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Preservation, origin and genetic imprint of extracellular DNA in permanently anoxic deep-sea sediments

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Abstract

Molecular approaches that target the total DNA pool recovered from permanently anoxic marine ecosystems have revealed an extraordinary diversity of prokaryotes and unicellular eukaryotes. However, the presence of gene sequences contained within the extracellular DNA pool is still largely neglected. We have investigated the preservation, origin and genetic imprint of extracellular DNA recovered from permanently anoxic deep-sea sediments of the Black Sea. Despite high DNase activities, huge amounts of total extracellular DNA were found in both the surface and subsurface sediment layers, suggesting reduced availability of the extracellular DNA pool to nuclease degradation. The reduced degradation of the total extracellular DNA was confirmed by its low decay rate and the high accumulation in the deeper sediment layers. The copy numbers of 16S and 18S rDNA contained within the extracellular DNA pool in both the surface and subsurface sediment layers was very high, indicating that permanently anoxic sediments of the deep Black Sea are hot spots of preserved extracellular gene sequences. The extracellular DNA recovered from these sediment layers also contained highly diversified 18S rDNA sequences. These were not only representative of the major protistan lineages, but also of new very divergent lineages, branching as independent clades at the base of the tree. Our findings indicate that the extracellular DNA pool is a major archive of present/past eukaryotic gene sequences, and they highlight the importance of integrating molecular cell-oriented approaches with molecular analyses of the extracellular DNA pool, for a better assessment of microbial diversity and temporal changes in marine benthic ecosystems.

Keywords: extracellular DNA, marine sediments, preserved gene sequences

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Introduction

The extracellular DNA in marine sediments (i.e. DNA that is not associated with living biomass) is by far the largest reservoir of DNA of the oceans of the World (Dell'Anno & Danovaro 2005). This pool exceeds the concentrations of DNA contained in the living biomass by at least 10-fold (Dell'Anno *et al.* 2002; Corinaldesi *et al.* 2007a).

The extracellular DNA pool in sediments derives from the flux of planktonic organisms and their remains that reach the seafloor by sedimentation (Dell'Anno & Danovaro 2005) and from *in situ* production that includes: (i) cellular exudation and excretion from viable cells; (ii) cellular autolysis (i.e. passive release following cell death); (iii) grazing processes; and (iv) cellular lysis because of viral infection (Danovaro *et al.* 2006; Corinaldesi *et al.* 2007b). In the sediment, the extracellular DNA pool undergoes complex biogeochemical transformations (e.g. interactions with sediment particles, DNase-mediated degradation, depurination), which influence the extent of its preservation with time (Corinaldesi *et al.* 2008).

Permanently anoxic conditions in the oceans are widespread, as they are present in all of the subsurface

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sediments (Lipp *et al.* 2008). Among these, there is the interior of the Black Sea (at depths > ca. 150 m; Kuypers *et al.* 2003). Anoxic environments are generally assumed to be exclusively inhabited by viruses, bacteria and archaea (Boetius *et al.* 2000; Kuypers *et al.* 2003; Danovaro *et al.* 2005; Lipp *et al.* 2008). The presence of unicellular eukaryotes (e.g. protozoan ciliates) in anoxic marine systems has been documented for decades (Fenchel & Finlay 1995). However, proof of the viability of benthic unicellular eukaryotes in permanently anoxic conditions has only been provided for a few specimens that belong to specific taxonomic groups (i.e. foraminifera) and that inhabit the upper few centimetres of the sediment (Bernhard *et al.* 2006; Risgaard-Petersen *et al.* 2006). Beside this, the presence of resting stages (i.e. cysts) of planktonic unicellular organisms (e.g. dinoflagellates) has been also documented in deeper anoxic sediment layers (Raghukumar *et al.* 2004; Coyne & Cary 2005; Coolen & Shtereva 2009).

Molecular analyses carried out on the total DNA pools in permanently anoxic marine ecosystems continue to reveal an extraordinary diversity of prokaryotes (D'Hondt *et al.* 2004; Parkes *et al.* 2005; van der Wielen *et al.* 2005; Teske & Sørensen 2008) and unicellular eukaryotes (Dawson & Pace 2002; Edgcomb *et al.* 2002, 2009; Stoeck *et al.* 2003; Takishita *et al.* 2005). Moreover, molecular tools targeting the total DNA pool extracted from permanently anoxic marine and lacustrine sediments have allowed the identification of the remains of specific photoautotrophic planktonic organisms (belonging both to the Bacteria and Eukarya domains) up to 217 000 years old (Coolen *et al.* 2004, 2006, 2007, 2009; Coolen & Overmann 2007; Manske *et al.* 2008).

Previous studies have reported high extracellular DNA concentrations also in permanently anoxic surface and subsurface sediments (Danovaro *et al.* 2005; Corinaldesi *et al.* 2008) suggesting that the lack of free dissolved oxygen can promote the preservation of extracellular DNA and the genetic information contained therein (Coolen & Overmann 2007; Coolen *et al.* 2007; Corinaldesi *et al.* 2008).

Amplifiable gene sequences of prokaryotic origin have been detected also within the extracellular DNA pool recovered from anoxic sediment layers of up to 10 000 years old, leading to hypothesize a hidden genetic information in this pool (Corinaldesi *et al.* 2008).

Because procedures commonly utilized to carry out molecular analyses from environmental samples are based on the extraction of the total DNA pool (where extracellular DNA is coextracted with intracellular DNA, Corinaldesi *et al.* 2005), these can provide an altered view of the actual microbial biodiversity.

Molecular fingerprinting analyses carried out on soil and marine sediments have indicated, indeed, that some of the prokaryotic phylotypes from extracellular DNA pool do not overlap with those contained within the intracellular DNA (i.e. associated with microbial cells) (Agnelli *et al.* 2004; Corinaldesi *et al.* 2008).

In this study, we have investigated the quantitative relevance and degradation rates of the total extracellular DNA pool in the permanently anoxic deep-sea sediments of the Black Sea, to determine the extent to which it has been preserved under these environmental conditions. Moreover, we have quantified the prokaryotic and eukaryotic gene sequences that were contained within the extracellular DNA pool, to provide new insights into its origins. Finally, we have explored the phylogenetic composition of eukaryotic small subunit (SSU) rDNA sequences preserved within the extracellular DNA pool, to improve our understanding of the diversity and changes that have occurred in the Black Sea ecosystem over time scales from decades to centuries.

Materials and methods

Study area and sampling

Sediment samples were collected in the north-western sector of the Black Sea, a sea that is highly eutrophic, as it is characterized by high phytoplankton biomass and primary productivity even in offshore waters (up to ca. 500 mgC m^{-2} day^{-1}; Yunev *et al.* 2002). Below ca. 150 m in depth, the Black Sea is characterized by permanently anoxic conditions, and as such, with the exception of the subsurface seafloor, it is the largest anoxic basin of the oceans of the World (Jørgensen *et al.* 2001). Undisturbed sediment samples were collected using a multiple corer at four stations, one located at the shelf break (195 m depth, 43° 59.4′ N, 29° 51.9′ E, hereafter referred to as Station 1), two on an open slope (at 539 m depth, 43° 56.8′ N, 23° 53.9′ E, hereafter referred to as Station 2 and 975 m depth, 43° 47.2′ N, 29° 94.3′ E, hereafter referred to as Station 3) and one on the bathyal plane of the basin (at 1970 m depth, 42° 99.8′ N, 31° 51.6′ E, hereafter referred to as Station 9). Immediately after retrieval, the three independent sediment cores from each station were sliced vertically under strictly anaerobic conditions (N_2 atmosphere) into three sediment layers: (i) the top 1 cm; (ii) 4–5 cm; and (iii) 9–10 cm. Moreover, the three independent sediment cores collected at Station 9 were sectioned from the first 1 cm down to 53 cm. The presence of sapropel (i.e. dark organic-rich layers with 5–20% wt organic carbon; Calvert & Karlin 1998) was identified from the 30-cm layer down to the deepest layers of the sediment core. The

base of the sapropel layer (which can be deeper than the 53 cm sampled in this study) was dated at ca. 7500 years B.P., whereas the upper boundary between the sapropel and the overlaying laminated sediment layer was dated at ca. 2700 years B.P. (Jones & Gagnon 1994; Coolen *et al.* 2009). Nine sediment layers were collected from the surface down to 17 cm (i.e. the coccolith-bearing layers, hereafter defined as Unit I; *sensu* Calvert & Karlin 1998; Coolen *et al.* 2006), and six sediment layers were collected from 31 to 53 cm (hereafter referred to as the sapropel layer; *sensu* Calvert & Karlin 1998). All of these sediment subsamples were stored at −80 °C, except for those for DNase activity determinations, which were analysed immediately after sediment collection under anaerobic conditions, as described later.

Total extracellular DNA determination

The working conditions, precautions and analytical details during the extracellular DNA analyses were as reported in Appendix S1. The concentrations of total extracellular DNA in the sediment were determined according to Dell'Anno *et al.* (2002). This procedure is based on the hydrolysis of the extracellular DNA (using commercial nucleases) and does not allow the recovery of the DNA for subsequent molecular studies (Corinaldesi *et al.* 2005, 2008); therefore, separate samples were also processed to provide extracellular DNA that was suitable for molecular analyses. The extracellular DNA concentrations were expressed as microgram total extracellular DNA per gram dry sediment.

DNase activity

The DNase activity was determined fluorometrically using a fluorescent DNA analogue [poly(dεA): polydeoxyribo-1-N^6 ethenoadenylic acid], as described by Dell'Anno & Corinaldesi (2004). The poly(dεA) substrate was prepared by chemical modification of poly(dA) (fragment length, 50 bases) using chloroacetaldehyde, according to Cazenave *et al.* (1983). The DNase activity was expressed as nanogram degraded DNA g^{-1} h^{-1} dry sediment (see details in Appendix S1).

Extractions of extracellular and total DNA pools

The extracellular DNA for molecular analyses was recovered from sediment samples using the procedure described by Corinaldesi *et al.* (2005). The purity of the DNA was checked by determining the ratio of absorbance at 260 nm to absorbance at 280 nm, and the DNA was quantified fluorometrically using SYBR Green I (Molecular Probes), according to Corinaldesi *et al.* (2005). The robustness and reliability of this protocol for extracellular DNA extraction has been shown to exclude any possible contamination because of cell lysis or to the coextraction of intracellular DNA (i.e. DNA from microbial cells; see Appendix S1 and Figs S1 and S2 for details). The total DNA pool was recovered from the sediments using the UltraClean soil DNA isolation kit (MoBio Laboratories Inc., California) according to the manufacture instructions and quantified fluorometrically using SYBR Green I (Corinaldesi *et al.* 2005). The total DNA concentrations were expressed as microgram total DNA per gram dry sediment.

Real-time PCR analysis

To provide information on the origin of the extracellular DNA and to quantify the relative importance of the 16S rDNA and 18S rDNA sequences contained within this pool, real-time PCR analysis was carried out on the extracellular DNA extracted from the different sediment layers collected at Station 9. Real-time PCR analyses targeting the 16S rDNA sequences were also carried out on the total DNA pools extracted from surface and subsurface layers (i.e. 0–1 cm and 9–10 cm) of sediments collected at the Station 9. Real-time PCR analysis was performed using the TaqMan technology, as described by Takai & Horikoshi (2000). The prokaryotic 16S rDNA sequences were amplified using the universal primers Uni340F (5′-CCTACGGGRBGCASCAG-3′) and Uni806R (5′-GGACTACNNGGGTATCTAAT-3′). The TaqMan probe was Uni516F (5′-TGYCAGCMGCCGCGGTAAHACVNRS-3′), which contained a fluorescent reporter dye (6-carboxyfluorescein) covalently attached to the 5′-end and a fluorescent quencher dye (6-carboxytetramethylrhodamine) attached six or more bases downstream of the reporter dye. The number in each primer or probe designation indicates the position of the 5′ end of the primer or probe in *Escherichia coli* 16S rRNA (Takai & Horikoshi 2000).

The eukaryotic 18S rDNA sequences were amplified using an equimolar mixture of two forward primers and two reverse primers (final concentration, 0.2 μM) and a TaqMan probe (final concentration, 0.1 μM), which were obtained from consensus alignment of eukaryotic sequences downloaded from the NCBI database (National Centre for Biotechnology Information; http://www.ncbi.nlm.nih.gov). In particular, we used Euk f1 (5′-CGC AAG GCT GAA ACT TAA AG-3′), Euk f2 (5′-GTT GCA AAG CTG AAA CTT AAA G-3′), Euk r1 (5′-ATC ACT CCA CCA ACT AAG AAC-3′) and Euk r2 (5′-ATC GCT CCA CCA ACT AAG AA-3′). The forward primers correspond to the position 1119–1140 in the *Saccharomyces cerevisiae* (GenBank accession number Z75578), whereas the reverse primers correspond to

the position 1281–1301. The TaqMan probe (Euk Probe; 5′-AAt TGA cGG AaG GgC-3′) corresponds to the position 1142–1156 in the *Saccharomyces cerevisiae*. This contained LNA (Locked Nucleic Acids represented by lower cases in the probe sequence) and was labelled with a fluorescent reporter dye (6-carboxyfluorescein) at the 5′end and a fluorescent quencher dye (BHQ1) at the 3′end. All of the real-time PCR was performed in a volume of 25 μL with an iQ5-icycler (Bio-Rad) using iQ Supermix (2×; Bio-Rad) containing 40 mM Tris-HCl, pH 8.4, 100 mM KCl, 0.4 mM each dNTP (dATP, dCTP, dGTP, dTTP), 50 U mL^{-1} hot-start iTaq DNA polymerase and 6 mM $MgCl_2$. The reaction mixtures contained 0.5–10 ng DNA (additional details on real-time PCR analyses are reported in Appendix S1). The copy number of ribosomal gene sequences determined by real-time PCR was normalized to sediment dry weight for a comparison with available literature information.

Clone library and phylogenetic analyses

The 18S rDNA gene sequences in the extracellular DNA extracted from the three different sediment layers (0–1 cm, 7–8 cm and 52–53 cm) collected at Station 9 were amplified using universal eukaryotic primers designed from consensus alignment of multiple sequences of the 18S rDNA (downloaded from NCBI database). These sediment layers were selected to investigate changes in eukaryotic diversity in relation to increasing sediment age.

Three primer sets were designed and checked with Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Based on preliminary data, a single set was used in this study: Euk3for 5′-GCATGGCCGTTCTTAGTTSG-3′, corresponding to positions 1273–1292 in *Saccharomyces cerevisiae* (GenBank accession number Z75578) and Euk3rev (5′-CTGATCCTTCYGCAGGTTCAC-3′) corresponding to positions 1778–1798 in *Saccharomyces cerevisiae* (GenBank accession number Z75578), derived from a modification of the universal primer designed by Sogin (1990). The amplicon length is ca. 520 bp. All of the PCRs were performed in 50 μL of solution, containing 5–10 ng extracellular DNA, 1× PCR buffer, 200 μM each dNTP and 0.5 μM each primer, and with a thermal cycler (Biometra, Germany) using the MasterTaq® kit (Eppendorf AG, Germany). Thirty PCR cycles were used, each consisting of 94 °C for 1 min, 52 °C for 1.5 min and 72 °C for 2 min, preceded by 5 min of denaturation at 94 °C and followed by a final extension of 10 min at 72 °C. To check for any contamination of the PCR reagents, negative controls containing the PCR solution without the DNA template were run during each amplification analysis. Positive controls were also run, with genomic DNA from herring testes (Sigma). The PCR products were checked on agarose–Tris-borate-EDTA (TBE) gels (1%), containing ethidium bromide for DNA staining and visualization. Amplifiable 18S rDNA sequences were obtained from the 0–1 cm and 7–8-cm sediment layers, whereas despite several attempts, amplification of the 18S rDNA from the deepest sediment layer (52–53 cm) was not successful.

The PCR products obtained from the 18S rDNA amplification were purified with spin columns (Wizard PCR purification kit, PROMEGA), according to the manufacturer protocol, and cloned, according to standard protocols (see Appendix S1 for details). Sequences were screened using VecScreen (http://www.ncbi.nlm.nih.gov/projects/VecScreen/) to remove the residual vector sequence, and visually inspected to remove any low-quality sequence readouts. The partial 18S rDNA sequences obtained in this study have been deposited in GenBank under Accession Nos. GU474024 to GU474200.

Pairwise comparisons were conducted of all of the sequences obtained, to identify the different phylotypes (with a sequence cut-off of 97% similarity) within each library prior to tree construction. The trees were produced by neighbour-joining (NJ), maximum-parsimony (MP) and maximum-likelihood (ML) methods using the PAUP 4.10 beta version programme (Swofford 1998). Additional details on the sequence analysis and tree construction are reported in Appendix S1 and Table S1.

Estimates of decay rate of total extracellular DNA

The decay rate of total extracellular DNA was estimated in the sediments collected at Station 9 using a kinetic model (Dell'Anno & Danovaro 2005). This kinetic model was applied to the sediment collected at Station 9 because the data on the total extracellular DNA pool along the sediment core were enough to provide robust estimates of its decay rate. Moreover, the model was applied to data obtained from the top 1 cm down, to 17 cm in depth, for a comparison with the available literature on decay rates of total extracellular DNA in deep-sea benthic ecosystems worldwide (Dell'Anno & Danovaro 2005). Additional details on the kinetic model are reported in Appendix S1.

Statistical analyses

Univariate analyses of variance (ANOVA) were carried out to test for differences in the investigated variables among the sites and depths of the sediment. Multivariate analyses of variance (PERMANOVA; McArdle & Anderson 2001) were also carried out to test for differences in the investigated variables in the in Unit I and sapropel layers (see additional details in Appendix S1). The

number of phylotypes, the expected number of phylotypes (estimated by nonparametric Chao1 estimator) and the Shannon diversity index of the two 18S rDNA libraries were calculated using the DOTUR software (Schloss & Handelsman 2005). The statistical significances of differences between the two 18S rDNA libraries were assessed using the ∫-LIBSHUFF software (Schloss *et al.* 2004).

Results

Total and extracellular DNA pools and DNase activity

The total extracellular DNA concentrations in the top 1 cm of the sediments from the Black Sea were high (range: 8.8 ± 0.7–32.5 ± 7.5 μg g^{-1} at Stations 3 and 1, respectively; Table 1), and they showed significant differences between the stations (Table S2). The total DNA pools in surface sediments of the Black Sea were also high and ranged from 9.8 ± 1.8–31.3 ± 6.3 μg g^{-1} at Stations 3 and 9, respectively (Table S3).

The surface sediments of the Black Sea were also characterized by high DNase activities (range: 94.1 ± 4.6–755.5 ± 128.8 ng degraded DNA g^{-1} h^{-1} at Stations 1 and 9, respectively; Table 1), which increased significantly with increasing water depth (Table S2). At all of the stations, the total extracellular DNA concentrations and DNase activities (Table 1) changed significantly with depth of sediment (Table S4), although without any consistent pattern across the investigated sites (Table S4). In particular, Stations 2 and 9 were characterized by significantly higher total extracellular DNA concentrations in the subsurface sediment layers, whereas Stations 1 and 3 had values significantly higher in the top 1 cm of sediment. The detailed analysis of the vertical profiles of the sediments collected at Station 9 revealed significant differences in the total extracellular DNA concentrations and DNase activities with depth of sediment (Fig. 1), as well as between the 0–17-cm layer (corresponding to the Unit I) and the 31–53 cm layer (corresponding to the sapropel layer) (Tables S5 and S6). In the Unit I, total extracellular DNA concentrations decreased significantly down to 10 cm depth and then increased down to 17 cm depth, whereas in the sapropel layer, there was a corresponding significant increase down to 47 cm depth. In the Unit I, the DNase activities were high at all sediment depths and did not show any consistent spatial pattern. Conversely, in the sapropel layer, the DNase activities were low and significantly decreased with increasing depth of sediment.

Table 1 Total extracellular DNA concentrations and DNase activities in the surface and subsurface layers of the sediments collected at the four sampling sites in the Black Sea

Sampling station	Sediment layer (cm)	Total extracellular DNA (μg g^{-1})	DNase activity (ng DNA degraded g^{-1} h^{-1})
Station 1	0–1	32.5 ± 7.5	94.1 ± 4.6
	4–5	2.3 ± 0.1	328.4 ± 56.9
	9–10	10.6 ± 4.3	154.7 ± 20.7
Station 2	0–1	15.3 ± 1.4	272.1 ± 37.9
	4–5	41.9 ± 10.5	587.8 ± 96.7
	9–10	3.6 ± 0.1	189.5 ± 19.4
Station 3	0–1	8.8 ± 0.7	541.0 ± 129.8
	4–5	2.8 ± 0.2	85.6 ± 8.1
	9–10	4.6 ± 0.3	220.6 ± 53.0
Station 9	0–1	31.1 ± 6.2	755.5 ± 128.8
	4–5	57.7 ± 13.3	525.7 ± 13.3
	9–10	6.3 ± 0.3	1240.4 ± 11.4

Data are means ± standard deviations from analyses of three independent sediment cores.

Quantitative relevance of 16S rDNA and 18S rDNA sequences within the extracellular DNA pool

The copy numbers of the 16S rDNA and 18S rDNA within the extracellular DNA pool in the sediments collected at the Station 9 were very high, in both the surface and subsurface layers (range: $0.34–9.9 \times 10^{10}$ 16S rDNA copies per gram, and $0.01–8.6 \times 10^{11}$ 18S rDNA copies per gram; Fig. 2). The copy number of 16S rDNA within the extracellular DNA pool accounted for 66 and 28% of the copy number of 16S rDNA determined in the total DNA pool extracted from surface and subsurface sediment layers ($3.94 \pm 0.33 \times 10^{10}$ 16S rDNA copies per gram and $2.59 \pm 0.28 \times 10^{10}$ 16S rDNA copies per gram in the 0–1 cm and 9–10 cm sediment layer, respectively). The detailed analysis of the 16S rDNA and 18S rDNA copy numbers along the vertical profile of the sediment revealed significant differences with depth in the sediment, as well as between the 0–17-cm layer and the 31–53-cm layer (Tables S5 and S6). In surface sediments, the 16S rDNA copy numbers were much higher than those of 18S rDNA and *viceversa* in deeper sediment layers.

Phylogenetic analyses of eukaryotic gene sequences contained within the extracellular DNA

From sequencing analyses of 89 and 88 clones in the surface (top 1 cm) and subsurface (7–8 cm) sediments, we identified 18 and 13 18S rDNA phylotypes, respectively; using the Chao 1 estimator, we identified 25 and 16 18S rDNA phylotypes, respectively. The higher numbers of 18S rDNA phylotypes in the top 1 cm of the sediment were reflected also by the higher value of

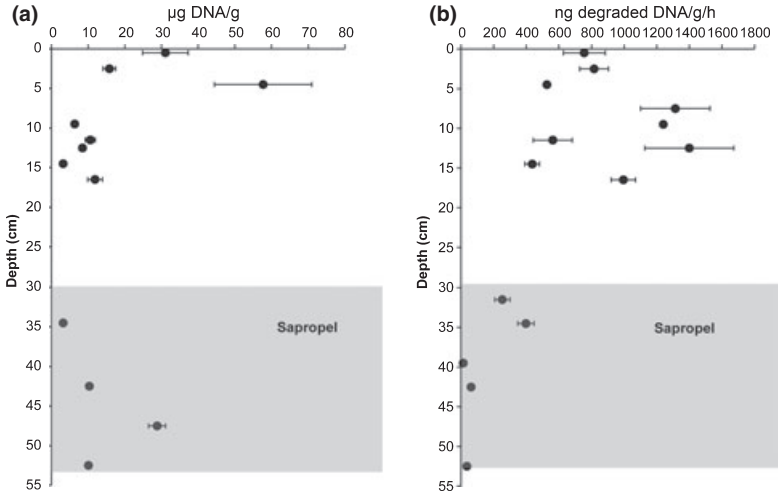

Fig. 1 Vertical distribution of total extracellular DNA concentrations (a) and DNase activities (b) along the sediment cores collected at Station 9 (1970 m depth). Data are means ± standard deviations (n = 3).

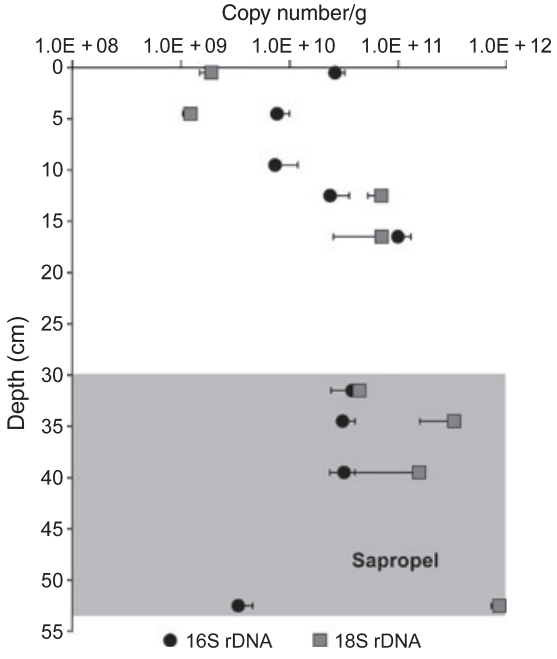

Fig. 2 Vertical distribution of 16S rDNA copy numbers and 18S rDNA copy numbers along the sediment cores collected at Station 9 (1970 m depth). Data are means ± standard deviations (n = 3). The X axis is a log scale.

diversity Shannon index (2.32 vs. 1.63). The rarefaction curves did not reach saturation in either the surface or subsurface sediment layers (Fig. S3). The 18S rDNA libraries of these two different sediment layers were significantly different (∫-LIBSHUFF, $P < 0.01$). Five 18S rDNA phylotypes were shared (i.e. showed ≥97% similarity) between these two sediment layers, and 14 18S rDNA phylotypes identified matched (i.e. showed ≥97% similarity) with sequences already contained in the data banks. In both the surface and subsurface sediment layers, the majority of the sequenced clones fell within Alveolates (top 1 cm, 61%; and 7–8 cm, 69%), followed by clones similar to phylotypes previously reported (Takishita *et al.* 2007) that cannot be assigned to known eukaryotic groups (top 1 cm, 17%; and 7–8 cm, 27%; Fig. 3). In the surface sediments, six clones belonging to the same phylotype have not been reported previously (accounting for 7% of the total clone number). The phylogenetic tree illustrated in Fig. 4 highlights the major differences among the sequences belonging to the Alveolates in these two sediment layers. In the surface sediment layer, only a few of the total sequences falling within the Alveolates are affiliated to the Dinoflagellates, whereas in the subsurface sediment layer, almost all of the total sequences of the Alveolates belong to the Dinoflagellates, close to the genus Gymnodinium (Fig. 3A, B). In the surface sediment layer, the less representative sequences were affiliated to Fungi (7%), Haptophytes (3%), Stramenopiles (3%), Ichthyosporea (1%) and Cercozoa (1%), whereas in the subsurface sediments, they were related to Fungi (2%), Stramenopiles (1%) and Chlorophytes (1%).

Discussion

Previous studies have reported that permanent anoxic conditions can favour the preservation of the extracellular DNA pool in marine sediments (Danovaro *et al.* 2005; Coolen & Overmann 2007; Corinaldesi *et al.* 2008). In the present study, we found high total extracellular DNA concentrations in the surface and subsurface anoxic sediments of the Black Sea, with levels that are

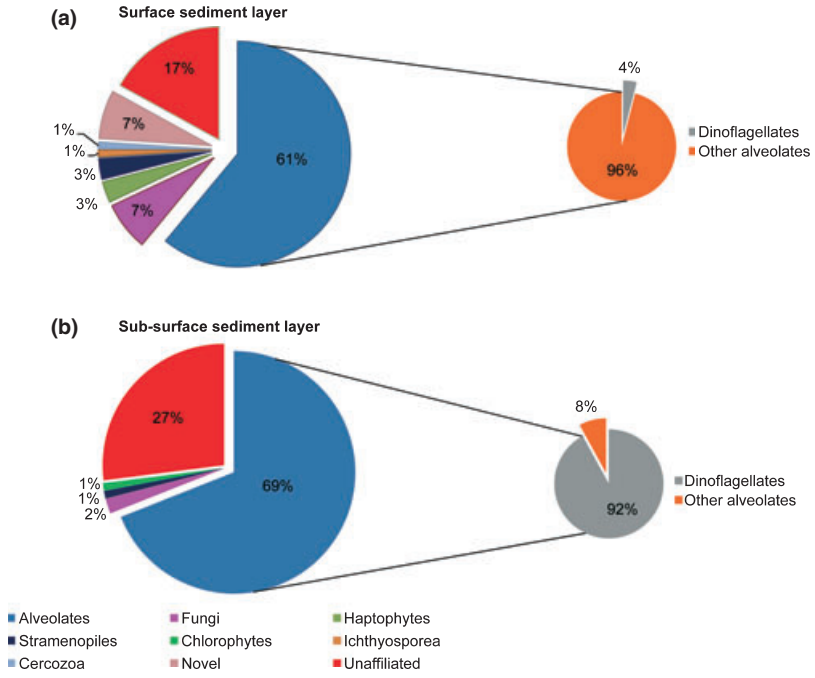

Fig. 3 Pie charts showing the relative abundance of clone numbers belonging to each major eukaryotic group retrieved from libraries obtained from the extracellular DNA recovered from the top 1 cm of sediment (a) and the 7–8-cm sediment layer (b) collected at Station 9 (1970 m depth).

comparable to those reported for highly productive marine ecosystems worldwide (Dell'Anno *et al.* 2002; Dell'Anno & Danovaro 2005; Corinaldesi *et al.* 2007a, 2008). The comparison between total extracellular DNA and total DNA concentrations (Table S3) revealed that the largest fraction of the entire DNA pool in the sediments was extracellular. The total extracellular DNA concentrations in deep-sea sediments of the Black Sea changed significantly across the different sampling sites, with the highest values at stations located at the shelf break (Station 1) and in the deep basin (Station 9). Such a high spatial variability can be dependent upon a complex array of factors, including changes in DNA input from the water column because of different sedimentation regimes (Dell'Anno *et al.* 2005), and DNA removal because of degradation processes mediated by DNase activities (Dell'Anno & Corinaldesi 2004; Corinaldesi *et al.* 2008).

Sedimentation rates in the Black Sea decrease ca. 10-fold from the shelf break (Station 1: 0.094 cm $year^{-1}$) to the deep basin (Station 9: 0.01 cm $year^{-1}$) (Teodoru *et al.* 2007). At the same time, the DNase activities are among the highest reported so far for marine sediments (see Corinaldesi *et al.* 2007a, for comparison), and they increased significantly with increasing water depth. These factors cannot, however, explain why high levels of extracellular DNA accumulate in the sediments of the deepest station of the Black Sea.

The paradox of the high levels of total extracellular DNA accumulated in the sediments that are associated with high DNase activities might arise as the interactions between the extracellular DNA and the sediment matrix can reduce its bioavailability to *in situ* DNase degradation, thus enhancing the DNA preservation with time (Coolen & Overmann 2007). To test this hypothesis, we applied a kinetic model that has been used previously to estimate the decay rate of total extracellular DNA (Dell'Anno & Danovaro 2005), here using the vertical profile of the sediments collected at the deepest station (Station 9). According to this model, we estimate that the decay rate of the total extracellular DNA pool in the deepest sediments of the Black Sea is very low (4.34×10^{-3} $year^{-1}$). This decay rate is ca. 10–20-fold lower than that previously reported for total extracellular DNA in oxic deep-sea systems characterized by similar or even lower sedimentation rates (Dell'Anno & Danovaro 2005). Moreover, the kinetic model revealed that the rate at which total extracellular DNA is potentially remineralized in deep sediments in the Black Sea is extremely low (integrated value for the 0–17-cm sediment layer: 3.8 ± 0.6 mg DNA m^{-2} $year^{-1}$). These data indicate that the anoxic conditions of the Black Sea may favour the preservation of extracellular DNA over the long term. This is further strengthened by our analyses of the total extracellular DNA concentrations in the older sediment layers that are

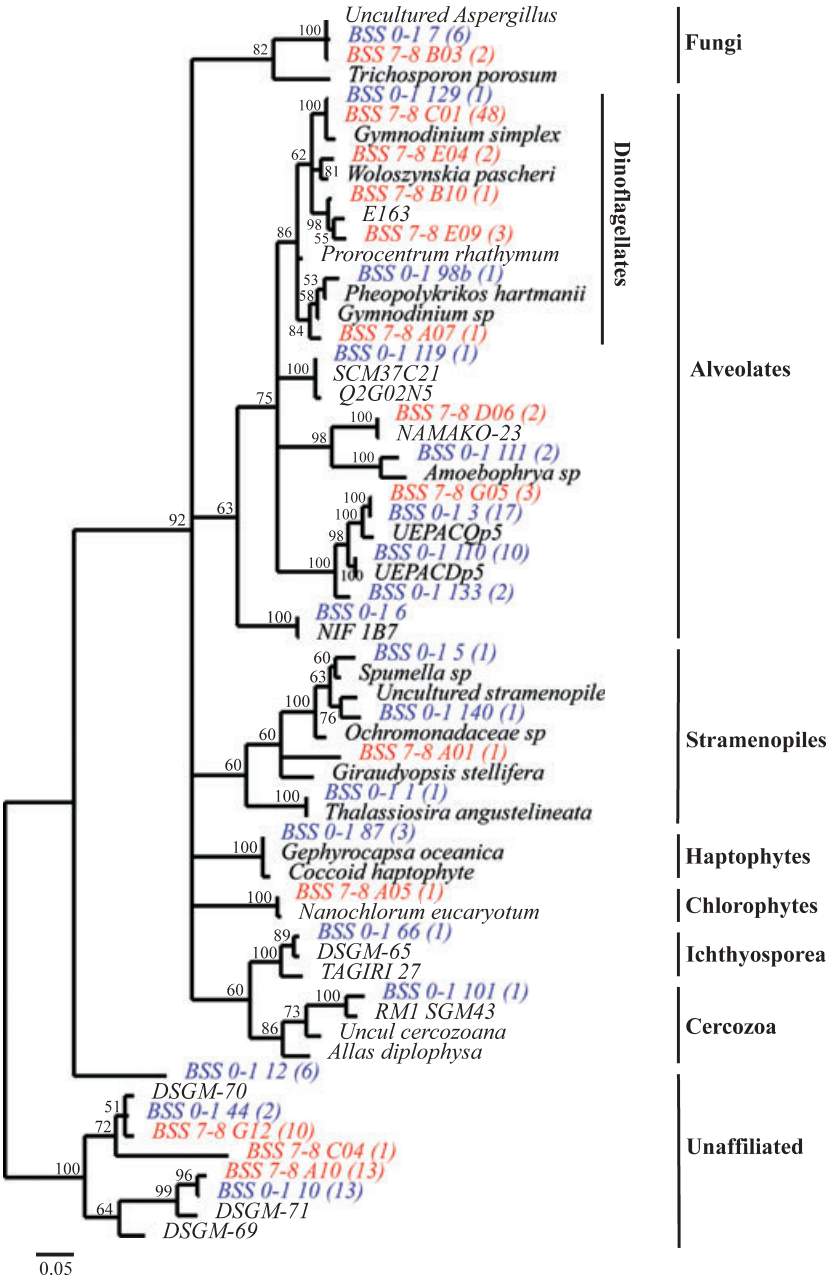

Fig. 4 Phylogenetic tree showing the relationships of the partial 18S rDNA sequences of phylotypes identified in the current study from the sediment layers of the Black Sea (blue and red text for surface and subsurface sediments, respectively) to reference sequences obtained from GenBank database (Table S1). Numbers in parenthesis indicate the number of sequences with sequence cut-off of 97% similarity. The tree was obtained by MP analysis. The numbers at the branches represent bootstrap estimates (>50%), and the scale bar indicates the expected substitutions per site. The trees constructed using ML and NJ analyses showed the same topology.

characterized by the presence of sapropel. Conversely to what is seen for the Unit I, in these older sediment layers, the total extracellular DNA content increased significantly with increasing depth of sediment. Such a depth-related pattern is opposite to that of the DNase activities, which decreased significantly with increasing depth of sediment. Overall, these findings indicate that sediments characterized by permanently anoxic conditions and a high organic matter load (such as the deeper sediment layers of the Unit I and the sapropel; Calvert & Karlin 1998; Coolen *et al.* 2006) can promote extracellular DNA accumulation in the subsurface

sediments, which thus represent an important system for exploring preserved gene sequences.

Previous studies based on analyses of the total DNA pool have provided evidence for the preservation of gene sequences belonging to photoautotrophic bacteria and algae in ancient lacustrine and marine environments including the Black Sea (Coolen *et al.* 2004, 2006, 2009; Coolen & Overmann 2007; Manske *et al.* 2008). In the present study, and for the first time, we have explored the quantitative relevance and composition of ribosomal gene sequences contained in the extracellular DNA pool, by real-time PCR and sequencing. The procedure used here for extracellular DNA extraction excludes possible contamination because of cell lysis or coextraction of intracellular DNA (see Appendix S1 for details and Figs S1 and S2). Therefore, the data from the real-time PCR and sequencing obtained here can only be attributed to sequences contained within genuine extracellular DNA.

The extracellular DNA pool from both the surface and subsurface sediments of the Black Sea contained a large number of 16S rDNA copies (range: 10^9–10^{10} copies g^{-1}). These values are up to four orders of magnitude higher that those reported for the extracellular DNA pool in oxic surface sediments (ca. 10^6 copies g^{-1}; Corinaldesi *et al.* 2008), and they are also higher than those determined for the total DNA pool extracted from deep-sea sediments (range 10^8–10^{10} copies g^{-1}; Schippers & Neretin 2006). Also, the number of 18S rDNA copies contained within the extracellular DNA pool was extremely high (range: 10^9 to $>10^{11}$ copies g^{-1}) and, indeed, up to four orders of magnitude higher than those reported for the surface and subsurface sediment layers of the deep Pacific Ocean (from the top 1 cm down to 34 cm in depth: 10^5–10^7 copies g^{-1}; Schippers & Neretin 2006). The number of 18S rDNA copies reported in the present study is also higher than the values previously recorded from an analysis of the total DNA pool extracted from surface and subsurface deep-sea sediments of the Black Sea (from the top 1 cm down to 35 cm in depth: 10^8–10^9 copies g^{-1}; Coolen *et al.* 2006). The comparison with available literature data should be considered with caution because of the different characteristics of the sampling sites and methodological approaches utilized. For instance, data reported by Coolen *et al.* (2006) were obtained from deep-sea sediment samples collected in the eastern sector of the Black Sea, which is an area less influenced by river outflows than that investigated here. Moreover, the copy number of ribosomal gene sequences determined by real-time PCR is influenced by the analytical procedures used for the DNA extraction and purification from the sediment (Lloyd *et al.* 2010). Despite this, we found that the copy number of 16S rDNA contained within the extracellular DNA pool accounted, indeed, for a large fraction of the copy number of 16S rDNA contained in the total DNA pool. Overall these findings indicate that the permanently anoxic sediments of the deep Black Sea are hot spots of microbial gene sequences that are preserved within the extracellular DNA.

We also observed that changes of the copy number of ribosomal gene sequences, determined along the vertical profile of the sediment, do not covary with changes of the total extracellular DNA concentrations. This can be because of the fact that the procedures used for quantifying the total extracellular DNA pool (by nuclease digestion) and for the recovery of the extracellular DNA suitable for molecular analyses are independent and provide different DNA yields (Table S7). Moreover, gene sequences contained within the extracellular DNA pool can be degraded at a different extent during burial in the sediment (Coolen *et al.* 2006).

In the present study, we observed clear differences of the extracellular DNA composition with increased depth of the sediment. In the surface sediment layers, the number of 16S rDNA copy was higher than that of 18S rDNA and *viceversa* in the deeper sediment layers. The increased dominance of 18S rDNA gene sequences with depth of the sediment does not depend upon an increased *in situ* release of eukaryotic extracellular DNA. In fact, the abundance and metabolism of specific benthic unicellular eukaryotes (i.e. foraminifera), able to live in permanently anoxic conditions, decrease exponentially with sediment depth (Bernhard *et al.* 2006; Risgaard-Petersen *et al.* 2006) and planktonic organisms, settled to the seafloor and buried in the sediment, can be viable only as resting stages (i.e. cysts; Raghukumar *et al.* 2004; Coyne & Cary 2005; Coolen & Shtereva 2009). Therefore, the higher copy number of 18S rDNA gene sequences, which we found in the deeper sediment layers, may reflect the temporal changes of DNA inputs from the water column to the seafloor and their progressive accumulation in the sediment. In particular, the accumulation of 18Sr DNA sequences in the deeper sediment layers is consistent with periods of enhanced primary productivity followed by massive deposition events to the seafloor of eukaryotic organisms (Glenn & Arthur 1985; Hay 1988; Huang *et al.* 2000).

Previous studies based on molecular approaches that have targeted the total DNA pool have reported that anoxic marine systems host a high diversity of unicellular eukaryotes (Dawson & Pace 2002; Edgcomb *et al.* 2002, 2009; Lopez-Garcia *et al.* 2003; Stoeck *et al.* 2003; Takishita *et al.* 2007). In the present study, we have seen for the first time that permanently anoxic deep-sea sediments of the Black Sea can be characterized by a high diversity of the eukaryotic rDNA sequences contained within the extracellular DNA pool. This high

genetic diversity might be even higher, as shown by the richness estimators and the rarefaction curves, which did not reach saturation (as previously reported also for other anoxic marine systems; Edgcomb *et al.* 2002, 2009). We found that the eukaryotic small subunit (SSU) rDNA sequences contained within the extracellular DNA pool are representative of the major protistan lineages. The two clone libraries contained only sequences that have affiliations to single cell organisms, and the majority of the sequenced clones fell within Alveolates, from both the surface and subsurface sediment layers. The dominance of Alveolates in molecular surveys of eukaryotic SSU rDNA sequences contained in the total DNA pool has been reported for a wide variety of anoxic marine systems (Edgcomb *et al.* 2002, 2009; Lopez-Garcia *et al.* 2003; Stoeck & Epstein 2003; Takishita *et al.* 2007) and, indeed, this also appears to be a specific feature of the genetic imprint contained within the extracellular DNA recovered from Black Sea sediments. Within Alveolates, sequences that were affiliated to photosynthesizing lineages (i.e. Dinoflagellates) were identified to a larger extent in the deeper sediment layers than in the surface sediment layers. The dominance of eukaryotic SSU rDNA sequences affiliated to Dinoflagellates has been already reported in deeper layers of sediments collected in the Black Sea, although data were obtained using different methodological approaches (i.e. DGGE analyses and sequencing from the total DNA pool) and different sediment cores (Coolen *et al.* 2006). This finding together with the presence of different phylotypes (branching with different sequences affiliated to all of the major protistan lineages), we found in the surface and subsurface sediment layers, suggests that in the Black Sea ecosystem, major changes have occurred in the eukaryotic diversity over time scales from decades to centuries (Coolen *et al.* 2006, 2009).

Previous studies based on phylogenetic analyses of eukaryotic SSU rDNA sequences recovered from anoxic systems worldwide have emphasized the presence of novel lineages that are not specifically related to any known organisms at the kingdom level. This has provided new insights into the diversity and patterns of eukaryotic evolution (Dawson & Pace 2002; Lopez-Garcia *et al.* 2003; Stoeck & Epstein 2003; Stoeck *et al.* 2003; Takishita *et al.* 2007). Moreover, the analysis of the total DNA pool recovered from subsurface sediments revealed, using selective PCR primers, the presence of remains of specific photoautotrophic planktonic organisms (belonging both to the Bacteria and Eukarya domains) up to 217 000 years old (Coolen *et al.* 2004, 2006, 2007, 2009; Coolen & Overmann 2007; Manske *et al.* 2008). These studies allowed the identification of species belonging to past planktonic communities, thus providing new insights into changes in paleoenvironmental conditions.

In our study, in addition to the identification of all of the major protistan lineages, new very divergent lineages were also discovered in both the surface and subsurface sediment layers, which should correspond to hitherto unknown eukaryotic kingdoms. These branches appear as independent clades at the base of the tree, and they are closely affiliated with rRNA sequences that have been detected previously in other anoxic marine ecosystems (Takishita *et al.* 2007).

The high number of copies of 18S rDNA that belong to the extracellular DNA pool in these anoxic sediments and their high diversity suggest that the extracellular DNA is a major archive of eukaryotic gene sequences from present-day (i.e. originating from recently dead planktonic and/or benthic organisms) to past communities. Overall, our data indicate the importance of integrating molecular cell-oriented approaches (i.e. molecular analyses on cells and organisms) with molecular analyses on the extracellular DNA pool, to provide a better assessment of the microbial diversity and the temporal changes that can occur in marine benthic ecosystems.

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Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1 Materials and methods: Details on Working conditions and precautions during extracellular DNA analyses, Total extracellular DNA determination, DNase activity, Testing for the absence of cell lysis, Real-time PCR analysis, Clone library and phylogenetic analyses, Decay rate of total extracellular DNA and Statistical analysis.

Table S1 The sequences used for the phylogenetic analysis and the respective accession numbers

Table S2 Output of the one-way analysis of variance and the *post-hoc* comparison testing for the differences in total extracellular DNA concentrations and DNase activities in the surface sediments (i.e. 0–1 cm) of the four investigated sites (i.e. Stations 1, 2, 3 and 9)

Table S3 Concentrations of total DNA in surface sediments of Stations 1, 2, 3 and 9 and in the different layers of sediments collected at Station 9

Table S4 Output of the two-way analysis of variance and the *post-hoc* comparison testing for differences in total extracellular DNA concentrations and DNase activities in the different sediment layers (i.e. 0–1 cm, 4–5 cm, 9–10 cm) of the four investigated sites (i.e. Stations 1, 2, 3 and 9)

Table S5 Output of the one-way analysis of variance and the *post-hoc* comparison testing for differences in total extracellular DNA concentrations, DNase activities, 16S rDNA copy numbers and 18S rDNA copy numbers in the Unit I (i.e. 0–17 cm in depth) and sapropel (i.e. 31–53 cm in depth) layers of the sediments collected at Station 9

Table S6 Output of the PERMANOVA analysis testing for differences in total extracellular DNA concentrations, DNase activities, 16S rDNA copy numbers and 18S rDNA copy numbers

between the Unit I (i.e. 0–17 cm in depth) and sapropel (i.e. 31–53 cm in depth) layers of the sediments collected at Station 9

Table S7 Concentrations of extracellular DNA (utilized for molecular analyses) in the different layers of sediments collected at Station 9

Fig. S1 Gel electrophoresis of the 16S rRNA gene amplified with primers 27f and 907r from samples utilized to test for the contamination of extracellular DNA with intracellular DNA because of cell lysis.

Fig. S2 Comparison between extracellular DNA concentrations (A) and 16S rDNA copy numbers (B) determined in fresh and frozen sediment samples.

Fig. S3 Saturation profile (phylotype accumulation curve) in the surface (0–1 cm) and subsurface (7–8 cm) sediments collected at Station 9.

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