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Molecular Tools for the Analysis of DNA in Marine Environments

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Abstract In the last decade, microbial ecologists have increasingly applied molecular techniques to investigate microorganisms in natural environments. The use of molecular tools has allowed the identification of new and uncultured microbial species, and has greatly advanced our knowledge on the diversity and functioning of microbial communities in aquatic ecosystems. At the same time, the discovery of large quantities of extracellular DNA in both seawater and sediments is opening up new questions on the role and significance of these components in biogeochemical cycles and, potentially, also in horizontal gene transfer. This chapter describes the most recent methods for the extraction, quantification and isolation of intracellular and extracellular DNA in water and sediment samples. An outline of methods currently used in marine molecular ecology and their limitations is presented. The application of various molecular tools for studying DNA associated with microorganisms and for investigating the extracellular fraction is critically discussed. Some recent discoveries and new perspectives for future research are highlighted.

Keywords Diversity · Extracellular DNA · Intracellular DNA · Molecular analysis · Sediment · Water column

1 DNA in Aquatic Ecosystems

In aquatic ecosystems, the organic carbon inventory is largely dominated by non-living materials (i.e. detrital carbon) present in both the dissolved and particulate states, whose cycling is primarily mediated by heterotrophic prokaryotes. The labile fraction of the organic carbon pool in the oceans is mainly composed of simple (i.e. monomeric) and combined biochemical compounds. Among the biochemical classes of organic compounds, DNA ranks fourth after carbohydrates, proteins and lipids [1]. In aquatic environments, DNA is present in different forms: (1) associated with living organisms (i.e. intracellular DNA); (2) encapsulated by proteins (i.e. viral DNA); (3) free (i.e. soluble DNA) and (4) adsorbed to detrital and/or mineral particles [2]. Since viruses are a group of biological entities with a genome [3], only the two latter forms can be considered genuine extracellular DNA. DNA associated with living biomass is the ultimate source of extracellular DNA because cell-free DNA synthesis is not known to occur. Potential pathways of extracellular DNA production include: (1) exudation and excretion from viable cells; (2) losses associated with grazing activities; (3) passive release following cell death and lysis; (4) release due to virus-induced cell lysis; and (5) desorption/adsorption of dissolved DNA from seston particles.

The presence of DNA as a constituent of the dissolved organic matter pool in aquatic systems has been known since the early 1970s [4, 5], but our current understanding of the dynamics and distribution of dissolved DNA in marine environments is largely due to the works of Paul and co-workers [6–9] and DeFlaun and co-workers [10, 11]. Studies carried out on a regional scale in the Gulf of Mexico indicated that dissolved DNA concentrations are highest in estuarine environments (from 5 to 44 mg m^{-3}) and decrease with increasing distance from land (2–15 and 1–5 mg m^{-3} in coastal and offshore oceanic environments, respectively) and with increasing water depth (up to < 1 mg m^{-3} at bathyal depths). In addition, these studies revealed that the molecular size of the dissolved DNA pool ranges from < 0.5 to > 23.0 kbp, with DNA in offshore environments at the lower end of this range.

The recent discovery by microbial ecologists of high viral abundances in seawater has raised scepticism about the existence of a large pool of genuine extracellular DNA [12]. However, although common procedures for quantifying dissolved DNA do not distinguish soluble DNA from encapsulated DNA (i.e. viral DNA), several authors have demonstrated that viral DNA accounts generally for less than 20% of dissolved DNA pools [9, 13–16]. Quantitative estimates of the dissolved DNA pool in the water column thus reflect, to a large degree, extracellular DNA concentrations.

Besides quantitative estimates, other studies have specifically addressed questions about the production, degradation and cycling of extracellular DNA in aquatic ecosystems. For instance, by using radioactive precursors, Paul

et al. [17] showed that heterotrophic bacterioplankton was the major source of dissolved DNA, while actively photosynthesizing phytoplankton did not contribute to this pool. However, subsequent studies by means of dot-blot hybridization allowed researchers to identify the presence of phytoplankton genes (i.e. ribulose biphosphate large subunit gene, rbcL) in the dissolved DNA fraction of freshwater and seawater samples [18].

Turnover times of extracellular DNA, calculated on the basis of estimates of degradation rates, range from 6.5 to 25 h [6, 8], indicating that in a pelagic environment extracellular DNA is a highly reactive macromolecule in the dissolved organic matter pool. Although Paul and co-workers showed that extracellular DNA is mainly a source of exogenous nucleotides, recycled by bacteria for the synthesis of new DNA, other studies suggested that extracellular DNA can be an important source of organic N and P for bacterioplankton metabolism [19–22]. Dissolved extracellular DNA alone may supply about 50% of the daily P requirements and about 10% of the daily N requirements of bacterioplankton [19, 20], and can play an even more important role in P-depleted ecosystems [23, 24]. Extracellular DNA can also have implications in horizontal gene transfer [25–29].

As far as particulate DNA is concerned, conceptual models indicate that more than 70% of the total particulate DNA pool in surface oceanic waters is accounted for by DNA associated with prokaryotes in the pico-plankton fraction (i.e. 0.2–1.0 μm, *sensu* [13]). However, particulate DNA pools may also include an extracellular fraction of detrital DNA (i.e. DNA adsorbed onto detrital particles; Fig. 1).

This is particularly evident in marine sediments, where these proportions may be inverted, the detrital fraction being largely dominant over the en-

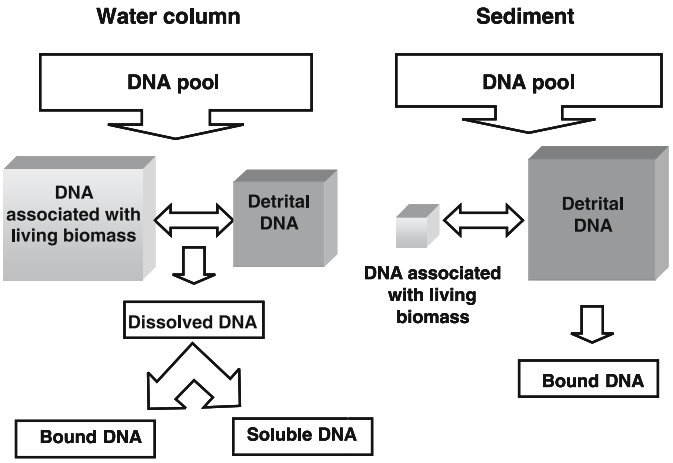

Fig. 1 Conceptual model of the different DNA pools in seawater (modified from [13]) and sediment (modified from [32, 60])

tire DNA pool. Particulate detrital DNA, estimated by the use of conversion factors, can account for a highly variable fraction of the total particulate DNA pool (0–93% [30, 31]). Estimates carried out on marine sediments highlighted that detrital DNA is the dominant component over the entire sedimentary DNA pool (up to 90% [1, 32, 33]). Concentrations of extracellular DNA in sediments from shallow depths down to the abyssal floor were 3 to 4 orders of magnitude higher than those in the water column. However, the use of conversion factors is questionable for providing accurate quantitative estimates of the relative importance of the extracellular DNA pool in different ecological compartments. Recently, Dell'Anno et al. [34] developed a new nuclease-based procedure for quantifying extracellular DNA concentrations in marine sediments. This procedure is highly specific for extracellular DNA, allowing one to obtain accurate quantitative estimates not biased by DNA contamination due to cell lysis or viral DNA. The results from this study clearly confirmed that extracellular DNA in marine sediments is the dominant fraction of the total sedimentary DNA pool.

The quantitative relevance of extracellular DNA in marine sediments is the result of complex interactions including DNA inputs from the photic layer through particle sedimentation, autochthonous DNA production, and degradation and/or utilization by heterotrophic organisms [35] (Fig. 2).

Extracellular DNA diagenesis in sediments is also influenced by DNA binding to complex refractory organic molecules and/or to inorganic particles, which protect DNA against nuclease degradation [36–38]. In this regard, Romanowski et al. [39] showed that DNA adsorbed on sand and clay becomes 100- to 1000-fold more resistant to DNase. Consequently, the half-life

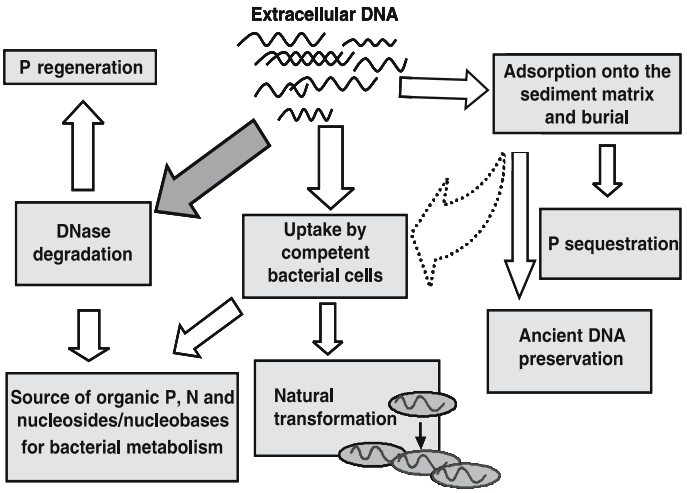

Fig. 2 Theoretical model of the fate and ecological significance of extracellular DNA in marine sediments

of extracellular DNA in sediments appears to be much longer than that in the water column [40]. The reduced degradability of extracellular DNA may explain why this molecule can also persist in deeper sediment layers (i.e. on geological timescales), thus representing a potential genetic marker of paleo-environments [35, 41, 42]. In fact, due to the higher resolving power of DNA sequences as compared to biomarkers, molecular characterization of ancient genetic material may improve the reconstruction of past communities and related paleo-environments. At the same time, since extracellular DNA production and accumulation in sediments represents a record of processes occurring in the pelagic and benthic domains at different temporal scales [32, 35, 41], analyses of sequences of structural and functional genes preserved in the extracellular DNA pool might provide new information about the ecological functioning of present-day ecosystems and paleo-ecosystems [34, 43].

Despite the overwhelming dominance of the detrital fraction in sedimentary DNA pools, in the last decade molecular techniques have been generally applied to the whole DNA pool, assuming that it is entirely associated with living biomass. The use of molecular techniques in the field of microbial ecology has greatly enhanced our understanding of the diversity and functioning of microorganisms in aquatic ecosystems [44], but questions related to potential biases due to the presence of extracellular DNA are still largely unsolved. Therefore, a crucial step for enhancing the accuracy of molecular tools is the development of reliable extraction methods able to separate intra- and extracellular DNA from a given environmental matrix.

2 Extraction and Quantification of Intracellular DNA in Water Samples

The standard approach utilized by most investigators for isolating particulate DNA is to concentrate cells on micropore membranes (0.2-μm pore size filters), after pre-filtration to avoid sample contamination with larger material, and then to lyse the cells retained on the filters [45, 46]. For example, Fuhrman et al. [45] described a method for DNA extraction based on filtration of marine and brackish waters (8–40 l) through 0.2-μm pore size filters. DNA was extracted directly from the filters in 1% sodium dodecyl sulphate (SDS) heated to 95–100 °C for 1.5–2 min. This procedure lyses essentially all bacterial cells and does not significantly denature the DNA, which is then purified by phenol extraction. DNA is quantified fluorometrically using Hoechst 33258 (i.e. a groove-binding DNA ligand that becomes brightly fluorescent when it binds to the double-strand form of the DNA).

Final yields are in the range of a few micrograms of DNA per litre and correspond, roughly, to 25–50% of the total bacterial DNA in the sample [45]. Although the bacterial community probably does not change during filtration,

the large volumes of the water samples and the extended time of filtration required by this procedure could restrict the application of molecular tools in extensive surveys of aquatic systems and in experiments with multiple treatments or repeated sampling.

These problems could be circumvented by alternative techniques, such as the use of cylindrical filter membranes [46], tangential-flow filtration (TFF) [47] or vortex-flow filtration [48]. The first of these techniques is largely utilized for concentrating microbial cells [46]. This technique requires a high-capacity cylindrical filter, through which water is pumped and in which cell lysis is finally achieved [46]. Lysozyme, SDS and proteinase K are used in this step. DNA from the lysate can be purified by ethanol precipitation or buoyant-density centrifugation and utilized for molecular studies.

Recent studies on aquatic microbial communities used small volumes of water for recovering DNA [49–52]. Kirchman et al. [51] developed a method based on filtration of 10 ml (or less) of seawater through a polycarbonate filter which is sectioned, and a section is directly amplified by the polymerase chain reaction (this method has been designated as "filter PCR"). Molecular analyses revealed little difference when comparing the 16S rRNA amplicons obtained by other techniques and the "filter PCR" protocol.

3
Isolation and Quantification of Dissolved DNA

During the last 15 years, the isolation and quantification of dissolved DNA from water samples has been addressed by several studies using different approaches [6, 7, 9–11, 17, 24, 53–58]. Filtration of water samples through 0.2-μm pore size filters is the first step required for the isolation and quantification of dissolved DNA from microbial cells and other particulate material.

Several authors [6, 10, 11] have utilized ethanolic precipitation to concentrate dissolved DNA from freshwater and seawater samples. Concentrated DNA was quantified by the fluorescence of dye–DNA complexes (using Hoechst 33258 dye). To correct for the fluorescence not caused by DNA or caused by packaged phage DNA, samples treated with DNase were measured in parallel. As an example, De Flaun et al. [10] found that up to 76% of the fluorescence remained after DNase treatment of estuarine and oceanic water samples. The effectiveness of this procedure for concentrating dissolved DNA was demonstrated by the efficient (> 90%) recovery of internal standards. Further purification by chromatography, polyvinylpolypyrrolidone (PVPP) treatment and CsCl buoyant-density centrifugation gave preparations of sufficient purity for determination of the molecular weight of the extracted DNA and for the detection of specific genes by hybridization [7, 11, 18, 57].

Ethanolic precipitation is widely used in molecular ecology, but is time-consuming and is limited by the specificity of Hoechst 33258 towards double-

stranded DNA. Karl and Bailiff [54] proposed an alternative technique based on addition of cetyltrimethylammonium bromide (CTAB) for concentrating dissolved DNA from water samples. The insoluble CTA-nucleic acid salts obtained are used to determine dissolved DNA concentrations by the fluorometric method using 3,5-diaminobenzoic acid (DABA). This procedure is compatible with rapid shipboard analyses, detects both single- and double-stranded DNA and, as opposed to ethanolic precipitation methods, proteins do not react with the precipitating agent.

Other compounds (such as diphenylamine, 4′-6-diamidino-2-phenylindole (DAPI), ethidium bromide and mithramycin) can, in theory, be used to quantify the dissolved DNA concentrated by the CTAB procedure. However, the investigation of Siuda and Güde [58] provided clear evidence that dissolved DNA concentrations were overestimated when determined by the CTAB–DAPI method in eutrophic freshwater samples. The CTAB technique, which causes the precipitation of DNA and other compounds [58], is not highly specific. This, together with the partial solubilization of various fluorescent components, might cause a significant alteration of the fluorescence during the assay [10, 58]. To avoid these problems, Siuda and Güde [58] estimated the DNA fraction that was hydrolysable by nucleases as the difference between the concentration of the DNA in samples with and without DNase treatment. The discrimination of the enzymatically hydrolysable DNA from the total dissolved DNA pool is important for a better understanding of the ecological role of extracellular DNA. In fact, the abundance of extracellular DNA in most aquatic systems makes it an important source of P and N, and/or nucleotides for aquatic microorganisms [59]. This discrimination can be achieved by using a nuclease-based procedure developed by Dell'Anno and Danovaro [60], in which extracellular DNA is cleaved into deoxynucleosides, which are then quantified fluorometrically by DABA or by HPLC. However, since this technique is based on nuclease hydrolysis of extracellular DNA, it does not allow the recovery of DNA for subsequent molecular studies, but only the quantification of the hydrolysis products (i.e. deoxynucleosides).

4 Intracellular DNA in Sediment Samples

Several protocols for DNA extraction from soils and sediments have been developed and improved in recent years [61–64], and a large effort has been devoted to purifying DNA and to enhancing DNA extraction efficiency [61, 62, 65, 66]. DNA extraction techniques usually involve a direct in situ lysis of cells and the subsequent release of DNA, and allow investigations of "community DNA" [67]. Other protocols involve the isolation of microbial cells from sediments prior to DNA extraction [68, 69]. Both these methods have advantages and disadvantages [66], but direct in situ lysis is more commonly

used, mainly because of its faster times of extraction and much higher DNA yields [66].

In situ cell lysis from sediments can be achieved by means of physical (e.g. bead-mill homogenization, ultra-sonication and freeze–thawing) or chemical procedures (e.g. using SDS or Sarkosyl [61, 66]), or a combination of both. Freeze–thawing [70–72] and bead-mill homogenization [69, 73, 74] are commonly utilized, although bead-mill homogenization yields more DNA than the freeze–thaw procedure [70, 74–76]. However, a larger amount of contaminating humic acids are recovered by bead-mill homogenization [75–77] and, in certain cases, it can increase DNA shearing [75]. Chemical lysis can be obtained using solutions that contain SDS [45, 64, 73, 78–80] or Sarkosyl [81, 82]. The modifications of these chemical techniques also include high-temperature (from 60 °C to boiling) incubation [69, 70, 73], a phenol [72, 76, 83] or chloroform [84] extraction step, and the incorporation of chelating agents (EDTA and Chelex 100) to inhibit nucleases and disperse soil/sediment particles [85].

DNA extraction can also utilize enzymatic lysis, which involves the use of lysozyme [72, 73, 81, 83, 84], proteinase K [79, 86, 87], chromopeptidase [88] or pronase E [81]. All these enzymes have been employed to promote cell lysis, but due the lack of comparative studies it is not clear whether the addition of the enzymatic lysis step to other extraction protocols increases DNA yields.

The purification of DNA from sediments can be achieved by agarose gel electrophoresis [64, 74, 79, 80, 89], Sephadex G-200 column chromatography [70, 71, 83, 84, 90] and silica-based DNA binding [64, 73, 74], used individually or in combination. The purification efficiency is usually estimated by the amount of DNA recovered and by the effectiveness of the methods used to remove any contaminant that might inhibit PCR and/or other enzymes utilized for molecular analyses [91].

5 Extracellular DNA in Sediment Samples

Although in situ lysis is the most commonly utilized technique for DNA extraction from sediments, with this procedure extracellular DNA is co-extracted with nucleic acids released from the lysed cells, possibly leading to misinterpretation of the composition of the target community derived from molecular analysis [92]. Discrimination between intracellular and extracellular DNA in marine sediments is essential to carry out simultaneous molecular studies of these two DNA fractions. However, until recently, the isolation of extracellular DNA from sediments was an unsolved task, because the available procedures for the extraction of nucleic acids adsorbed on organic and inorganic particles disrupt living cells [40, 64, 91]. An attempt has been made to isolate extracellular DNA from aquatic sediments [77]. This protocol involves

several washings of wet sediment samples with sodium phosphate, precipitation with ethanol and then purification by hydroxylapatite chromatography. However, this protocol has not been tested for possible contamination by intracellular DNA due to cell lysis during sediment handling. Moreover, the procedure of Ogram et al. [77] obtains extracellular DNA yields at least one order of magnitude lower than those obtained using the nuclease-based procedure of Dell'Anno and Danovaro [60]. Although extracellular DNA concentrations obtained by the nuclease-based procedure are not biased by DNA contamination due to cell lysis or by viral DNA [34, 60], this technique does not allow the recovery of DNA for subsequent molecular studies.

Recently, a new protocol has been developed to recover simultaneously DNA associated with microbial cells and extracellular DNA from the same sediment sample [93]. This protocol is an adaptation of the procedures extensively used for the isolation of microbial cells from sediments [67, 69, 94]. To date, this procedure has been applied only for extracting intact microbial cells from sediments, without considering the presence of extracellular DNA, which may be co-extracted. In order to recover simultaneously extracellular DNA and DNA associated with microbial cells, three washing steps of the samples using an isotonic solution of sodium phosphate buffer, supplemented with PVPP and low SDS concentrations (ten times lower than those generally used for in situ lysis), are required to improve the extraction efficiency of both extracellular and intracellular DNA pools. This protocol is suitable for molecular studies of extracellular DNA because it avoids any contamination by DNA released from cell lysis during handling and extraction, and provides adequate DNA yield and purity.

6 Degradation and Turnover of Extracellular DNA

Bacteria-mediated degradation of organic matter plays a key role in carbon cycling and nutrient regeneration in the world's oceans [39]. This process is largely mediated by extracellular enzymatic hydrolysis, which converts high molecular weight compounds into low molecular weight ones suitable for bacterial uptake [95–97]. The removal and cycling of extracellular DNA from marine environments occur through two basic mechanisms. The first is carried out by competent bacterial cells, which are able to internalize DNA fragments [40, 98]. The second is mediated by both cell-associated and free DNases, which are present in all aquatic environments and convert extracellular DNA into nucleosides and nucleobases [7, 57, 99]. This process is expected to be the main route for extracellular DNA cycling [40].

Nucleic acid turnover time can be defined as the ratio of the ambient (i.e. extracellular) DNA concentration and the velocity of its removal (i.e. degradation rates [6, 8]). The quantification of ambient extracellular DNA and

the measurement of its degradation rates [34] are, therefore, indispensable in order to provide accurate estimates of extracellular DNA turnover rates in seawater and sediment samples. Previous studies carried out in marine environments estimated degradation rates of DNA in aquatic environments by analysing: (1) the decrease of acid-precipitable labelled DNA [8, 57, 100]; (2) the conversion of supercoiled into relaxed-circular or linear plasmid DNA [101]; and (3) the loss of hybridization signals of plasmid DNA in Southern transfer or dot blots [53]. However, these methods have mainly been applied for understanding the survival of specific DNA sequences [25, 40]. Moreover, being only a minor fraction of culturable marine bacteria [102], estimates of DNase activity based on isolates do not reflect the actual degradation of extracellular DNA in marine systems.

A new procedure for estimating extracellular DNA degradation rates in marine systems (seawater and sediment) is based on fluorometric detection of nuclease activity by means of a fluorescent DNA analogue [poly(dεA), polydeoxyribo-1-N_6ethenoadenylic acid (6 in apice)] [103]. This method, which was developed for in vitro studies, is based on the increase of the fluorescence of poly(dεA) due to degradation of polynucleotides [104] and it is highly specific for detecting exonuclease activity [105]. By this procedure it is possible to quantify the amount of nucleotides released from the degradation of the fluorescent DNA analogue. The conversion of extracellular DNA into nucleotides represents the key step for the subsequent bacterial uptake of nucleosides and nucleobases [7, 57]. The results of these studies indicate that poly(dεA) is effectively degraded into etheno-monomeric residues (i.e. dεAMP), and that this procedure can be routinely utilized for estimating extracellular DNA degradation rates in marine environments. In addition, since quantitative estimates of extracellular DNA do not necessarily reflect its actual bioavailability [13, 34], in order to calculate accurate turnover rates of extracellular DNA in seawater and sediment samples it has been suggested that the actual bioavailable fraction of extracellular DNA (i.e. hydrolysable by nucleases; [60, 103]) should be quantified. Turnover estimates of the bioavailable fraction of extracellular DNA are important for clarifying bacterial utilization pathways of extracellular DNA and provide new elements for a better comprehension of the mechanisms controlling DNA preservation in aquatic environments.

7
Molecular Tools for the Analysis of Nucleic Acids in Marine Environments

A wide variety of molecular techniques can be utilized for the analysis of nucleic acids (both DNA and RNA) in the marine environment. The choice of different molecular tools is clearly dependent upon the ecological objec-

tive. Basically there are two possible approaches for studying target genes or genomes of aquatic microorganisms: the first consists of the identification of target gene sequences within intact cells, without any extraction step of nucleic acids, while the second is based on cell lysis and nucleic acid isolation and recovery. If the target is a gene sequence within cells, it is possible to use probes (both fluorescent and radiolabelled) for its hybridization. The most common technique in marine microbial ecology is fluorescent in situ hybridization (FISH) [106], which utilizes fluorescent probes to hybridize complementary rRNA sequences (see [107] for details). For prokaryotes, the FISH technique allows one to identify and count, by epifluorescence microscopy, specific target genera or groups (e.g. *Cytophaga*, α-, β- and γ-Proteobacteria, Archaea [106]).

PCR is now a routinely used tool in marine molecular ecology. Once extracted and purified, the nucleic acid is amplified by PCR (polymerase chain reaction, in the case of DNA) or RT-PCR (reverse transcription-PCR in the case of RNA), thus allowing the production in vitro of large numbers of identical copies of a specific nucleic acid sequence [107]. For this purpose, it is necessary to know the sequences of the regions (primers: usually 15–20 nucleotides in length) flanking the two ends of the gene target. The PCR reaction is a very powerful tool as the reaction can theoretically proceed with just one single copy of the gene.

However, a review dealing with PCR amplification and the associated difficulties indicates that PCR is not completely free from pitfalls [108]. For example, PCR is strongly limited by the availability of known sequences in the database, which limits the design of gene-specific PCR primers: this means that primers may not include all the relevant naturally occurring genes, leading to an underestimation of gene diversity. Some authors [109] showed that biases can occur in the amplification step, caused by template annealing of mixtures of 16S rRNA genes, and showed how such biases were strongly dependent upon the number of cycles of replication. A potential bias can be introduced by the formation of chimeric products or artefacts. Another problem deals with the inhibition of the polymerase activity. The presence of inhibitors can often cause false-negative reactions (i.e. a target gene is present but not amplified). This is a frequent problem when working with marine sediment samples, where high concentrations of potential inhibitors (e.g. humic acids) are present [88]. However, in recent years several techniques have addressed and solved this problem [41, 42].

PCR generally utilizes primers for the 16S rRNA gene but other genes or gene families can also be employed for studying microbial diversity and phylogeny. Moreover, PCR allows investigation of the presence of mobile genetic elements, such as bacterial plasmids (in both the water column and marine sediments; [110–112]). Plasmid-encoded genes are a pool of mobile DNA, which is known to contribute significantly to genetic adaptation of natural microbial communities.

PCR does not allow recovery of intact genes, but only portions of them and only one single gene can be investigated each time. Different molecular tools must, therefore, be used if the target is a multiple gene or the entire genome. The recently proposed DNA microarray (or microchip) technology provides a platform for genome-wide hybridization experiments that can be utilized for identifying DNA sequences, for comparing different genomes and for monitoring gene expression [113–116].

DNA microarrays consist of thousands of unique DNA sequences connected to a small, solid surface (such as a glass slide [114]). Fluorescent or radioactivity-labelled mRNA or DNA derived from mRNA by RT-PCR or genomic DNA can bind to these sequences, thus producing a pattern indicative of nucleic acid sequences that can be qualitatively and quantitatively analysed [114]. Microarray technology has the advantage of rapid detection, automation [116, 117] and lower costs than conventional membrane-based hybridization (such as FISH; [107]). These characteristics make microarray technologies extremely useful for molecular characterization of mixed populations of microorganisms and their biological functions. Microarray technologies can theoretically be utilized for screening for the presence and expression of both prokaryotic and eukaryotic genes.

Another method for characterizing nucleic acids and their molecular diversity in the environment employs bacterial artificial chromosomes (BACs). BACs are capable of sustaining DNA inserts larger than 300 kbp [114] and allow construction of libraries of community DNA ("metagenomic libraries"). The "metagenome" is the whole genome pool within an environmental sample [118]. Subsequent sequencing or hybridization of BAC libraries allows the phylogenetic and genomic analysis of the entire microbial assemblage. To date, metagenomic libraries have been constructed from environmental DNA recovered from terrestrial soils [118, 119] and biofilms in drinking waters [120].

All these approaches have been utilized for studying a wide variety of genes associated with the DNA of living cells, but they could also be used for studying genes potentially present in the extracellular DNA pool. There is only molecular study that has addressed concomitantly, but separately, both intracellular and extracellular DNA in sediment [93]. This is particularly important in marine sediments, where extracellular DNA is characterized by high molecular weights, and is apparently also well preserved in subsurface sediment layers [34, 60].

Despite the advancement in molecular techniques, several questions still remain unanswered. What is the contribution of extracellular DNA to the metagenome? Does extracellular DNA contribute significantly to horizontal gene transfer through natural transformation in marine sediments? What is the fate of genes potentially released by genetically modified organisms (GMO) in the marine environment? What information is contained in the extracellular DNA preserved in the deeper sediment layers?

We do not know if the extracellular DNA pool contains functional sequences and we have no information on its origin. The extracellular DNA could represent a sort of "unexplored gene pool". Molecular studies specifically examining extracellular DNA from different environments could improve our comprehension of mechanisms controlling the persistence of nucleic acid molecules in the marine environment.

8 Analysis of Microbial Diversity

The origin of the molecular approach for identifying microorganisms can be traced back to the early work of Zuckerkandl and Pauling in the 1960s [121] and later to Woese's advances in microbial phylogeny [122]. Pace and coworkers [123, 124] are, however, considered to be the first to appreciate the power of molecular phylogeny for studying the diversity of microbial communities in the environment. Traditionally, the approaches for a taxonomic identification of bacteria were based on the identification of metabolic, phenotypic and physiological traits [124]. This required that bacteria had to be isolated on agar before being identified. The discovery that often less than 1% of the microbes in a sample can grow on agar plates, known as a central dogma in aquatic microbial ecology, demonstrated the need for new methodologies and approaches that were able to resolve the complex and diverse array of species making up the "black box" of marine microbial communities.

The most common approach for studying prokaryote diversity in the marine environment is based on the 16S rRNA gene. The rationale is to use a phylogenetic approach for establishing evolutionary relationships among microorganisms, and to use this as a framework for making inferences of community structure and biodiversity [125]. This target gene is particularly useful for studying microbial biodiversity [126] because it is present in all prokaryotes [127], and contains diagnostic variable regions (together with highly conserved regions), which are unique to specific populations or closely related groups [125]. Moreover, rRNA genes are thought to lack inter-specific horizontal gene transfer, in contrast to many other prokaryotic genes [127]. The methods based on 16S rRNA genes involve DNA extraction from the sample (sediment or seawater), followed by a PCR step using universal or specific primers (i.e. targeting all prokaryotic microorganisms or specific taxa), and then screening of the PCR products by means of one of the following techniques:

1. Cloning and sequencing [128, 129]: the PCR product is cloned into vectors and then randomly chosen clones are sequenced and their sequences aligned with those presented in databases. This permits the identification of prokaryotes, by assigning clones a phylogenetic identity.

2. DGGE (denaturing gradient gel electrophoresis [130]): an electrophoretic method allowing the separation of DNA fragments having the same length but different nucleotide sequences, which become visible as separate bands. These bands can be recovered for further cloning and sequencing. This method allows a molecular fingerprint of the microbial community in a sample to be obtained.
3. T-RFLP (terminal restriction fragment length polymorphisms [131]): a semi-quantitative technique allowing the estimation of the number and relative abundance of microbial ribotypes (often defined as OTU, operational taxonomic units) in a sample. In brief, the method involves a PCR amplification with fluorescently labelled primers, followed by digestion with restriction enzymes and screening of the number and types of restriction fragments. Screening and sizing of fragments can be performed on high-resolution (± 1 base) sequencing gels or on capillary electrophoresis systems providing digital outputs. Each fragment represents a single microbial ribotype. When compared with DGGE, the T-RFLP method has the advantage of being more sensitive and reproducible, but does not allow the recovery (and thus the cloning and sequencing) of the final product. Alternatively, using automated rRNA intergenic spacer analysis (ARISA) it is possible to track the presence and abundance of putative phylotypes over time, and compare community structures.
4. SSCP (single strand conformation polymorphisms [132]), ARDRA (amplified rDNA restriction analysis [133]) and heteroduplex mobility assay [134] are methods that are used less frequently.

In addition, real-time PCR allows the quantification of specific genes encountered in a sample. This methodology is based on the use of fluorescence reporters, which allow monitoring of the PCR reaction in a continuum.

Although the most utilized molecular marker for studying microbial diversity in the marine environment is the 16S rRNA gene for prokaryotes [135], 18S rRNA is increasingly used for eukaryotic microorganisms [136]. New genes have been proposed for studying microbial diversity in the marine environment, with special attention on microorganisms involved in specific biogeochemical processes [137–140]. For instance, much effort has been devoted to the study of denitrifying bacteria through functional genes, such as cd1-nir and Cu-nir genes, which encode for two forms of nitrite reductase [141].

The gene nosZ (encoding for nitrous oxide reductase) has been used as a molecular marker for studies of microbial diversity of benthic denitrifying bacteria [142]. Other genes such as nitrogenase reductase (NifH), cytochrome cd1-containing nitrite reductase (NirS), and Cu-containing nitrite reductase (NirK) have been used for studying the biodiversity of denitrifying and dinitrogen-fixing bacteria in terrestrial soils [143]. The conserved photosynthetic psbA gene (coding for the protein D1 of photosystem II re-

action centre) has been utilized as a diversity indicator of marine oxygenic picophytoplankton, including cyanobacteria and eukaryotic algae [144]. The NH_3-monooxygenase subunit A gene (amoA) has been utilized for studying the diversity of ammonia-oxidizing bacteria [145]. Attempts to study the diversity of methanotrophic bacteria have been made by studying pmoA (a gene encoding the subunit of the particulate methane monooxygenase), mmoX (coding for subunits of soluble methane monooxygenase) and mxaF (methanol dehydrogenase; [146]).

The gene pufM (encoding the M subunit of the photosynthetic reaction centre) has been used for studying the diversity of anoxygenic phototrophs [147]. The diversity of autotrophic microorganisms can be assessed by studying genes encoding the ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) enzyme, and attempts have been made using the ribulose bisphosphate carboxylase/oxygenase form I gene (rbcL; [148]) and the RuBisCO form II cbbM gene [149].

These new insights gained from the molecular approach are opening fresh ecological perspectives on microbial biodiversity in the marine environment and its interactions with ecosystem functioning, and not only for prokaryotes. Moreover, these techniques are providing important elements in the field of evolutionary biology [150].

The tree of life has been significantly revised after the recognition of Archaea as the third domain of life. A three-domain model, rather than one based on five kingdoms, has been proposed [151]. In a few years, the number of known major divisions within the two prokaryotic domains, Bacteria and Archaea, has doubled [152]. Indeed, 36 divisions have already been identified within the domain Bacteria, and 13 of them are known only from phylotypes [152].

Investigations based on 16S rRNA genes have revealed a previously unexpected microbial diversity in almost all aquatic ecosystems. Novel and yet-uncultured phylogenetic lineages have been discovered to be widely distributed in the marine environment [153]. For instance, among heterotrophic bacteria, those belonging to groups such as the Proteobacteria and the Cytophaga–Flavobacteria cluster have been shown to be extremely common in many oceanic habitats, accounting for as much as half of all bacteria identified with molecular microscopic techniques (i.e. FISH [154, 155]). Indeed, these bacteria have been demonstrated to be important consumers of dissolved organic matter in aquatic environments [156]. Molecular methods have shown how bacteria belonging to new and uncultured bacterial divisions, such as the SAR11 cluster (a phylogenetic group within the α-Proteobacteria) or W6, are often numerically important components of the marine picoplankton [129]. Members of the bacterial kingdom Acidobacterium, which has only one cultured member, has been found to be widespread, being present in most marine and freshwater sediments worldwide [157].

Archaea have been discovered only very recently to be common in marine ecosystems [158]. Initially known as Archebacteria [159], they represented until a decade ago a small group of atypical prokaryotes inhabiting unusual or extreme niches, such as those at high temperature, high salinity, extreme values of pH and/or in strictly anaerobic niches [160]. Recently, a wide number of studies, based on sequencing and comparison of 16S rRNA prokaryotic genes, have radically changed our view of the Archaea, revealing their ubiquitous distribution and their capability of thriving in aquatic and terrestrial temperate environments [160]. Studies of microbial diversity have revealed archaeal ribotypes to be a significant component of marine picoplankton assemblages [158, 161, 162], and even to dominate the mesopelagic prokaryotic communities of the north Pacific Ocean [163].

Culture-independent studies have shown that marine Archaea, which belong to the kingdom Crenarcheota (one of the three recognized kingdoms of the Archaeal domain), can be one of the most abundant cell types in the global ocean [163]. Archaea have been widely reported to also inhabit the benthic domain, including continental shelf anoxic sediments [164], freshwater sediments [165], and deep-sea and hydrothermal vent sediments [126, 160, 166]. Indeed, Archaea have been shown to possess important functional roles in marine carbon cycling. In this regard, Ouverney and Fuhrman [167] suggested that free-living plankton marine Archaea are involved in the heterotrophic uptake of dissolved amino acids, with activities comparable to those of their bacterial counterparts.

9 Concluding Remarks

Molecular tools will be increasingly useful in the future for gathering additional information on marine biodiversity and ecosystem functioning that traditional biogeochemical markers could not detect. For instance, lipid markers are useful tracers of organic matter sources [168]; in particular fatty acids having a great diversity of structures have been used as indicators of photosynthetic activity in surface water and phytoplankton taxonomic composition [169]. The relative abundances of individual fatty acids are also useful in evaluating the respective importance of inputs from bacteria, microalgae, marine fauna and continental higher plants [170]. Isotope analysis has been shown to be a powerful technique for distinguishing the sources of such compounds and tracing their metabolic pathways within organisms and food webs [171]. Pigment analysis by HPLC has also proven to be a valuable method for evaluating phytoplankton biomass, providing essential information regarding taxonomy, food-chain relationships, zooplankton grazing and detritus formation [172]. However, the fact that many fatty acid biomarkers and phytopigment products can originate from more than one source empha-

sizes the need for caution in assigning their biological origins based only on one single approach.

Analyses of nucleic acids through a variety of molecular techniques are a useful complement to all these biochemical approaches, and provide additional insights into the specific origin of DNA encountered in the ecosystem and into the functioning of microbes that play a key role in biogeochemical cycles. Moreover, molecular tools for the analysis of DNA in marine environments can be successfully applied to fossilized organic components, thus providing an archive of ancient aquatic microbial communities. Hence, they can be used to reconstruct variations in climate and their impacts on biodiversity. The combined stratigraphy on lipid and DNA analyses is opening the opportunity to reconstruct the paleo-microbiology and hence the paleoecosystem functioning with unprecedented detail [173].

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