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Multi-marker eDNA metabarcoding survey to assess the environmental impact of three offshore gas platforms in the North Adriatic Sea (Italy)

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ABSTRACT

The environmental DNA (eDNA) metabarcoding represents a new promising tool for biomonitoring and environmental impact assessment. One of the main advantages of eDNA metabarcoding, compared to the traditional morphotaxonomy-based methods, is to provide a more holistic biodiversity information that includes inconspicuous morphologically non-identifiable taxa. Here, we use eDNA metabarcoding to survey marine biodiversity in the vicinity of the three offshore gas platforms in North Adriatic Sea (Italy). We isolated eDNA from 576 water and sediment samples collected at 32 sampling sites situated along four axes at increasing distances from the gas platforms. We obtained about 46 million eDNA sequences for 5 markers from nuclear 18S V1V2, 18S V4, 18S 37F and mitochondrial 16S and COI genes that cover a wide diversity of benthic and planktonic eukaryotes. Our results showed some impact of platform activities on benthic and pelagic communities at very close distance (< 50 m), while communities for intermediate (125 m, 250 m, 500 m) and reference (1000 m, 2000 m) sites did not show any particular biodiversity changes that could be related to platforms activities. The most significant community change along the distance gradient was obtained with the 18S V1V2 marker targeting benthic eukaryotes, even though other markers showed similar trends, but to a lesser extent. These results were congruent with the AMBI index inferred from the eDNA sequences assigned to benthic macrofauna. We finally explored the relation between various physicochemical parameters, including hydrocarbons, on benthic community in the case of one of the platforms. Our results showed that these communities were not significantly impacted by most of hydrocarbons, but rather by macro-elements and sediment texture.

1. Introduction

The environmental DNA (eDNA) metabarcoding is emerging as a new promising tool for biodiversity surveys and environmental impact assessments (EIA) (Baird et al., 2012; Bohmann et al., 2014; Valentini et al., 2016). It consists in analysing the diversity of a target group of organisms based on their DNA isolated from water, soil and/or sediment samples (Taberlet et al., 2018, 2012). The eDNA is a mixture of genomic DNA present in living cells as well as preserved in cellular organelles or as extra-cellular molecules in tissue fragments, organic secretions or other biological materials. By using high-throughput sequencing (HTS) and bioinformatic pipelines, it is possible to obtain and analyse millions of DNA sequences present in environmental samples (Bàlint et al., 2016).

There are multiple advantages of using eDNA metabarcoding compared to traditional methods based on visual observation of organisms and morphology-based identification. The most commonly mentioned arguments supporting the adoption of DNA-based methods are the costs and time-effectiveness of automated molecular protocols and the lack of taxonomic expertise, which is necessary in classical biodiversity surveys (Leese et al., 2018; Pawlowski et al., 2018). Another major advantage of metabarcoding is the possibility to expand the range organisms used for EIA, by including various inconspicuous, mainly microbial and meiofaunal taxa, and taking advantage of their genetic variations (Pawlowski et al., 2016). Compared to the conventional approaches that are focused on selected morphologically identifiable biological

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quality elements (fish, macro-invertebrates, diatoms), the eDNA metabarcoding can provide information about all organisms that are present at a given ecosystem. Such global assessment provides overview of ecosystem functioning at different trophic levels that can be particularly valuable for environmental managers and stakeholders involved in EIA.

The current applications of eDNA metabarcoding are still largely limited to academic studies. The results of these studies led to spectacular advances in our knowledge of global patterns of diversity in aquatic ecosystems, including marine plankton (de Vargas et al., 2015), freshwater invertebrates (Deiner et al., 2016). Practical applications of metabarcoding in marine environment concern mainly the benthic monitoring of fish farms (Cordier et al., 2017, 2018a; Dowle et al., 2015; Keeley et al., 2018; Lejzerowicz et al., 2015; Pawlowski et al., 2014; Pochon et al., 2015; Stoeck et al., 2018b, 2018a). The results of these studies have shown general congruence between biotic indices inferred from metabarcoding and morphology-based macro-invertebrates data (Lejzerowicz et al., 2015; Pawlowski et al., 2016; Pochon et al., 2015; Stoeck et al., 2018b, 2018a), and led to the development of new methods predicting biotic indices from metabarcoding data (Cordier et al., 2018a, 2018b, 2017).

The eDNA metabarcoding has also been applied to the EIA of offshore oil and gas platforms, either in relation to measuring the impact of oil spills (Bik et al., 2012; Brannock et al., 2016; Coelho et al., 2016; Smith et al., 2015), or as a complementary tool for benthic monitoring of offshore platforms (Lanzén et al., 2016; Laroche et al., 2018b, 2018a, 2016, 2017). These studies demonstrate the strengths of metabarcoding as a tool to detect the changes in bacterial and eukaryotic benthic communities in relation to oil hydrocarbon pollution, either in natural (Bik et al., 2012; Smith et al., 2015) or controlled laboratory conditions (Coelho et al., 2016; Frontalini et al., 2018). These studies also explore the possibility to extend the range of bioindicator taxa by using metabarcoding of foraminifera (Laroche et al., 2016), or other eukaryotes, including meiofauna (Lanzén et al., 2016) as complement to traditional macrofauna-based benthic monitoring. However, up to our knowledge, only one study (Laroche et al., 2018b) proposed a global, multi-taxa approach to assess the impact of offshore oil and gas platforms in New Zealand.

Here, we use eDNA metabarcoding to assess the impact of three offshore gas platforms on eukaryotic biodiversity in the North Adriatic Sea (Italy). This semi-enclosed basin is strongly affected by the land-based industrial and agriculture activities as well as those related to gas production (Alessio Gomiero et al., 2011a). Rapid expansions of drilling activities have taken place in the central and northern Adriatic Sea since the 1960s with the construction of more than 110 offshore gas platforms representing ca. 90% of the offshore platforms existing in the Mediterranean Sea (A. Gomiero et al., 2011b; Scarcella et al., 2011; Spagnolo et al., 2014). Several investigations have been performed to evaluate the impact of platforms along the Italian coast using a wide range of bioindicators like fish, macrofauna, and polychaetes (i.e. (Manoukian et al., 2010; Punzo et al., 2017, 2015; Scarcella et al., 2011; Spagnolo et al., 2014), as well as biomarkers and bioassays (i.e., (Alessio Gomiero et al., 2011a; Gomiero et al., 2015, 2013; Gorbi et al., 2008; Tornambè et al., 2012)). However, none of these studies used eDNA metabarcoding to assess the impact of gas production in the Adriatic Sea.

The objective of the present study is to obtain a global overview of benthic and pelagic eukaryotic biodiversity in the area of three gas platforms. To achieve this aim, we generated eDNA metabarcoding data from 288 water and 288 sediment samples. We hypothesized that potential impacts related to drilling activities would remain in the close vicinity of the platform structure, and that these impacts would be mostly detectable in the sediment. In total, we analysed about 46 million DNA sequences coming from five genetic markers spanning the full benthic and pelagic eukaryotic community. The taxonomic description of these communities, as well as results of alpha and beta-diversity analyses are presented and discussed in relation to the potential impact of platforms.

2. Material and methods

2.1. Sampling

The sampling stations were chosen according to the 'gradient design' approach that is suitable when stressor or disturbance grades with the distance from the point source of impact (Ellis and Schneider, 1997). Three offshore gas platforms, namely Agostino B, Garibaldi A, and Armida were considered. For each platform, 32 sites along four axes at 0 m, 25 m, 50 m, 125 m, 250 m, 500 m, 1000 m and 2000 m were sampled in July–August 2017. For each station, three replicates of water samples and three replicates of sediment samples were collected. The total sum of 288 water and 288 sediment samples were analysed. Samples were labelled as platform (Agostino B: AB, Garibaldi A: GA, and Armida: AA), axes (A1: north, A2: west, A3: south, and A4: east), and distance from the structure (0, 25, 50, 125, 250, 500, 1000, and 2000). Sampling locations were determined with Global Position System (Table S1).

Sediment samples were collected using a box core. Three replicates of about 10 g of surface sediment were sampled using sterile spoon and placed in a tube with DNA preservation solution (LifeGuard, MOBio). Samples were kept frozen until processing. For one of the platforms (Armida), additional sediment samples were collected to measure various physico-chemical parameters, including sediment texture, macro-elements and a set of 16 hydrocarbons compounds.

Water samples were collected using Niskin bottles (2–3 litters). Each sample was filtered with glass microfiber filters (Whatman, GF/F, No. 1825-025). The filters were housed in sterile filter cases and half a liter of sea water was pushed through the filters using 50 ml sterile syringes. Once a day during sampling, negative control using sterile distilled water were performed. After filtration, each filter was rolled with tweezers, placed in a sterile 1.5 mL tube and stored at −20 °C until DNA extraction. All equipment used during filtration was treated with 10% household bleach and sterilized with a 30-min UV treatment.

2.2. eDNA extraction, PCR amplification and high-throughput sequencing (HTS)

For water samples, each filter was extracted using the DNeasy Blood and Tissue kit (Qiagen) with an incubation in the lysis buffer for 48 h at 56 °C. The kit was used according to the manufacturer's instructions excepted for the final DNA elution that was done in 100 µl instead of 200 µl.

Sediment samples were extracted using the DNeasy Power Soil Kit (Qiagen). For each sediment sample, three extractions were performed. For each extraction, 500 µl of sediment (suspended in lifeguard preservation buffer) were treated according to the manufacturer's instructions with bead beating at 6,5 m/s for 45 s in high-speed homogenizer (MP biomedicals).

For each DNA extraction session (water and sediment samples), negative controls were included to exclude possible contamination of the DNA samples during extraction. DNA extracts were stored at −20 °C until PCR amplifications.

We used five genetic markers to explore the full taxonomic range of eukaryotic biodiversity. We selected markers that are commonly used in biodiversity survey to cover eukaryotic diversity as much as possible and to test whether they would provide a similar pattern of variation. These markers included: mitochondrial cytochrome oxidase 1 gene (COI, the BOLD standard), mitochondrial 16S small subunit of rRNA gene for vertebrates (Kitano et al., 2007), and three hypervariable regions of nuclear 18S rRNA gene: V1V2 (mainly used for meiofaunal zoobenthos, (Fonseca et al., 2010), V4 (standard marker for targeting planktonic eukaryotes, Tara Ocean), and 37 + 41F (targeting benthic

foraminifera). The sequences of primers used for amplification of each marker and the conditions of PCR amplifications are indicated in Table S2. Tagged primers bearing 8 nucleotides attached at each primer's 5′-extremity were used to enable the multiplexing of PCR products in sequencing libraries (Esling et al., 2015).

PCR reactions were performed in three replicates for each water sample, including filtration negative controls. For the sediment samples, one PCR reaction was performed for each of the three extraction replicates. PCR negatives controls were included in each session to ensure that no contaminations occurred. PCR products were verified by agarose gel electrophoresis. Then, the PCR products obtained for the three sediment extraction replicates as well as the three water PCR replicates were combined for each sample.

The pooled replicate PCR products for each sample were quantified by high-resolution capillary electrophoresis using QIAxcel System (Qiagen). Equimolar pools of PCR products were performed for each library. Each library was purified using High Pure PCR Product Purification kit (Roche Applied Science) and the libraries preparation was performed using Illumina TruSeq® DNA PCR-Free Library Preparation Kit. The libraries were then quantified with qPCR using KAPA Library Quantification Kit and sequenced on a MiSeq instrument (Illumina) using paired-end sequencing for 600 cycles with kit v3. The raw data was submitted to the SRA public database under the accession PRJEB29469.

2.3. Bioinformatics

Raw sequencing data were quality-filtered by removing any sequence with a mean quality score below 30, as well as all sequences with ambiguous bases or any mismatch in the tagged primer. These stringent parameters ensured to keep only high-quality data. Then, paired-end reads were merged by aligning them into a contiguous long-length sequence using the VSEARCH v2.8 toolkit (Rognes et al., 2016), with a minimum overlap of 40 bp and five mismatches allowed. Potential chimeras were removed by using the default settings of the UCHIME *de novo* algorithm (Edgar, 2010). Filtered reads were then dereplicated and clustered into OTUs using the SWARM v2.1.8 algorithm (Mahé et al., 2015), with the default resolution ($d = 1$). OTUs representative sequences were used as input of the *assign_taxonomy.py* function of the QIIME v1.9.1 toolkit (Caporaso et al., 2010) with default parameters for taxonomic assignment (*uclust* method, Edgar, 2010). Representatives sequences were compared against curated reference sequence database for taxonomic assignments. For the nuclear ribosomal 18S (benthic V1V2 and water V4), we used SILVA v128 (Quast et al., 2013). For the COI marker (both water and sediment), we used the MIDORI v1.1 database (Machida et al., 2017). Finally, for the mitochondrial 16S, we used the GenBank database. Matrices with OTUs as rows and samples as columns were generated for each of the five markers analysed and served for downstream statistical analyses in the R programming environment (R Core Team).

2.4. Statistics

Samples with less than 10000 reads were discarded as well as rare OTUs (below 100 reads). To investigate the variation of alpha-diversity metrics with distance from the platforms, we rarefied 100 times our matrices at a 10000 reads sequencing depth using the *rrarefy* function of the vegan package (Oksanen et al., 2016), and averaged the OTU richness, the Shannon and the Chao index. The effect of distance on these averaged diversity metrics were then tested using linear models. Difference of diversity between platform and axis were also tested.

In order to investigate the variation of composition, the OTUs matrices were then normalized to account for variation in sequencing depth across samples, using the cumulative-sum scaling method (CSS) implemented in the metagenomeSeq v1.16.0 R package (Paulson et al., 2013). We investigated communities' structure with Non-metric MultiDimensional Scaling (NMDS) ordinations by using Bray-Curtis pairwise dissimilarity matrices as input of the *metaMDS* function of the vegan package, with default settings. Ordinations were then plotted with the sampled stations grouped in three classes of distance, e.g. close to platforms (0, 25 and 50 m), intermediate (125 and 250 m) and reference (1000 and 2000 m). We also investigated whether communities could be different between axes. We tested for significant difference of communities with nested models in Permutational Analysis of Variance (PERMANOVA) using the *adonis* function of the vegan R package (Oksanen et al., 2016) using the *strata* option to constrain permutations within each platform to test for difference between axes and along the distance gradient.

Finally, we investigated the effect of various sediment physico-chemical parameters on benthic communities for the Armida platform, by fitting environmental vectors to the ordinations using the *env_fit* function of the vegan R package. The *p*-values for both *adonis* and *env_fit* functions were computed by permuting the data 999 times. We used the BIO-ENV procedure (Clarke and Ainsworth, 1993), with the *bioenv* function of the vegan package, to select the subset of environmental variables (up to 6 variables) that produces an Euclidian matrix that best correlates (best R^2 in mantel Spearman test) the communities Bray-Curtis dissimilarity matrix.

From the V1V2 OTUs matrix, we extracted OTUs assigned to benthic macro-invertebrates to compute the AMBI index (Borja et al., 2000) using the BBI R package (Cordier and Pawlowski, 2018). This biotic index, among others (review in (Pawlowski et al., 2018), is a widely used measure of ecological quality through organic enrichment in marine environments. The AMBI values were plotted against distance to the platforms to investigate the effect of platform activities on the organic enrichment of the marine sediments. These values were also used to make an interpolation map of the surrounding of the three platforms, using the Inverse Distance Weighted (IDW) method implemented in the ArcMap v10.2 software (see (de Mesnard, 2013)).

3. Results

3.1. Sequencing data

The total amount of raw sequences generated for this study was 100,409,268, from which 33,268,657 were kept for downstream analysis after stringent quality filtering (Table S3). The OTU-to-sample of the matrices, including the taxonomic assignments of the OTUs are available in Tables S4–S9.

3.2. Taxonomic composition of sedimentary eDNA

3.2.1. 18S V1V2 (zoobenthos)

No PCR products was detected in the negative controls. The VIV2 dataset is dominated by metazoans (Fig. 1, 34% of reads). It also comprises a broad range of different groups of single-cell eukaryotes (protists). Among them, the most abundant are the super-groups of Stramenopiles (18%, mainly diatoms) and Rhizaria (12%, mainly cercozoans). Many of them live in the water column and it is difficult to distinguish those representing benthic and planktonic communities. Hence, our analyses of V1V2 dataset focus on metazoans (zoobenthos). The V1V2 assemblage of metazoans comprises all common macro-invertebrate taxa, such as annelids, molluscs, nemerteans, cnidarians and echinoderms (Fig. S1). The assemblage also consists of large number of meiofaunal taxa mainly represented by copepods, gastrotrichs, nematodes and Platyhelminthes. The sequences of minor phyla, such as calcareous sponges, kinorhynches and acoeles also remain unassigned to lower taxonomic level.

The distribution of macro- and meiofaunal species varies from station to station. Most of taxonomic groups are present in all stations, even though some of them are clearly absent or very rare in stations close to the platform. In particular, this is the case of Brachiopoda and

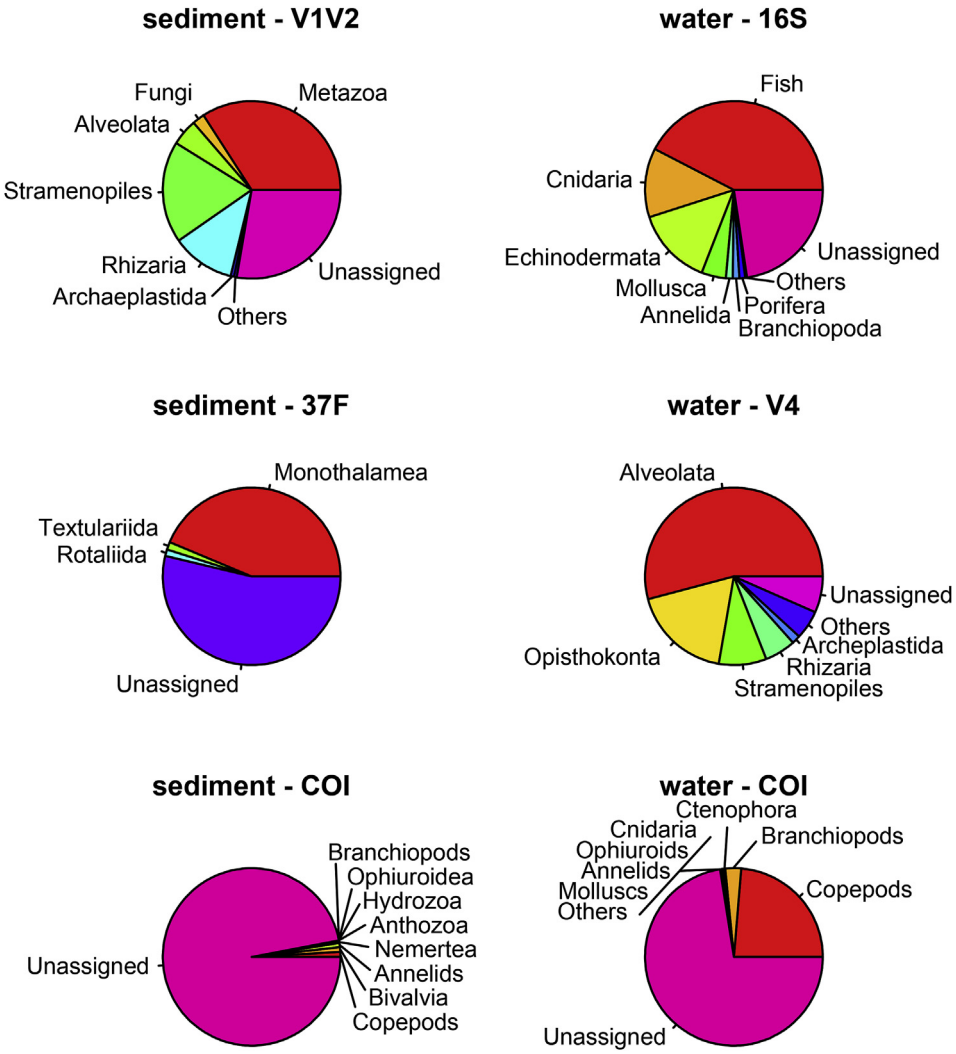

Fig. 1. Taxonomic composition of benthic, planktonic and pelagic communities obtained with different markers in relative abundance of the total amount of good-quality reads.

Echinodermata, whose DNA is absent at 0 m and 25 m stations. This is also the case of Anthozoa (soft corals) much rarer and generally absent from stations close to the platforms. Nevertheless, the metazoan assemblage in these stations is quite variable. It is generally dominated by annelids and copepods, but it also contains large proportions of nematodes in Agostino, gastrotrichs in Armida and molluscs and gastrotrichs in Garibaldi.

3.2.2. COI (zoobenthos)

No PCR products was detected in the negative controls. The COI marker is here used to identify marine zoobenthos (Fig. 1, Fig. S2). Although the large majority of OTUs (97%) remains unassigned, all assigned OTUs are identified to species level. Among these OTUs that could be assigned using GenBank/BOLD database, the most abundant are assigned to *Corbula gibba* (Bivalvia), *Acartia clausii* (Calanoida), *Pseudomystides limbata* (Polychaeta) and *Hubrechtella dubia* (Nemertea).

3.2.3. 18S 37f/41f (benthic foraminifera)

No PCR products was detected in the negative controls. The eDNA assemblage of foraminifera comprises 1116 OTUs assigned to 50 morphospecies. Like in other metabarcoding studies, the foraminiferal assemblage is dominated by reads assigned to the organic-walled and agglutinated monothalamous taxa (Fig. 1, Fig. S3). Many of monothalamids are undescribed, which explains the large number of unassigned species.

All common Adriatic and Mediterranean coastal foraminiferal species are present in our datasets. The monothalamiids are dominated by genera *Micrometula, Bathysiphon, Vellaria, Saccammina, Hippocrepinella*, and *Psammophaga* as well as several unidentified allogromiid species. The dominant rotaliids genera are *Ammonia, Bulimina, Nonionella, Epistominella*, and *Stainforthia*, while the most represented textulariid species are *Leptohalysis scotti* and *Textularia gramen*.

3.3. Taxonomic composition of water eDNA

3.3.1. 18S V4 (Planktonic eukaryotes)

No PCR products was detected in the negative controls. In total, 1416 OTUs represented by more than 100 reads for V4 dataset are retained for analysis. Compared to other markers, the majority of V4 OTUs are already present in the database, however most of them are represented by an environmental sequence, not assigned to any particular morphospecies.

The V4 dataset is largely dominated by alveolates (mainly Dinophyceae) represented by more than 50% of all reads (Fig. 1, Fig. S4). Among them the most abundant are *Gyrodinium* and *Scripsiella* genera. Another very abundant group is copepods dominating the major part of zooplankton. We also identified DNA of other planktonic metazoans including cnidarians, ctenophores and tunicates. Some

benthic metazoans are also present in water DNA, although their diversity is much lower compared to V1V2 dataset from sedimentary DNA. The distribution of high-level taxa in V4 dataset does not significantly changed among stations and platforms, the proportion of taxonomic groups remains stable (Fig. S2).

3.3.2. COI (Zooplankton and animals)

Some PCR products were detected in the negative controls. We sequenced those controls and removed the OTUs (representing 323575 reads, 0,3% of the total) from the dataset. Like in the case of sedimentary DNA, the COI marker provides very limited taxonomic information about planktonic eukaryotes (Fig. 1, Fig. S5). Only 11 OTU could be assigned to the known species. Among them, 8 represent arthropods, dominated by copepod genera *Paracalanus* and *Pseudodiaptomus*. In addition, we identify COI sequences of hydrozoan *Obelia dichotoma*, ctenophore *Mnemiopsis leidyi* and ophiuroid *Ophiotrix fragilis*.

3.3.3. 16S rRNA (Vertebrates)

No PCR products was detected in the negative controls. The mitochondrial 16S marker targets vertebrates and some marine invertebrates. The 16S data assigned to bacteria have been removed from the analyses. We also removed sequences of humans and non-marine domestic mammals as well as birds that originate most probably from food products and contamination.

After removing these sequences, the 16S dataset was dominated by marine fish (Fig. 1, Fig. S6) represented by 52 assigned species. Among them, the highest number of reads is obtained for such species as seabream (*Boops boops*), Atlantic bonito (*Sarda sarda*), bluefish (*Pomatomus saltatrix*), European anchovy (*Engraulis encrasicolus*), sardine (*Sardina pilchardus*), and mackerel (*Trachurus* sp.). Remarkably, among other vertebrates the DNA sequences of striped dolphin (*Stenella coeruleoalba*) and loggerhead sea turtle (*Caretta caretta*) were recognized.

The 16S datasets also contain DNA of common marine macro-invertebrates, dominated by pelagic medusae (*Zanclea giancarloi, Aurelia limbata, Obelia geniculata*), as well as common benthic species, such as genera of bivalves of genera *Venerupis* and *Chlamys*, branchiopodes *Pleopis*, sea urchin *Abatus*, and sponge of genus *Mycale*.

3.4. Alpha-diversity patterns

The linear models showed that OTU richness and diversity in the sediment were not significantly different among platforms, axes, and along the distance gradient for the V1V2 and the COI markers (Table 1). For the 37F marker, significant differences in OTU richness ($p = 0.037$) and Shannon diversity ($p = 0.023$) were detected, with the closest stations to the platforms showing commonly a less enriched and diversified foraminiferal communities (Table 1, Fig. S7).

Regarding the water samples, highly significant difference in richness and diversity were detected among platforms, axes, and to a lower extent along the distance gradient to the platform (Table 1).

Table 1
Analysis of variance of alpha-diversity metrics for the six markers.

Marker	Treatment	OTU richness	Shannon	Chao	AMBI
sediment - V1V2	Platform	0.189ns	0.165ns	0.606ns	0.443ns
	Axis	0.329ns	0.891ns	0.079ns	0.837ns
	Distance	0.822ns	0.113ns	0.729ns	**0.001*****
sediment - 37F	Platform	0.060ns	**0.043***	0.183ns	–
	Axis	**0.014***	**0.001*****	**0.005****	–
	Distance	**0.037***	**0.023***	0.056ns	–
sediment - COI	Platform	0.171ns	0.497ns	**0.048***	–
	Axis	0.365ns	0.917ns	0.154ns	–
	Distance	0.392ns	0.406ns	0.241ns	–
water - V4	Platform	**0.004****	**0.001*****	0.058ns	–
	Axis	**0.005****	0.082ns	0.001***	–
	Distance	0.161ns	0.239ns	0.134ns	–
water - 16S	Platform	**0.001*****	**0.001*****	**0.001*****	-
	Axis	**0.001*****	**0.001*****	**0.001*****	-
	Distance	0.630ns	0.844ns	0.838ns	–
water - COI	Platform	0.094ns	**0.007****	0.204ns	–
	Axis	**0.006****	**0.013***	**0.011***	–
	Distance	**0.011***	0.051ns	**0.005****	–

3.5. Beta-diversity patterns

The normalization of the matrices did not allow to completely remove an uneven sequencing depth bias (Table S10). Rarefaction were also performed but could not alleviate the bias neither (Table S10). However, given the extremely low R^2 value (from 0.01 to 0.03), we performed the downstream analysis on the normalized version of the matrices. The NMDS ordinations plots showed a relatively similar trends across the three markers for the sediment samples, with the stations close to the platforms forming a separate group from the intermediate and the references stations for each of the three platforms (Fig. 2). This was particularly evident for V1V2 and COI, while 37F showed a more overlapping pattern among groups of samples (Fig. 2). Water samples showed a more variable pattern. Agostino samples coming from the three distance classes overlapped, while Armida and Garibaldi tended to have relatively similar pattern as for the sediment samples, i.e. the close samples forming a separated group from the more distant ones. Even though the samples close to the platforms were relatively separated from the others ones, the variation within each class of distance was high (the size of the hull on the NMDS), corresponding to the compositional variability between platforms and axis and between axis. This means that the distance to the platform only explains partially the compositional variation of communities captured by our sampling. This trend was confirmed by the results of the PERMANOVA nested models (Table 2). Indeed, R^2 values showed that, even if the distance explained the most the compositional variation among the models terms, the unexplained variation (residuals) was still dominant, with R^2 values ranging from 0.58 to 0.69. This residual variation may correspond to both natural variation of communities (patchiness) at the scale of our sampling area, and sampling effect. However, at a given sampling station, the variation between replicates of sediment samples or water samples was usually much lower than the variation captured at the scale of an axis of a platform or at the scale of a platform (Fig. S8).

3.6. Benthic invertebrates index

From the total of 4,752,529 V1V2 reads kept after filtering for the eukaryotic V1V2 marker of the sediment samples, 1,329,657 (28%) matched benthic macrofaunal taxa for which an ecological weight is available in the AMBI index database. The values of the AMBI index were highly variable between samples, ranging from 0 (no reads assigned to benthic macrofauna) to 6 (all the reads assigned to benthic macrofauna were to a pollution bioindicator). However, on average, the AMBI index showed a highly significant correlation with distance to the platform, while remaining similar between platforms and axis (Table 1, Fig. 3, Fig. 4). AMBI values were the highest at the closest stations to the platforms, which mean that these stations were the most subject to organic enrichment as well as other stressors (Fig. 3). The AMBI values quickly decreased with distance to reach a plateau 50 m away from the platforms (Fig. 3).

The interpolation map of the AMBI index within the geographical context of the three platforms showed a relatively high variation between locations close to one another. However, on average, the stations close to the platform were the most organically polluted (Fig. 4).

3.7. Physicochemical parameters variation and their effect on benthic community composition

For the Armida platform, the physico-chemical parameters, including macro-elements, sediment texture and a set of 16 hydrocarbons

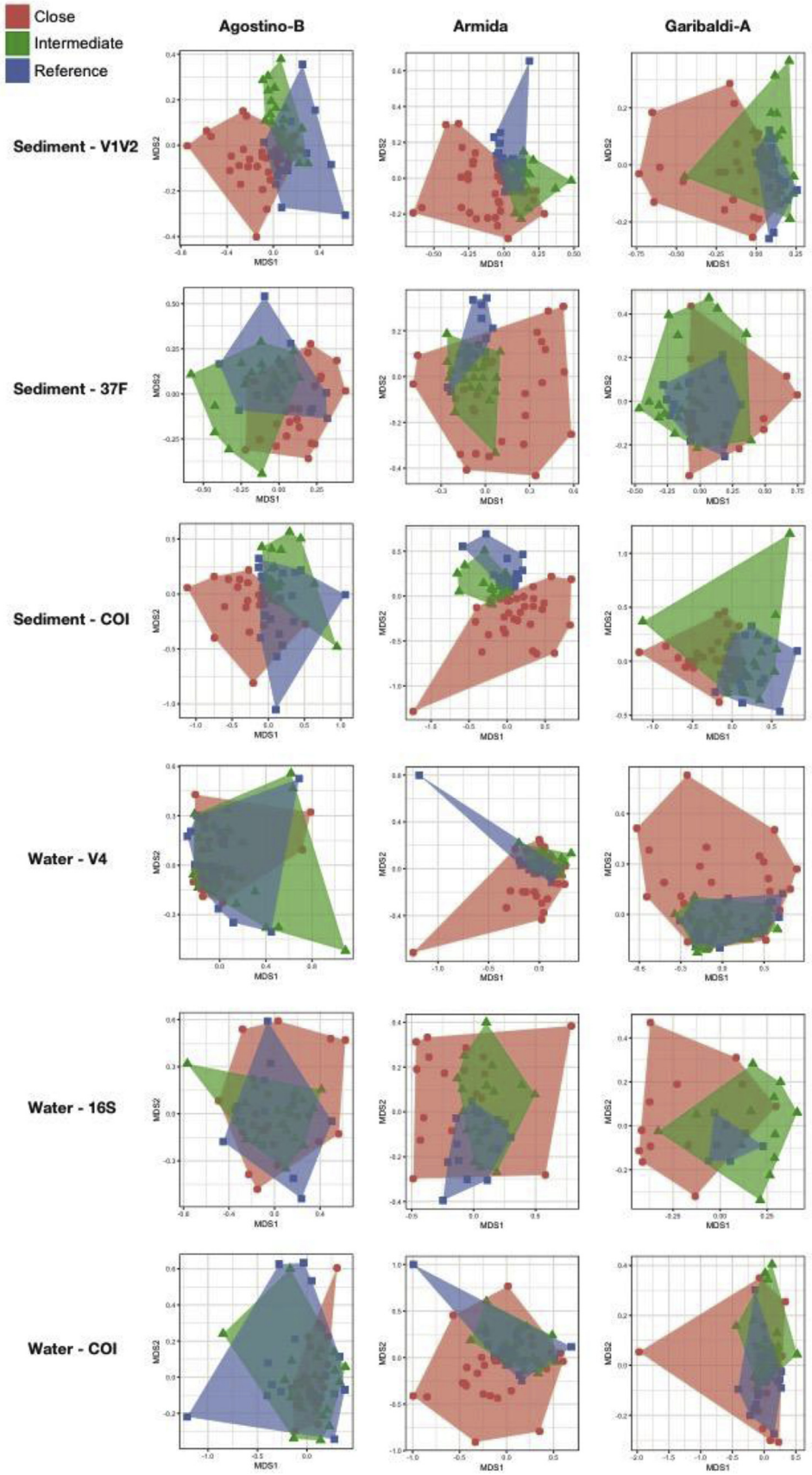

Fig. 2. NMDS plots of Bray-Curtis dissimilarity matrices for water and sediment samples from 3 platforms showing the differences in communities between close, intermediate and reference sites.

Table 2
Permutational analysis of variance of Bray-Curtis dissimilarity matrices for the six markers in nested PERMANOVA models. The difference between platform was tested within simple one-term models while for the axes and distance, we constrained the permutations within each platform using the strata option of the adonis function.

			Adundance data				Binary data			
Marker	Treatment	Df	MeanSqs	F.Model	R^2	P	MeanSqs	F.Model	R^2	P
sediment - V1V2	Platform	2	1.14638	13.3602	0.09208	0.001***	0.90158	12.7275	0.08909	0.001***
	Platform/Axis	9	0.21064	2.4549	0.07614	0.001***	0.17009	2.4011	0.07563	0.001***
	Platform/Axis/Distance	24	0.19436	2.2651	0.18734	0.001***	0.15247	2.1524	0.18079	0.001***
	Residuals	187	0.08581		0.64444		0.07084		0.65448	
	Total	222			1.00000				1.00000	
sediment - 37F	Platform	2	1.76290	15.9556	0.13180	0.001***	1.38003	14.1436	0.12059	0.001***
	Platform/Axis	9	0.25513	2.3091	0.08583	0.001***	0.21264	2.1793	0.08362	0.001***
	Platform/Axis/Distance	24	0.20455	1.8513	0.18351	0.001***	0.16942	1.7363	0.17765	0.001***
	Residuals	145	0.11049		0.59886		0.09757		0.61815	
	Total	180			1.00000				1.00000	
sediment - COI	Platform	2	1.85066	10.4794	0.07744	0.001***	1.55517	10.1401	0.07627	0.001***
	Platform/Axis	9	0.31042	1.7578	0.05845	0.001***	0.24770	1.6151	0.05467	0.001***
	Platform/Axis/Distance	24	0.33744	1.9108	0.16945	0.001***	0.27525	1.7947	0.16199	0.001***
	Residuals	188	0.17660		0.69466		0.15337		0.70706	
	Total	223			1.00000				1.00000	
water - V4	Platform	2	2.40288	32.645	0.16994	0.001***	1.65389	27.8334	0.15023	0.001***
	Platform/Axis	9	0.35122	4.772	0.11178	0.001***	0.26693	4.4922	0.10911	0.001***
	Platform/Axis/Distance	24	0.12560	1.706	0.10659	0.001***	0.09766	1.6436	0.10645	0.001***
	Residuals	235	0.07361		0.61168		0.05942		0.63421	
	Total	270			1.00000				1.00000	
water - 16S	Platform	2	0.92654	8.0130	0.08957	0.001***	0.76822	7.2123	0.08402	0.001***
	Platform/Axis	8	0.37250	3.2215	0.14405	0.001***	0.31338	2.9421	0.13710	0.001***
	Platform/Axis/Distance	21	0.18234	1.5770	0.18510	0.001***	0.15074	1.4152	0.17311	0.001***
	Residuals	104	0.11563		0.58128		0.10652		0.60577	
	Total	135			1.00000				1.00000	
water - COI	Platform	2	1.97967	16.4017	0.12262	0.001***	1.52869	14.4237	0.11129	0.001***
	Platform/Axis	9	0.29476	2.4421	0.08216	0.001***	0.23176	2.1868	0.07593	0.001***
	Platform/Axis/Distance	24	0.17976	1.4893	0.13361	0.001***	0.14873	1.4033	0.12993	0.001***
	Residuals	177	0.12070		0.66162		0.10599		0.68285	
	Total	212			1.00000				1.00000	

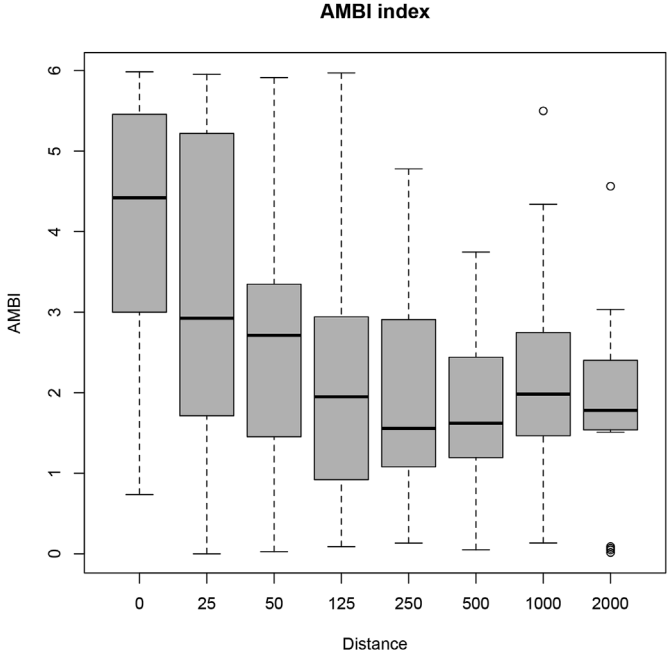

Fig. 3. AMBI values computed from benthic macro-invertebrates obtained from the eDNA V1V2 metabarcoding dataset as a function of distance to the three platforms. The vertical axis indicates the AMBI scale, from 0 (very bad ecological state) to 6 (very good ecological state).

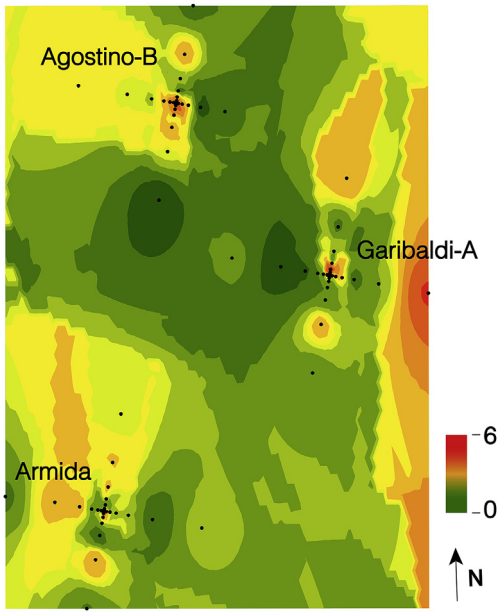

Fig. 4. AMBI values interpolation map of the surrounding of the three sampled platforms using the Inverse Distance Weighting method. The scale indicates the range of AMBI values obtained from the benthic macro-invertebrates OTUs extracted from the eDNA V1V2 metabarcoding dataset.

molecules, were available. We tested for difference between axis and along the distance to the platform for each of the parameters category (macro-elements, sediment texture and hydrocarbons) as well as when including all the available parameters with PERMANOVA analysis on Euclidian matrices (Table S11). This analysis showed that hydrocarbons compounds were not significantly differently present between axis and along the distance to the platform. Also, the environmental parameters

Table 3
Results of sediment physicochemical parameters fitting on NMDS ordinations for the Armida platform. R^2 values are given and significant correlated variables are in bold. The best subset of parameters selected by the BIO-ENV procedure are underlined.

Parameters	V1V2	37F	COI
Al	**0.2299*****	**0.2820*****	**0.2175*****
As	**0.0763***	**0.1743****	0.0240
Cd	0.0500	**0.1553****	**0.0988***
Cr	**0.2132****	**0.2120*****	**0.2406*****
Pb	0.0358	0.0555	0.0042
Ni	**0.1954****	**0.3140*****	**0.1828****
Cu	0.0410	**0.1380***	0.0567
V	**0.2557*****	**0.2711*****	**0.2571*****
Zns	**0.2852*****	**0.3826*****	**0.3614*****
Fe	**0.3001*****	**0.3213*****	**0.2099*****
Hg	**0.0977***	0.0379	**0.0802***
TOT.PAH	0.0240	0.0361	0.0057
TOC	**0.3000*****	**0.2916*****	**0.2867*****
clay	**0.2695*****	**0.2465****	**0.2131*****
silt	**0.3904*****	**0.3143*****	**0.4500*****
mud	**0.4158*****	**0.3322*****	**0.4257*****
sand	**0.3484*****	**0.3724*****	**0.2848*****
gravel	**0.4098*****	**0.3043*****	**0.4316*****
Fluoranthene	0.0299	0.0190	0.0063
Naphthalene	**0.0797***	**0.2639*****	**0.0941***
Anthracene	0.0582	0.0004	0.0139
Benzo[a]pyrene	0.0139	0.0149	0.0051
Benzo[e]acephenanthrylene	0.0359	0.0558	0.0132
Benzo[k]fluoranthene	0.0327	0.0294	0.0105
Benzo[ghi]perylene	0.0708	**0.0953***	0.0608
Acenaphthene	0.0009	0.0050	0.0122
Fluorene	0.0049	**0.1419****	0.0001
Phenanthrene	0.0110	0.0782	0.0015
Benzo[a]pyrene	0.0338	0.0306	0.0081
Benzo[a]anthracene	0.0228	0.0220	0.0043
Chrysene	0.0533	0.0630	0.0176
Dibenz[a,h]anthracene	0.0180	0.0206	0.0092
Indeno(1.2.3-C.D)pyrene	0.0402	0.0364	0.0166
Hydrocarbon C10—C40	**0.0987***	**0.1592****	**0.1377****

were generally not significantly different between axis, except for sediment texture. Instead, both the concentration of macro-elements and sediment texture were different along the distance to the platform (Table S11, Figs. S9–S11).

These environmental parameters were fitted on the ordinations for each of the three markers amplified from the sediment samples (Table 3). Among the 16 tested hydrocarbons compounds, only the Naphthalene, Fluorene, Benzo[ghi]perylene and the C10—C40 hydrocarbon ratio showed a significant correlation with the variation of benthic communities. These molecules seemed to have the strongest impact, i.e. higher R^2, on the foraminiferal communities. However, the variation of benthic communities was in general more correlated to variation in macro-elements and sediment texture rather than hydrocarbons pollutants (Table 3).

When performing variable selection using the BIO-ENV procedure, three variables seemed to be the most important for benthic communities. These variables included the zinc concentration in the sediment, the total organic matter content (TOC) and Naphthalene. These three variables were indeed selected for at least two markers, 37F and COI in the case of Zn, and the V1V2 and 37F in the case of TOC and Naphthalene. The fitting of the selected variables on the ordinations showed that macro-elements, sediment texture and hydrocarbons were only partially explaining the variation between class of distances to the platform (Fig. 5) for both V1V2 and 37F marker. For the COI marker, the Zn content alone was the best environmental vector to explain the ordination.

4. Discussion and conclusion

In this paper, we report an extensive eDNA dataset obtained from the area surrounding three offshore platforms in the North Adriatic Sea (Italy). By analysing water and sediment samples, using five different genetic markers, we provide a global survey of marine eukaryotic biodiversity, ranging from pelagic fish, to benthic macroinvertebrates and various groups of planktonic and benthic protists. Compared to other offshore drilling sites surveys (Lanzén et al., 2016; Laroche et al., 2018b, 2016), our sampling design allows us to obtain data for three very densely sampled sites with 32 stations per platform. We also analysed both sediment and water eDNA samples, while the previous studies only surveyed benthic diversity.

Taking together various components of marine biodiversity revealed by our study, we do not observe any striking changes in richness and taxonomic composition along the increasing distance from the platforms. In sediment samples, the alpha diversity patterns show significant differences only in the case of benthic foraminifera, whose assemblage was less rich and diverse in the stations closest to the platforms; the result that was confirmed by microscopic analysis of foraminiferal assemblage from the same sites (Frontalini et al., in prep). Interestingly, we also observed significant differences in richness and diversity of planktonic and pelagic communities between platforms and sampling axes. These differences could be ascribed to water mass movement or to the time effect, as the samples from different platforms were collected during the period of more than one month.

The patterns of beta-diversity across different taxa also show relatively limited impact of platforms (Fig. 2). Although the closest stations tend to be different from intermediate and reference stations, the distance to the platform does not explain most of the compositional variation. This is particularly true for plankton and pelagic communities, which seem less affected by distance than benthic communities. However, compared to other studies, the observed changes in benthic communities along the distance are relatively small. For example, Laroche et al. (2018a,b) show clear distinction of bacterial and foraminiferal communities between near- and far-field stations, situated at less or more than 100–200 m from the rig, depending on the site. In our study, the transition between communities is situated at about 50 m from the rig.

There are several lines of evidence that the impact being is limited to 50 m zone. As mentioned above, both sediment and water eDNA samples show distinct communities in close stations (< 50 m), compared to intermediate and distant stations (Figs. 2 and 3). However, the extent of these differences depends largely on the platform and the marker. The Armida platform shows much higher difference between communities, compared to Agostino and Garibaldi ones. These differences seem also more significant in benthic communities compared to the plankton or pelagic fauna. However, the most compelling evidence comes from the analysis of AMBI index, which is based on the occurrence of benthic macro-invertebrates' species extracted from the V1V2 dataset. Because of the limited taxonomic assignments obtained with the COI marker (probably due to gaps in the curated database), we could not compute the AMBI index with the COI dataset. The values of AMBI index obtained with the V1V2 dataset shows high correlation with distance to the platform, indicating poor to bad conditions close to the rig (0–25 m) and poor to moderate conditions at 50 m (Table 1). These results are consistent with morphological study of benthic macroinvertebrates conducted on other platforms in the Adriatic Sea that also show the change of AMBI values related to the distance (Spagnolo et al., 2014). Interestingly, the difference of AMBI values observed in our eDNA data is much more significant than in morphology-based study (Spagnolo et al., 2014), possibly due to limited number of macrofauna specimens that have been morphologically analysed. An additional evidence for the 50 m impact zone is brought by microscopic analysis of benthic foraminifera from the same sites (Frontalini et al. in prep). Although, we did not screen bacterial diversity as it has been done by

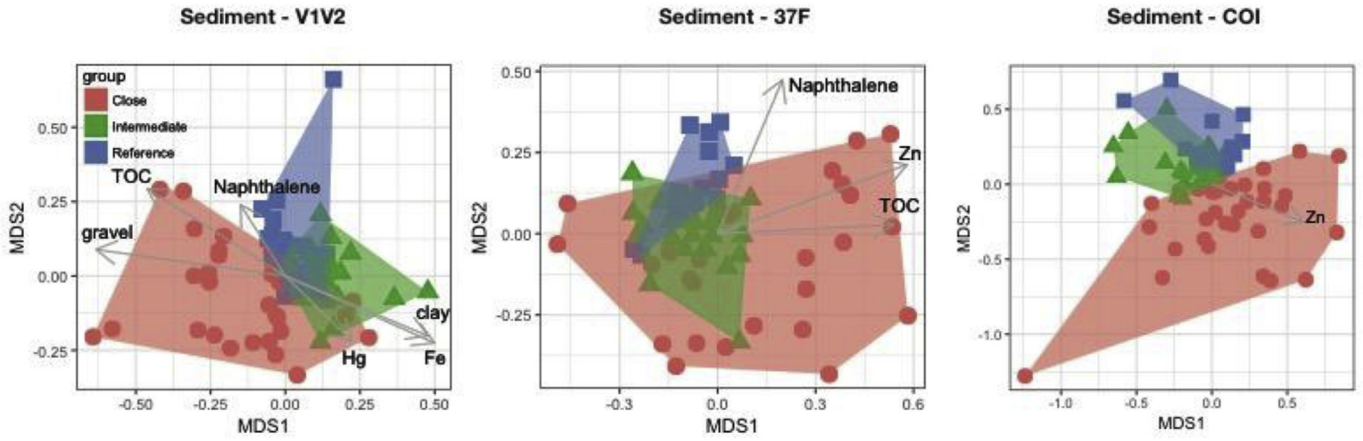

Fig. 5. NMDS ordinations of three molecular markers generated from the sediment samples collected in the vicinity of the Armida platform. The arrows represent the selected physico-chemical parameters that best explained the pairwise benthic community dissimilarity matrix using the BIO-ENV procedure.

Laroche et al. (2018b), we assume that adding bacteria would have led to the same conclusions.

The results of eDNA analyses are also congruent with physico-chemical analyses. Focusing on Armida platform, for which physico-chemical parameters were available, we found that several parameters are significantly correlated with the variation of benthic communities. High correlations were observed for trace elements such as Zn, Fe, Ni, Cr and Hg, as well as for few hydrocarbon compounds (e.g. Naphthalene). However, compared to chemical elements, the correlation of benthic communities with sediment texture and organic matter (TOC) seems much more significant. This may suggest that observed differences in benthic communities at 50 m zone result from different sediment properties in the vicinity of the platform rather than from hydrocarbon pollution.

Our study confirms the usefulness of eDNA metabarcoding to detect biodiversity changes in the environment. Our selection of markers shows that universal eukaryotic, ribosomal ones (here V1V2 for the sediment and V4 for the water) yield more taxonomically assigned OTUs (Fig. 1) than mitochondrial or foraminiferal ones. Such result can likely be explained by the fact that eukaryotic 18S reference sequences are largely represented in sequences databases. On one hand, this is of particular interest when the aim is to compute biotic benthic indices from those assigned sequences, using the AMBI or any other biotic indices formula. On the other hand, all of the taxonomic marker tested here appears suitable for conducting a biodiversity survey that would focus solely on communities' variation. However, we think that universal eukaryotic markers are most likely the best choice at hand when it comes to biomonitoring, because they combine high taxonomic spectrum, relatively high taxonomic assignments yield, and are easier to PCR amplify than single-copy mitochondrial markers.

Even if the level of chemical pollution was relatively low, the variation of benthic and pelagic communities detected in eDNA data may reflect their cumulative effects and allowed here to delineate an impact zone. In our case, this zone was very close to the platforms (50 m), indicating their limited and possible indirect impact on marine biodiversity. However, more research is needed to draw general conclusions about the level of pollution in this area. Up to our knowledge no comparable eDNA metabarcoding study was conducted in the North Adriatic Sea. There is general lack of eDNA data that would allow us to establish baseline conditions for unimpacted reference sites. Future eDNA studies would need to include such reference sites and also take in consideration seasonal changes that may have considerable impact on marine biodiversity.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marenvres.2018.12.009.

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