

Diversity and abundance of sulfate-reducing microorganisms in a Mediterranean lagoonal complex (Amvrakikos Gulf, Ionian Sea) derived from *dsrB* gene

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ABSTRACT: Sulfate-reducing microorganisms (SRMs) are a phylogenetically and physiologically diverse group of microorganisms, responsible for the dissimilatory reduction of sulfate. SRMs thrive under anaerobic conditions with high availability of organic matter. Such conditions characterize lagoonal ecosystems which experience regular dystrophic crises. The aim of the present study was to explore the biodiversity patterns of SRMs and to examine the extent to which these patterns are associated with biogeographic and environmental factors. Sediment samples were collected from 5 lagoons in the Amvrakikos Gulf (Ionian Sea, western Greece). DNA was extracted from the sediment and was further processed through pyrosequencing of a region of the dissimilatory sulfite reductase β -subunit (*dsrB*). The results of this exploratory study show that the majority of the observed operational taxonomic units (OTUs) belong to the *Deltaproteobacteria* super-cluster and more specifically, to the *Desulfobacteraceae* family. Salinity and ammonium ions are the environmental factors that best correlated with the SRM community pattern. Furthermore, the SRM community of the brackish lagoons is differentiated from that of the brackish-marine lagoons and the studied lagoons have distinct SRM communities.

KEY WORDS: *dsrB* gene · Amvrakikos Gulf · Lagoon · Pyrosequencing · Sediment · Sulfate-reducing microorganisms

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INTRODUCTION

Sulfate-reducing microorganisms (SRMs) are a taxonomically diverse group involved in the biogeochemical cycles of carbon, sulfur (Jørgensen 1982) and mercury (Gilmour et al. 1998). They are anaerobically respiring microorganisms, which couple the degradation of organic compounds to the reduction of sulfate as a terminal electron acceptor (Rabus et al. 2006), resulting in the production of sulfide (Muyzer

& Stams 2008). SRMs have been found in a variety of ecosystems, including freshwater wetlands (Li et al. 1999, Pester et al. 2012), estuarine sediments (Jiang et al. 2009) and extreme environments (Dhillon et al. 2003, Fishbain et al. 2003); they have also been found in high abundances in polluted sites (Pérez-Jiménez & Kerkhof 2005) and associated with metals (Nakagawa et al. 2002).

Six phylogenetic lineages constitute the cultured representatives of known SRMs, with 4 of them

belonging to the bacterial (*Delta-proteobacteria*, *Nitrospirae*, *Firmicutes*, *Thermodesulfobacteria*) and 2 to the archaeal domain (*Euryarchaeota*, *Crenarchaeota*) (Muyzer & Stams 2008, Müller et al. 2015). Due to their polyphyletic nature, 16S rRNA gene based analysis cannot sufficiently describe the SRMs and functional gene markers should be used instead for the assessment of their abundance and diversity (Wagner et al. 2005).

Sulfate reduction is a reaction found in sulfate-reducing prokaryotes and other organisms, such as plants, algae and fungi, and it can be further distinguished into assimilatory and dissimilatory sulfate reduction (Madigan et al. 2012). The final step of the latter, namely the reduction of (bi)sulfite to sulfide, is catalyzed by the dissimilatory (bi)sulfite reductase (*dsr*) which is encoded by the *dsrAB* gene (Kondo & Butani 2007, Liu et al. 2009). Therefore, the *dsrAB* gene is considered a key functional marker for molecular analysis and detection of SRMs (Wagner et al. 1998, Dar et al. 2007) and its application has revealed a great diversity of organisms that are not closely related to known and recognized SRMs. However, it should be interpreted with caution since *dsrAB*, in reverse, is also involved in the oxidative steps of the biogeochemical sulfur cycle (Müller et al. 2015).

In the present study, the chosen technique for the identification and enumeration of SRMs was pyrosequencing (Ronaghi et al. 1998) of the dissimilatory sulfite reductase β -subunit (*dsrB*) gene. Pyrosequencing is a molecular technique that has been widely applied in the field of microbial ecology, mostly targeting hypervariable regions of the 16S rRNA gene (e.g. Thompson et al. 2011, Wang et al. 2012) instead of functional marker genes (e.g. Pelikan et al. 2016).

The site under investigation was the lagoonal complex of the Amvrakikos Gulf (Ionian Sea, western Greece), one of the largest semi-enclosed embayments of the Mediterranean Sea, which is characterized by a fjord-like oceanographic regime (Ferentinos et al. 2010). The structure and function of lagoonal ecosystems is largely determined by the input of organic matter from terrestrial and marine environments, as well as from the atmosphere (e.g. Viaroli et al. 2008). The subsequent decomposition and removal of organic matter (Tagliapietra et al.



Fig. 1. Amvrakikos Gulf in the Ionian Sea, indicating the location of lagoons (Logarou, Rodia, Tsoukalio, Mazoma and Tsopeli) and sampling stations (2 per lagoon) used for study of the diversity and abundance of sulfate-reducing microorganisms. Filled symbols are stations inside the lagoons; empty symbols are stations in the channel connecting each lagoon to the gulf

2012) defines the structure and function of lagoonal ecosystems.

The study aimed to identify SRMs, and specifically to test (1) whether the SRM communities in the studied lagoons exhibit biogeographic patterns and (2) the extent to which these patterns are associated with environmental factors.

MATERIALS AND METHODS

Location and general characteristics of the lagoons and sampling sites

The lagoons of the Amvrakikos Gulf (38° 59' N, 20° 57' E) are protected by the Ramsar convention and are listed in the Natura 2000 network. The Gulf is connected with the Ionian Sea via a narrow channel, the Preveza (Aktio) Strait (Kapsimalis et al. 2005). The lagoonal complex at the northwest part of the Amvrakikos Gulf is formed by the rivers Arachthos and Louros (Poulos et al. 1995).

Five lagoons of the Amvrakikos Gulf were sampled for the purposes of this study: Logarou, Rodia, Tsoukalio, Mazoma and Tsopeli. In each lagoon, 2 sampling stations were chosen at the extremes of the confinement gradient: the first station was located at the inner part of the lagoon and the second near the channel connecting the specific lagoon with the Gulf (Fig. 1). Sampling was carried out in February 2011.

Sampling methodology

Sediment samples were collected from all stations by means of a modified manually operated box-corer, with a sampling surface of 156.25 cm² and a sediment penetration depth of 25 cm. Cylindrical sampling corers, with an internal sampling surface of 15.9 cm², were placed inside the box-corer and sub-samples of the sediment's upper layer (0 to 0.2 cm) were collected from them. Three replicate units were taken from each sampling station, to determine variability within and among stations. Samples for molecular analysis (each consisting of about 15 cm³ of sediment) were placed in 50 ml falcon tubes (Sarstedt) and were stored at -20°C until further processing in the laboratory.

In addition, a variety of environmental variables were measured both in the sediment and in the water column (for a detailed description see Pavloudi et al. 2016, Vasileiadou et al. 2016).

DNA extraction, PCR amplification and pyrosequencing of the *dsrB* gene

DNA was extracted using the UltraClean® Soil DNA Isolation Kit (MO BIO Laboratories), according to the 'alternative protocol for maximum yields', as recommended by the manufacturer. About 0.5 g (± 0.2 g) of wet sediment from each sample were used.

PCR amplification was performed with newly designed primers, based on genomes of known SRMs and targeting part of the *dsrB* gene. Primer design was done in accordance with the recommendations of Roche (manufacturer of the GS FLX Titanium) and specifically guided by the advice that amplicons should cover the sequence of interest within the first 400 bp of sequencing. Primer coverage was tested *a posteriori* with the ARB Probe Match tool (Ludwig et al. 2004) against the 1292 core nucleotide sequences from the reference database (Müller et al. 2015), using perfect match and one weighted mismatch.

The primers used were 1595f (5'-YCA YGA RAT CCT BGA RCC-3') and 1905r (5'-CTG GGT RTG RAC GAT RTT G-3'). The primers were complemented with the 454 adapters (Lib-A Chemistry) and with sample-specific 5 bp barcodes (nucleotide 'keys') downloaded from VAMPS (Huse et al. 2014). Six different primer pairs were used, each one bearing a specific barcode which enabled the separation of the different samples after the sequencing (de-multiplexing).

The amplification reaction mix contained 6 μ l 5X KAPAHiFi Fidelity buffer, 0.9 μ l KAPA dNTP Mix (10 mM), 1.5 μ l from each primer (10 μ M), and 0.6 μ l KAPAHiFi HotStart DNA polymerase (1 U μ l⁻¹) in a final volume of 30 μ l per reaction. DNA template concentration was about 50 ng μ l⁻¹. The PCR protocol used as follows: 95°C for 5 min; 30 cycles at 98°C for 20 s, 54°C for 15 s, 72°C for 30 s; 72°C for 5 min. Amplifications were carried out using MyCycler (BIORAD) and DNA Engine DYAD (Peltier Thermal Cycler, MJ Research). Samples were purified using Agencourt AMPure XP (Becker Coulter). Amplicons were quantified with the Picogreen assay (Molecular Probes), mixed in equimolar amounts and sequenced using the 454 GS FLX Titanium Series (Roche) hosted at IMBBC (HCMR), in compliance with the recommendations of the manufacturer and using 4 lanes of the sequencing plate. Sequencing of one amplicon (sample M_01_A; see Table S1 in Supplement 1 at www.int-res.com/articles/suppl/a079p209_supp1.pdf) was considered to be faulty, resulting in a high number of errors. Therefore this sample was not included in further analyses.

All raw sequence files of this study were submitted to the European Nucleotide Archive (ENA) (Leinonen et al. 2011) with the study accession number PRJEB3370 (available at www.ebi.ac.uk/ena/data/view/PRJEB3370).

Sequence processing

The raw sequence reads retrieved from all sediment samples were processed with the Amplicon-Noise algorithm for removal of 454 sequencing errors, PCR single base errors and chimeras (Quince et al. 2011), as described in Pavloudi et al. (2016).

In addition to filtering and denoising, a further level of error correction was employed by translating nucleotide sequences into amino acids. The clustered high quality nucleotide sequences, using the 90% nucleotide similarity cut-off (Kjeldsen et al. 2007, Angermeyer et al. 2016), were translated to protein using FrameBot (Wang et al. 2013); detection and correction of frameshift errors in the reads were done using a set of known *dsrB* protein sequences.

As described in Pelikan et al. (2016), the translated amino acid sequences of the present study were aligned to the *dsrAB* reference sequence alignment in MEGA 6 (Tamura et al. 2013) using MUSCLE (Edgar 2004) and they were placed into the reference tree using the Evolutionary Placement Algorithm (EPA; Berger & Stamatakis 2011) in RAxML (version

8.0.23) (Stamatakis 2014) and the PROTGAMMA-AUTO option which automatically chooses the best protein model for the data provided. The EPA derived OTUs classification was compared to the classification of the core *dsrAB* dataset of Pelikan et al. (2016) for the taxonomic inference of the OTUs.

Furthermore, the nucleotide sequences of the OTUs that were translated into protein were used as an input to the Seqenv pipeline (Sinclair et al. 2016), using the unique isolation option, in an attempt to link the 100 most abundant OTUs with descriptive environmental terms and determine in which environments they have been previously observed.

Statistical processing

Lagoons were assigned to salinity categories based on their salinity ranges following the Venice system (International Symposium on the Classification of Brackish Waters 1958), with the (mixo-) mesohaline domain (salinity 5 to 18 psu) further divided as described by Pavlouidi et al. (2016). Hence, the 3 categories comprised the (1) (mixo-) polyhaline (salinity 18-30 psu), (2) (mixo-) b-mesohaline (salinity 8 to 18 psu) and (3) (Mixo-) a-mesohaline (salinity 5 to 8 psu) domains.

The number of sequences belonging to each OTU was considered representative of OTU abundance. Subsequently, a matrix of the OTU abundance was constructed, with the microbial OTUs as variables and sampling stations as samples. Nucleotide sequences that failed to translate to amino acids were excluded from the matrix. The OTU abundance matrix was used for the calculation of the triangular similarity matrix using the Bray-Curtis similarity coefficient (e.g. Clarke & Warwick 1994). In order to investigate the bacterial community pattern in the area under study, non-metric multidimensional scaling (nMDS) (Clarke 1993) and permutational multivariate analysis of variance (PERMANOVA) (Anderson 2001) were performed. The design considered 4 factors: 'lagoon', 'location', 'lagoon and location' and 'salinity category' (999 permutations). Due to data limitations that did not allow for a successful 4-factor design to be tested, each factor was tested separately (under a design of unrestricted permutation of raw data).

In order to test the second hypothesis, an abundance matrix was constructed with the sampling stations as samples and the 38 environmental terms found associated with the OTUs as variables, which was also treated as mentioned previously. In addition, BIO-ENV analysis was applied, thus permitting

investigation of all potential correlations between the biotic and abiotic matrices, by employing the weighted Spearman rank coefficient ρ_w (Clarke & Ainsworth 1993). Environmental variables that were highly correlated ($-0.9 > \rho_w > 0.9$, $p < 0.05$) were mutually excluded from further analyses (Clarke & Ainsworth 1993). The RELATE routine (Clarke & Gorley 2006) was applied to test for the significance of the correlated patterns, as calculated by the BIO-ENV analysis. This was performed between the biotic similarity matrices and those produced by subsets of the environmental parameters, as identified by the BIO-ENV analysis in each case.

OTU richness was estimated via extrapolation using the Chao-1 (Chao 1987, Chiu et al. 2014) and the Abundance Coverage Estimator (ACE) (O'Hara 2005). In addition, a suite of diversity indices (Margalef's species richness, Pielou's evenness, Shannon-Wiener; Pielou 1969) was calculated. The diversity indices, as well as the relative abundance percentages of the SRM OTUs, were tested for significant differences between the different salinity categories and lagoons by means of the nonparametric analysis of variance Kruskal-Wallis test (Kruskal & Wallis 1952). The nonparametric Mann-Whitney *U*-test (Mann & Whitney 1947) was used for the post hoc pairwise comparisons; a Bonferroni-correction was applied and the level of significance for the results of the Mann-Whitney pairwise tests was lowered from 0.05 to 0.017 in the case of the salinity categories, and from 0.05 to 0.01 in the case of the lagoons. In addition, the Mann-Whitney *U*-test was used to test for significant differences between the locations.

The DIVERSE routine (Clarke & Warwick 1994) of the PRIMER (v.6) package (Clarke & Gorley 2006) was used for the calculation of diversity indices. The same software was used for the BIO-ENV analysis and the RELATE routine. nMDS and PERMANOVA were performed with the R virtual laboratory (RvLab) (Varsos et al. 2016). Chao-1 and ACE estimator were calculated using the EstimateR function of the vegan package (Oksanen et al. 2016). Mann-Whitney *U* and Kruskal-Wallis tests were conducted with the IBM SPSS Statistics for Windows (Version 22.0). The Venn diagrams were constructed using the jvenn JavaScript library (Bardou et al. 2014).

RESULTS

The coverage of the primers is presented in Table 1. The forward primer did not have as many positive hits as the reverse one; however, a substan-

Table 1. Coverage of the primers used to detect the presence of sulfate-reducing microorganisms (SRMs) in the lagoons of Amvrakikos Gulf (Ionian Sea). Design of the primers was based on genomes of known SRMs, targeting the β -subunit of the dissimilatory sulfite reductase gene (*dsrB*). Coverage of the primers was calculated using the ARB Probe Match Tool. Degeneracy is given as the number of oligonucleotides that comprise the primer. Coverage is calculated as the percentage of positive hits against the 1292 core dataset

Primer name	Direction	Sequence (5'–3')	Length (nt)	Degeneracy	Coverage of core dataset <i>dsrAB</i> (%)	Average coverage of core dataset <i>dsrAB</i> (%)
1595f	Forward	YCAYGARATCCTBGARCC	18	48	3.17–14.86	8.01
1905r	Reverse	CTGGGTRTGRACGATRTTG	19	8	12.93–40.94	23.90

tial percentage of the *dsrAB* core dataset could have been amplified when tested *in silico*. Therefore, one could make the assumption that the results of the present study are indicative of the SRM community in the study area, although representatives of the community may exist for which amplification was not successful.

The results of the processing of the sequences during the noise removal are shown in Table S1 in Supplement 1. The 148 626 initial raw sequences were clustered into 18 655 high quality sequences, which corresponded to 5912 OTUs at the 90% similarity cut-off; out of those, 2167 were translated to amino acid sequences. The automatic protein model assignment algorithm of RAXML resulted in a log likelihood of $-247\,061.75$. The labelled reference tree including branch labels and query sequences (Supplement 3 at www.int-res.com/articles/suppl/a079p209_supp3.pdf) and the classification results (Table S2 in Supplement 2 at www.int-res.com/articles/suppl/a079p209_supp2.xls) show that the majority of the observed OTUs (74%) belong to the *Deltaproteobacteria* supercluster, within which the most abundant is the family *Desulfobacteraceae* (33%), with the Environmental supercluster 1 being second in terms of abundance (25%) (Fig. 2a). However, when the abundance of the OTUs was taken into consideration, the difference between the groups was augmented (Fig. 2b); the most abundant OTUs belong, as expected, to the *Deltaproteobacteria* supercluster (83%) followed by the Environmental supercluster 1 (16%).

This pattern was similar when each lagoon was regarded separately (Fig. S1 in Supplement 1). Although representatives from the Environmental supercluster 1 were present in all lagoons (21 to 25%), their abundance was relatively lower (11 to 20%), while the *Deltaproteobacteria* supercluster showed higher abundance, which reached 88% in the case of Rodia lagoon. In addition, although the *Desulfatiglans anilini* lineage was present in similar percentages in all

lagoons (18 to 27%), its abundance was greater in the a-mesohaline lagoons, i.e. in Tsoukalio and Rodia, and especially in the latter (40%), while levels were the same in the polyhaline and b-mesohaline sampling stations. In the case of the *Desulfobacteraceae* family, the lowest presence (25 to 28%) and abundance (12 to 17%) were found in the a-mesohaline lagoons; by contrast, in the other sampling stations the aforementioned family exhibited a higher presence (33 to 38%) and abundance (22 to 39%). Although the presence of the *Syntrophobacteraceae* family was very low in all lagoons (1 to 2%), it showed higher abundance in Logarou (11%) and Rodia (8%) lagoons. The abundance of the unclassified OTUs of the *Deltaproteobacteria* supercluster was higher in all lagoons (12 to 27%) than their presence (7 to 9%). However, none of the aforementioned variations in the relative abundance percentages of the SRM OTUs were statistically significant (Kruskal-Wallis: $p > 0.05$ for all cases); this could be attributed to the use of data at the phylum level for the Kruskal-Wallis test while differences may be exhibited at a lower taxonomic level.

The number of OTUs that were commonly shared among the lagoons (90% similarity cut-off) is shown in Fig. 3. A total of 149 OTUs were commonly shared by all 5 lagoons, corresponding to less than 7% of the total number of observed OTUs.

nMDS of the bacterial OTUs spatial pattern (Fig. 4) showed that the bacterial community pattern differs by lagoon and salinity category. The PERMANOVA test produced significant results for the factors 'lagoon' (F.Model = 3.5936, $p < 0.01$), 'lagoon and location' (F.Model = 3.3443, $p < 0.01$) and 'salinity category' (F.Model = 4.0402, $p < 0.01$). This is also depicted in the Venn diagram for the 3 salinity categories (Fig. 5). In addition, salinity and ammonium ions (NH_4^+) (Table 2) were the abiotic variables with the highest correlation to the SRM community pattern ($\rho_w = 0.575$).



Fig. 2. Taxonomic classification of the sulfate-reducing microorganism operational taxonomic units (SRM OTUs) retrieved from all the samples based on (a) the presence/absence and (b) the abundance of OTUs

Based on nMDS of the environmental terms that are associated with the SRM OTUs (Fig. S2 in Supplement 1), and according to the PERMANOVA test, the samples can be differentiated by all tested factors (lagoon: $F_{\text{Model}} = 2.2625$, $p < 0.01$; location: $F_{\text{Model}} = 2.5827$, $p < 0.05$; lagoon and location: $F_{\text{Model}} = 4.0728$, $p < 0.01$; salinity category: $F_{\text{Model}} = 5.0393$, $p < 0.01$). This is also evident from Table 3, where it is shown that the abundance of the associated environmental terms varied among the sampling stations. Undoubtedly, the most abundant term in all the

lagoons was 'sediment' (~44%). The SRM OTUs found in the a-mesohaline lagoons, i.e. in Tsoukalio and Rodia, were associated with the term 'wetland' in higher abundances (~14 to 25%) compared with the other lagoons (~1 to 3%). Similarly, the terms 'hydrothermal vent' and 'acid mine drainage' were found in much lower abundance in the polyhaline and b-mesohaline lagoons (~2 to 5%) than in Tsoukalio and Rodia (~16 to 25%). In addition, Tsopeli lagoon was the only lagoon associated with the terms 'lake' and 'reservoir' (~12%).

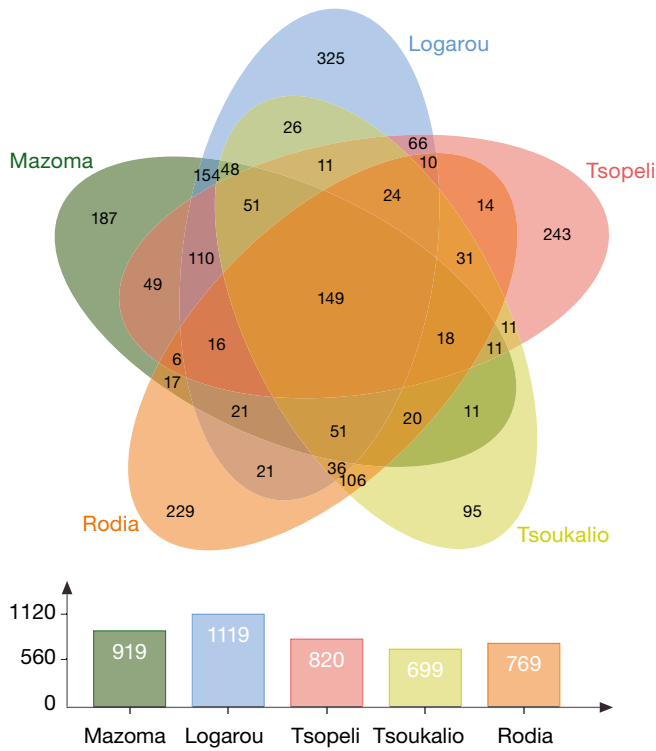


Fig. 3. Number of OTUs commonly shared among all possible combinations of 2 or more of the 5 studied lagoons (see Fig. 1) (90% similarity cut-off). The bar chart indicates the total number of OTUs retrieved from each lagoon

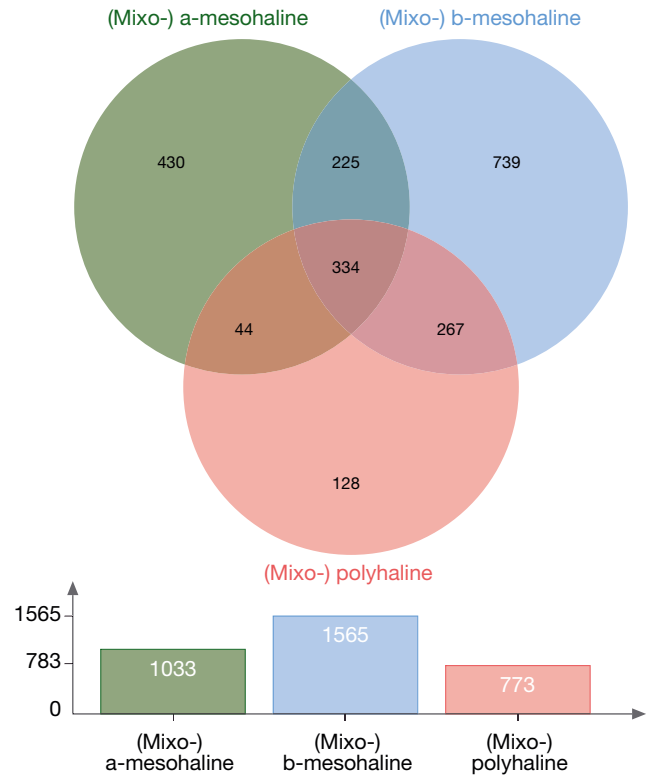


Fig. 5. Number of OTUs of each salinity category shared with those of the other categories (90% similarity cut-off). The bar chart indicates the total number of OTUs found in each salinity category

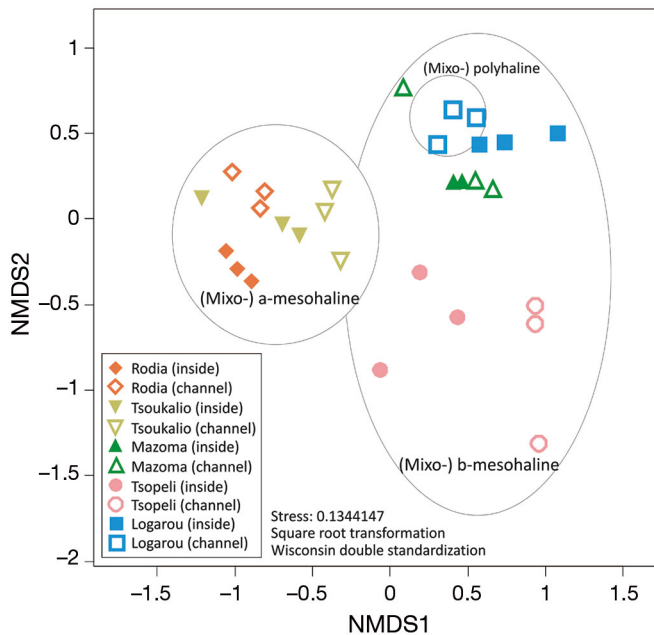


Fig. 4. Multidimensional scaling of the SRM OTUs (90% similarity cut-off). Symbols indicate sampling stations (2 per lagoon in 5 lagoons; see Fig. 1). Salinity categories are encircled (see 'Materials and methods: Statistical processing')

All diversity indices, except for the total SRM relative abundance values (N), were significantly different between the 3 salinity categories and the 5 lagoons (Kruskal-Wallis: $p < 0.05$ for all cases) (Tables S3 & S4 in Supplement 1). The post hoc comparisons showed that the diversity indices, in the case of salinity categories, did not differ significantly between (mixo-) b-mesohaline and (mixo-) polyhaline samples (Table S5 in Supplement 1). However, only Pielou's evenness index (J') was significantly different between the 2 locations (Mann-Whitney: $p < 0.05$) (Table S6 in Supplement 1).

DISCUSSION

The majority of the observed OTUs were identified as uncultured; although there are known representatives of sulfate-reducing microorganisms for which sequence data are deposited in public databases, there are still many more that remain to be cultured and described. As expected, the vast majority of SRMs were affiliated to the *Deltaproteobacteria*.

Table 2. Environmental variables best correlated with the sulfate-reducing microorganism community diversity pattern ($p < 0.01$). NH_4^+ : ammonium ion; NO_2^- : nitrite ion; MD: median diameter; σ_1 : sorting coefficient; ρ_w : weighted Spearman rank correlation coefficient

	Water			Sediment		ρ_w	
	Salinity	NH_4^+	NO_2^-	MD	Phaeo-pigments		
90% cut-off	+	+				0.575	
	+					0.558	
	+	+		+		0.537	
	+	+	+			0.523	
	+	+	+	+		0.521	
	+		+			0.519	
	+	+	+	+	+	0.518	
	+	+			+	0.518	
	+	+				+	0.513
	+	+		+	+		0.510

teobacteria supercluster, and in particular *Desulfobacteraceae*, which have been shown to exhibit high abundances in marine (Leloup et al. 2009), saline and hypersaline (Foti et al. 2007) and deep

sea sediments (Kaneko et al. 2007). However, the abundance of the *Desulfobacteraceae* family fluctuated according to the salinity, i.e. its abundance was lower in the a-mesohaline lagoons and higher in the more saline lagoons, although it has also been reported from freshwater sediments (Wang et al. 2012).

The high abundance of sequences belonging to Environmental supercluster 1, which comprises sequences from uncultured microorganisms (Müller et al. 2015), is indicative of the lack of knowledge of SRM diversity in the sampling sites and in lagoonal habitats in general. In addition, the effect of salinity on the distribution of SRM in the studied habitat is evident from the abundance of family-level Lineage 9 that is composed of many sequences from the marine environment (Müller et al. 2015) and from the absence (Lineages 6 and 10) or very low abundance (Lineage 8) of lineages often detected from freshwater wetlands (Pester et al. 2012). Apart from these findings, when analyzing each lagoon separately, there were certain differences in the abundance of the groups present. Specifically, the *Desul-*

Table 3. Abundance percentages of the environmental terms based on Environment Ontology (ENVO) vocabulary associated with the SRM OTUs (90% similarity cut-off) at each lagoon and at all lagoons combined. depression: includes the ENVO terms 'canyon', 'continental shelf', 'drainage basin', 'trough', 'back-arc basin'. lentic water body: includes the ENVO terms 'lake', 'reservoir'. lotic water body: includes the ENVO terms 'hydrothermal vent', 'acid mine drainage'. marine biome/sediment/water body: includes the ENVO terms 'sea', 'marine habitat', 'ocean water'. saline water: includes the ENVO terms 'saline water', 'sea water'. sea coast: includes the ENVO terms 'bay', 'fjord'. wetland: includes the ENVO terms 'saline marsh', 'fen'

ENVO terms	Mazoma	Logarou	Tsopeli	Tsoukalio	Rodia	Lagoons combined
aquifer	7.24	5.75	3.66	9.65	5.19	6.05
biofilter	0.28	0.62		0.08	0.06	0.19
borehole	6.87	5.68	3.24	9.65	5.16	5.95
coast	0.05	0.03	0.12	0.02		0.02
depression	2.57	1.39	0.75	0.46	0.52	0.93
seamount		0.23	0.18		0.02	0.07
ground water		0.08	0.36	0.67	0.24	0.26
harbor	0.78	0.23	1.26	2.15	0.29	0.68
inlet	1.96		0.09	0.24	0.39	0.45
landfill		0.08	0.36	0.67	0.24	0.26
leachate		0.08	0.36	0.67	0.24	0.26
lentic water body	0.78	0.04	12.20		0.36	1.26
lotic water body	3.57	1.72	5.40	16.11	24.92	15.21
marine biome/sediment/water body	10.51	4.00	2.25	4.14	1.41	3.40
microbial mat	0.23	0.04		0.40		0.09
gold mine	6.87	5.68	3.24	9.65	5.16	5.95
mud	0.16	0.08				0.03
saline water	0.16				0.03	0.03
sea coast	1.96	0.16	0.09	0.24	0.42	0.49
sediment	52.35	72.47	64.54	31.05	30.41	43.76
sludge		0.04			0.15	0.08
soil	0.08			0.10	0.22	0.13
terrestrial biome/habitat	0.72	0.16	0.84		0.06	0.21
wetland	2.87	1.45	1.08	14.04	24.52	14.24

fatiglans anilini lineage exhibited greater abundance in lower salinities; it has been previously found in both riverine (Suzuki et al. 2014) and marine sediments (Schnell et al. 1989, Ahn et al. 2009), although it has been isolated from marine enrichment cultures, inoculated with mud from the North Sea coast, using brackish water medium (Schnell et al. 1989).

However, these results were undoubtedly affected by the reference dataset used for the classification of the sequences, where most of the included sequences are derived from marine environments, followed by freshwater and other environments (Müller et al. 2015). This succession of environments is also depicted in the retrieved environmental terms that were found to be associated with the sequences of the present study; samples were mainly characterized by environmental descriptive terms that could be broadly classified to the marine biome, while terms belonging to the freshwater or brackish biome were found to a lesser extent. This could suggest that in environments of intermediate salinity concentrations, there is still an unknown component of the SRM diversity that remains to be investigated and incorporated to our knowledge of SRM communities. However, there were certain environmental descriptive terms, such as the term 'wetland', that contributed more to the specificity of the lower salinity lagoons. In addition, the influence of Louros river in the SRMs of Tsopeli lagoon may be deciphered from the association of the OTUs found in this lagoon with the terms 'lake' and 'reservoir'.

The SRM community diversity pattern seems to differ in each lagoon, a finding which is in accordance with previous reports of the total community diversity pattern from the same study sites (Pavloudi et al. 2016). This finding also concurs with those of previous studies, which have shown that SRM communities exhibit biogeographic distribution patterns at small spatial scales and that a homogeneous distribution is not unlikely (Pérez-Jiménez & Kerkhof 2005). In addition, the SRM community diversity pattern can be clustered according to broad salinity categories; this indicates that salinity is one of the major factors influencing the SRM communities in this habitat which is at the interface of marine and fresh water. This can be also concluded from the significant differentiation of the diversity indices between the salinity categories.

Although sulfate reducers are named after their ability to use sulfate as a terminal electron acceptor, they can in fact use many different electron acceptors, such as nitrate and nitrite (Dalsgaard & Bak

1994, Moura et al. 1997) or other sulfur compounds (thiosulfate, sulfite and sulfur) (Muyzer & Stams 2008). Therefore, the detection of *dsrAB* gene sequences in environmental samples should not be regarded per se as actual physiological capability for dissimilatory sulfate/sulfite reduction (Pester et al. 2012, Müller et al. 2015), i.e. the occurrence of high abundance of SRMs does not necessarily reflect the occurrence of sulfate reduction in the respective environment (Muyzer & Stams 2008). This may be the reason why our results suggest that the concentration of nitrite ions is correlated to the SRM community pattern. In addition, SRMs might be linked to ammonium on a secondary level, by using products of anaerobic protein degradation, which releases acetate, H₂ and ammonium. The lack of correlation with the concentration of total reduced inorganic sulfur (TRIS), which was also among the tested variables, may be explained by the fact that it is the sum of hydrogen sulfide (H₂S), iron sulfide (FeS), elemental sulfur (S⁰) and iron pyrite (FeS₂) (Fossing & Jørgensen 1989) and thus it cannot reflect only the biogenically produced sulfide (S²⁻) (Jong & Parry 2003). However, although there is no conclusive evidence to support this, it can be suggested that the sulfate reducers found at this particular study site and at this particular time point, probably were using sulfate and/or nitrogen compounds as electron acceptors.

Further investigation is needed to shed light on the functionality of SRMs in lagoonal ecosystems, especially in terms of their viability and competition with each other for the available sulfate, when the latter is insufficient for complete oxidation of organic compounds. In addition, the seasonality of SRMs should be investigated given that the environmental variables that primarily influence the community pattern are subjected to seasonal changes.

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LITERATURE CITED

- Ahn YB, Chae JC, Zylstra GJ, Häggblom MM (2009) Degradation of phenol via phenylphosphate and carboxylation to 4-hydroxybenzoate by a newly isolated strain of the sulfate-reducing bacterium *Desulfobacterium anilini*. *Appl Environ Microbiol* 75:4248–4253
- Anderson MJ (2001) A new method for non-parametric multivariate analysis of variance. *Austral Ecol* 26:32–46
- Angermeyer A, Crosby SC, Huber JA (2016) Decoupled distance-decay patterns between *dsrA* and 16S rRNA genes among salt marsh sulfate-reducing bacteria. *Environ Microbiol* 18:75–86
- Bardou P, Mariette J, Escudié F, Djemiel C, Klopp C (2014) jvrenn: an interactive Venn diagram viewer. *BMC Bioinformatics* 15:293
- Berger SA, Stamatakis A (2011) Aligning short reads to reference alignments and trees. *Bioinformatics* 27:2068–2075
- Chao A (1987) Estimating the population size for capture-recapture data with unequal catchability. *Biometrics* 43:783–791
- Chiu CH, Wang YT, Walther BA, Chao A (2014) An improved nonparametric lower bound of species richness via a modified good-turing frequency formula. *Biometrics* 70:671–682
- Clarke K (1993) Non-parametric multivariate analyses of changes in community structure. *Aust J Ecol* 18:117–143
- Clarke K, Ainsworth M (1993) A method of linking multivariate community structure to environmental variables. *Mar Ecol Prog Ser* 92:205–219
- Clarke K, Gorley R (2006) PRIMER v6: user manual/tutorial. PRIMER-E, Plymouth
- Clarke KR, Warwick R (1994) Similarity-based testing for community pattern: the two-way layout with no replication. *Mar Biol* 118:167–176
- Dalsgaard T, Bak F (1994) Nitrate reduction in a sulfate-reducing bacterium *Desulfovibrio desulfuricans*, isolated from rice paddy soil: sulfate inhibition, and regulation. *Appl Environ Microbiol* 60:291–297
- Dar SA, Yao L, van Dongen U, Kuenen JG, Muyzer G (2007) Analysis of diversity and activity of sulfate-reducing bacterial communities in sulfidogenic bioreactors using 16S rRNA and *dsrB* genes as molecular markers. *Appl Environ Microbiol* 73:594–604
- Dhillon A, Teske A, Dillon J, Stahl DA, Sogin ML (2003) Molecular characterization of sulfate-reducing bacteria in the Guaymas Basin. *Appl Environ Microbiol* 69:2765
- Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 32:1792–1797
- Ferentinos G, Papatheodorou G, Geraga M, Iatrou M and others (2010) Fjord water circulation patterns and dysoxic/anoxic conditions in a Mediterranean semi-enclosed embayment in the Amvrakikos Gulf, Greece. *Estuar Coast Shelf Sci* 88:473–481
- Fishbain S, Dillon JG, Gough HL, Stahl DA (2003) Linkage of high rates of sulfate reduction in Yellowstone hot springs to unique sequence types in the dissimilatory sulfate respiration pathway. *Appl Environ Microbiol* 69:3663–3667
- Fossing H, Jørgensen BB (1989) Measurement of bacterial sulfate reduction in sediments: evaluation of a single-step chromium reduction method. *Biogeochemistry* 8:205–222
- Foti M, Sorokin DY, Lomans B, Mussman M and others (2007) Diversity, activity, and abundance of sulfate-reducing bacteria in saline and hypersaline soda lakes. *Appl Environ Microbiol* 73:2093–2100
- Gilmour CC, Riedel GS, Ederington MC, Bell JT, Gill GA, Stordal MC (1998) Methylmercury concentrations and production rates across a trophic gradient in the northern Everglades. *Biogeochemistry* 40:327–345
- Huse SM, Mark Welch DB, Voorhis A, Shipunova A, Morrison HG, Eren AM, Sogin ML (2014) VAMPS: a website for visualization and analysis of microbial population structures. *BMC Bioinformatics* 15:41
- International Symposium on the Classification of Brackish Waters (1958) The Venice system for the classification of marine waters according to salinity. *Limnol Oceanogr* 3:346–347
- Jiang L, Zheng Y, Peng X, Zhou H, Zhang C, Xiao X, Wang F (2009) Vertical distribution and diversity of sulfate-reducing prokaryotes in the Pearl River estuarine sediments, Southern China. *FEMS Microbiol Ecol* 70:249–262
- Jong T, Parry DL (2003) Removal of sulfate and heavy metals by sulfate reducing bacteria in short-term bench scale upflow anaerobic packed bed reactor runs. *Water Res* 37:3379–3389
- Jørgensen BB (1982) Mineralization of organic matter in the sea bed—the role of sulphate reduction. *Nature* 296:643–645
- Kaneko R, Hayashi T, Tanahashi M, Naganuma T (2007) Phylogenetic diversity and distribution of dissimilatory sulfite reductase genes from deep-sea sediment cores. *Mar Biotechnol* 9:429–436
- Kapsimalis V, Pavlakis P, Poulos SE, Alexandri S and others (2005) Internal structure and evolution of the Late Quaternary sequence in a shallow embayment: The Amvrakikos Gulf, NW Greece. *Mar Geol* 222–223:399–418
- Kjeldsen KU, Loy A, Jakobsen TF, Thomsen TR, Wagner M, Ingvorsen K (2007) Diversity of sulfate-reducing bacteria from an extreme hypersaline sediment, Great Salt Lake (Utah). *FEMS Microbiol Ecol* 60:287–298
- Kondo R, Butani J (2007) Comparison of the diversity of sulfate-reducing bacterial communities in the water column and the surface sediments of a Japanese meromictic lake. *Limnology* 8:131–141
- Kruskal WH, Wallis WA (1952) Use of ranks in one-criterion variance analysis. *J Am Stat Assoc* 47:583–621
- Leinonen R, Akhtar R, Birney E, Bower L and others (2011) The European Nucleotide Archive. *Nucleic Acids Res* 39:D28–D31
- Leloup J, Fossing H, Kohls K, Holmkvist L, Borowski C, Jørgensen BB (2009) Sulfate-reducing bacteria in marine sediment (Aarhus Bay, Denmark): abundance and diversity related to geochemical zonation. *Environ Microbiol* 11:1278–1291
- Li J, Purdy KJ, Takii S, Hayashi H (1999) Seasonal changes in ribosomal RNA of sulfate-reducing bacteria and sulfate reducing activity in a freshwater lake sediment. *FEMS Microbiol Ecol* 28:31–39
- Liu XZ, Zhang LM, Prosser JI, He JZ (2009) Abundance and community structure of sulfate reducing prokaryotes in a paddy soil of southern China under different fertilization regimes. *Soil Biol Biochem* 41:687–694
- Ludwig W, Strunk O, Westram R, Richter L and others (2004) ARB: a software environment for sequence data. *Nucleic Acids Res* 32:1363–1371

- Madigan MT, Martinko JM, Stahl DA, Clark DP (2012) Brock biology of microorganisms, 13th edn. Benjamin Cummings, San Francisco, CA
- Mann HB, Whitney DR (1947) On a test of whether one of two random variables is stochastically larger than the other. *Ann Math Stat* 18:50–60
- Moura I, Bursakov S, Costa C, Moura JJ (1997) Nitrate and nitrite utilization in sulfate-reducing bacteria. *Anaerobe* 3:279–290
- Müller AL, Kjeldsen KU, Rattei T, Pester M, Loy A (2015) Phylogenetic and environmental diversity of *DsrAB*-type dissimilatory (bi)sulfite reductases. *ISME J* 9:1152–1165
- Muyzer G, Stams AJM (2008) The ecology and biotechnology of sulphate-reducing bacteria. *Nat Rev Microbiol* 6: 441–454
- Nakagawa T, Hanada S, Maruyama A, Marumo K, Urabe T, Fukui M (2002) Distribution and diversity of thermophilic sulfate-reducing bacteria within a Cu-Pb-Zn mine (Toyoha, Japan). *FEMS Microbiol Ecol* 41:199–209
- O'Hara RB (2005) Species richness estimators: how many species can dance on the head of a pin? *J Anim Ecol* 74: 375–386
- Oksanen J, Blanchet FG, Friendly M, Kindt R and others (2016) vegan: Community Ecology Package. R package version 2.4-1. <https://CRAN.R-project.org/package=vegan>
- Pavloudi C, Oulas A, Vasileiadou K, Sarropoulou E, Kotoulas G, Arvanitidis C (2016) Salinity is the major factor influencing the sediment bacterial communities in a Mediterranean lagoonal complex (Amvrakikos Gulf, Ionian Sea). *Mar Genomics* 28:71–81
- Pelikan C, Herbold CW, Hausmann B, Müller AL, Pester M, Loy A (2016) Diversity analysis of sulfite- and sulfate-reducing microorganisms by multiplex *dsrA* and *dsrB* amplicon sequencing using new primers and mock community-optimized bioinformatics. *Environ Microbiol* 18: 2994–3009
- Pérez-Jiménez J, Kerkhof L (2005) Phylogeography of sulfate-reducing bacteria among disturbed sediments, disclosed by analysis of the dissimilatory sulfite reductase genes (*dsrAB*). *Appl Environ Microbiol* 71:1004–1011
- Pester M, Knorr KH, Friedrich MW, Wagner M, Loy A (2012) Sulfate-reducing microorganisms in wetlands—fameless actors in carbon cycling and climate change. *Front Microbiol* 3:72
- Pielou EC (1969) An introduction to mathematical ecology. Wiley Interscience, New York, NY
- Poulos SE, Collins MB, Lykousis V (1995) Late Quaternary evolution of Amvrakikos Gulf, Western Greece. *Geo-Mar Lett* 15:9–16
- Quince C, Lanzen A, Davenport RJ, Turnbaugh PJ (2011) Removing noise from pyrosequenced amplicons. *BMC Bioinformatics* 12:38
- Rabus R, Hansen TA, Widdel F (2006) Dissimilatory sulfate- and sulfur-reducing prokaryotes. In: Dworkin M, Falkow S, Rosenberg E, Schleifer KH, Stackebrandt E (eds) *The prokaryotes*, Vol 2: Ecophysiology and biochemistry. Springer, New York, NY, p 659–768
- Ronaghi M, Uhlén M, Nyrén P (1998) A sequencing method based on realtime pyrophosphate. *Science* 281:363–365
- Schnell S, Bak F, Pfennig N (1989) Anaerobic degradation of aniline and dihydroxybenzenes by newly isolated sulfate-reducing bacteria and description of *Desulfobacterium anilini*. *Arch Microbiol* 152:556–563
- Sinclair L, Ijaz UZ, Jensen LJ, Coolen MJL and others (2016) Seqenv: linking sequences to environments through text mining. *PeerJ* 4:e2690
- Stamatakis A (2014) RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30:1312–1313
- Suzuki D, Li Z, Cui X, Zhang C, Katayama A (2014) Reclassification of *Desulfobacterium anilini* as *Desulfatiglans anilini* comb. nov. within *Desulfatiglans* gen. nov., and description of a 4-chlorophenol-degrading sulfate-reducing bacterium, *Desulfatiglans parachlorophenolica* sp. nov. *Int J Syst Evol Microbiol* 64:3081–3086
- Tagliapietra D, Sigovini M, Magni P (2012) Saprobity: a unified view of benthic succession models for coastal lagoons. *Hydrobiologia* 686:15–28
- Tamura K, Stecher G, Peterson D, FilipSKI A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol* 30:2725–2729
- Thompson FL, Bruce T, Gonzalez A, Cardoso A and others (2011) Coastal bacterioplankton community diversity along a latitudinal gradient in Latin America by means of V6 tag pyrosequencing. *Arch Microbiol* 193:105–114
- Varsos C, Patkos T, Oulas A, Pavloudi C and others (2016) Optimized R functions for analysis of ecological community data using the R virtual laboratory (RvLab). *Biodivers Data J* 4:e8357
- Vasileiadou K, Pavloudi C, Kalantzi I, Apostolaki ET and others (2016) Environmental variability and heavy metal concentrations from five lagoons in the Ionian Sea (Amvrakikos Gulf, W Greece). *Biodivers Data J* 4:e8233
- Viaroli P, Bartoli M, Giordani G, Naldi M, Orfanidis S, Zaldívar JM (2008) Community shifts, alternative stable states, biogeochemical controls and feedbacks in eutrophic coastal lagoons: a brief overview. *Aquat Conserv* 18:S105–S117
- Wagner M, Roger AJ, Flax JL, Brusseau GA, Stahl DA (1998) Phylogeny of dissimilatory sulfite reductases supports an early origin of sulfate respiration. *J Bacteriol* 180: 2975–2982
- Wagner M, Loy A, Klein M, Lee N, Ramsing NB, Stahl DA, Friedrich MW (2005) Functional marker genes for identification of sulfate-reducing prokaryotes. *Methods Enzymol* 397:469–489
- Wang Q, Quensen JF, Fish JA, Lee TK, Sun Y, Tiedje JM, Cole JR (2013) Ecological patterns of *nifH* genes in four terrestrial climatic zones explored with targeted metagenomics using FrameBot, a new informatics tool. *MBio* 4: e00592–13
- Wang Y, Sheng HF, He Y, Wu JY, Jiang YX, Tam NFY, Zhou HW (2012) Comparison of the levels of bacterial diversity in freshwater, intertidal wetland, and marine sediments by using millions of illumina tags. *Appl Environ Microbiol* 78:8264–8271