The Impact of Selenium on an Archaea-Dominated, Methanogenic Granular Sludge Consortium

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1 Introduction

A major challenge for applied microbiology is the transition from pure/axenic microbes, investigated under sterile conditions, to the real case scenario of dealing with unsterile matrices. This is particularly relevant in the context of wastewater treatment, where large amounts of water cannot be sterilized to ensure the proliferation of pure cultures. In practice, the wastewater is biologically treated (bioremediation) using mixed microbial communities, defined as multi-species aggregates with high resilience and wide metabolic spectrum. An interesting type of mixed microbial consortia is anaerobic granular sludge, developing in upflow bioreactors by self-aggregation of microbial cells (McHugh et al. 2003; Trego et al. 2021). Granular sludge is comprised of small, dense microbial aggregates (0.2–4 mm in diameter), is spherical in shape, is extracellular polymeric substance (EPS)-embedded, and has well-settling properties (Mills et al. 2021; Trego et al. 2021). Hulshoff Pol et al. (2004) and Show et al. (2020) provide a detailed review of the granulation process and the bioreactors using these microbial consortia. Sludge granules contain numerous microbial ecological groups including heterotrophs, methanogens, sulfate-reducing bacteria, denitrifiers, selenium respirers, and selenium reducers, presenting a spatial, 3D distribution based on their nutritional and growth (e.g., redox potential) requirements (van Lier et al. 2016; Staicu and Barton 2021). The presence of

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methanogens is of particular interest as certain waste streams contain high levels of organic pollution that can be converted to biogas for energy generation.

Bioreactors treating industrial and municipal wastewater based on anaerobic granular sludge have been tested against organic and inorganic contaminants, such as the oxyanions of selenium (Se): selenite, SeO_3^{2-}, and selenate, SeO_4^{2-} (Staicu et al. 2017a). The two oxyanions have high water solubility and display toxicity against aquatic biota, leading to documented environmental disasters (e.g., in North America, see Gerson et al. 2022). The treatment of Se-laden wastewaters aims at reducing/converting Se oxyanions to solid elemental Se and Se^0, and volatile Se chemical species (e.g., hydrogen selenides and methyl selenides). Se^0, owing to its solid state, displays limited toxicity for aquatic ecosystems (Staicu et al. 2017b; Ruiz-Fresneda et al. 2023). Figure 1 presents anaerobic sludge granules exposed to selenite, yielding red Se^0 that covers its surface.

However, using mixed microbial communities poses an important challenge to wastewater treatment facilities in terms of ensuring efficient and constant removal of target pollutants because of the inherent interspecific competition and dynamics present in such communities. Therefore, understanding the complex structure and the development of these communities is critical for providing consistent performance of the bioreactors. For this study, we used an anaerobic granular sludge produced by a full-scale bioreactor treating dairy wastewater and we aimed to investigate the presence of selenium detoxification and respiratory genes/operons. The primers were designed based on the available sequences and refined using the UniPriVal algorithm developed by our group at the University of Warsaw (Górecki et al. 2019). Apart from selenium detoxification and respiration, the study also investigated the change in bacterial diversity using 16S rRNA genes as a function of incubation time

Fig. 1 Anaerobic sludge granules exposed to 10 mM sodium selenite collected at day 7 of incubation (growth conditions presented in Sects. 2.1 and 2.2) (Legend: Scale bar represents 100 μm) (personal archive of Dr. Staicu)

and exposure to Se oxyanions, as well as the impact of Se on the methanogens present in the granular sludge. An additional focus of the study explored the biomineralization of Se^0 as this process is poorly understood (Staicu et al. 2022).

2 Materials and Methods

2.1 Reagents and Growth Medium

Sodium selenate (Na_2SeO_4, 98.0%) and sodium selenite (Na_2SeO_3, 98.0%) were purchased from Sigma-Aldrich, and fresh solutions were prepared before each experiment. All other reagents were of analytical grade, and all solutions were prepared using deionized water.

Incubations were done using basal mineral medium (BMM) containing (g L^{-1}) NH_4Cl (0.3), NaCl (0.3), $CaCl_2.2H_2O$ (0.11), $MgCl_2.6H_2O$ (0.1), 1 mL L^{-1} acid trace element solution, 1 mL L^{-1} basic element solution, and 0.2 mg L^{-1} vitamin solution (Stams et al. 1993). 10 mM sodium selenite/selenate and 10 mM lactate (as sodium L-lactate, 60%, Sigma) were amended to BMM.

2.2 Inoculum and Incubation

The anaerobic granular sludge was kindly provided by Dr. Gavin Collins from the Department of Microbiology, National University of Ireland, Galway (NUI Galway), and was used as inoculum. The granular sludge was collected from a full-scale bioreactor in Ireland treating dairy wastewater. 1% of the final volume of sludge was added to 0.5-L serum bottles (see Fig. 2). The bottles were closed with butyl rubber septa and aluminum caps, and the headspace was flushed with nitrogen gas for 5 min through a 0.22-µm filter to ensure sterility. The incubations were performed for 7 days at 30 °C, pH ~ 7.5, in the dark, and under static conditions. Table 1 provides information on the biotic incubations and the genetic analysis carried out in this study (Fig. 3).

2.3 DNA Extraction

2 ml of raw sludge sample and 2 ml of cultures were centrifuged at 8000 rpm, for 10 min, at 4 °C. The anaerobic granular samples are detailed in Table 1. After centrifugation and removal of the supernatant, all pellets were used for DNA extraction. Total DNA was extracted using a FastDNA SPIN Kit for Feces (MP Biomedicals, Illkirch, France) according to the manufacturer's instructions. The DNA concentration was determined using the Qubit™ 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA).

Fig. 2 Anaerobic incubation using granular sludge and selenium oxyanions. (1) Abiotic control [10 mM SeO_4^{2-} and 10 mM lactate], (2) abiotic control [10 mM SeO_3^{2-} and 10 mM lactate], (3) [10 mM SeO_4^{2-} without electron donor + granular sludge], (4) [10 mM SeO_3^{2-} without electron donor + granular sludge], (5) [10 mM SeO_4^{2-} and 10 mM lactate + granular sludge], and (6) [10 mM SeO_3^{2-} and 10 mM lactate + granular sludge]. Abiotic controls: 1 and 2; biotic incubations: 2–6

Table 1 Description of the sludge samples used for metagenomics and selenium respiration analysis

ID	Description	Time point (day)	Community	Selenium respiration
S0	Sludge before experiment		Y	Y
S1	Sludge +10 mM SeO_3	3	Y	Y
S2	Sludge +10 mM SeO_3 + 10 mM lactate	3	Y	Y
S3	Sludge +10 mM SeO_4	7	Y	Y
S4	Sludge +10 mM SeO_3	7	Y	Y
S5	Sludge +10 mM SeO_4 + 10 mM lactate	7	Y	Y
S6	Sludge +10 mM SeO_3 + 10 mM lactate	7	Y	Y

Legend: *Y* yes (performed analysis)

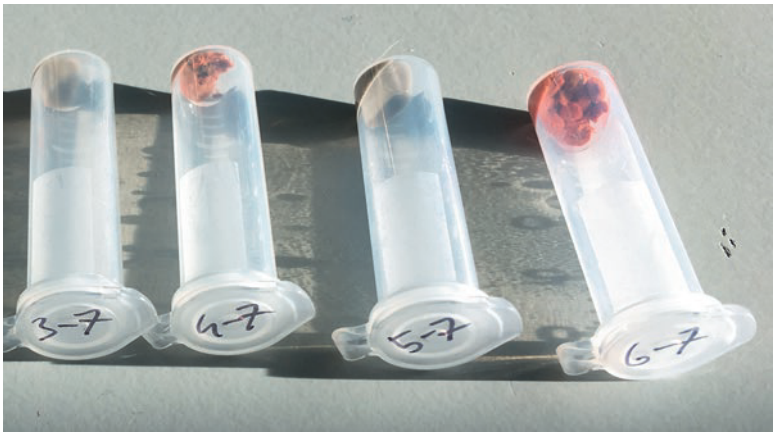

Fig. 3 The granular sludge recovered after 7 days of incubation from the biotic experiments. *3–7* [10 mM SeO_4, no lactate], *4–7* [10 mM SeO_3, no lactate], *5–7* [10 mM SeO_4 + 10 mM lactate], and *6–7* [10 mM SeO_3 + 10 mM lactate]. The first number indicates the incubation bottle, and the second number indicates the incubation day (7) when the experiment was stopped. The protocol implied the recovery of 2 mL of homogenous sludge, followed by centrifugation at 6000 rpm for 10 min

2.4 Primer Design

In total, 13 genes from three operons involved in selenium respiration were used as a template for designing the novel primer pairs: two for selenate respiration and one for selenite respiration.

Selenate Respiration Operons.

SerABCD (GenBank: AJ007744.1) [*Thauera selenatis*; Krafft et al. 2000].
SrdBCA (GenBank: AB534554.1) [*Bacillus selenatarsenatis* SF-1; Kuroda et al. 2011].

Selenite Respiration.

SrrEABCDF (GenBank locus tags: BSEL_RS07625, BSEL_RS07630, BSEL_RS07635, BSEL_RS07640, BSEL_RS17060, BSEL_RS07650) [*Bacillus selenitireducens* MLS10; Wells et al. 2019].

For each gene, its nucleotide sequence was downloaded from the GenBank database and run through Primer3 software, in order to construct a large number of PCR primer pairs that could be used to amplify a particular gene (Untergasser et al. 2012). Next, the UniPriVal algorithm was used to reduce large quantities of previously obtained primer pairs to the ones that could amplify only the gene of interest, even in a metagenomic sample (Górecki et al. 2019). Briefly, UniPriVal takes reference gene sequences, primer pairs, and the GenBank NT database as input. Using sequence alignment tools, it performs in silico PCR in order to determine the number of possible amplification products on the NT database. Based on the results, it computes three parameters for each primer: specificity (S), which shows whether the primer pair amplifies only the gene reference sequences; efficacy (E), which shows what percentage of supplied reference sequences gets amplified; and taxonomic efficacy (TE), reflecting the usefulness of given PCR primer pair for amplification of the targeted gene in bacteria classified within particular taxa. For in vitro analysis, we selected one primer pair for each gene that had all of the above coefficients equal to 1 (Table 2).

2.5 Screening for Selenium Respiration Genes

The occurrence of selected antibiotic resistance genes in the collected culture samples (Table 1) was investigated using PCR assays. All PCRs were performed using 10 ng of template DNA and 0.1 pmol of each primer (Table 2), with DreamTaq PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). The PCR thermocycler program employed depended on the recommended annealing temperature for each primer pair (Table 2). PCR products were resolved by electrophoresis in 1.5% agarose gels.

2.6 Bacterial Community Structure

The bacterial 16S rRNA genes were amplified using the primers targeting the variable V3-V4 region (Mizrahi-Man et al., 2013). Each reaction was prepared using KAPA HiFi polymerase in a concentration of 1 U (KAPA Biosystems) and 0.3 pmol

Table 2 List of primers used in the present study

Gene	Forward sequence[a]	Reverse sequence[a]	Amplicon size (bp)	Denaturation	Annealing	Extension	Reference gene GenBank accession no.	Ref.
serA	CGACTTCCTCAAATATCCCCTGAAG	CTTGAGGTAATCGGCCTTGTACTTCTT	553	95 °C—60 s	57.7 °C—30 s	72 °C—40 s	CAB53372.1	This study
serB	CAGAGACAGTTGGCCTACGTATTC	GTAGAAGTAATGATTGTTCGGGAACTC	378		56.7 °C—30 s	72 °C—40 s	CAB53373.1	
serC	GAGAACATCCAGCATGATGAAGC	TCGGTATCCTTGATTTCCGTATTGG	345		55.3 °C—30 s	72 °C—40 s	CAB53375.1	
serD	CAAGGCTACGGTCTCCCTCTA	TCATTGCAATTCTCCCAGTTGC	400		53.0 °C—30 s	72 °C—40 s	CAB53374.1	
srdA	TAAACTGGATTGAAAAGACGACATTGT	TATTGCTAGATTGCTGTTTTCTGGATC	581		53.7 °C—30 s	72 °C—50 s	BAJ83594.1	
srdB	ACGATGTTAGGAGGGCGCTA	ATGTTCTCGCACCATACGGG	354		53.8 °C—30 s	72 °C—50 s	BAJ83592.1	
srdC	TTGCTCTTCGTACTAATTGATTTAGGC	TAATTACCATTCCGATTTGTCCAATCC	670		55.2 °C—30 s	72 °C—45 s	BAJ83593.1	
srrA	ATTCTTCCGGAGAACTACTATCTTGAG	TGTATTTTTCCGTGATTCTGAGAAGTG	535		55.2 °C—30 s	72 °C—45 s	WP_013172412.1	
srrB	GAAGTTTCATATAACCGGTTGGAGTTT	TTCAGGGACTAATTGAACAGTGTTGAA	411		55.2 °C—30 s	72 °C—45 s	WP_013172413.1	
srrC	TCATGATCATCTTTGGTATCATCGGTA	ACATTTGGTGTTACAAGAATGTAGACC	304		55.2 °C—30 s	72 °C—45 s	WP_013172414.1	
srrD	ACAATCCTTTTCATCATTACGCGATAA	GACATCGTCTATTTCCTGATGTTTCTT	385		53.7 °C—30 s	72 °C—20 s	WP_013172415.1	
srrE	TTACTTTATCCTCGACGTCAGAGATAC	TTTGAGAAATCAAACATATGCTCACCA	744		53.7 °C—30 s	72 °C—50 s	WP_013172411.1	
srrF	GTACCATGAGAACTGGAACTATATCGA	ATTCAGGTGTCACGGTAAAATAGTTTC	395		55.2 °C—30 s	72 °C—50 s	WP_013172416.1	

serABCD (identified in *Thauera selenatis*, Krafft et al. 2000), SrdABC (identified in *Bacillus selenatarsenatis* SF-1; Kuroda et al. 2011), and SrrABCDEF (from *Bacillus selenitireducens* MLS10; Wells et al. 2019)

[a]Sequences are shown in the 5′ to 3′ orientation. *bp* base pairs

primers (forward: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTAC GGGNGGCWGCAG, reverse: GTCTCGTGGGCTCGGAGATGTGTATAAGA GACAGGACTACHVGGGTATCTAATCC).

A template of 10 ng of the extracted DNA was used. Annealing was performed at 63 °C, and a total of 27 cycles were used (Mastercycler nexus GX2 Thermal Cycler, Eppendorf). Three technical replicates for each experimental variant were pooled and sent for library preparation and sequencing to Oligo.pl (Polish Academy of Sciences).

Amplicon libraries were prepared using MiSeq Reagent Kit v3 (2x300bp), and their sequencing was performed using Illumina MiSeq machine (Illumina, San Diego, USA). Obtained reads were subjected to quality control using FASTQC and fastp (Andrews 2010; Shifu et al. 2018).

Next, reads were analyzed using the QIIME 2 platform (Bolyen et al. 2019). Briefly, reads that passed the initial quality control step were subjected to additional quality filtering, merging, denoising, and de novo chimera removal using the DADA2 pipeline (Callahan et al. 2016).

After that, only the reads longer than 200 bp were retained. Obtained unique reads were analyzed using the amplicon sequence variant (ASV) approach. Each ASV was annotated with its putative taxonomy using a classifier trained on the SILVA database (v132) (Yilmaz et al. 2014). Raw sequencing data are available at the European Nucleotide Archive (ENA) under BioProject accession number PRJEB71082 (https://www.ebi.ac.uk/ena/browser/view/PRJEB71082).

Alpha diversity of samples was assessed using the following metrics: species richness, Faith's phylogenetic diversity, Shannon's index, and Pielou's evenness. Diversity metrics were generated and evaluated using the following QIIME 2 plugins: q2-diversity and q2-phylogeny (including MAFFT aligner and FastTree tool) (Price et al. 2009; Katoh and Standley 2013; Vázquez-Baeza et al. 2013).

2.7 *The In Silico Identification of Taxa Hosting Selenium Genes*

A set of 24 genes related to selenium metabolism (Table 3) was queried against the NCBI NT database with specific criteria (98.00% percent identity and 98.00% query coverage). The standard BLASTn output format was extended by incorporating *-sscinames* flag, extracting additional details about taxonomic annotation for each identified sequence. The resulting taxonomic information, highlighting the organisms potentially hosting the analyzed selenium metabolism genes, is presented in Table 3.

Subsequently, the identified taxonomic names underwent a cross-reference with the outcomes of bacterial 16S rRNA gene amplification, as detailed in Sect. 2.6. This analysis provided insights into the relative abundances of taxa potentially encoding selenium metabolism proteins within the analyzed samples (S0-S6; see Table 1) and was illustrated in Fig. 4.

Table 3 Genes involved in the non-respiratory reduction or in the non-specific respiration of Se oxyanions

Gene	Locus tag	Microorganism
dmsD	*b1591*	*Escherichia coli*, *Salmonella* sp., *Shigella boydii*, *Shigella* sp.
FccA	*SBAL678_RS41755*	*Shewanella baltica*
frdC	*b4152*	*Escherichia coli*, *Escherichia* sp., *Salmonella* sp., *Shigella boydii*, *Shigella dysenteriae*, *Shigella sonnei*, *uncultured bacterium*
frdC	*PMI_RS17840*	*Proteus mirabilis*, *Proteus vulgaris*
frdD	*b4151*	*Escherichia coli*, *Escherichia* sp., *Klebsiella oxytoca*, *mixed culture*, *Salmonella* sp., *Shigella flexneri*, *uncultured bacterium*
frdD	*PMI_RS17835*	*Proteus mirabilis*, *Proteus vulgaris*
moeA	*b0827*	*Escherichia coli*, *Escherichia* sp., *Salmonella* sp., *Shigella flexneri*
sr_1	*L867_RS0103850*	*Ciceribacter selenitireducens*
sr_2	*RHIZ70P_RS16955*	*Ciceribacter selenitireducens*
sr_3	*L867_RS0106850*	*Ciceribacter selenitireducens*
sr_4	*L867_RS0109050*	*Ciceribacter selenitireducens*
sr_5	*AAY72_00755*	*Alishewanella* sp.
sr_6	*AQ984_RS16555*	*Clostridium pasteurianum*
sr_7		*Bacillus aerophilus*, *Bacillus altitudinis*, *Bacillus pumilus*
trxA	*PMI_RS16470*	*Proteus mirabilis*
trxB	*SBAL678_RS35010*	*Proteus mirabilis*
trxR	*MJ_RS08205*	*Methanocaldococcus jannaschii*
ygfK	*b2878*	*Escherichia coli*, *Escherichia* sp., *Salmonella* sp., *Shigella flexneri*
ygfL	*b2879*	*Escherichia coli*, *Escherichia* sp., *Salmonella* sp., *Shigella boydii*, *Shigella flexneri*, *Shigella* sp.
ygfM	*b2880*	*Escherichia coli*, *Escherichia* sp., *Salmonella* sp., *Shigella sonnei*
ygfN	*b2881*	*Escherichia coli*, *Escherichia* sp., *Salmonella* sp., *Shigella boydii*, *Shigella flexneri*
ynfE	*b1587*	*Escherichia coli*, *Escherichia* sp., *Salmonella* sp., *Shigella flexneri*
ynfG	*b1589*	*Escherichia coli*, *Salmonella* sp., *Shigella flexneri*, *Shigella* sp.
ynfH	*b1590*	*Escherichia coli*, *Escherichia* sp., *Salmonella* sp., *Shigella boydii*, *Shigella flexneri*

Compiled from Staicu and Barton (2021) and the NCBI NT database

2.8 Analytics

Scanning electron microscopy (SEM): Field emission scanning electron microscopy (FE-SEM) (Carl Zeiss, Oberkochen, Germany) analysis was performed at 2–5 kV range accelerating voltage.

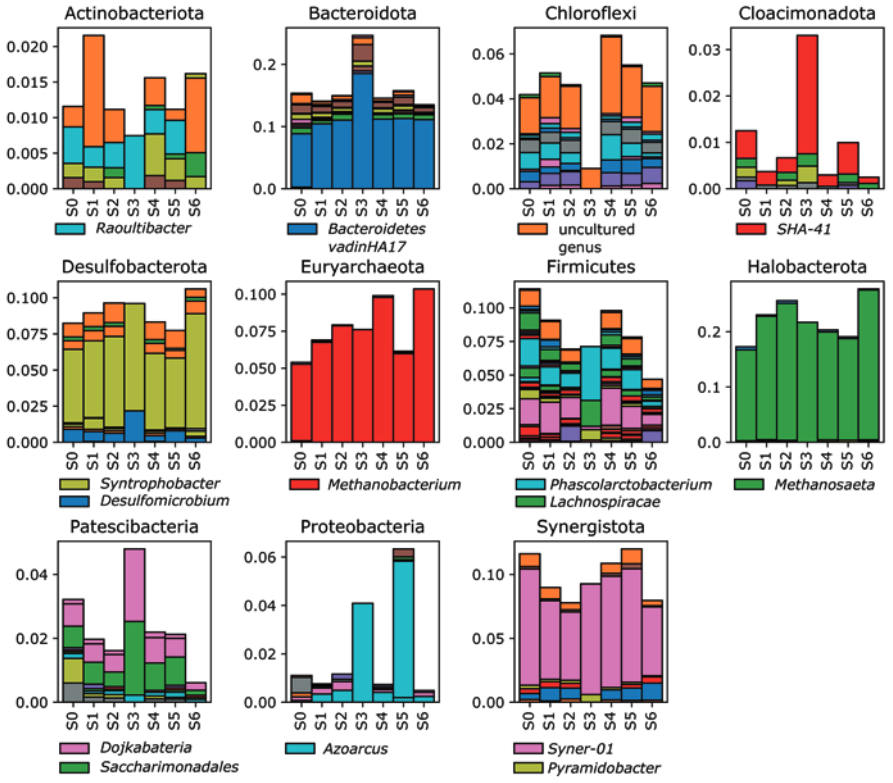

Fig. 4 Microbial community dynamics based on 16S rDNA in samples S0-S6. The x-axis indicates samples. S0: sludge unexposed to Se oxyanions; S1: sludge +10 mM SeO_3; S2: sludge +10 mM SeO_3 + 10 mM lactate; S3: sludge +10 mM SeO_4; S4: sludge +10 mM SeO_3; S5: sludge +10 mM SeO_4 + 10 mM lactate; and S6: sludge +10 mM SeO_3 + 10 mM lactate. The y-axis corresponds to relative abundance of a particular genus within the phylum (values range from 0 to 1)

3 Results and Discussion

3.1 Selenium Respiration or Detoxification?

The reduction of Se oxyanions to red Se^0 is a result of detoxification or respiratory (dissimilatory or energy-generating) strategies. However, distinguishing between these two strategies is not easy because Se detoxification appears to be genetically constitutive in most bacteria, while the respiration based on Se was identified only in a limited number of bacteria and archaea (Wells and Stolz 2020; Staicu and Barton 2021). Although selenium is one of the most energy-dense substrates used in anaerobic respiration, its limited use in bacteria might indicate its scarcity in the environment. In contrast, the oxyanions of sulfur, due to their environmental abundance, are widely used in respiration by phylogenetically diverse bacteria in spite of

the limited amount of cellular energy provided (SeO_4^{2-}, 556 kJ/mol of lactate; SeO_3^{2-}, 530 kJ/mol of lactate vs. SO_4^{2-}, 48 kJ/mol of lactate) (Muyzer and Stams 2008; Staicu and Barton 2021; Barton et al. 2023).

The incubation results are presented in Fig. 2. A progressive conversion of SeO_3 to red Se^0 can be observed in bottles 4 and 6, whereas the negative controls lacking the granular sludge (bottles 1 and 2) remained colorless. This indicates the contribution of the biotic factor in the reduction of selenite and the accumulation of red Se^0. In contrast to bottle 6, which contained 10 mM lactate, bottle 4 lacked it, and this was reflected in the intensity of the red color as well as in the amount of red sediment recovered from the settled granular sludge after 7 days of incubation (Fig. 3). This indicates that lactate is an important factor boosting the production of Se^0 and, probably, also of the bacterial growth. Due to the high density of the granular sludge, which leads to its settling, measuring bacteria growth by optical density is not feasible. However, lactate is enzymatically oxidized and used as an electron donor (e-donor) in anaerobic respiration. This might also explain the higher amount of Se^0 produced when lactate was present. Se^0 produced in bottle 4 indicates that the granular sludge contains an endogenous amount of organic molecules that can be used either for heterotrophic growth or as an e-donor (Wilén et al. 2018). To date, selenite respiration was documented only in *Bacillus selenitireducens* MLS10 (encoding the SrrEABCDF operon), a haloalkaliphilic Firmicutes member isolated from Mono Lake, California (Wells et al. 2019). Given this limited known genetic diversity for selenite respiration, the presence of other genes/operons capable of this transformation cannot be excluded from the inoculum used in this study. However, because of the high toxicity exhibited by selenite, most bacteria contain genes capable of reducing it in a detoxification manner to solid Se^0, a less/non-toxic valence state of Se. In a future study, we plan to apply a transcriptomic approach and check for the genes involved in selenite detoxification (e.g., glutathione reductase and thioredoxin) (a detailed presentation of selenite detoxification, including the genetic determinants, can be found in Staicu et al. 2017a).

In contrast to the selenite-amended bottles, those containing selenate (3 and 5) did not show the production of red Se^0, as evident from Figs. 2 and 3. Indeed, selenate reduction by bacteria was rarely reported [its reduction using physical-chemical methods is even more difficult, being currently an important limitation for the efficient treatment of selenium-laden industrial effluents (Staicu et al. 2017b; Holmes et al. 2022)]. To this end, the known genes reported for selenate respiration (Table 2) were screened in the granular sludge inoculum used in this study. The bioinformatics investigation of the sequenced genes in the granular sludge (bottles 3 to 7) did not identify any of the selenate-respiring genes/operons (namely *serABCD* from *Thauera selenatis* and *SrdABC* from *Bacillus selenatarsenatis* SF-1) listed in Table 2. The conversion of selenate to volatile Se(-II), the most reduced Se species, is unlikely, as this transformation is accompanied by the formation of Se^0 at the concentrations used in the current study (Liu et al. 2023). Moreover, the stepwise reduction to selenite can also be ruled out, because this chemical species would be reduced to red Se^0. Overall, it appears that selenate was not transformed during these incubations.

3.2 Microbial Community Dynamics

The V3-V4 16S rDNA region sequencing produced an average of 179,158 reads per sample. Quality control procedures, encompassing sequence trimming, denoising, merging, and chimera removal, led to the removal of an average of 91,909 reads per sample. After these steps, an average of 87,248 reads per sample remained, representing 47.62% of the initial reads. Notably, sample S3 was excluded from the biodiversity index analysis due to a relatively lower sequence count remaining after quality control (20%).

Figure 4 illustrates taxonomic distributions within two archaeal and nine bacterial phyla. The archaeal phyla, Euryarchaeota and Halobacterota, exhibited remarkable homogeneity, with a single genus representing over 95% of the total phylum abundance. Notably, these genera, known for methane production, collectively constituted a substantial portion, ranging from 19.4% to 37.7%, of the overall microbial abundance in the examined samples. Samples S0 to S6 show an increase in the abundance of the archaeal (Euryarchaeota and Halobacterota) taxa with incubation time. This indicates not only the high resilience of archaea in this system but also its tendency to progressively overcompete other microbial/ecological groups present in the consortium.

In contrast, bacterial phyla displayed much higher diversity (inside its domain), evident not only at the phylum level but also down to the genus level. Within 7 of the 9 bacterial phyla, a clear dominant genus emerged consistently across all samples. These were *Raoultibacter* in Actinobacteriota, *Bacteroidetes* vadinHA17 group in Bacteroidota, a SHA-41 genus in Cloacimonadota, *Syntrophobacter* in Desulfobacterota, *Azoarcus* in Proteobacteria, and Synger-01 group in Synergistota. Furthermore, an unidentified genus, annotated in the Silva database as "uncultured genus," dominated in the Chloroflexi phylum. For instance, Azoarcus is a physiologically versatile beta-Proteobacteria reported to biosynthesize selenium nanoparticles (Fernández-Llamosas et al. 2016).

The overall trend in the relative abundance of identified genera exhibited a relatively stable pattern across samples S0, S1, S2, S4, and S6, corresponding to the sludge before the experiment, sludge +10 mM SeO_3 (day 3), sludge +10 mM SeO_3 + 10 mM lactate (day 3), sludge +10 mM SeO_3 (day 7), and sludge +10 mM SeO_4 + 10 mM lactate (day 7), respectively. Notably, the most substantial alterations were evident in samples S3 and S5, the ones supplemented with SeO_4. In these instances, marked shifts were observed in the Actinobacteria, Chloroflexi, Cloacimonadota, Patescibacteria, and Proteobacteria phyla (Fig. 4).

The biodiversity indices offered valuable information about the impact of Se oxyanions on the microbial community (Table 4). Shannon's index (*H*) measures the diversity of species in a community (the higher the value of H, the higher the diversity of species in a particular community). For the Se-unexposed consortium (S0), H was 9.28. The addition of SeO_3^{2-} resulted in a diversity decrease to 8.93 (day 7), in the absence of lactate. When lactate was present, the diversity decrease was higher: 8.65 (day 7). H also decreased in the SeO_4^{2-}amended incubation, but to a lower degree (9.10).

Table 4 Microbial diversity indices as a function of incubation time

Sample	Time (days)	Se oxyanion	Evenness	Shannon (*H*)	Faith (*PD*)	Richness (*S*)
S0	0	–	0.95	9.28	32.16	863
S1	3	SeO_3^{2-}	0.94	9.34	32.16	976
S2[a]	3	SeO_3^{2-}	0.94	8.97	28.72	748
S3	7	SeO_4^{2-}	n/a	n/a	n/a	n/a
S4	7	SeO_3^{2-}	0.95	8.93	25.68	677
S5[a]	7	SeO_4^{2-}	0.94	9.10	27.48	798
S6[a]	7	SeO_3^{2-}	0.94	8.65	24.95	584

Sample S3 was excluded from the analysis due to the low number of sequencing reads remaining after QC. *S0*: sludge unexposed to Se oxyanions; *S1*: sludge +10 mM SeO_3; *S2*: sludge +10 mM SeO_3 + 10 mM lactate; *S3*: sludge +10 mM SeO_4; *S4*: sludge +10 mM SeO_3; *S5*: sludge +10 mM SeO_4 + 10 mM lactate; and *S6*: sludge +10 mM SeO_3 + 10 mM lactate. *n/a* not available
[a]Legend: S2, S5, and S6 (+ lactate)

Faith's phylogenetic diversity (*PD*) uses phylogenetic distance to calculate the diversity of a given sample. PD of Se-unexposed consortium was 32.16, while the addition of SeO_3^{2-} resulted in a decrease of this index to 25.68 (day 7). This again indicates the loss of diversity in the presence of selenite, a known toxicant for microbial metabolism. In the case of SeO_4^{2-}, *PD* decreased to 27.48, reflecting the lower toxicity of selenate vs selenite.

Richness (*S*) is another valuable index used to assess the diversity. *S* showed a marked decrease from 863 (S0) to 677 (day 7) in the presence of selenite and without lactate. The addition of lactate led to an even higher decrease, 584 (day 7). In the case of selenate, the richness was also diminished, but to a lesser degree, 748 (day 7).

Overall, three diversity indices (Shannon, Faith, and richness) showed a decrease in microbial diversity in the presence of both selenite and selenate, with a higher impact induced by selenite. Lactate also appears to have a negative effect on decreasing the microbial diversity, probably due to the proliferation of lactate-consuming microorganisms over other heterotrophic or chemolithotrophic microorganisms (lactate is also an electron donor in anaerobic respiration). Species evenness describes the rarity of a species. This index did not show a significant change in this study (0.94–0.95), potentially indicating the high resilience (stability) of the microbial consortium against the emergence of highly dominant (invasive) taxa.

3.3 Screening for Taxa Involved in Non-Respiratory Selenium Reduction

As the PCR screening targeting known genes associated with respiratory selenium reduction yielded no positive results, we subsequently turned our attention to screening amplicon results for taxa that might engage in non-respiratory selenium reduction. To achieve this, we compiled a list of genes implicated in non-respiratory selenium reduction or in the non-specific respiration of Se oxyanions and aligned

them against the NCBI NT database to ascertain their taxonomic distribution. The outcomes of this screening are detailed in Table 3. Intriguingly, upon cross-referencing the identified taxonomic ranges with those obtained from 16S rDNA sequencing, *Shewanella* sp. emerged as the sole taxon present in both datasets. This is particularly relevant as the Shewanella is known for its plastic metabolism with regard to selenium (Tam et al. 2010; Jiang et al. 2012; Staicu et al. 2021, 2022; Edwards et al. 2023). For instance, *Shewanella* sp. O23S (belonging to *Shewanella baltica*) is capable of reducing selenite and selenate to red Se^0 both under oxic and anoxic conditions in a respiratory and non-respiratory fashion (unpublished results).

3.4 Electron Microscopy: A Glimpse into a Hidden World

The granular sludge inoculum viewed under the electron microscope reveals a diverse consortia of microorganisms of various shapes and forms (Fig. 5). The micrograph identifies tubular, Bacillus-shape microorganisms, coiled, Spirillum-shape, cell debris and microbial aggregates, and, potentially, mineral

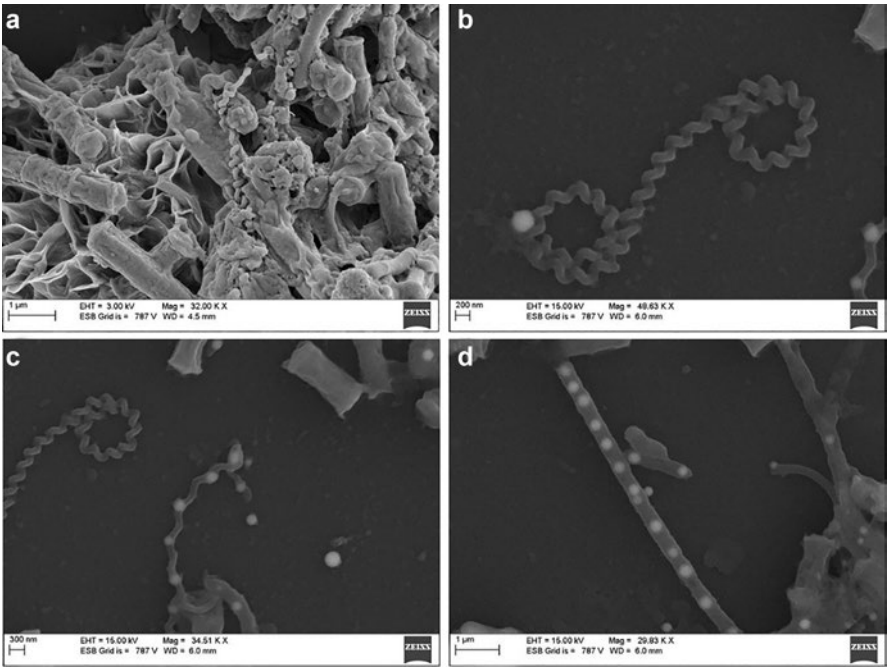

Fig. 5 Scanning electron micrographs (SEMs) of the biomineralization of Se^0 by the granular sludge consortium (S6—day 7). (**a**) The microbial phenotypic diversity of the granular sludge; (**b**) extracellular Se^0 attached to a Spirillum-type microorganism; (**c**) Spirillum- and bacillus-type microorganisms with extracellular Se^0; and (**d**) intracellular Se^0 associated with stalk morphology microorganisms

formations. Because we lack an elemental mapping and a mineralogical analysis to complement the electron microscopy, we cannot speculate on the distribution of chemical elements and the nature of mineral formations. It is well established that granular sludges contain various amounts of iron minerals conferring them a black color (Hulshoff et al. 2004).

Microbial biomineralization of Se often leads to the amorphous, globular, red Se^0 allotrope (Figs. 1, 2 and 3). There is an ongoing debate about the nucleation site of biogenic Se^0 particles because they have a considerable diameter (100–500 nm) relative to the bacterial size (Staicu and Barton 2021). An intracellular nucleation and growth of biogenic Se^0 to such an extent raise the challenge to export such particles to the extracellular environment or to handle them intracellularly. Both of these instances would affect the integrity and viability of the microbial cells. A number of microorganisms seem to have found a solution to this problem by performing extracellular respiration (Bird et al. 2021). In this process, the electrons are conveyed extracellularly to the electron acceptor (e.g., SeO_3^{2-} or SeO_4^{2-}), yielding Se^0 particles outside of the cell. Extracellular respiration has been studied extensively in the genus Shewanella, and a recent article showed extracellular polydisperse Se^0 particles in *Shewanella* sp. O23S (belonging to *Shewanella baltica*) (Staicu et al. 2022). Interestingly, Se^0 appears to be present both intracellularly (Fig. 5c, d) and outside the cell (in its vicinity and in the extracellular milieu) (Fig. 5b, c). This may indicate a complex metabolism involving both respiratory and detoxification processes. Another particular aspect is the relative uniformity of Se^0 in terms of size distribution (~200 nm). Previous investigations using both pure (Staicu et al. 2022) and mixed microbial communities (Staicu et al. 2015) have reported polydisperse Se^0 particles of diameters ranging from 50 to 250–300 nm or more. This observation deserves further investigation in a follow-up study with micrographs recorded at various incubation times.

4 Summary and Perspectives

This study provides a first attempt to study the impact of Se on a methanogenic archaeal consortium from a genetic and biomineralization perspective. The results indicate the high resilience of archaeal methanogens when exposed to toxic Se, having particular relevance in the context of biomethane production in anaerobic digestion systems (ADs). AD is a growing trend at the international level for the valorization of wastes in the form of energy (biogas/methane) and digestate (fertilizers) (Garuti et al. 2023). In a follow-up study, we plan to have a deeper understanding of the impact of Se in such systems by using a qPCR approach (investigate the gene expression level of methanogens and Se reducers), as well as quantify the amount of lactate (and other electron donors) consumed and methane produced (including the purity of the biogas) using model systems and organic waste materials. The fact that the biomineralization process of Se proceeded in parallel with

biomethane production shows promise for the co-harvest of both scarce minerals (e.g., Se) and energy (biomethane).

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