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Project Report

Culture-independent assessment of dissolved DNA as a nutrient source in the marine environment

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ABSTRACT

Extracellular DNA is widely distributed in the marine environments, where it has been proposed to substantially contribute to the carbon, nitrogen and phosphorus supply for marine microorganisms. Despite this potential, the actual role of DNA as a nutrient source remains poorly understood. Especially, it is not clear whether the extracellular DNA represent a nutrient source accessible to the majority of the marine microbial population or, instead, it is exploited by just a subset of specialists 'DNA-eaters'. Previous investigations have addressed this question by a cultivation-based approach, which provided valuable insights about DNA-degrading bacteria, but are limited by the fact that just a small fraction of the microbial universe can be cultured in the laboratory. The objective of this project was to test the possibility of studying the role of DNA as nutrient source by culture-independent methods. Based on the results obtained, DNA Stable Isotope Probing using ^{13}C - ^{15}N -labeled DNA as a substrate has been established as a promising tool to investigate the identity and diversity of DNA-eaters in the marine environment.

INTRODUCTION

It is nowadays largely acknowledged that microorganisms play a fundamental role in shaping and driving biogeochemical cycles in aquatic environments (1). Nonetheless, this ability depends on the availability and quality of resources, such as dissolved organic matter. DNA released from marine organism, through either active (secretion) or passive (cell lysis, viral predation, grazing) processes, is a considerable component of dissolved organic matter and might constitute a readily-accessible and energy-rich source of preformed nucleotides, carbon, nitrogen and phosphorus. The latter is of special importance given that marine environments are often phosphorus-limited (2). Several lines of evidence support the hypothesis that marine ecosystems rely, to a certain extent, on dissolved DNA as a source of energy and organic matter:

- 1- Dissolved DNA concentrations in the water column are often higher than cell-associated DNA (3). Sediments display an even more pronounced scenario, with extracellular DNA accounting for more than 90% of total DNA (4), possibly due to enhanced preservation after DNA adsorption to the sediment matrix. The recent discovery of high viral abundances in the seawater has raised skepticisms about the existence of a large pool of genuine extracellular DNA (5). However, even though common procedures for quantifying dissolved DNA do not distinguish soluble DNA from encapsulated DNA, several authors have demonstrated that viral DNA generally accounts for less than 20% of dissolved DNA pools (6-9). Quantitative estimates of the dissolved DNA pool in the water column thus reflect, to a large degree, extracellular DNA concentrations.
- 2- Turnover times, calculated on the basis of estimates of degradation rates, range from 6.5 to 25 hours (3, 10). Dissolved DNA is therefore a dynamic component of dissolved organic matter and it has been estimated that it might supply almost 50% of the P demand of deep-sea prokaryotic population worldwide (4).
- 3- The ability to uptake DNA from the extracellular environment by natural competence is a well characterized phenomenon, mostly due to its implications for lateral gene transfer. It has been proposed that natural competence, in addition to playing a role in genetic recombination, might also, if not primarily, serve to allow the use of extracellular DNA for nutritional purposes (11-14).
- 4- Marine bacteria capable of degrading and growing on DNA as a primary C, N and P source have been successfully isolated, and most of them belong to abundant marine *γ-Proteobacteria* and *Bacteroidetes* such as *Vibrio*, *Alteromonas* and *Pseudoalteromonas* (15). In addition, the ability to grow on DNA has been shown to be widespread among the metal-reducing bacteria of the *Shewanellae* group (16), possibly playing a major role in nutrient supply in P-starved environments where metal-reducing bacteria reside.

So far, DNA-eating bacterioplankton has solely been investigated by cultivation-dependent methods. Although providing valuable information, culture dependent methods have the limitation that only the microorganisms capable of growing in standard media can be studied (a limitation often referred to as 'the great plate count anomaly'), possibly resulting in a considerable underestimation of the actual diversity of the DNA-eating

microbial population. DNA Stable Isotope Probing (DNA-SIP) is a powerful technique that allows linking the taxonomic identity of microorganisms with their function in the environment, bypassing the requirement of previous cultivation. Stable-isotope-labeled carbon (^{13}C) or nitrogen (^{15}N) sources are assimilated into microbial biomass of environmental samples. Separation and molecular analysis of labeled nucleic acids (DNA or RNA), achieved by isopycnic centrifugation through a cesium chloride gradient, reveals phylogenetic and functional information about the microorganisms responsible for the metabolism of a particular substrate (17).

In this project, I applied DNA-SIP to investigate the identity and diversity of DNA-eating bacterioplankton. The study was conducted on sweater samples from Eel Pond (Woods Hole, MA) using DNA enriched with both ^{13}C and ^{15}N , prepared by growing *Saccharomyces cerevisiae* (baker's yeast) in the presence of labeled glucose and ammonium chloride as sole carbon and nitrogen sources. In addition, another culture independent method based on 454-pyrosequencing was applied and the results compared to those from the DNA-SIP experiment.

MATERIALS AND METHODS

Strains and growth conditions

Saccharomyces cerevisiae was obtained from the local baker's shop as lyophilized pellets. Pellet grains were dispersed in sterile drinking water and streaked onto YPD (yeast extract 10 g/L; peptone 20 g/L, glucose 10 g/L). An isolated colony was selected and checked for ability to grow on minimal Yeast Nitrogen Base (YNB) with 2% Glucose, 4 g/L ammonium chloride, and 5.37 g/L sodium sulfate as carbon, nitrogen and sulfur source, without additional requirements. For ^{13}C - ^{15}N -labeled DNA preparation, the yeast was grown on the same medium containing ^{13}C -Glucose and ^{15}N ammonium chloride (Cambridge Isotope Laboratories, Andover, MA, USA). Liquid cultures (typically 100 ml) were incubated until saturation into one-liter Erlenmeyer flasks at 30 degrees C with shaking.

Field characteristics and sampling

This study has been conducted on sweater samples collected from Eel pond, Woods Hole, MA. Eel Pond is a shallow body of salt water almost land-locked but with a strong tidal circulation. According to previous measurements (15), salinity temperature and conductivity are constant throughout the water column, while oxygen concentration decreases with depth from 7.3 to 3.6 mg/L; dissolved DNA concentration was found to be $26 \pm 0.3 \mu\text{g/L}$. Cell abundance, determined by DAPI staining and fluorescent microscopy, was $1.24 \pm 0.23 \times 10^7$ (this study). Water samples were collected from the surface layer (top 10 cm) using sterile 6 liters-

Erlenmeyer flasks, transported immediately to the laboratory and aseptically transferred to sterile 1 or 2 liter screw cap bottles. Incubation experiments were started immediately after sampling.

Total DNA extraction from S. cerevisiae

Individual isolated colonies were picked and transferred into 100 ml of YNB and incubated at 30 degrees C with shaking. When the culture reached the stationary phase, cells were harvested by centrifugation at 5000 g for 15 minutes at 4°. Up to 100 mg of cell pellet were mixed with 0.2 ml of Phenol:Chloroform:Isoamyl alcohol (25:24:1) and 0.2 ml of Lysis solution (1% SDS; 2% Triton X-100; 100 mM NaCl; 10 mM Tris-Cl pH 8.0; 1 mM Na₂EDTA). Cells were bead-beated for 1 minute into a PowerBiofilm DNA isolation Kit bead-beating tube. The aqueous phase was transferred to a new tube and the DNA precipitated by adding 2.5 volumes of 95% ethanol. Precipitated DNA was centrifuged at 13000 g for 10 minutes, air dried and resuspended in 0.4 ml of TE. RNase was added to a final concentration of 100 µg/ml and incubated at 37°C for 10 minutes. DNA was precipitated again by adding ammonium acetate (2M final concentration) and 2.5 volumes of 95% ethanol. Precipitated DNA was centrifuged at 13000 g for 10 minutes, washed once with 70% ethanol, air dried and dissolved in a suitable volume of sterile, distilled water. DNA concentration and quality were assessed spectrophotometrically (Nanodrop 2000C, Thermo Scientific) and by agarose gel electrophoresis. Protein contamination in the DNA extracts was checked by Bradford assay and protein concentration was found to be below the detection limit of the assay (data not shown).

Cell count

10-ml seawater samples were fixed for 24 hours in the presence of 1% paraformaldehyde. 0.5 ml of fixed sample were mixed with 9.5 ml of sterile PBS 1X and filtered through 25-mm diameter, 0.2-µm pore size polycarbonate filters (Millipore) under a gentle pressure (ca. 200 mBar). Filters were washed with 10 ml distilled water and air-dried. One quarter sections were cut from the filters using a scalpel, lied on a glass microscope slide and soaked with 10 µl of mounting solution (Citifluor:Vectashield:PBS 5.5:1:0.5) containing 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI). Cells were counted on a fluorescent microscope under 1000X magnification. At least 1000 cells were counted on each filter.

SIP incubation

Three screw-cap glass bottles (2 liters each) were each filled with 1.7 liters of freshly sampled water from Eel pond. The water from one bottle was filtered immediately onto 47 mm diameter, 0.2 um pore size polycarbonate filters (Millipore) using a sterilized filtration tower; of the other two, one was amended with 1.7 mg of unlabeled yeast DNA, and the other with the same amount of ¹³C-¹⁵N-labeled yeast DNA. The bottles were incubated at room temperature (ca. 25°C) under natural light with loose caps. 6, 12 and 24 hours after

the addition of substrate DNA, 550 ml of water from each of the two bottles were filtered onto 47 mm-diameter, 0.2 µm-pore size filters (type GTTP, Millipore). Filters were air-dried, cut in halves with a sterile scalpel and store at -20 °C. For DNA extraction, filters were folded into a bead-beating vial (cell side inward) and processed with the PowerBiofilm DNA isolation kit (MoBio Laboratories), according to manufacturer's instructions.

SIP gradient fractionation

From each timepoint DNA extracts, 4 µg of total DNA were added to cesium chloride (CsCl) solutions for isopycnic ultracentrifugation and gradient fractionation similarly to a previously described protocol (18). Briefly, CsCl was dissolved in gradient buffer (15 mM Tris-Cl pH 8.0, 15 mM EDTA, 15 mM KCl) to achieve a density of 1.762 g/ml. 4 µg of DNA in a volume of 0.45 ml were then added to achieve a final density of 1.69 g/ml. Samples were centrifuged at 55000 rpm (164000 g) in a 30° fixed angle rotor (type TLA 110) at 20°C for at least 60 hours. A total of 40 fractions (100 µl each) were collected using a syringe pump into Acroprep 96-well 30000 Da molecular weight cutoff filter plates (Pall Corporation) and the refractive index of each fraction was measured immediately on a digital refractometer. Fractions were supplemented with 250 µl of TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and plates were centrifuged at 500 g for 10 minutes at room temperature. To avoid CsCl inhibition of downstream molecular applications, the DNA retained on the membranes was wash 6 more times with 300 µl of TE. After the last wash, the DNA in each fraction was eluted from the membranes with 50 µl of TE, transferred to a clean 96 well plate and stored at -20°C.

Quantification of 16S gene copy number in SIP fraction by quantitative real-time PCR analysis (qPCR)

To determine the 16S rRNA gene copy number in each of the SIP fractions, qPCR assays were conducted in a reaction volume of 25 µl containing 2 µl of template DNA, 1 µl of 515F primer (25 µM), 1 µl of 907R primer (25 µM) and 12.5 µl of QuantiFast SYBR Green PCR Master Mix 2X (Qiagen). The primer sequences are the same as reported in Table 1, but without adapters, barcodes and linkers. A 10-fold dilution series (from 10⁷ to 10¹ gene copies/µl) in irradiated sheared salmon sperm DNA (2µg/µl) of a pCR4-TOPO plasmid containing a bacterial 16S rRNA gene from a clone library generated during the course was used for standard curves. Reactions were run in a StepONE Plus Real Time PCR System (Applied Biosystems) with the following thermocycling program: [95°C, 5 min] x 1 cycle, [(95°C, 10 sec)(60°C, 30 sec)] x 35 cycles. Due to reagents limitations, only the samples for the standard curve were run in duplicates.

Clone-library construction and analysis

PCR was carried out on SIP fraction using bacterial 16S rRNA gene-specific primers 8F and 1492R (19) in a reaction mixture consisting of: 2 µl template DNA, 12.5 µl PCR Master Mix (Promega), 2 µl 8F primer 7.5 µM, 2

μl 1492R primer 7.5 μM, and 6.5 μl nuclease-free water. The thermocycling program used was: [95°C, 5 min] x 1 cycle; [(95°C, 30 sec)(46°C, 30 sec)(72°C, 1.5 min)] x 30 cycles; [72°C, 5 min] x 1 cycle. PCR reactions were loaded on a standard agarose gel (1%). At the end of the run, bands of interest were excised from the gel and the DNA extracted using the DNA Gel Extraction Kit (Millipore). The cloning reaction was performed using the TOPO TA cloning kit (Invitrogen), following manufacturer's instructions. 4 μl of the cloning reaction were used to transform electrocompetent *E. coli* TOP10 cells (Invitrogen) on a BioRad GenePulser instrument. Cells were then plated onto LB agar containing 50 μg/ml ampicillin and incubated at 37°C for 12 hours. Individual colonies were picked, subcultivated in 1.5 ml of liquid LB medium containing ampicillin into 96-well plates and sent for sequencing. Sequences were assigned to OTUs using QIIME.

454-pyrosequencing experiment

Three liters of seawater from Eel Pond were dispensed into three 1-liter sterile screw cap bottles. The water from one bottle (time zero) was filtered immediately as described above, while, of the other two, one was amended with 1 mg/L (final concentration) yeast DNA (experimental) and the other was left unamended (control). After 24 hours incubation at room temperature (ca. 25°C) under natural light with loose caps, the experimental and control bottles were also filtered and the DNA extracted as described in the "SIP incubation" paragraph. Partial SSU rRNA genes were amplified with primers 515F and 907R, modified for 454 pyrosequencing as follows:

	Adapter	Barcodes	Linker	SSU rRNA complementary region	Ref.
515F	5'-CGTATCGCCTCCCTCGCGCCATCAG	ACTTCGGTT TAAGGTTGT TTCCTCGTT	GA	GTGYCAGCMGCCGCGGTAA-3'	(19)
907R	5'-CGTATCGCCTCCCTCGCGCCATCAG		GG	CCGYCAATTCMTTTRAGTTT-3'	(25)

Table 1. Primers used to PCR-amplify partial SSU rRNA sequences for 454-pyrosequencing.

PCR reactions consisted of 15 μl Phusion 2X Master mix (New England Biolabs), 2.4 μl DMSO 100%, 2.4 μl 515F primer 6.25 μM, 0.6 μl 907R primer 25 μM, 4 μl of 10-fold diluted DNA extracts and 5.6 μl of nuclease-free water. The following thermocycling program was applied: [98°C, 1 min]x1; [(98 C, 5 sec) (68°C, 10 s) (72°C, 7 sec)]x10; [(98°C, 5 sec) (58°C, 10 sec) (72°C, 7 sec)]x12, [72°C, 10 min]x1. The RDP classifier as implemented in the QIIME pipeline for microbial community analysis tool was used to assign taxa to all sequences (20, 21). Heat map was constructed using Python.

RESULTS AND DISCUSSION

DNA extraction from S. cerevisiae

A representative sample of DNA extracted from cultures of *S. cerevisiae* grown in YNB in either labeling or normal condition (i.e. in the presence of ^{13}C -Glucose + ^{15}N -Ammonium chloride or ^{12}C -Glucose + ^{14}N -Ammonium chloride, respectively) is shown in the gel picture in figure 1. The bulk of the DNA appears to be in the 10-3 Kb size range, with a smear of sheared DNA extending down to the 1 kb band of the ladder.

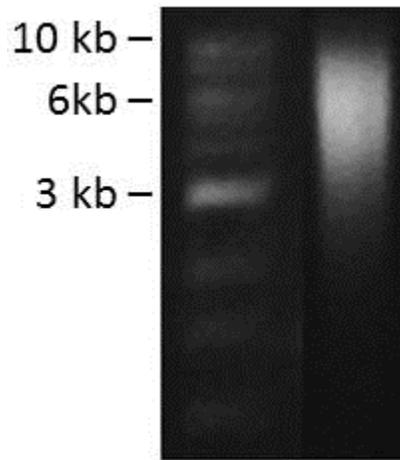


FIGURE 1. Total DNA extracted from *S. cerevisiae* analyzed on a standard agarose gel electrophoresis. Marker: 1Kb DNA Ladder, New England Biolabs.

1.2 to 1.7 mg of DNA were typically extracted from 100-ml saturated yeast cultures in YNB.

Community analysis of seawater incubated with S. cerevisiae DNA by 454-pyrosequencing

After 24 hours of incubation, the bottle containing 1 mg/L yeast DNA appeared slightly more turbid than the unamended bottle. The concentration of spiked DNA exceeds the *in situ* DNA concentration by at least 40 times, and this probably led to the enrichment of microorganisms that are highly efficient in exploiting DNA as a nutritional resource. Microbial growth in Eel Pond water in the presence of 1 mg/L DNA was confirmed in an independent experiment by performing cell count on subsamples taken 0, 3, 6, 12 and 24 hours after the addition of 1 mg/L yeast DNA. The result (Figure 2) shows that the cell density in the sample incubated in the presence of an additional DNA source is double as much as the value obtained from the unamended sample at

the end of the incubation period, indicating that DNA is actually a valuable nutrient source for the microbial population in the sample seawater.

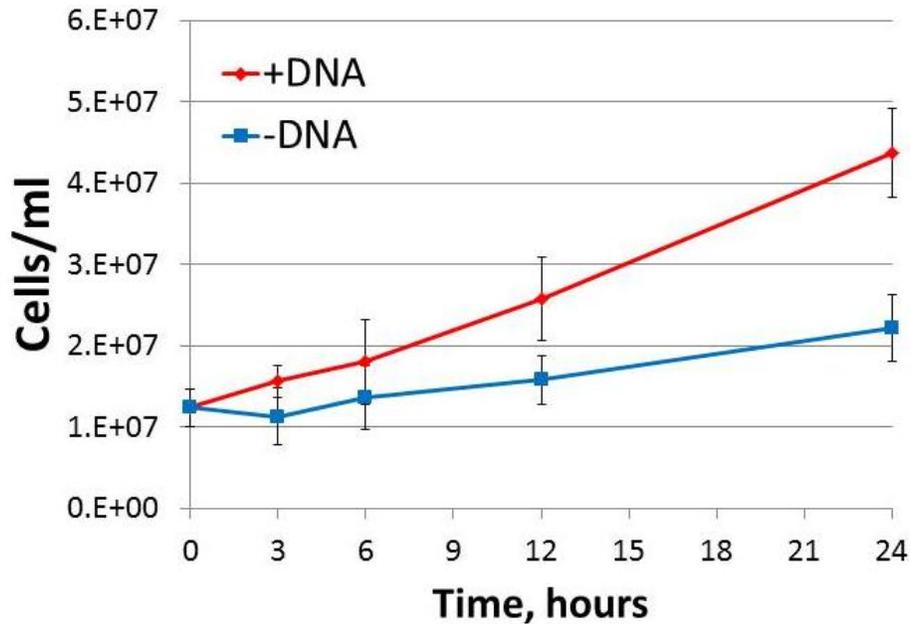


FIGURE 2. Trend in cell abundance in Eel Pond water incubated in the presence of 1 mg/L (red line) compared to the unamended control (blue line), determined by direct cell count over an incubation period of 24 hours.

The heat map in Figure 3 shows a remarkable change in the community composition induced by the presence of added DNA. Even though I didn't calculate any diversity index, a clear loss of diversity occurred in the sample incubated with DNA, in favor of a subgroup of microorganisms (e.g. *Vibrio*, *Alteromonas*, *Neptunibacter*) that became highly enriched. The heat map also shows that a bottle effect (the change in the community composition simply due to the confined incubation in bottles) probably occurred, because the '-DNA' condition is more similar to the '+DNA' condition than to the 't₀' control. If no bottle effect occurred, the 't₀' would have clustered with the '-DNA' control.

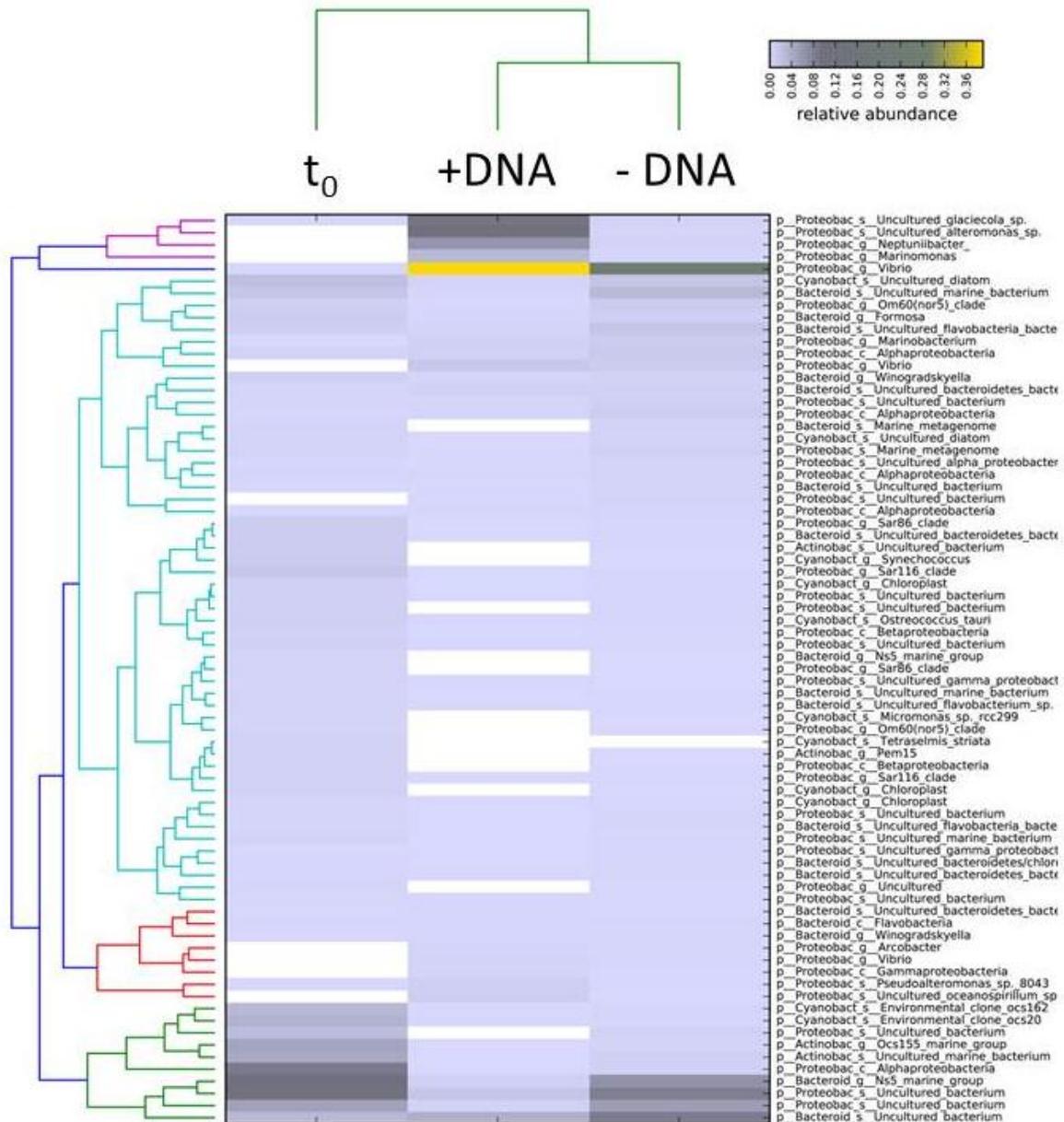


FIGURE 3. Heat map generated in Python showing the change in the overall OTU abundance across the three experimental conditions.

Comparison of OTUs abundance at the phylum level (Figure 4A) shows that, in the presence of added DNA, Proteobacteria become strongly enriched. Conversely, cyanobacteria almost disappear, although this is also observed in the control without added DNA, indicating that the bottle effect might be responsible for this.

From the analysis of OTUs abundance at the class level (Figure 4B), it is clear that the increased abundance of Proteobacteria is mostly due to the class γ -Proteobacteria.

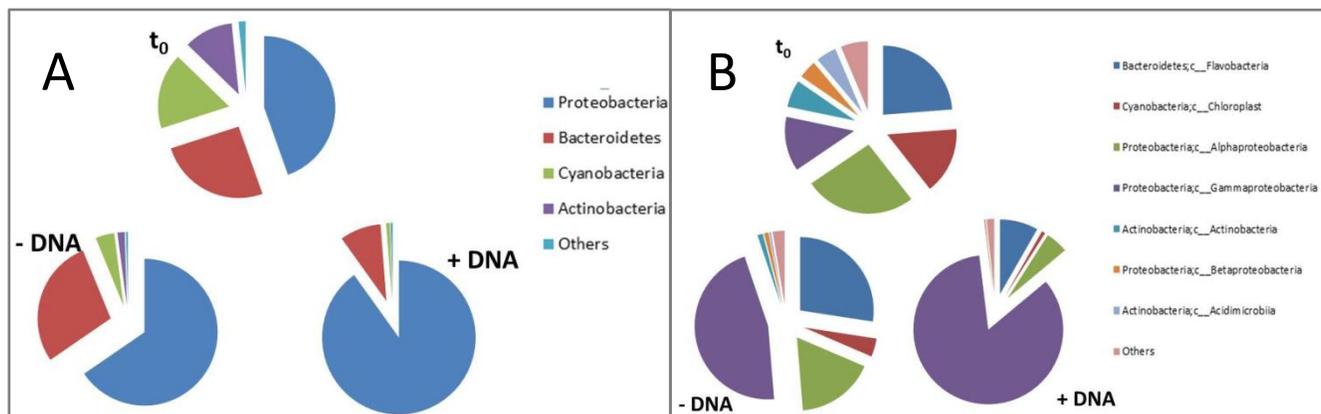


FIGURE 4. Comparison of community composition at the phylum (Panel A) and class (Panel B) level, at the beginning (t_0) and after 24 hours of incubation in the presence (+ DNA) or absence (- DNA) of added DNA.

For comparison to available data on the phylogenetic identity of DNA-eating microorganisms in Eel pond, the analysis of OTUs abundance was pushed to the genus level within the class γ -Proteobacteria (Figure 5). OTU abundances in the '+DNA' condition were normalized by subtracting the abundances of the same OTUs in the '-DNA' condition. The most abundant genera, namely *Vibrio* and *Alteromonas*, were also found to dominate clone libraries generated from microorganisms cultivated in the presence of DNA as a sole C, N, and P source (15).

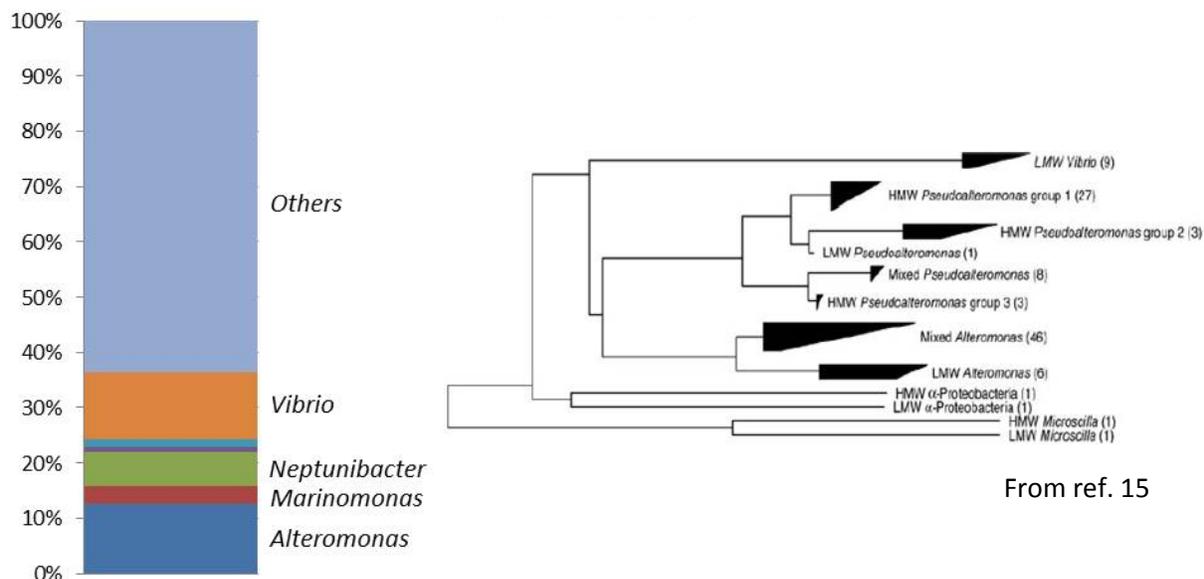


FIGURE 5. Normalized OTU abundance at the genus level within the class γ -Proteobacteria in the '+DNA condition' obtained from 454-pyrosequencing (left, this study) compared to a clone library results generated from cultured microorganisms from Eel pond (Ref. 15)

Overall, the result from the 454-pyrosequencing experiment show that the ability to exploit DNA as a nutrient source is widespread among the γ -Proteobacteria.

Stable Isotope Probing of DNA-eaters

The incubation of seawater with ^{13}C - ^{15}N labeled yeast DNA and unlabeled control proceeded for a total of 24 hours; this time limit was chosen based on previous estimates of DNA turnover times discussed in the Introduction. Subsamples were withdrawn from the incubation bottles at 0, 6, 12 and 24 hours after the addition of DNA and processed as described in section 'Material and Methods' in order to monitor the incorporation of the labeled substrate over time.

The DNA content was first screened on fraction pools (created by mixing 1 μl from 4 adjacent fractions) by standard PCR using universal primers 8F and 1492R. The result (Figure 6) shows that the elution peak from the samples incubated with labeled DNA gradually shifts toward higher buoyant densities, indicating incorporation of heavy isotopes from the substrate DNA into the DNA of DNA-eating microorganisms. Due to time constraints, clone libraries were generated immediately from pooled fraction 5-8 and 9-12, time point 24 hours (both labeled and unlabeled, highlighted in green in Figure 6), before more detailed analyses. A total of 120 and 60 clones from the labeled and unlabeled pools, respectively, were sent for sequencing.

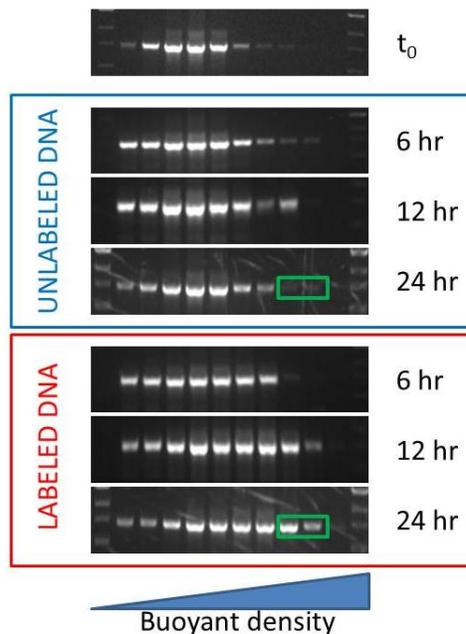


FIGURE 6. Standard PCR screening of pooled fractions from the SIP experiment. Pools used for clone library generation are highlighted in green.

In the meantime, individual fractions were further analyzed by qPCR assays with primers 515F and 907R (Figure 7). The curve corresponding to the sample incubated with labeled DNA overlaps the one from the unlabeled control after 6 hours of incubation, although a shoulder in the heavier region starts being visible. After 12 and 24 hours of incubation, a complete separation was achieved. The shape of the curve also shows that the labeled DNA was uptaken by just a subfraction of the population, indicating that the ability of utilizing DNA as a nutrient source does not belong to the whole microbial population.

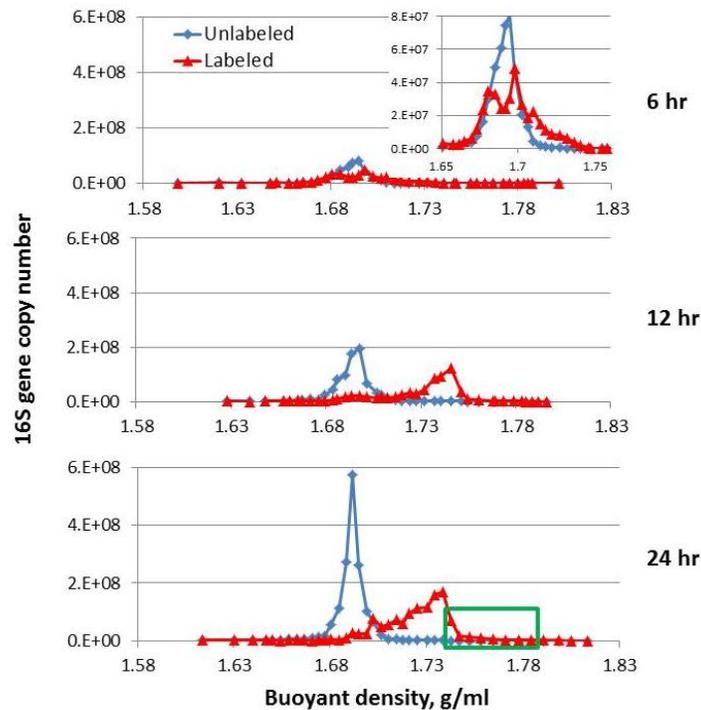


FIGURE 7. qPCR analysis of individual fractions from the SIP experiment. Fractions used to construct clone libraries are highlighted in green.

Clone library analysis

The result of the analysis of the clone libraries generated from the ‘heavy’ and ‘light’ fractions from the timepoint 24 hours is shown in Figure 8. Surprisingly, the clone library from the labeled fractions has a high occurrence of *Bacteroidetes*-related sequences and almost no *Proteobacteria* sequences. This contrasts with the results from the 454-sequencing experiment, although this could be partly due to cloning biases or undersampling of the clone library. Also, *Proteobacteria* tend to have a fairly low GC content, therefore they might not be well represented in the fraction chosen for clone library

construction, which fall in the ‘heavier’ part of the peak, which is enriched in high-GC-content sequences (due to their higher buoyant density).

Notably, one OTU from the genus *Winogradskyella* (Taxon #2 in figure 8) seems to be highly enriched in the clone library from the heavy fraction. Interestingly, cultured species from this genus have been shown to be able to degrade DNA by extracellular nucleases (22-24).

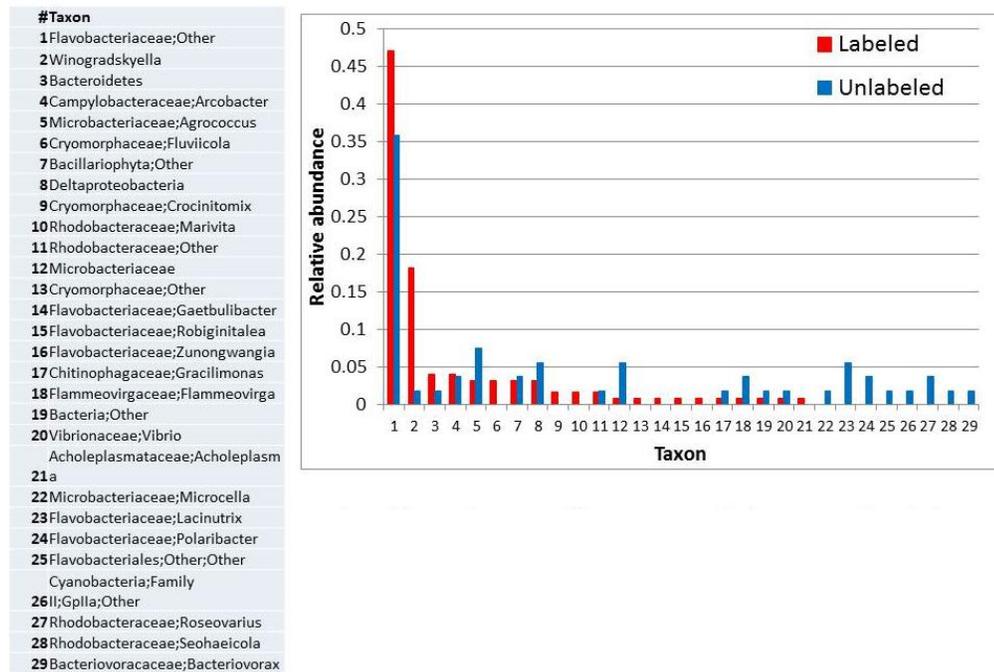


Figure 8. OTU abundances in the clone libraries generated from the labeled and unlabeled fractions retrieved from timepoint 24 hours.

CONCLUSIONS

This work describes the first successful attempt to use DNA as a substrate for DNA-SIP experiments, providing the proof of concept for the possibility to study the role of DNA as a nutrient source in the marine environment by a culture-independent approach. The results presented here also show that the ability to live on DNA is not widespread among the marine microbial population. However, it is not possible to answer the ultimate question “who is eating the DNA out there?” based on these results. Indeed *Proteobacteria* seem to be the major players according to the 454 experiment, while the SIP experiment indicates a more important contribution from the *Bacteroidetes*. Given that SIP is specifically designed for targeting functional groups within a population, I personally tend to give

more credit to the SIP results, but more experiments are definitely required. Also 24 hours of incubation are probably too long, and the both the 454 and the SIP results are probably affected by an enrichment effect. Therefore, it will be worth analyzing the SIP fraction from the 6 and 12 hour timepoints, which are probably less affected. Incubation with less DNA might also help avoiding the enrichment effect. Finally, it would be interesting to extend this approach to the benthic environment. Sediments play a large role in the biogeochemical cycle in the marine environment and have been shown to contain the largest reservoir of DNA in the world's oceans. Furthermore, due to adsorption processes to the mineral matrix, extracellular DNA dynamics are probably very different from those of the water column.

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