate

The Bradford assay, was performed as a routine assay to quantify the whole cell protein to monitor the growth of bacteria, through heat induced cell disruption method of our anerobic cultures typically with lower biomass (optimized to a working range: 0.5-4 µg/ml) when compared to aerobic cultures. Coomassie, is already known to be a robust reagent, and has a high tolerance to many chemicals, commonly found in lysate matrices (ThermoFischerScientific; Bradford, 1976). Hence, this method was also tested for compatibility with our Fe rich lysate samples. Similar to the BCA spike test, the BSA-

spiked SRB lysate concentration was also measured photometrically using Bradford assay (Working range: 0.5-4 µg/ml). However, in contrast to the BCA method the sensitivity was much higher and thus the concentration range of the spike was comparatively lower. Table 8 shows the measured concentrations of the BSA spiked lysate. The recovered concentrations of the BSA spike were found to be 90-150% for concentrations from 10 µg/ml to 0.625 µg/ml. Although the smallest spike concentration, 0.325 µg/ml was below our LOQ, 90% recovery was observed. Furthermore, the sensitivity of the Bradford assay was further assessed for much lower concentrations with another BSA spike test (0.063-4 µg/ml), and the SRB lysate was also diluted 100 times, so that concentration does not exceed 4 µg/ml. In this test, we found recoveries with a recovery concentration of 102% for 0.063 µg/ml BSA. Therefore, based on the results of the BCA and Bradford spike test, the Coomassie reagent (Bradford) is comparatively more robust to the Fe-minerals than to the bicinchoninic acid (BCA) and the sensitivity of the Bradford method was considerably higher. Nevertheless, BCA assay is especially good for concentrations above 25 µg/ml, and it has a higher compatibility with detergents (SDS ~0.125%). Therefore, based upon the two tests, we implemented the methods in our proteomics workflow, specifically with the detergent compatible versions because the protein extractions hereafter would be performed with 1% SDS. We used BCA test for pure culture experiments with lactate, with higher biomasses (~400 µg/ml) and the lower biomass and high Fe-mineral autotrophic cultures e.g. enrichment culture were estimated with Bradford, (~1-5 µg/ml). Overall, both the methods were implemented to quantify lysate proteins for further processing (reduction, alkylation and digestion). Additionally, in terms of quantification we subjected the digested peptides to Zip tip-cleanup with C-18 column, so that the peptides were desalted and concentrated. Thereafter, a final quantification with Qubit was performed before injection. Hence, through the quantification of lysate with BCA and Bradford, helped us to ensure that the necessary modifications (reduction, alkylation and digestion) of the proteins are optimal and also inject approximately the same amount of peptides for LC/MS measurement.

Table 8: Measured BSA and lysate concentrations using Bradford assay

BSA- spike concentration (Theoretical) (µg/ml)	Spiked sample concentration (µg/ml)	Spiked sample concentration- unspiked SRB lysate (µg/ml)	% Recovery BSA
10	23.9	13.0	130.2
5	18.2	7.2	144.9
2.5	14.7	3.8	151.2
1.25	12.9	1.9	150.6
0.625	11.9	0.9	154.7
0.325	11.2	0.3	90.3
0	10.9	0	-

4.6.3 FeS spike test with *E. Coli*

The pure culture studies were designed to study the electron transfer pathway of sulfate reducing bacteria under corrosive and non-corrosive conditions. And, sulfate reduction is the primary energy metabolism among sulfate reducing bacteria, hence, sulfides are produced collateral to sulfate reduction. Sulfides with iron form iron sulfides that interact with proteins and hinder the extraction and post-modifications of the proteins (Benndorf et al., 2009). We aimed to extract FeS spiked, *E.coli* cultures and determine the influence the matrix effect of FeS on *E.coli*. The salts Na₂S·9H₂O and FeSO₄·7H₂O

were used to generate 0-20mM of FeS reactions. 20mM was chosen to be the maximum concentration based on the maximum sulfate (20mM) concentrations provided in our media of the microcosm experiments. With respect to the *E.coli* biomass, definite amounts of *E.coli* (whole cell protein) were spiked with FeS and subjected to extraction. The detailed experimental set-up is explained in 3.17.5.3. The protein concentrations of the *E.coli*-FeS spike lysates were determined by detergent compatible BCA assay (Table 9). The protein concentrations of the non-spiked lysates or controls were observed to be almost 4 times ($R^2=0.99$) lower than the initial cell biomass (Bradford assay), the reduction is typically due to extraction losses and cell lysates mostly include only the cytoplasmic proteins unlike whole cell quantifications. Furthermore, the FeS spiked samples showed higher concentrations than the respective control samples. Table 9 indicates the matrix effect due to the FeS spike on the protein concentration. Matrix effect here is defined as the influence of FeS (matrix) on the BCA reagent, causing an alteration in the actual absorbance values, thereby resulting in an overestimation or underestimation or both of the protein concentrations. The vertical trend of the table shows that, the protein concentration of the spiked samples concentration was overestimated, and the concentration increases with the FeS spike levels. And the horizontal trend indicates that the matrix effect of FeS is higher with the decreasing *E.coli* biomass (2.49-0.16 mg/l). Since, a trend was observed between the protein concentrations and the FeS spikes, regression curves of the measured concentrations for *E. coli* (2.49-0.16) mg/ml and the corresponding FeS spike concentration (20-4) mM were plotted. The slope was observed to be almost 5% for all the concentrations, proving a correlation between the FeS spike and the protein concentration. Furthermore, in order, to test if the interaction of FeS and the BCA reagent was systematic, the measured O.D. values of the “pure” FeS mineral (referred to as blind values) were plotted against the spike levels (20-4) mM. We observed a strong positive correlation (R^2 is 0.99) between the FeS spike concentrations and the blind values (O.D). Hence, FeS reaction with Bicinchoninic reagent was evident and a proportionality factor exists between the FeS concentration and the O.D. values. Nevertheless, in a corrosive environment with sulfate reducers, there could be other Fe- phases that contribute to the matrix effect. But, FeS is normally one of the main corrosion products in the anaerobic zones. Also, we observed FeS as a greyish residue optically during the extraction of the lysate (even in the pre-experiment). Hence, the reaction of FeS with bicinchoninic reagent is co-relatable. A plausible approach to quantify proteins in our Fe-mineral rich lysates of future samples would be to prepare BSA calibration standards with FeS spike and thereby estimate the unknown concentration from the regression plot of these standards.

Table 9: Measured protein concentrations in the *E. coli*- FeS spiked lysates. The FeS spike concentrations range from 0-20 mM.

	Measured concentrations [mg/ml]					
FeS spikes (mM)	<i>E.Coli</i> [2.49mg/ml]	<i>E.Coli</i> [1.24mg/ml]	<i>E.Coli</i> [0.62mg/ml]	<i>E.Coli</i> [0.31mg/ml]	<i>E.Coli</i> [0.16mg/ml]	<i>E.Coli</i> [0 mg/ml]
20	1.59	1.37	1.23	1.16	1.27	1.22
16	1.29	1.08	1.06	1.06	0.91	1.00
12	0.95	0.99	0.90	0.84	0.85	0.70
8	0.92	0.65	0.59	0.63	0.39	0.43
4	0.78	0.57	0.45	0.34	0.25	0.26
0	0.56	0.30	0.18	0.08	0.02	-

Table 10: The matrix effect of FeS on *E. coli* protein is depicted below. The first row indicates the biomass of *E. coli* (whole cell protein) subjected to extraction with and without FeS spikes (column 1). The colour scheme from red through green corresponds with high to low matrix effect.

FeS spikes (mM) (Theoretical)	Matrix effect %				
	<i>E.Coli</i> [2.49 mg/ml]	<i>E.Coli</i> [1.24 mg/ml]	<i>E.Coli</i> [0.62 mg/ml]	<i>E.Coli</i> [0.31 mg/ml]	<i>E.Coli</i> [0.16 mg/ml]
20.00	184.55	355.52	603.86	1267.94	5837.83
16.00	130.98	260.08	505.34	1147.16	4165.65
12.00	68.82	230.21	412.37	890.59	3869.13
8.00	63.79	116.06	236.70	638.18	1727.39
4.00	39.44	89.92	157.43	298.03	1083.91
0.00	0.00	0.00	0.00	0.00	0.00

4.6.4 Optimisation of proteomic sample preparation methods

We have established from the previous sections 4.6.1, 4.6.2, 4.6.3 that the sulphides and iron sulphides in our SRB samples have an influence on protein quantification. Further on, in this section and section 4.6.5 different protein preparation methods were tested for SRB culture samples (dsv-lac), in order to determine the best method that would result in matrix removal and better protein identification. The protein preparation methods were assessed based on the number of protein identifications and the peptide abundance. In this section, we discuss the routinely used in-solution method with in-gel method, and the latter is known for protein separation through gel-electrophoresis, leading to protein purification (Dupree et al., 2020). Thereby, we wanted to test the method for our matrix-loaded protein samples. Additionally, SDT buffer was used alongside TRIS A buffer for the preparation of lysate, since SDS is a well-known surfactant that aids in denaturation and solubilization of the proteins (Scheerlinck et al., 2015). Subsequently, DTT was also added as an extra step to ensure that the proteins are denatured and hence the extraction of lysate is maximised. However, care must be taken that the SDS concentrations do not exceed 0.01%, due to its incompatibility with LC/MS instrument causing ion suppression (Crowell, 2015). The in-solution digestion was performed as stated in section 3.17.2.1 and the in-gel method was adapted from (Starke, 2017). The protein counts and abundances are shown in Figure 69. Overall, in-solution method functioned better than in-gel method based on the protein counts, twice as much peptide abundance resulted with in-solution compared to in-gel. This could be attributed to the fact that protein losses in the in-gel method are higher because the proteins are encompassed within the gel matrix and digestion mainly takes place on the surface of the gel matrix, and some of the inaccessible protein remains undigested. In terms of the extraction buffer, SDT buffer was evidently more successful in stabilizing the protein thereby leading to more identifications in both the methods. Hence, higher SDS (or SDT) buffer (1%) would be implemented in the further lysate extractions, in order to result in higher solubilization of proteins, which is all the more imperative with our matrix samples. And, pertaining to the extraction methods, in-solution method is a relatively uncomplicated protocol and protein concentrations ranging from 0.025 - 10 µg can be processed, but samples with higher SDS concentrations and more matrix effect require the implementation of additional clean-up processes. In-gel digestion was much more laborious and requires a comparatively higher protein concentrations for better recoveries. In conclusion, through this preliminary experiment, in-solution approach proved to be better than in-gel furthermore, experiments in the next section would be explored for matrix and SDS removal.

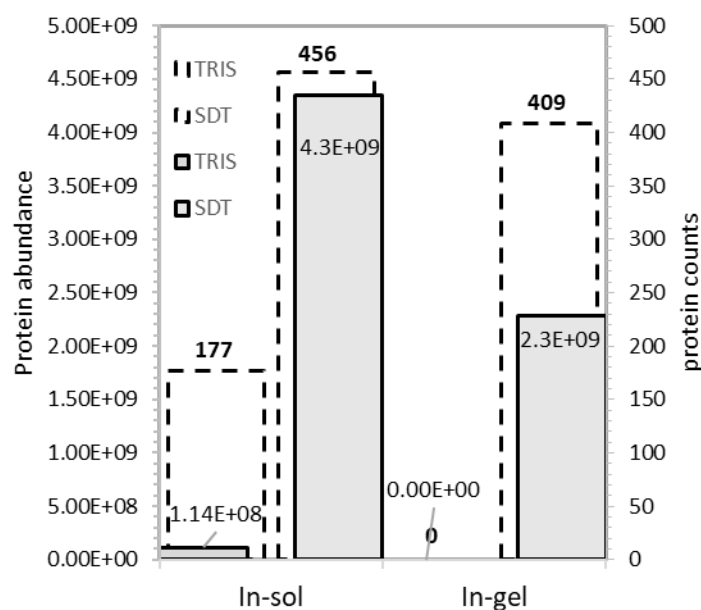


Figure 69: The protein counts and peptide abundances are indicated as columns for Tris and SDT buffer respectively, extracted using in-solution and in-gel approaches.

4.6.5 Comprehensive analyses of proteomics preparation methods on sulfate-rich cultures

This experiment involves the comparison of three protein preparation methods (IS-modified, in-gel and SP3 method). The in-solution digestion method was modified, by the integration of FASP (filter aided (30KDa) sample preparation) after lysate extraction. And a new method, using magnetic beads was also tested (SP3 extraction). The detailed experimental set up is stated in section 3.16.5.5. The aim of the experiment was to assess the three extraction methods for sulphide rich pure cultures of *D. ferrophilus* with two biomass thresholds 1mg/ml and 10 mg/ml based on the protein counts and abundance. Therefore, the methods were compared not only based on the recoveries (protein identifications) but also on their efficiency with lower biomasses, typical to autotrophic growth patterns. The samples used for this experiment were sulfide-rich *D. ferrophilus* cultures. This strain was particularly chosen because in the pre-experiment we observed that the cultures were more sensitive to the nutrient conditions, and therefore yielded lower biomasses, and were relatively more difficult to culture compared to *D. vulgaris*. In addition, proteins had not yet been extracted and analysed from the *D. ferrophilus* cultures of the pre-experiment. In any case, proteomics was unsuccessful with *D. vulgaris* strains from the pre-experiment. However, dsv-lac was subsequently used extensively to optimize the proteomics method (4.6.1, 4.6.2, 4.6.4). Therefore, *D. ferrophilus* was used for this experiment to implement the proteomics methods and to observe the corresponding protein profiles. The protein results were analysed according to the biomass range for each method. The protein counts and total abundances are indicated in Figure 70a and b. Comparing the three methods, the SP3 method was more compatible, followed by the FASP-in-solution method as shown in Figure 70a and b. The SP3 method is known to remove detergents such as SDS, chaotropes and other contaminants, by serial washes with organic solvents such as ethanol. Thereafter, the proteins are alkylated, reduced, digested and eluted in a single tube (Hughes et al., 2019). As a result of the high reproducibility and sensitivity of the method, it was proven to be efficient for lower biomasses, one such study identified more than 15,000 unique peptides from 1000 HeLa cells (Sielaß et al., 2017). In our case, this method proved to be the most efficient method of the three, yielding higher peptide abundances even with 1 µg of starting material. The FASP in-solution method, on the other hand was modified specifically for the removal of SDS and protein purification, using urea buffer. The principle here is that the concentrated urea (8M) allows the dissociation of the protein and SDS complex (buffer exchange principle), and also denatures the protein, thereby stabilizing the protein. Finally, after several spins, the proteins retained on the column are eluted with a protein-stabilizing

buffer, in our case 50 mM TRIS buffer. This method also proved to be efficient for the lower biomass threshold. However, compared to the SP3 method the total peptide abundances are lower due to the protein loss caused by the buffer exchange during each spin. Overall, we concluded that the SP3 method was less laborious and more efficient for the proteomic analysis of our sulfide-rich samples. And SP3 method was implemented for the enrichment cultures.

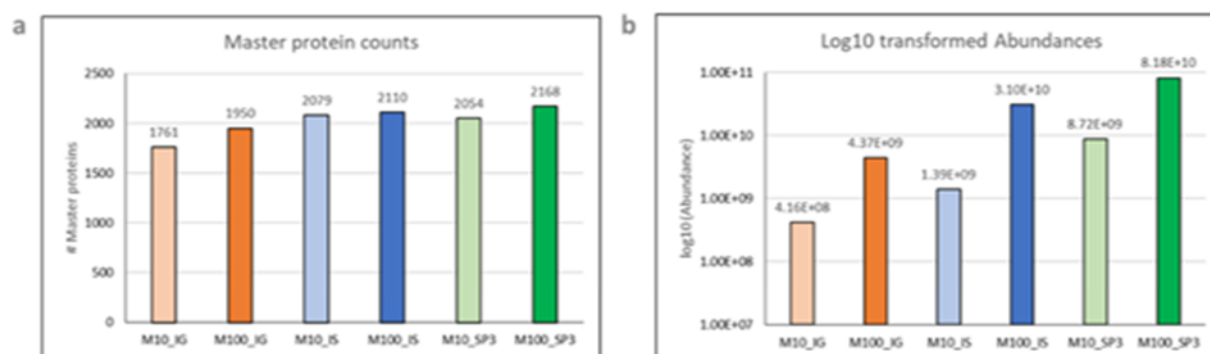


Figure 70: The proteomic results are depicted (a) as the protein counts and (b) the $\log_{10}$ transformed total peptide abundances of the two biomasses, 10 μg (M100) and 1 μg (M10) for in-gel digestion (IG), in-solution digestion (IS) and SP3 methods (SP3).

4.6.6 Overall outcome of proteomics optimization

Based on the above optimization trials, for protein quantification, proteomics sample preparation methods of our mineral rich cultures, the following flowchart (Figure 71) depicts the key results pertaining to each optimization trial. Therefore, the SP3 and FASP-IS method were the most efficient for our samples, and lysate extractions done with SDT buffer yielded more protein identifications

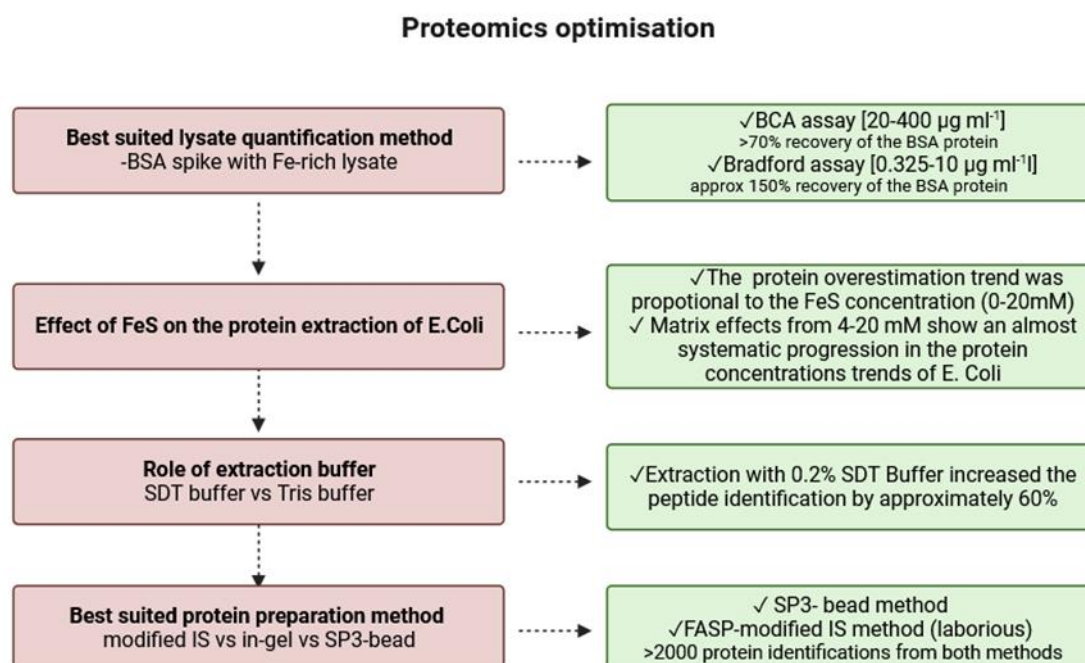


Figure 71: Overall outcome of proteomic optimisation of SRB lysate with Fe-rich minerals, created from Biorender.com.

5 Conclusions

5.1 Conclusions German

Die Kombination von Feldstudien, einschließlich *in-situ*-Messungen und Expositionsexperimenten, mit Laboranreicherungs- und Reinkulturstudien unter kontrollierten Bedingungen trug dazu bei, unser Verständnis von MIC (Microbial Induced Corrosion) im Süßwasser zu verbessern. Nur ein mehrphasiger Ansatz mit Methoden, die von der 3D-Struktur- und Mineralanalyse über Amplikonsequenzierung und Mikrosensormessungen bis hin zur Metaproteomik reichen, ermöglichte es uns, die MIC-Mechanismen in Süßwassersystemen erfolgreich zu untersuchen.

Durch den Vergleich einer großen Anzahl von Proben von verschiedenen Standorten in Bundeswasserstraßen konnten wir zeigen, dass sich die mikrobielle Gemeinschaft je nach Probenotyp stärker unterscheidet als nach geografischer Lage und entsprechenden abiotischen Faktoren. Mikrobielle Gemeinschaften, die mit Korrosionsschäden assoziiert sind, unterschieden sich deutlich von denen der Wasserproben. Einige mikrobielle Taxa wiesen in den Tuberkelproben im Vergleich zu den Wasserproben eine deutlich höhere relative Häufigkeit auf. Dabei handelte es sich unter anderem um Sulfat-reduzierer aus den *Desulfovibrionaceae* (insbesondere *Desulfovibrio*), Eisenoxidierer aus den *Gallionellaceae* (insbesondere *Gallionella*), Nitritoxidierer aus den *Nitrospiraceae* (*Nitrospira*) und Eisenreduzierer aus den *Holophagaceae* (insbesondere *Geothrix* und *Holophaga*). Diese mikrobiellen Taxa könnten als mikrobielle Indikatoren für mikrobiell induzierte Korrosion geeignet sein.

Bei der genaueren Untersuchung von Rosttuberkeln haben wir außerdem festgestellt, dass diese eine sehr komplexe innere Struktur mit verschiedenen Schichten, Kammern und Poren aufweisen. Sie zeichnen sich durch starke geochemische Gradienten aus und beherbergen eine metabolisch äußerst vielfältige mikrobielle Gemeinschaft, die in vielerlei Hinsicht zur Korrosion beitragen kann. Starke Gradienten deuten auf einen fortlaufenden Korrosionsprozess trotz einer dicken Schicht von Korrosionsablagerungen hin. Die Korrosion wurde durch anaerobe Atmung alternativer Elektronenakzeptoren wie CO₂ und Sulfat, (Re-)Oxidation von Fe(II) und reduzierten Schwefelverbindungen sowie elektrochemischer O₂-Reduktion an der Tuberkeloberfläche angetrieben, die durch elektrisch leitende Mineralien wie Magnetit (mit) angetrieben wird. Zusätzlich zur Primärproduktion durch chemolithoautotrophes Wachstum, das durch Elektronen aus Fe(0) angetrieben wird, versorgen phototrophe Mikroorganismen die Biofilmgemeinschaft mit organischem Kohlenstoff. Wir haben gezeigt, dass sich das Milieu im Inneren eines Rosttuberkels völlig von der umgebenden Luft- oder Wasserphase unterscheidet. Aus diesem Grund und weil die Unterschiede zwischen den Standorten geringer waren als zwischen den Probenotypen, ist es (bisher) nicht möglich, standortbezogene Umweltrisikofaktoren für MIC wie Redoxpotential, O₂-Konzentrationen, DOC und pH-Wert zu ermitteln.

Indem wir Stahlproben direkt im Feld exponierten, konnten wir die zeitliche Entwicklung der mikrobiellen Gemeinschaften auf der Stahloberfläche verfolgen und die Korrosionsraten quantifizieren. Dabei führten kurze Inkubationszeiten (Hochskalierung auf ein Jahr (mm/Jahr)) zu deutlich höheren Korrosionsraten als längere Inkubationszeiten. Auch wenn dies bekannt ist und nicht sonderlich überrascht, ist es wichtig, dies beim Vergleich der in verschiedenen Studien ermittelten Korrosionsraten zu berücksichtigen. Darüber hinaus basieren die in Laborstudien ermittelten Raten häufig auf kürzeren Inkubationszeiten und finden bei höheren Temperaturen statt und sind daher nur schwer auf die Praxis übertragbar. Obwohl die Korrosionsraten im Laufe der Zeit abnahmen, kam die Korrosion nicht zum Stillstand, sondern näherte sich einem konstant niedrigen Niveau an. Diese Ergebnisse haben auch Auswirkungen auf die Wartung von Spundwänden. Es ist davon auszugehen, dass die regelmäßige Entfernung von Korrosionsprodukten und Biofilmen von Spundwänden (wenn die Reinigung nicht zur Bestimmung der Spundwanddicke und zur Begutachtung von Schadensfällen bzw. zum Auftragen von

Korrosionsschutzbeschichtungen erforderlich ist) nicht per se von Vorteil ist. Wir haben auch gezeigt, dass sich die Zusammensetzung der mikrobiellen Gemeinschaft je nach Alter des Tuberkels stark unterscheidet, und zwar in einem solchen Ausmaß, dass einige Taxa dominant oder überhaupt nicht vorhanden sind. Zusätzlich zu den chronologischen und saisonalen Verschiebungen in der Zusammensetzung der mikrobiellen Gemeinschaft in Rosttuberkeln machen es die hohe Diversität und inhärente Komplexität natürlicher mikrobieller Gemeinschaften schwierig, die wichtigsten mikrobiellen Korrosionstreiber zu identifizieren.

Insgesamt konnten wir zeigen, dass die dominanten mikrobiellen Taxa, die in Feldproben von 13 verschiedenen Standorten gefunden wurden, auch als die dominanten Arten in den Couponexpositionsversuchen identifiziert wurden. Darüber hinaus konnten wir die wichtige Rolle der dissimilatorischen Sulfatreduktion bei MIC-Prozessen auch in Süßwassersystemen trotz niedriger Sulfatkonzentrationen nachweisen, wobei Mitglieder der Gattung *Desulfovibrio* die dominierenden Sulfatreduzierer in den Feldproben waren. Dieses Taxon wurde auch unter sulfatreduzierenden Bedingungen in den Feldproben angereichert und als Modellorganismus für die Reinkulturstudien verwendet, um die MIC-Mechanismen zu entschlüsseln (siehe unten). Außerdem sehen wir eine wichtige Rolle von (mikroaerophilen) Fe(II)-Oxidierern wie *Gallionella*, neben der abiotischen Oxidation durch O₂. Darüber hinaus spielen Schwefeloxidierer eine wichtige Rolle bei der Auffüllung des Sulfatpools für Sulfatreduzierer.

Anreicherungskulturstudien zeigten, dass Süßwasser-Korrosionstuberkel, Prokaryoten beherbergen, die zur dissimilatorischen Reduktion von Nitrat, Sulfat und CO₂ mit Fe(0) als einziger Elektronenquelle fähig sind. Im Falle der dissimilatorischen Nitrat- und Sulfatreduktion waren die Korrosionsraten im Vergleich zu abiotischen Kontrollen deutlich erhöht, was darauf hindeutet, dass diese Prozesse potenziell zu MIC in Süßwassersystemen beitragen können. Nitratreduzierer tragen hauptsächlich durch die Produktion von hochkorrosivem Nitrit und die weitere Oxidation von Eisen zu Eisen(III) bei. Letzteres kann auch als Korrosionsmittel dienen, so dass Nitratreduzierer hauptsächlich durch CMIC wirken. Die Bedeutung der Fe(II)-Oxidation wurde auch durch die Prävalenz von aeroben Fe(II)-oxidierenden *Gallionella* in Feldproben belegt. Die relative Häufigkeit von nitratreduzierenden Fe(II)-Oxidierern, die mit *Thermomonas* und *Acidovorax* in Verbindung gebracht werden, war in Feldproben gering. Diese fakultativen anaeroben Mikroorganismen sind jedoch metabolisch sehr vielfältig und ubiquitär vorhanden. Daher können sie in Aktion treten, sobald anoxische, nitratreduzierende Bedingungen herrschen. Außerdem wurde gezeigt, dass die angereicherten Nitratreduzierer H₂ verbrauchten und der Elektronentransfer von Fe(0) höchstwahrscheinlich durch H₂ vermittelt wurde (HIMET). Um herauszufinden, ob enzymatische Katalyse hier eine Rolle spielt, wäre eine Metaproteomanalyse hilfreich. Voraussetzung für MIC unter nitratreduzierenden Bedingungen war eine organische C-Quelle, die in der natürlichen Umgebung durch den anaeroben Abbau von organischem Material (Fermentation) oder durch Acetogenese leicht verfügbar wäre. Die Verfügbarkeit einer organischen C-Quelle könnte auch die Prävalenz von *Desulfovibrio spp.* in den Feldproben sowie in den mit Acetat angereicherten Anreicherungskulturen erklären. Die Mitglieder dieser Gattung sind heterotroph, und einige von ihnen sind dafür bekannt, dass sie stark korrosiv sind und eine Schlüsselrolle in marinen Lebensräumen und - wie hier bestätigt wurde - höchstwahrscheinlich auch in Süßwasserhabitaten spielen. Der Mechanismus des Elektronentransfers wird noch diskutiert. Die Fähigkeit, auf H₂ zu wachsen, und die große Menge an periplasmatischen Ni-Fe-Hydrogenasen aus der metaproteomischen Analyse deuten auf einen durch H₂ vermittelten Elektronentransfer von Fe(0) hin (HIMET). Eine extrazelluläre Hydrogenase wurde nicht nachgewiesen; allerdings wurde die extrazelluläre Proteinfraction in der Metaproteomanalyse auch nicht gezielt untersucht. Neben *Desulfovibrio spp.* waren autotrophe sulfatreduzierende Bakterien aus den Familien *Desulfocapsaceae/Desulfobulbaceae* und den Gattungen *Desulfomicrobium* und *Desulfosporosinus* in Anreicherungskulturen vorherrschend. Dies deutet darauf hin, dass die dissimilatorische Sulfatreduktion im Freiland auch in Abwesenheit von organischem C ablaufen kann. Neben periplasmatischen Hydrogenasen waren in sulfatreduzierenden Anreicherungskulturen mit Fe(0) als Elektronenquelle auch Porinproteine der Außenmembran in großer Zahl vorhanden. Diese Kanalproteine, die mit einem Cytochromtransport gekoppelt sind, könnten einen direkten

Elektronentransfer von Fe(0)-Elektronen in die Zelle ermöglichen (DIMET). Dies ist von besonderem Interesse, da ein direkter Elektronentransfer-Mechanismus immer als Indikator für hochkorrosive Organismen diskutiert wurde. Daher wird die weitere Erforschung dieser Mechanismen durch Metaproteomik für die Entwicklung von Diagnoseinstrumenten zum Nachweis von MIC im Feld nützlich sein. Wir fanden auch heraus, dass die Methanogenese und Acetogenese mit Fe als einziger Elektronenquelle stattfinden kann. Sequenzen, die mit Methanogenen in Verbindung stehen, wurden auch überwiegend in Rosttuberkeln nahe der Stahloberfläche unter stark reduzierenden Bedingungen gefunden, die für die Methanogenese erforderlich sind.

Die Mikrokosmos-Experimente in den Reinkultur gaben uns einen Einblick in die möglichen Energiegewinnungsmechanismen, die Sulfatreduzierer für ihr Wachstum nutzen, und insbesondere wurde die Rolle von Fe(0) als potenzieller Elektronendonator untersucht. Reinkulturversuche zeigten, dass die Stämme *D. vulgaris* und *D. ferrophilus* sowohl in Süßwasser- als auch in Salzwassermedien Stahl korrodieren. Dies deutet auf eine erhebliche Schädigung von Stahl durch diese beiden Bakterienstämme hin. Insgesamt war es eine Herausforderung, die metaproteomische Analyse der Fe-reichen Proben zu optimieren. Die SP3-Bead-Extraktionsmethode erwies sich als am besten geeignet für die Extraktion von Proben mit geringer Biomasse und hohem Matrixeffekt. Der klassische Ansatz der Proteinanalyse unter Verwendung der Metagenomik-Ergebnisse unserer Proben hätte als ideale Datenbank für die Proteomik dienen können, aber die Qualität und Quantität der extrahierten DNA, insbesondere aus gemischten Anreicherungsproben, reichte aufgrund der geringen Biomasse und der Interferenz von Fe-Mineralien, die dazu neigen, sich an die DNA zu binden und dadurch Verluste zu verursachen, für eine gute Analyse nicht aus. Daher war die Datenbank Uniprot der TOP10-Familien für die Auswertung von Mischkulturproben unsere nächstbeste Lösung. Uniprot ist eine gute Datenbank, die jedoch noch Lücken in Bezug auf Proteine aufweist, die an den von sulfatreduzierenden Bakterien verwendeten Atmungssystemen beteiligt sind. Als Erweiterung dieser Studie wäre es interessant, Proteine aus jeder Zellkomponente zu extrahieren, d.h. aus dem Periplasma, dem Zytoplasma, der äußeren Membran und der Zytoplasmamembran. Dadurch würde eine genaue Lokalisierung der Proteine unsere Rückschlüsse auf die Elektronentransportketten untermauern. Dennoch wäre es eine Herausforderung, kompartimentierte Proteine aus biomassearmen und Fe-reichen Proben zu extrahieren, weshalb ein ausgiebiges Ausprobieren der verfügbaren Proteinkits unabdingbar ist. Außerdem sollten Experimente zur weiteren Validierung und Aufklärung der Elektronentransportmechanismen durchgeführt werden, beispielsweise durch Gendeletion von Genen, die für elektronenübertragende Proteine kodieren, z. B. Ni-Fe-Hydrogenase, OM-Porine und spezifische Cytochrome.

5.2 Conclusions English

The combination of field studies, including *in-situ* measurements and exposition experiments, with laboratory enrichment and pure culture studies under controlled conditions helped to deepen our understanding of freshwater MIC. Only a polyphasic approach using methods ranging from 3D structure and mineral analysis over amplicon sequencing and microsensor profiling to metaproteomics allowed us to successfully investigate MIC mechanisms in freshwater systems.

By comparing a large number of samples from different sites within German federal waterways we demonstrated that the microbial community is diverging stronger according to sample type than according to the geographical setting and corresponding abiotic factors. Microbial communities associated with corrosion damage clearly differed from those of the water samples. Some microbial taxa showed a significantly higher relative abundance in the tubercle samples compared to water samples. Those were, among others, sulfate reducers from the *Desulfovibrionaceae* (especially *Desulfovibrio*), iron oxidizers from the *Gallionellaceae* (especially *Gallionella*), nitrite oxidizers from the *Nitrospiraceae* (*Nitrospira*) and iron reducers from the *Holophagaceae* (especially *Geothrix* and *Holophaga*). These microbial taxa might be suitable as microbial indicators for microbial induced corrosion.

Furthermore, by investigating corrosion scales (i.e. rust tubercles) in more detail, we found that rust tubercles possess a very complex inner structure, containing distinct layers, chambers, and pores. They are characterised by strong geochemical gradients and harbor a metabolically highly diverse microbial community which may contribute to corrosion in many ways. Steep gradients suggested an ongoing corrosion process despite a thick layer of corrosion scale. Corrosion was driven by anaerobic respiration of alternative electron acceptors such as CO₂ and sulfate, (re)oxidation of Fe(II) and reduced sulfur compounds, and electrochemical reduction of O₂ at the tubercle surface mediated by electroconductive minerals such as magnetite. In addition to primary production by chemolithoautotrophic growth fuelled by electrons from Fe(0), phototrophic microorganisms supplied organic carbon to the biofilm community. We showed that the environment inside a rust tubercle differs completely from the surrounding air or water phase. Due to that and because site differences were smaller than between sample type, it (so far) remains elusive to identify site based environmental risk factors for MIC such as redox potential, O₂ concentrations, DOC and pH. By exposing steel coupons directly in the field, we were able to follow the temporal development of the microbial communities on the steel surface and to quantify corrosion rates. Hereby short incubation times led to significantly higher corrosion rates when upscaled to one year (mm/year) than longer incubation times. Even though this is known and not particularly surprising, it is important to keep this in mind when comparing corrosion rates determined in different studies. Furthermore, rates obtained from laboratory studies are often based on shorter incubations at higher temperatures and are, therefore, difficult to extrapolate to the field. Even though rates decreased over time, corrosion did not come to a halt but approached a constant low level. These results have also implications for sheet pile maintenance management. It is likely that regular removal of corrosion scales and biofilm from sheet piles (if not needed for determining sheet pile thickness/damage and or application of corrosion protection coatings) is not advantageous per se. We also showed that the microbial community composition strongly differs according to tubercle age, to such an extent where some taxa are dominant or not present at all. In addition to the chronological as well as seasonal shifts in the composition of microbial community in rust tubercles, the high diversity and inherent complexity of natural microbial communities, makes it difficult to pin point the main microbial corrosive agents.

Overall, we showed that the dominant microbial taxa found in field samples collected from 13 different sites were also identified as the dominant species in the coupon exposition experiments. Furthermore, we verified the important role of dissimilatory sulfate reduction in MIC processes also in freshwater systems despite low sulfate concentrations, with members of the genus *Desulfovibrio* being the dominant sulfate reducers in the field samples. This taxon was also enriched under sulfate reducing conditions from field samples and it was used as model organism for the pure culture studies to unravel the MIC mechanisms (see below). Furthermore, we see an important role of (microaerophilic) Fe(II) oxidizers such as *Gallionella* next to the abiotic oxidation by O₂. In addition, sulfur oxidizers play an important role in replenishing the sulfate pool for sulfate reducers.

Enrichment culture studies showed that freshwater corrosion scales harbor prokaryotes capable of the dissimilatory reduction of nitrate, sulfate, and CO₂ with Fe(0) as sole electron source. In case of dissimilatory nitrate and sulfate reduction, corrosion rates were significantly enhanced compared to abiotic controls, suggesting that these processes have a potential for contributing to MIC in freshwater systems. Nitrate reducers mainly contribute by producing highly corrosive nitrite and oxidizing ferrous iron further to ferric iron. The latter may also serve as corrosive agent, hence, nitrate reducers act mostly by CMIC. The importance of Fe(II) oxidation has also been shown by the prevalence of aerobic Fe(II) oxidizing *Gallionella* in field samples. The relative abundances of nitrate reducing Fe(II) oxidizers affiliated to *Thermomonas* and *Acidovorax* were low in field samples. However, these facultative anaerobes are metabolically highly diverse and ubiquitously present. Therefore, they may come to action as soon as anoxic, nitrate reducing conditions are established. Furthermore, it was shown that the enriched nitrate reducers consumed H₂ and the electron transfer from Fe(0) was most likely H₂ mediated (HIMET). In order to find out whether enzymatic catalysis played a role here, metaproteome

analysis would be helpful. An organic C source was prerequisite for MIC under nitrate reducing conditions which in the natural environment would be readily available by the anaerobic degradation of organic matter (fermentation) or by acetogenesis. The availability of an organic C source may also explain the prevalence of *Desulfovibrio* spp. in the field samples as well as in enrichment cultures amended with acetate. Members of this genus are heterotrophic and some of them are known to be highly corrosive being key players in marine habitats and – as confirmed here – most likely also in freshwater habitats. The mechanism of electron transfer is still under debate. The ability to grow on H_2 and the high abundance of periplasmic Ni-Fe hydrogenases from the metaproteomic analysis indicate a H_2 mediated electron transfer from Fe(0) (HIMET). An extracellular hydrogenase was not detected; however, the extracellular protein fraction was also not specifically targeted in metaproteome analysis. Next to *Desulfovibrio* spp., autotrophic sulfate reducing bacteria affiliated to the families *Desulfocapsaceae/Desulfobulbaceae* and the genera *Desulfomicrobium*, and *Desulfosporosinus* were predominant in enrichment cultures. This indicated that dissimilatory sulfate reduction in the field could also proceed even in the absence of organic C. Apart from periplasmic hydrogenases, outer membrane porin proteins were also highly abundant in sulfate reducing enrichment cultures with Fe(0) as electron source. These barrel-shaped, channel proteins coupled with a cytochrome transport may allow a direct electron transfer from Fe electrons to the cell (DIMET). This is of special interest as a direct electron transfer mechanism has always been discussed as indicative for highly corrosive organisms. Therefore, further exploration of these mechanisms by metaproteomics will be useful in the development of diagnostic tools to detect MIC in the field. We also found a potential of methanogenesis and acetogenesis to occur with Fe(0) as sole electron source. Sequences affiliated to methanogens were also found predominant inside rust tubercles close to the steel surface under highly reducing conditions, which are required for methanogenesis to proceed.

The pure culture microcosm experiments gave us an insight into the possible energy gain mechanisms that sulfate reducers employ to grow, and specifically the role of Fe as a potential electron donor was investigated. Pure culture experiments showed that the strains *D. vulgaris* and *D. ferrophilus* were corrosive to steel in both freshwater and marine media. This indicated the significant damage of steel by these two strains of bacteria.

Overall, it was a challenge to optimize metaproteomic analysis of the Fe-rich samples. The SP3-bead extraction method was found to be best suited for the extraction of lower biomass and high matrix effected samples. The classical approach of performing protein analysis using metagenomics results of our samples, could have served as an ideal databank for proteomics, but the quality and quantity of the DNA extracted especially from mixed enrichment culture samples were not sufficient for a good analysis, due to low biomass and the interference of Fe-minerals, which tend to bind to DNA, thereby rendering losses. Hence, the databank, Uniprot of the TOP10 families, for the evaluation of mixed culture samples was our next best solution. Uniprot is good database, however still with sufficient gaps pertaining to proteins involved in respiration systems employed by sulfate reducing bacteria. As an extension to this study, it would be interesting to extract proteins from each cell component that are, periplasm, cytoplasm, outer membrane and cytoplasmic membrane. Thereby, an accurate localization of the protein would reinforce our inferences of the electron transport chains. Nevertheless, it would be a challenge to extract compartmentalized proteins, from low biomass and Fe-rich samples, hence extensive trial and error of the available protein kits would be imperative. Also, experiments to further validate and elucidate the electron transport mechanisms should be performed, for example by gene deletion of genes coding for electron transferring proteins e.g. Ni-Fe hydrogenase, OM porins and specific cytochromes.

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Falls auffällige Korrosionserscheinungen beobachtet wurden: Konnte eine vertikale Zonierung (Zunahme der Anzahl/Dichte an Pusteln bzw. Stellen mit Lochfraß/Mulden) von der WWZ zur UWZ z. B. an Spundwänden oder Kantenschutzblechen festgestellt werden?

	ja			nein			Nicht bekannt
Platz für kurze Hinweise/Kommentare:							

Als Anhaltspunkt für das Erscheinungsbild der oben beschriebenen Erscheinungen sollen nachfolgende Beispielbilder 1) - 4) von realen betroffenen Standorten dienen.

1) Auffällige Rostpusteln im Bereich der Wasserwechselzone (WWZ)



2) Bei Bauwerksinspektion beobachtete Fälle im Bereich der UWZ (vertikale Zonierung sichtbar)



3) Unvermutet starker muldenförmiger Abtrag unter auffälligen Pusteln / teilweise auch bei Tauchinspektionen in UWZ zu beobachten



4) Von wasserseitiger MIC betroffene Einbauteile aus andersartigen Werkstoffen, wie z. B. Edelstahlbauteile, feuerverzinkte Bauteile...



Edelstahlblech in WWZ



Edelstahlenschutzblech mit Zonierung (Be-
reich in UWZ stärker betroffen)



Feuerverzinktes Kantenschutzblech

Landseitiges Auftreten

Landseitige MIC wird oftmals erst nach Eintritt eines Schadensfalles nachgewiesen, da die Landseite von Spundwänden i. d. R. nur sehr eingeschränkt bzw. gar nicht während z. B. der Bauwerksinspektion zugänglich ist. Folgende Beobachtungen können auf eine Beteiligung durch MIC (i. d. R. lokale Durchrostungen) am Korrosionsgeschehen hindeuten:

- Relativ schnell auftretende Trichterbildung aufgrund von ausgespültem Hinterfüllungsmaterial, verursacht durch unerwartete, lokale Durchrostungen in UWZ (teilw. auch oberhalb), nach vergleichsweise kurzer Standzeit
- Unerwartet hoher, lokaler Materialdickenverlust (hohe Abrostungsraten) bei bspw. Bauwerksinspektion (Ultraschallmessungen), bei gleichzeitig unauffälligem (normale, gleichmäßige Korrosion) Erscheinungsbild auf der Wasserseite.
- „Frische“ ockerfarbige Rostfahnen (z. B. mit schleimigen Bakterienrasen) an künstlichen Öffnungen in Spundwänden (Entlastungsöffnungen, Ankerlagen), an welchen anstehendes (Grund-)Wasser von hinten austritt.
- Augenscheinlich auffälliges, sehr inhomogenes Hinterfüllungsmaterial (z. B. Schlackereste, Holzreste, Gips, Kohlereste etc.) im Bereich von vorhandenen Spundwandschäden.
- An betroffenen(?) Spundwandrückseiten auffälliger „Bakterien“boden (in Nähe bzw. direkt an der Stahloberfläche der SW), welcher im frischen, feuchten Zustand sehr dunkel/schwarz ist und beim Lagern an Luft deutlich nachoxidiert (hell wird)

Anzahl bekannter <u>Standorte</u> im Amtsbereich an denen obige Erscheinungsformen/Schäden beobachtet (bitte weißes Feld links ankreuzen):							
☐	☐	☐	☐	☐	☐	☐	☐
	> 15			1-15			Keine / nicht bekannt
Platz für kurze Hinweise/Kommentare:							
Treten die landseitigen Schäden gehäuft an Standorten einer bestimmten Region auf oder ist das Auftreten (falls vorhanden) im gesamten Amtsbereich des WSA relativ gleich verteilt?							
☐	☐			☐	☐		
	In bestimmten Regionen gehäuft				Keine Aussage möglich		
Platz für kurze Hinweise/Kommentare:							
An welchen Bauwerken/teilen wurden die auffälligen Korrosionserscheinungen festgestellt?							
☐	☐		☐	☐		☐	☐
	Spundwände freie Kanalstrecke			Spundwände in Schleusen/Vorhäfen			Düker
☐	☐						
	Nur an Ankerlagen						
Konnte ergänzend ein erhöhter Materialabtrag von der Landseite her durch Ultraschallmessungen von Restwanddicken festgestellt bzw. u. U. bestätigt werden?							
☐	☐	☐	☐	☐	☐	☐	☐
	ja			nein			Keine Aussage möglich / Keine US-Messungen an auffälligen Bauwerk./Bauteilen durchgeführt
Platz für kurze Hinweise/Kommentare:							

Als Anhaltspunkt für das Erscheinungsbild der oben beschriebenen Erscheinungen sollen nachfolgende Beispielbilder 5) – 8) von realen, von landseitiger MIC betroffenen Standorten dienen:

5) Absacken bzw. Ausspülen von Material der Hinterfüllung durch lokale Durchrostung in UWZ



6) Auffällige Rostfahnen beim Austritt von anstehendem Wasser z. B. an Ankerlagen, Öffnungen





7) Inhomogenes Hinterfüllungsmaterial im Bereich von lokalen Durchrostungen



8) Schwarze Belegung / Böden (i. d. R. sulfidhaltig) von Spundwandrückseiten, welche sich bei Luftzutritt schnell hell verfärben



Frisch, äußerlich u. U.
schon oxidiert.



Getrocknetes Material

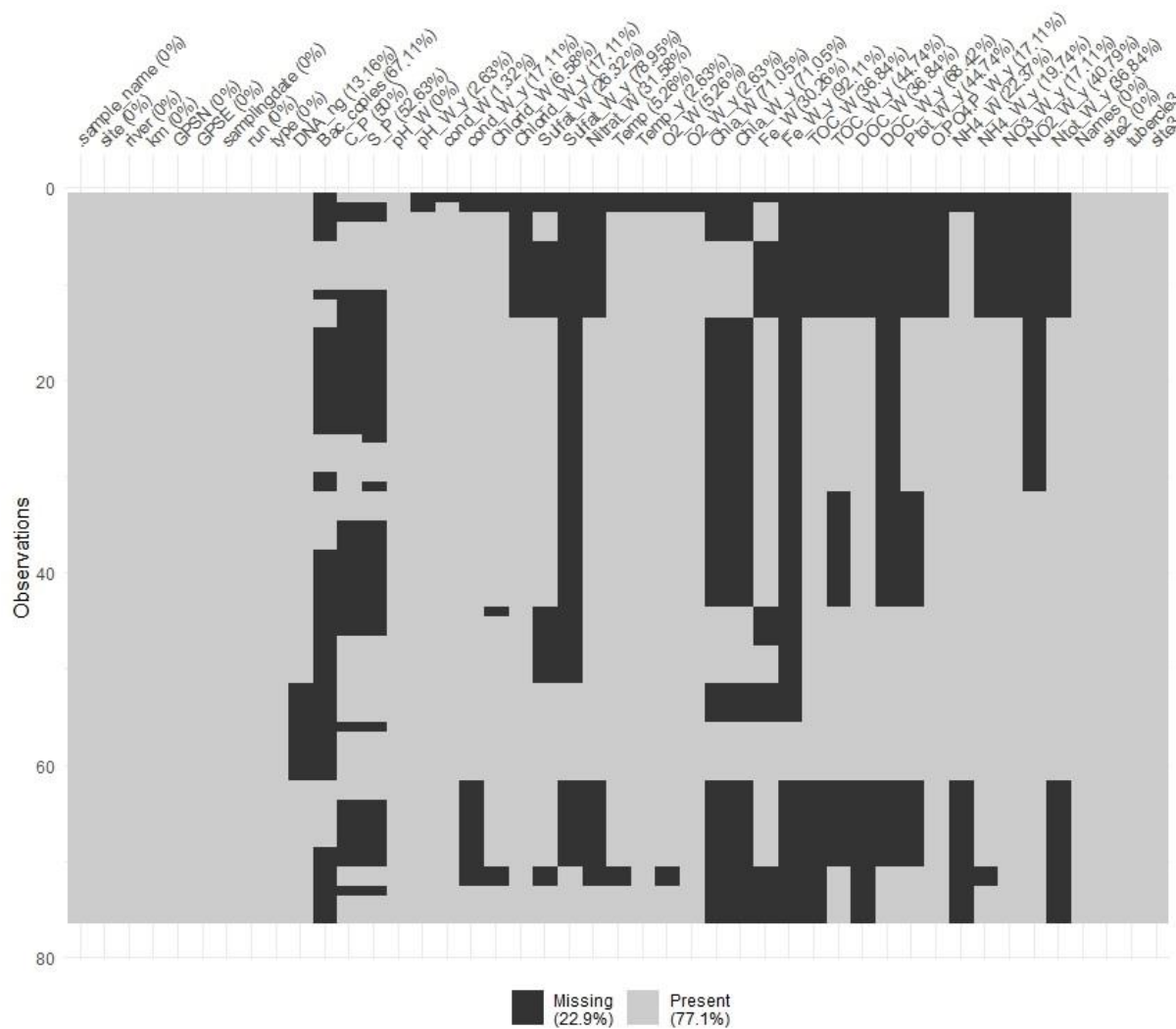
2. S2

	Recknagel coupons		Sheet pile steel plate		Xometry coupons	
Element	Mean	±	Mean	±	Mean	±
Fe	98.140	0.026	98.027	0.052	97.910	0.024
C	0.159	0.003	0.146	0.012	0.037	0.001
Si	0.161	0.005	0.202	0.011	0.367	0.008
Mn	1.422	0.030	0.822	0.010	0.327	0.003
P	<0.005	0.000	<0.005	0.000	0.008	0.001
S	<0.003	0.000	0.016	0.004	<0.003	0.000
Cr	0.034	0.001	0.117	0.004	0.826	0.002
Mo	<0.004	0.001	0.047	0.004	<0.004	0.000
Ni	0.017	0.002	0.160	0.006	0.220	0.005
Al	0.022	0.001	0.002	0.001	0.022	0.001
Co	<0.002	0.000	0.011	0.002	0.005	0.001
Cu	0.013	0.001	0.415	0.019	0.261	0.007
Nb	0.011	0.001	0.005	0.001	<0.003	0.000
Ti	0.016	0.001	0.001	0.000	<0.002	0.000
V	<0.002	0.000	0.003	0.001	<0.002	0.000
W	<0.025	0.000	0.025	0.000	<0.025	0.000
Pb	<0.015	0.000	<0.015	0.000	<0.015	0.000
Sn	0.004	0.000	0.025	0.003	0.013	0.000
B	<0.001	0.000	<0.001	0.000	<0.001	0.000
Zr	<0.003	0.000	<0.003	0.000	<0.003	0.000
As	<0.005	0.000	<0.005	0.000	<0.005	0.000
Bi	<0.025	0.000	<0.025	0.000	<0.025	0.000

3. S3

[illegible]

4. S4



5. S5

ID	site	km	GPSN	GPSE	samplingdate	t	start	d	type	origin	weightloss [%]	DNA_ng	BAC gene copies [nr/g]	C [%]	S [%]
FBL_t1_BF	Lahn	136	50.308	7.617	05.05.2021	t1	31.03.2021	35	biofilm	bouye	NA	41113			
FBL_t2_BF	Lahn	136	50.308	7.617	02.06.2021	t2	31.03.2021	63	biofilm	bouye	NA	36218			
FBL_t3_BF	Lahn	136	50.308	7.617	07.07.2021	t3	31.03.2021	98	biofilm	bouye	NA	77612			
FBL_t4_BF	Lahn	136	50.308	7.617	04.08.2021	t4	31.03.2021	126	biofilm	bouye	NA	16396		25.2	0.5
FBL_t5_biofilm	Lahn	136	50.308	7.617	03.09.2021	t5	31.03.2021	156	biofilm	bouye	NA	27154			
FBL_t6_BF	Lahn	136	50.308	7.617	07.10.2021	t6	31.03.2021	190	biofilm	bouye	NA	51703		21.5	0.5
FBL_t7_BF	Lahn	136	50.308	7.617	27.10.2021	t7	31.03.2021	210	biofilm	bouye	NA	9281			

FBL_t8_BF	Lahn	136	50.308	7.617	01.12.2021	t8	31.03.2021	245	biofilm	bouye	NA	24343			
FBL_t9_BF	Lahn	136	50.308	7.617	15.12.2021	t9	31.03.2021	259	biofilm	bouye	NA	17310		66.3	0.6
FBL_t10_BF	Lahn	136	50.308	7.617	16.03.2022	t10	31.03.2021	350	biofilm	bouye	NA	41223			
FBL_t11_BF	Lahn	136	50.308	7.617	21.04.2022	t11	31.03.2021	386	biofilm	bouye	NA	38056		16.3	0.8
FBL_t1_N2N3	Lahn	136	50.308	7.617	05.05.2021	t1	31.03.2021	35	tubercle	coupon	1.61	31851	7.18E+08	5.69	0.1
FBL_t2_O1O3	Lahn	136	50.308	7.617	02.06.2021	t2	31.03.2021	63	tubercle	coupon	2.26	17485	7.54E+08	2.05	0.2
FBL_t3_P2_P3	Lahn	136	50.308	7.617	07.07.2021	t3	31.03.2021	98	tubercle	coupon	2.53	18795	8.20E+08		
FBL_t4_Q1Q2	Lahn	136	50.308	7.617	04.08.2021	t4	31.03.2021	126	tubercle	coupon	2.94	42176	1.42E+09		
FBL_t5_R1_R2	Lahn	136	50.308	7.617	03.09.2021	t5	31.03.2021	156	tubercle	coupon	3.92	31265	1.87E+09	2.59	0.4
FBL_t6_S1S3	Lahn	136	50.308	7.617	07.10.2021	t6	31.03.2021	190	tubercle	coupon	4.12	26006	1.54E+09		
FBL_t7_T1T2	Lahn	136	50.308	7.617	27.10.2021	t7	31.03.2021	210	tubercle	coupon	3.91	41630	3.36E+09	7.83	0.1
FBL_t8_U1U2	Lahn	136	50.308	7.617	01.12.2021	t8	31.03.2021	245	tubercle	coupon	3.87	23120	4.73E+09	5.65	0.1
FBL_t9_V1V2	Lahn	136	50.308	7.617	15.12.2021	t9	31.03.2021	259	tubercle	coupon	3.44	27322	3.09E+09	5.48	0.2
FBL_t10_W1W3	Lahn	136	50.308	7.617	16.03.2022	t10	31.03.2021	350	tubercle	coupon	4.80	62159	4.66E+09	5.5	0.3
FBL_t11_X1X3	Lahn	136	50.308	7.617	21.04.2022	t11	31.03.2021	386	tubercle	coupon	5.17	33345	3.58E+09	3.08	0.2
FBL_t12_Y3	Lahn	136	50.308	7.617	18.05.2022	t12	31.03.2021	413	tubercle	coupon	5.46	50361	2.45E+09	5.65	0.2
FBL_t1_SpWS	Lahn	136	50.308	7.617	05.05.2021	t1	31.03.2021	35	tubercle	plate	NA	25604	1.96E+09	1.97	0
FBL_t2_SpWS	Lahn	136	50.308	7.617	02.06.2021	t2	31.03.2021	63	tubercle	plate	NA	37847	2.46E+09	2.4	0.1
FBL_t3_SpWS	Lahn	136	50.308	7.617	07.07.2021	t3	31.03.2021	98	tubercle	plate	NA	30864	1.70E+09	1.66	0.5
FBL_t4_SpWS	Lahn	136	50.308	7.617	04.08.2021	t4	31.03.2021	126	tubercle	plate	NA	33017	2.02E+09	3.28	0.5
FBL_t5_SpWS	Lahn	136	50.308	7.617	03.09.2021	t5	31.03.2021	156	tubercle	plate	NA	25881	1.14E+09	2.37	0.6
FBL_t6_SpWS	Lahn	136	50.308	7.617	07.10.2021	t6	31.03.2021	190	tubercle	plate	NA	43707	3.88E+09		
FBL_t7_SpWS	Lahn	136	50.308	7.617	27.10.2021	t7	31.03.2021	210	tubercle	plate	NA	29930	3.57E+09	1.77	0.4
FBL_t8_SpWS	Lahn	136	50.308	7.617	01.12.2021	t8	31.03.2021	245	tubercle	plate	NA	45413	3.89E+09	2.86	0.4
FBL_t9_SpWS	Lahn	136	50.308	7.617	15.12.2021	t9	31.03.2021	259	tubercle	plate	NA	16528	2.97E+09	1.43	0.6
FBL_t10_SpWS	Lahn	136	50.308	7.617	16.03.2022	t10	31.03.2021	350	tubercle	plate	NA	31374	1.78E+09	2.03	0.6
FBL_t11_SpWS	Lahn	136	50.308	7.617	21.04.2022	t11	31.03.2021	386	tubercle	plate	NA	27779	9.50E+08		
FBL_t12_SpWS	Lahn	136	50.308	7.617	18.05.2022	t12	31.03.2021	413	tubercle	plate	NA	23858		1.91	0.6
FBL_t2_P_So	Lahn	136	50.308	7.617	02.06.2021	t2	31.03.2021	63	tubercle	sonde	NA	12109			
FBL_P_cMS	Lahn	136	50.308	7.617	07.10.2021	t6	NA	NA	tubercle	wall	NA	11203	1.70E+09		
FBL_P_MS	Lahn	136	50.308	7.617	07.10.2021	t6	NA	NA	tubercle	wall	NA	13615	1.30E+09		
LT_1	Lahn	136	50.308	7.617	27.10.2021	t7	NA	NA	tubercle	wall	NA	30388	1.90E+09		

RG_t1_H1H2	HO	0.3	53.177	13.230	08.04.2021	t1	19.11.2020	140	tubercle	coupon	1.48	27520	5.72E+08	7.33	0.1
HO_t2_E2E3	HO	0.3	53.177	13.230	29.07.2021	t2	19.11.2020	252	tubercle	coupon	2.51	15056	1.92E+08	5.45	0.1
HO_t3_F1F2	HO	0.3	53.177	13.230	24.11.2021	t3	19.11.2020	370	tubercle	coupon	2.81	24278	8.71E+08	5.2	0.4
HO_t4_G4G1	HO	0.3	53.177	13.230	23.03.2022	t4	19.11.2020	489	tubercle	coupon	3.41	31133	1.86E+09	7.49	0.2
HO_W	HO	0.3	53.177	13.230	29.07.2021	t2	NA	NA	water	lock	NA	4	4.52E+05		
HO_t1_P_SpWS	HO	0.3	53.177	13.230	08.04.2021	t1	19.11.2020	140	tubercle	plate	NA	11761	8.09E+08	1.72	0.2
HO_t3_SpWS	HO	0.3	53.177	13.230	24.11.2021	t3	19.11.2020	370	tubercle	plate	NA	16834	6.24E+07		
HO_t4_SpWS	HO	0.3	53.177	13.230	23.03.2022	t4	19.11.2020	489	tubercle	plate	NA	14351	1.45E+09	2.49	0.5
HO_t1_BF_g	HO	0.3	53.177	13.230	08.04.2021	t1	19.11.2020	140	biofilm	wall	NA	37367	NA		
HO_t1_BF_w	HO	0.3	53.177	13.230	08.04.2021	t1	19.11.2020	140	biofilm	wall	NA	13675	NA		
HO_t1_BF_w_B	HO	0.3	53.177	13.230	08.04.2021	t1	19.11.2020	140	biofilm	wall	NA	16426	NA		
HO_t1_P_SpW	HO	0.3	53.177	13.230	08.04.2021	t1	19.11.2020	140	tubercle	wall	NA	6353	NA	0.17	0.1
HO_t2_BF	HO	0.3	53.177	13.230	29.07.2021	t2	19.11.2020	252	biofilm	wall	NA	11532	NA	21.3	0.2
HO_t2_Pa_SpW	HO	0.3	53.177	13.230	29.07.2021	t2	19.11.2020	252	tubercle	wall	NA	17699	NA		
HO_t2_Pb_SpW	HO	0.3	53.177	13.230	29.07.2021	t2	19.11.2020	252	tubercle	wall	NA	16567	NA		
HO_t2_Pc_SpW	HO	0.3	53.177	13.230	29.07.2021	t2	19.11.2020	252	tubercle	wall	NA	11159	NA		
HO_t3_BF	HO	0.3	53.177	13.230	24.11.2021	t3	19.11.2020	370	biofilm	wall	NA	21525	NA		0.2
HO_t3_P2_SpW	HO	0.3	53.177	13.230	24.11.2021	t3	19.11.2020	370	tubercle	wall	NA	11661	NA	2.44	0.4
HO_t4_BF	HO	0.3	53.177	13.230	23.03.2022	t4	19.11.2020	489	biofilm	wall	NA	28745	NA	20.6	0.4
HO_t4_GB	HO	0.3	53.177	13.230	23.03.2022	t4	19.11.2020	489	biofilm	wall	NA	11092	1.99E+09		
Mosel_t1	Mosel	2	50.366	7.579	01.09.2021	t1	25.08.2021	7	tubercle	coupon	0.66	10356	1.95E+08		
Mosel_t2	Mosel	2	50.366	7.579	08.09.2021	t2	25.08.2021	14	tubercle	coupon	0.86	18772	5.50E+08		
Mosel_t3	Mosel	2	50.366	7.579	15.09.2021	t3	25.08.2021	21	tubercle	coupon	0.98	26860	1.04E+09		
Mosel_t4	Mosel	2	50.366	7.579	22.09.2021	t4	25.08.2021	28	tubercle	coupon	1.29	35265	1.22E+09		
Mosel_t5	Mosel	2	50.366	7.579	29.09.2021	t5	25.08.2021	35	tubercle	coupon	1.04	41813	1.52E+09		
RG_t1_A1A2	RG	42	52.717	13.238	07.04.2021	t1	08.12.2020	120	tubercle	coupon	1.91	16425	5.76E+08	5	0.1
RG_t2_B1B2	RG	42	52.717	13.238	28.07.2021	t2	08.12.2020	232	tubercle	coupon	3.33	36713	8.31E+08	4.44	0.3
RG_t3_C1C4	RG	42	52.717	13.238	25.11.2021	t3	08.12.2020	352	tubercle	coupon	4.49	61085	1.45E+09	3.77	0.4
RG_t4_D4D3	RG	42	52.717	13.238	24.03.2022	t4	08.12.2020	471	tubercle	coupon	5.00	97238	1.88E+09	7.8	0.4
RG_W	RG	42	52.717	13.238	28.07.2021	t2	NA	NA	water	lock	NA	4	4.43E+05		
RG_t1_PaSpWS	RG	42	52.717	13.238	07.04.2021	t1	08.12.2020	120	tubercle	plate	NA	12719	1.09E+09	2.2	0.3
RG_t1_PbSpWS	RG	42	52.717	13.238	07.04.2021	t1	08.12.2020	120	tubercle	plate	NA	14465	3.24E+09		

RG_t2_SpWS	RG	42	52.717	13.238	28.07.2021	t2	08.12.2020	232	tubercle	plate	NA	21445	2.02E+09	5	0.2
RG_t3_SpWS	RG	42	52.717	13.238	25.11.2021	t3	08.12.2020	352	tubercle	plate	NA	43058	1.22E+09		
RG_t4_SpWS	RG	42	52.717	13.238	24.03.2022	t4	08.12.2020	471	tubercle	plate	NA	78000	8.26E+09	7.71	0.3
RG_t1_P_SpW	RG	42	52.717	13.238	07.04.2021	t1	08.12.2020	120	tubercle	wall	NA	17089	NA	3.24	0.1
RG_t2_BF	RG	42	52.717	13.238	28.07.2021	t2	08.12.2020	232	biofilm	wall	NA	32360	NA	26.4	0.3
RG_t3_BF	RG	42	52.717	13.238	25.11.2021	t3	08.12.2020	352	biofilm	wall	NA	52836	NA		
RG_t3_P1_SpW	RG	42	52.717	13.238	25.11.2021	t3	08.12.2020	352	tubercle	wall	NA	21358	NA	2.79	0.4
RG_t3_P3_SpW	RG	42	52.717	13.238	25.11.2021	t3	08.12.2020	352	tubercle	wall	NA	48423	NA	2.09	0.3
RG_t4_BF	RG	42	52.717	13.238	24.03.2022	t4	08.12.2020	471	biofilm	wall	NA	78506	NA	9.88	0.4
RG_t4_P2	RG	42	52.717	13.238	24.03.2022	t4	08.12.2020	471	tubercle	wall	NA	31169	NA	2.43	0.6
RG_t4_P3	RG	42	52.717	13.238	24.03.2022	t4	08.12.2020	471	tubercle	wall	NA	37695	NA	2	1.4
Rhein_t1	Rhein	590	50.350	7.600	18.08.2021	t1	11.08.2021	7	tubercle	coupon	0.26	87159	5.79E+08		
Rhein_t2	Rhein	590	50.350	7.600	25.08.2021	t2	11.08.2021	14	tubercle	coupon	0.54	41546	3.39E+08		
Rhein_t3	Rhein	590	50.350	7.600	01.09.2021	t3	11.08.2021	21	tubercle	coupon	0.95	30634	7.34E+08		
Rhein_t4	Rhein	590	50.350	7.600	08.09.2021	t4	11.08.2021	28	tubercle	coupon	1.16	21106	1.97E+09		
Rhein_t5	Rhein	590	50.350	7.600	15.09.2021	t5	11.08.2021	35	tubercle	coupon	1.74	15647	3.03E+09		
VW_t1_I3I4	VW	88	53.321	13.032	08.04.2021	t1	12.12.2020	117	tubercle	coupon	1.13	18422	6.71E+08	6	0.1
VW_t2_J1J3	VW	88	53.321	13.032	29.07.2021	t2	12.12.2020	229	tubercle	coupon	2.48	16808	3.31E+08	3.8	0.3
VW_t3_K4K3	VW	88	53.321	13.032	24.11.2021	t3	12.12.2020	347	tubercle	coupon	3.37	32014	1.85E+09	6.49	0.3
VW_t4_L2L4	VW	88	53.321	13.032	23.03.2022	t4	12.12.2020	466	tubercle	coupon	4.14	40444	2.46E+09	7.57	0.3
VW_W	VW	88	53.321	13.032	29.07.2021	t2	NA	NA	water	lock	NA	13	1.46E+06		
VW_t1_P_SpWS	VW	88	53.321	13.032	08.04.2021	t1	12.12.2020	117	tubercle	plate	NA	21333	1.18E+09	2.39	0.1
VW_t2_P_SpWS	VW	88	53.321	13.032	29.07.2021	t2	12.12.2020	229	tubercle	plate	NA	15758	2.61E+08	2.16	0.4
VW_t3_SpWS	VW	88	53.321	13.032	24.11.2021	t3	12.12.2020	347	tubercle	plate	NA	25347	2.04E+09	2.64	0.3
VW_t4_SpWS_B	VW	88	53.321	13.032	23.03.2022	t4	12.12.2020	466	tubercle	plate	NA	47707	3.33E+09		
VW_t4_SpWS	VW	88	53.321	13.032	23.03.2022	t4	12.12.2020	466	tubercle	plate	NA	24953	1.27E+09	3.29	0.3
VW_t2_BF	VW	88	53.321	13.032	29.07.2021	t2	12.12.2020	229	biofilm	wall	NA	49861	NA	22.6	0.9
VW_t3_BF	VW	88	53.321	13.032	24.11.2021	t3	12.12.2020	347	biofilm	wall	NA	37112	NA		0.9
VW_t4__BF	VW	88	53.321	13.032	23.03.2022	t4	12.12.2020	466	biofilm	wall	NA	21848	NA	16.6	0.7
VW_t1_Pa_SpW	VW	88	53.321	13.032	08.04.2021	t1	12.12.2020	117	tubercle	wall	NA	17590	NA	3.48	0.3
VW_t1_Pb_SpW	VW	88	53.321	13.032	08.04.2021	t1	12.12.2020	117	tubercle	wall	NA	10950	NA		
VW_t2_P_SpW	VW	88	53.321	13.032	29.07.2021	t2	12.12.2020	229	tubercle	wall	NA	6380	NA		

VW_t2_P1_SpW	VW	88	53.321	13.032	29.07.2021	t2	12.12.2020	229	tubercle	wall	NA	16009	NA	3.21	0.2
VW_t2_P2_SpW	VW	88	53.321	13.032	29.07.2021	t2	12.12.2020	229	tubercle	wall	NA	7465	NA	3.09	0.2
VW_t3_P3_SpW	VW	88	53.321	13.032	24.11.2021	t3	12.12.2020	347	tubercle	wall	NA	28328	NA		
VW_t3_Pa_SpW	VW	88	53.321	13.032	24.11.2021	t3	12.12.2020	347	tubercle	wall	NA	23448	NA		

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ID	site	t	AirT_lab	WT_Lab	O2_lab	O2_lab_p	pH_lab	cond_lab	turbi- dity_lab	Chla_Lab	N_diss_lab	NH4_lab	NO2_lab	NO3_lab	P_diss_lab
FBL_t1_BF	Lahn	t1	8	13.1	13.63	131	8.85	418	6	98.3	1.8	0	0	1.6	0.02
FBL_t2_BF	Lahn	t2	20	19.1	8.63	92.7	7.73	373	6	19.5	2.2	0	0	2.1	0.09
FBL_t3_BF	Lahn	t3	16	19.4	7.99	86.8	7.86	446	12	20.4	2.5	0.1	0	2.1	0.09
FBL_t4_BF	Lahn	t4	15.5	19.7	8.91	97.4	7.98	466	4	50.3	2.3	0	0	1.8	0.07
FBL_t5_biofilm	Lahn	t5	18	17.5	8.81	90.7	7.79	509	7	17.2	2.3	0.1	0	2.2	0.11
FBL_t6_BF	Lahn	t6	8	12	9.1	83.3	7.81	560	4	5.62	2.9	0.1	0	2.3	0.08
FBL_t7_BF	Lahn	t7	13	9.9	10.09	88	8.05	544	3	2.37	3.2	0.1	0	2.5	0.1
FBL_t8_BF	Lahn	t8	7	9.7	12.09	98	7.85	537	16	11.5	0.2	0	0	2.7	0.08
FBL_t9_BF	Lahn	t9	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
FBL_t10_BF	Lahn	t10	9	6.8	11.98	96.9	7.86	437	4	4.14	NA	0	0	4.9	0.05
FBL_t11_BF	Lahn	t11	8	11.8	10.5	96	8.17	421	22	71.3	NA	0	0	3.3	0.04
FBL_t1_N2N3	Lahn	t1	8	13.1	13.63	131	8.85	418	6	98.3	1.8	0	0	1.6	0.02
FBL_t2_O1O3	Lahn	t2	20	19.1	8.63	92.7	7.73	373	6	19.5	2.2	0	0	2.1	0.09
FBL_t3_P2_P3	Lahn	t3	16	19.4	7.99	86.8	7.86	446	12	20.4	2.5	0.1	0	2.1	0.09
FBL_t4_Q1Q2	Lahn	t4	15.5	19.7	8.91	97.4	7.98	466	4	50.3	2.3	0	0	1.8	0.07
FBL_t5_R1_R2	Lahn	t5	18	17.5	8.81	90.7	7.79	509	7	17.2	2.3	0.1	0	2.2	0.11
FBL_t6_S1S3	Lahn	t6	8	12	9.1	83.3	7.81	560	4	5.62	2.9	0.1	0	2.3	0.08
FBL_t7_T1T2	Lahn	t7	13	9.9	10.09	88	8.05	544	3	2.37	3.2	0.1	0	2.5	0.1
FBL_t8_U1U2	Lahn	t8	7	9.7	12.09	98	7.85	537	16	11.5	0.2	0	0	2.7	0.08
FBL_t9_V1V2	Lahn	t9	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
FBL_t10_W1W3	Lahn	t10	9	6.8	11.98	96.9	7.86	437	4	4.14	NA	0	0	4.9	0.05
FBL_t11_X1X3	Lahn	t11	8	11.8	10.5	96	8.17	421	22	71.3	NA	0	0	3.3	0.04
FBL_t12_Y3	Lahn	t12	NA	17.8	12.04	126.9	8.83	458	8	100	NA	0	0	2.4	0.02

FBL_t1_SpWS	Lahn	t1	8	13.1	13.63	131	8.85	418	6	98.3	1.8	0	0	1.6	0.02
FBL_t2_SpWS	Lahn	t2	20	19.1	8.63	92.7	7.73	373	6	19.5	2.2	0	0	2.1	0.09
FBL_t3_SpWS	Lahn	t3	16	19.4	7.99	86.8	7.86	446	12	20.4	2.5	0.1	0	2.1	0.09
FBL_t4_SpWS	Lahn	t4	15.5	19.7	8.91	97.4	7.98	466	4	50.3	2.3	0	0	1.8	0.07
FBL_t5_SpWS	Lahn	t5	18	17.5	8.81	90.7	7.79	509	7	17.2	2.3	0.1	0	2.2	0.11
FBL_t6_SpWS	Lahn	t6	8	12	9.1	83.3	7.81	560	4	5.62	2.9	0.1	0	2.3	0.08
FBL_t7_SpWS	Lahn	t7	13	9.9	10.09	88	8.05	544	3	2.37	3.2	0.1	0	2.5	0.1
FBL_t8_SpWS	Lahn	t8	7	9.7	12.09	98	7.85	537	16	11.5	0.2	0	0	2.7	0.08
FBL_t9_SpWS	Lahn	t9	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
FBL_t10_SpWS	Lahn	t10	9	6.8	11.98	96.9	7.86	437	4	4.14	NA	0	0	4.9	0.05
FBL_t11_SpWS	Lahn	t11	8	11.8	10.5	96	8.17	421	22	71.3	NA	0	0	3.3	0.04
FBL_t12_SpWS	Lahn	t12	NA	17.8	12.04	126.9	8.83	458	8	100	NA	0	0	2.4	0.02
FBL_t2_P_So	Lahn	t2	20	19.1	8.63	92.7	7.73	373	6	19.5	2.2	0	0	2.1	0.09
FBL_P_cMS	Lahn	t6	8	12	9.1	83.3	7.81	560	4	5.62	2.9	0.1	0	2.3	0.08
FBL_P_MS	Lahn	t6	8	12	9.1	83.3	7.81	560	4	5.62	2.9	0.1	0	2.3	0.08
LT_1	Lahn	t7	13	9.9	10.09	88	8.05	544	3	2.37	3.2	0.1	0	2.5	0.1
RG_t1_H1H2	HO	t1	NA	7.2	11	90.7					0.4	0	0	0.3	0.01
HO_t2_E2E3	HO	t2	NA	23.8	7.77	91.9	7.38				0.5	0	0	0	0.01
HO_t3_F1F2	HO	t3	NA	8.6	10.16	87.12	7.836				0.5	0.2	0	0	0.01
HO_t4_G4G1	HO	t4	NA												
HO_W	HO	t2	NA	23.8	7.77	91.9	7.38				0.5	0	0	0	0.01
HO_t1_P_SpWS	HO	t1	NA	7.2	11	90.7					0.4	0	0	0.3	0.01
HO_t3_SpWS	HO	t3	NA	8.6	10.16	87.12	7.836				0.5	0.2	0	0	0.01
HO_t4_SpWS	HO	t4	NA												
HO_t1_BF_g	HO	t1	NA	7.2	11	90.7					0.4	0	0	0.3	0.01
HO_t1_BF_w	HO	t1	NA	7.2	11	90.7					0.4	0	0	0.3	0.01
HO_t1_BF_w_B	HO	t1	NA	7.2	11	90.7					0.4	0	0	0.3	0.01
HO_t1_P_SpW	HO	t1	NA	7.2	11	90.7					0.4	0	0	0.3	0.01
HO_t2_BF	HO	t2	NA	23.8	7.77	91.9	7.38				0.5	0	0	0	0.01
HO_t2_Pa_SpW	HO	t2	NA	23.8	7.77	91.9	7.38				0.5	0	0	0	0.01
HO_t2_Pb_SpW	HO	t2	NA	23.8	7.77	91.9	7.38				0.5	0	0	0	0.01
HO_t2_Pc_SpW	HO	t2	NA	23.8	7.77	91.9	7.38				0.5	0	0	0	0.01

HO_t3_BF	HO	t3	NA	8.6	10.16	87.12	7.836				0.5	0.2	0	0	0.01
HO_t3_P2_SpW	HO	t3	NA	8.6	10.16	87.12	7.836				0.5	0.2	0	0	0.01
HO_t4_BF	HO	t4	NA												
HO_t4_GB	HO	t4	NA												
Mosel_t1	Mosel	t1	18	19.3	8.26	88.4	7.98	1002	2	5.03	3.6	0	0	2.6	NA
Mosel_t2	Mosel	t2	18	20.6	10.47	116.1	8.04	1015	1	3.26	2.9	0	0	2.5	NA
Mosel_t3	Mosel	t3	21	20.9	10.18	114.1	7.95	1031	4	3.55	3.4	0	0	2.4	NA
Mosel_t4	Mosel	t4	9	18.6	8.88	93.4	8.04	1005	1	3.35	3.5	0	0	2.6	NA
Mosel_t5	Mosel	t5	9	17.6	8.94	93.2	7.98	1028	2	2.66	3.7	0	0	2.5	NA
RG_t1_A1A2	RG	t1	16.3	6.0	60.0						1.2	0	0	0	0.01
RG_t2_B1B2	RG	t2	25.0	5.0	60.3		7.55				0.8	0.1	0	0.1	0.04
RG_t3_C1C4	RG	t3	8.6	7.5	64.2		7.474				0.9	0.2	0	0.2	0.03
RG_t4_D4D3	RG	t4													
RG_W	RG	t2	25.0	5.0	60.3		7.55				0.8	0.1	0	0.1	0.04
RG_t1_PaSpWS	RG	t1	16.3	6.0	60.0						1.2	0	0	0	0.01
RG_t1_PbSpWS	RG	t1	16.3	6.0	60.0						1.2	0	0	0	0.01
RG_t2_SpWS	RG	t2	25.0	5.0	60.3		7.55				0.8	0.1	0	0.1	0.04
RG_t3_SpWS	RG	t3	8.6	7.5	64.2		7.474				0.9	0.2	0	0.2	0.03
RG_t4_SpWS	RG	t4													
RG_t1_P_SpW	RG	t1	16.3	6.0	60.0						1.2	0	0	0	0.01
RG_t2_BF	RG	t2	25.0	5.0	60.3		7.55				0.8	0.1	0	0.1	0.04
RG_t3_BF	RG	t3	8.6	7.5	64.2		7.474				0.9	0.2	0	0.2	0.03
RG_t3_P1_SpW	RG	t3	8.6	7.5	64.2		7.474				0.9	0.2	0	0.2	0.03
RG_t3_P3_SpW	RG	t3	8.6	7.5	64.2		7.474				0.9	0.2	0	0.2	0.03
RG_t4_BF	RG	t4													
RG_t4_P2	RG	t4													
RG_t4_P3	RG	t4													
Rhein_t1	Rhein	t1		21.1	8.6	96.2	8.03	445	8	8.29	2.4	0	0	1.4	NA
Rhein_t2	Rhein	t2	11	20.5	8.99	98.8	8	462	6	3.55	2	0	0	1.6	NA
Rhein_t3	Rhein	t3	18	19	9.3	98.6	8.07	502	7	4.14	2.8	0	0	1.7	NA
Rhein_t4	Rhein	t4	18	20.8	9.41	104.5	8.04	475	4	6.51	2.2	0	0	1.6	NA
Rhein_t5	Rhein	t5	21	21.1	9.03	101.7	8	478	4	6.22	NA	NA	NA	NA	NA

VW_t1_I3I4	VW	t1	NA	7.13	11.87	98.14					1	0	0	0	0.01
VW_t2_J1J3	VW	t2	NA	24	6.7	79.27	7.7				1.5	0	0	0	0.01
VW_t3_K4K3	VW	t3	NA	6.85	NA	NA	7.542				1.3	0	0	0.1	0.01
VW_t4_L2L4	VW	t4	NA												
VW_W	VW	t2	NA	24	6.7	79.27	7.7				1.5	0	0	0	0.01
VW_t1_P_SpWS	VW	t1	NA	7.13	11.87	98.14					1	0	0	0	0.01
VW_t2_P_SpWS	VW	t2	NA	24	6.7	79.27	7.7				1.5	0	0	0	0.01
VW_t3_SpWS	VW	t3	NA	6.85	NA	NA	7.542				1.3	0	0	0.1	0.01
VW_t4_SpWS_B	VW	t4	NA												
VW_t4_SpWS	VW	t4	NA												
VW_t2_BF	VW	t2	NA	24	6.7	79.27	7.7				1.5	0	0	0	0.01
VW_t3_BF	VW	t3	NA	6.85	NA	NA	7.542				1.3	0	0	0.1	0.01
VW_t4__BF	VW	t4	NA												
VW_t1_Pa_SpW	VW	t1	NA	7.13	11.87	98.14					1	0	0	0	0.01
VW_t1_Pb_SpW	VW	t1	NA	7.13	11.87	98.14					1	0	0	0	0.01
VW_t2_P_SpW	VW	t2	NA	24	6.7	79.27	7.7				1.5	0	0	0	0.01
VW_t2_P1_SpW	VW	t2	NA	24	6.7	79.27	7.7				1.5	0	0	0	0.01
VW_t2_P2_SpW	VW	t2	NA	24	6.7	79.27	7.7				1.5	0	0	0	0.01
VW_t3_P3_SpW	VW	t3	NA	6.85	NA	NA	7.542				1.3	0	0	0.1	0.01
VW_t3_Pa_SpW	VW	t3	NA	6.85	NA	NA	7.542				1.3	0	0	0.1	0.01

6. S6

