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Artificial Seaweed Substrates Complement ARMS in DNA Metabarcoding-Based Monitoring of Temperate Coastal Macrozoobenthos

Barbara R. Leite [1,2,3], Sofia Duarte [1,2], Jesús S. Troncoso [3,4] and Filipe O. Costa [1,2,*]

1 Centre of Molecular and Environmental Biology (CBMA) and ARNET-Aquatic Research Network, Department of Biology, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal
2 Institute of Science and Innovation for Bio-Sustainability (IB-S), University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal
3 UVIGO Marine Research Centre (CIM-UVIGO), ECIMAT Marine Station, Toralla Island, S/N, 36331 Vigo, Spain
4 Department of Ecology and Animal Biology, Marine Sciences Faculty, University of Vigo, Campus Lagoas-Marcosende, 36310 Vigo, Spain
* Correspondence: fcosta@bio.uminho.pt

Abstract: We used DNA metabarcoding to compare macrozoobenthic species colonization between autonomous reef monitoring structures (ARMS) and artificial seaweed monitoring systems (ASMS). We deployed both substrates in two different locations (Ría de Vigo and Ría de Ferrol, NW Iberian coast) and collected them after 6, 9, and 12 months to assess species composition of the colonizing communities through high-throughput sequencing of amplicons within the barcode region of the mitochondrial cytochrome c oxidase I (COI-5P) and the V4 domain of the 18S rRNA genes. We observed a consistently low similarity in species composition between substrate types, independently of sampling times and sites. A large fraction of exclusive species was recorded for a given substrate (up to 72%), whereas only up to 32% of species were recorded in both substrates. The shape and structural complexity of the substrate strongly affected the colonization preferences, with ASMS detecting more exclusive crustacean and gastropod species and a broader diversity of taxonomic groups (e.g., Entoprocta and Pycnogonida were detected exclusively in ASMS). We demonstrate that despite the customary use of ARMS for macrozoobenthos monitoring, by using ASMS we complemented the recovery of species and enlarged the scope of the taxonomic diversity recorded.

Keywords: substrate type; artificial reef monitoring structures (ARMS); artificial seaweed monitoring system (ASMS); environmental DNA; COI; 18S

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1. Introduction

Species interactions within marine communities are responsible for the maintenance of a biological network (i.e., producers, predators, and decomposers) highly important in ecosystem processes (e.g., energy flow, primary and secondary production, nutrient recycling [1,2]). However, the functioning and ability of marine ecosystems to provide services can be severely compromised due to the effects of global impacts (e.g., multiple stressors, and other human pressures [3,4]).

The Lusitanian biogeographic province [5] constitutes a particularly interesting spot for marine research since it harbors a high diversity of macrofauna from various adjacent regions, and many species have their northern or southern range limits in this area [6]. Monitoring these communities is particularly relevant to assess the impact of global change on marine biodiversity and ecosystems (e.g., shifts in species range expansions or alteration of dispersal patterns) and changes in species interactions [1,7].

However, large-scale biodiversity assessment and hard-bottom community sampling are challenging, mostly due to difficulties in the assessment and retrieval of organisms for identification [8,9]. Implementing innovative and standardized methods is, thus, essential [10,11] to make species data more accessible and facilitate spatial-temporal comparisons [12]. Technical advances in monitoring through the implementation of innovative molecular approaches [13], namely DNA metabarcoding, provide an opportunity to rapidly improve the accuracy and throughput for marine biodiversity assessment and monitoring [14,15]. The combined employment of artificial substrates together with DNA metabarcoding, may be a valuable replicable and standardized methodology to monitor marine macrozoobenthos, using a more cost-effective and less challenging approach (i.e., faster and with greater throughput and accuracy) [16]. Although artificial substrates have already been used to promote colonization and monitor marine communities (e.g., [17–19]), the implementation of such a strategy in large-scale comparisons is difficult due to the low level of standardization of the monitoring methodologies [20].

Autonomous reef monitoring structures (ARMS), originally developed to mimic coral reef diversity, have a structure with cavities influenced by high and low light spaces and various flow regimes [21]. These characteristics provide shelter for small invertebrates (e.g., protecting against predation) and surfaces for sessile organisms' settlement [22,23]. Deployed over the long term, ARMS allow to assess and interpret the diversity, distribution, and structure of hard-bottom marine communities [24], and has been frequently applied in the assessment of diversity in a variety of geographic regions (e.g., Caribbean and Indo-Pacific [25], Singapore [26], Europe [27], Iberian Coast [28]). An artificial seaweed monitoring system (ASMS) is an alternative artificial substrate that mimics macroalgae, and that has been previously employed to study macrozoobenthos colonization in Ría de Ferrol (NW Iberian Peninsula) [29,30]. Using morphological approaches to compare the taxa composition between dendritic substrates (ASMS) and ARMS deployed side-by-side, the authors concluded that the substrates supported different and complementary macrofauna assemblages [30]. The observed differences reflect the differential attractiveness of the more complex tridimensional structure of ASMS to shelter a distinct set of species, particularly highly mobile fauna [30].

Thus, the combined monitoring using ARMS and ASMS could potentially provide more comprehensive and comparable assessments of a broader spectrum of the biodiversity of hard-bottom communities. However, the research effort required for such a monitoring scheme using morphology-based approaches would be probably too demanding and logistically unfeasible. Alternatively, the employment of DNA metabarcoding for species identifications would allow high-throughput monitoring and greater accuracy, including the capability to discriminate cryptic species, or specimens that can be damaged during sample processing, as well as taxa more recalcitrant to identification through morphology [11,18,31–33]. Despite the increasing implementation of ARMS in different geographical locations for hard-bottom marine monitoring using molecular approaches [27,34], the comparison between different types of artificial substrates and their influence on the assessment of macroinvertebrate species has not been performed yet. In this study, we compared the macrozoobenthic species colonizing ARMS and ASMS, in order to investigate the impact of their shape complexity on the recovery of species and the thoroughness of coastal monitoring. To this end, we deployed ARMS and ASMS side-by-side in two locations on the NW Iberian coast, and using DNA metabarcoding we monitored changes in species composition at three time points over a period of 12 months.

2. Materials and Methods

2.1. Study Area

This study was carried out at two locations on the NW Iberian Atlantic coast: Bajo Tofiño (42°13′42.3″ N 8°46′43.2″ W, Ría de Vigo, Spain) and San Cristovo (43°27′53.8″ N 8°18′00.7″ W, Ría de Ferrol, Spain). Ría de Vigo and Ría de Ferrol are fully marine environments structurally composed of semi-enclosed bays, which include both hard and soft

substrata and have high primary productivity [35–37]. Both regions are busy harbors and ports directly impacted by human activities (e.g., sewage runoff or harvesting [38,39]).

2.2. Sampling Design

We selected two types of artificial substrates: ARMS (Figure 1a), which are small, tiered platforms, composed of 9 piled-up plates (23 × 23 cm) of grey type I PVC separated by spacers and affixed to the seafloor, and ASMS (Figure 1b), which are plastic commercial artificial plants (IKEA, Delft, The Netherlands), with 28 cm height and composed by green polyethylene with a complex structure formed by different orientation of the plant branches (Figure 1). Three replicates of the two substrates were deployed in June 2018 anchored to a cement plate (60 × 60 cm) and fixed to the bottom (approximately 11 m of depth), in the two study sites. After 6, 9, and 12 months of deployment, one replicate of each substrate was collected in both study sites. Previous studies have shown that one replicate processed by metabarcoding, and using two molecular targets, is sufficient to capture the same, or higher, macrozoobenthos species diversity when compared with the cumulative number of species detected using triplicates and morphology-based assessments e.g., [33,40]. Therefore, one replicate of each substrate was used at each site/sampling time combination.

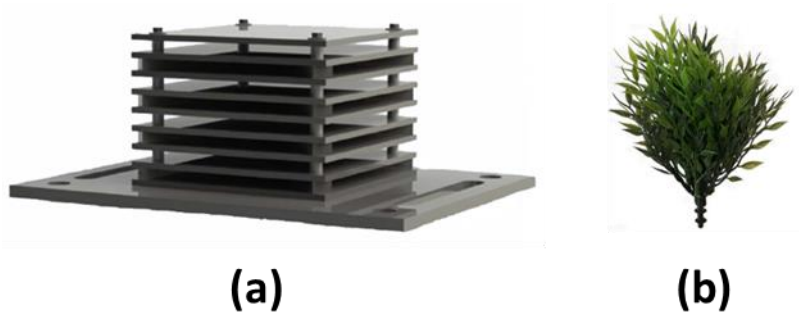

Figure 1. Artificial substrates used for marine macrozoobenthic colonization: (**a**) ARMS and (**b**) ASMS.

2.3. ARMS and ASMS Collection and Processing

In order to limit the loss of motile organisms, ARMS were enclosed in a labeled plastic box by scuba divers that lifted it onto the boat. For ASMS, each sample was carefully enclosed in a 500 µm mesh bag and then introduced in a hermetic plastic bag before being released from the substratum with a scraper.

At the laboratory, samples were photographed and then processed. We disassembled ARMS plate by plate following the procedure of Leray and Knowlton [22], and each branch of ASMS was also detached and processed individually. Then, each sample (plate or branch) was carefully washed using filtered sea water and shaken vigorously, and the representative mobile and sessile fauna were separated. The mobile fauna was brushed and sieved (500 µm). After collecting the mobile fauna, the sessile fauna was scraped with a spatula into a tray. The water in the container of each substrate was also sieved (500 µm), and the retained organisms were preserved with mobile fauna. All samples were then preserved in absolute ethanol and stored at −20 °C until further analysis.

2.4. DNA Metabarcoding

Samples collected from both locations after 6 (T1), 9 (T2), and 12 (T3) months were used to assess the species composition of the colonizing communities in both substrates, through high-throughput sequencing (HTS) of amplicons from the mitochondrial cytochrome c oxidase I (COI) and the 18S rRNA (18S) genes.

The mobile and sessile fauna were processed, amplified, and sequenced individually. DNA extraction procedures were adapted from Ivanova et al. [41] silica-based method, as described by Steinke et al. [42]. Ethanol-preserved samples were first filtered to retain the biomass and the ethanol was discarded. Then, based on the wet weight of each sample [42], an appropriate volume of a lysis buffer solution was added (100 mM NaCL, 50 mM Tris-

HCL pH 8.0, 10 mM EDTA, 0.5% SDS) and each sample was incubated overnight at 56 °C, while gently mixed on a shaker (60 rpm). Negative controls were included through all DNA extraction procedures. To maximize diversity recovery, two aliquots of each lysate were used, totaling two DNA extractions per sample. After extraction, the aliquots of genomic DNA for the same sample were pooled together.

The production of amplicon libraries and the HTS were carried out at Genoinseq (Cantanhede, Portugal), as described below. For COI, the primer pair mICOIintF (5′-GGWACWGGWTGAACWGTWTAYCCYCC-3′) [43] and LoboR1 (5′-TAAACYTCWGGRTGWCCRAARAAYCA-3′) [44] was selected to amplify an internal segment with 313 bp. The primer pair TAReuk454FWD1 (5′-CCAGCASCYGCGGTAATTCC-3′) and TAReukREV3 (5′-ACTTTCGTTCTTGATYRA-3′) [45,46] was used to amplify 400 bp of the V4 region of the 18S rRNA gene. PCR reactions were performed using KAPA HIFI HotStart PCR Kit according to manufacturer instructions, 0.3 μM of each PCR primer and 50 ng of template DNA in a total volume of 25 μL. For the mICOIintF/LoboR1 primer pair, the PCR conditions involved a 3 min denaturation at 95 °C, followed by 35 cycles of 98 °C for 20 s, 60 °C for 30 s, and 72 °C for 30 s and a final extension at 72 °C for 5 min. For the TAReuk454FWD1/TAReukREV3 primer pair, the PCR conditions involved a 3 min denaturation at 95 °C, followed by 10 cycles of 98 °C for 20 s, 57 °C for 30 s, and 72 °C for 30 s and 25 cycles of 98 °C for 20 s, 47 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min.

Second PCR reactions added indexes and sequencing adapters to both ends of the amplified target region according to manufacturer's recommendations [47]. PCR products were then one-step purified and normalized using SequalPrep 96-well plate kit (ThermoFisher Scientific, Waltham, MA, USA) [48], pooled and pair-end sequenced in an Illumina MiSeq® sequencer with the V3 chemistry, according to manufacturer's instructions (Illumina, San Diego, CA, USA).

PCR amplification failed with the COI primers for the following samples and fractions: T1, for ARMS in Ría de Ferrol (both mobile and the sessile fauna) and in Ría de Vigo (sessile fauna), and ASMS in Ría de Vigo (mobile fauna); T2, for ARMS in Ría de Vigo (sessile fauna) (Table S1). With the exception of T1 for ARMS in Ría de Ferrol, where only 18S data were produced, for all other samples at least one macrozoobenthic fraction (i.e., either sessile or mobile) was successfully recovered with COI. Therefore, for all samples, except T1/ARMS/Ferrol, we opted to use merged lists of species obtained together with 18S and COI, in subsequent analyses.

2.5. Data Processing

Raw reads, extracted from Illumina MiSeq® System in fastq format, were size- (<100 bp for COI region and <150 bp for 18S) and quality filtered to remove sequencing adapters (PRINSEQ v.0.20.4 [49]). Bases with an average quality lower than Q25 in a window of 5 bases were trimmed. The filtered forward-R1 and reverse-R2 reads were merged (make.contigs function) by overlapping pair-end reads using mothur 1.39.5 [50,51] and primers sequences were removed (trim.seqs function).

For COI, the usable reads were then processed and submitted to mBrave-Multiplex Barcode Research and Visualization Environment (www.mbrave.net, accessed on 28 August 2020, [52]) for filtering (maximum 313 bp, minimum 150 bp, QV > 10) and subsequent queries using the sample batch function which is linked with the Barcode of Life Data System (BOLD) [53]. Curated regional reference libraries e.g., [54,55] were given priority for taxonomic assignments in mBRAVE. Reads were taxonomically assigned at species level using 97% similarity threshold and were only retained for further analysis when an OTU was composed of at least eight sequences.

The 18S usable reads were processed and quality controlled (maximum 400 bp, >150 aligned nucleotides, <2% ambiguities or homopolymers, 50 alignment identity, 40 alignment score), aligned using the SILVA Incremental Aligner (SINA v1.2.10 for ARB SVN (revision 21008) [56] against the SILVA SSU rRNA SEED, and analyzed in SILVAngs database (https://ngs.arb-silva.de/silvangs/, accessed on 27 February 2023 [57]),

to generate the OTU tables and taxonomic assignments. After these initial steps, identical reads were identified (dereplication), the unique reads were clustered (OTUs) on a per-sample basis, and the reference read of each OTU was then taxonomically classified. VSEARCH (version 2.15.1; https://github.com/torognes/vsearch, accessed on 27 February 2023) [58] was used for dereplication and clustering, applying identity criteria of 1.00 and 0.99, respectively. The taxonomic classification was performed using BLASTn (2.2.30+; http://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 27 February 2023) [59] with standard settings and the non-redundant version of the SILVA SSU Ref dataset (release 138.1; http://www.arb-silva.de, accessed on 27 February 2023). The taxonomic classification of each OTU reference read was mapped onto all reads that were assigned to the respective OTU using 99% similarity threshold. Reads without any or weak classifications, where the function "(% sequence identity + % alignment coverage)/2" did not exceed the value of 70, remained unclassified, and were assigned to "No Taxonomic Match".

For both markers, the obtained reads were analyzed separately for mobile and sessile fauna samples, and then combined for data analysis. The status of the species names was verified in the World Register of Marine Species (WoRMS) database (http://www.marinespecies.org/, accessed on 7 march 2023).

2.6. Statistical Analyses

Only OTUs with matches at species level (>97% for COI and >99% for 18S) and composed by at least eight sequences were retained for further data analyses. Any cases of ambiguous assignments (e.g., more than one species name >97% or >99% threshold) were inspected and resolved individually. Only assignments to morphospecies were used to allow accurate and standardized comparisons, given that OTUs may not always correspond with morphospecies.

For each marker, the number of reads from OTUs with a match to the same species were summed up together, and presence(1)/absence(0) species tables were constructed in Microsoft Excel (for Windows) for each marker (COI, Table S2; 18S, Table S3) and both markers together (Table S4), for subsequent analyses. Qualitative data of species distribution among taxonomic groups was displayed through bar graphs (GraphPad Software, Inc.). The proportion of overlapping and unique species detected among sampling times, for each substrate, and between substrates for each sampling time was determined for both sampling locations and displayed using Venn diagrams, using the web tool InteractiVenn [60].

Multivariate analyses were carried out considering presence/absence of the taxa due to the qualitative nature of the molecular data. Non-metric multidimensional scaling (nMDS) analyses were performed using PAleontological STatistics (PAST) version 4.03 [61] for Windows and based on Bray–Curtis resemblance coefficient between samples to visualize community distribution from the two sampled locations (Ría de Vigo and Ría de Ferrol), for all substrates and sampling times, for 18S and 18S + COI.

3. Results

A total of 190 species, representing 11 phyla (Annelida, Arthropoda, Bryozoa, Chordata, Cnidaria, Echinodermata, Entoprocta, Mollusca, Nemertea, Platyhelminthes, and Porifera; Table S4) were detected in this study combining data from ARMS and ASMS, and all sampling sites and times, in both locations. Through the observation of photographs, differences between sampling sites and times were patent within each substrate (Figure 2).

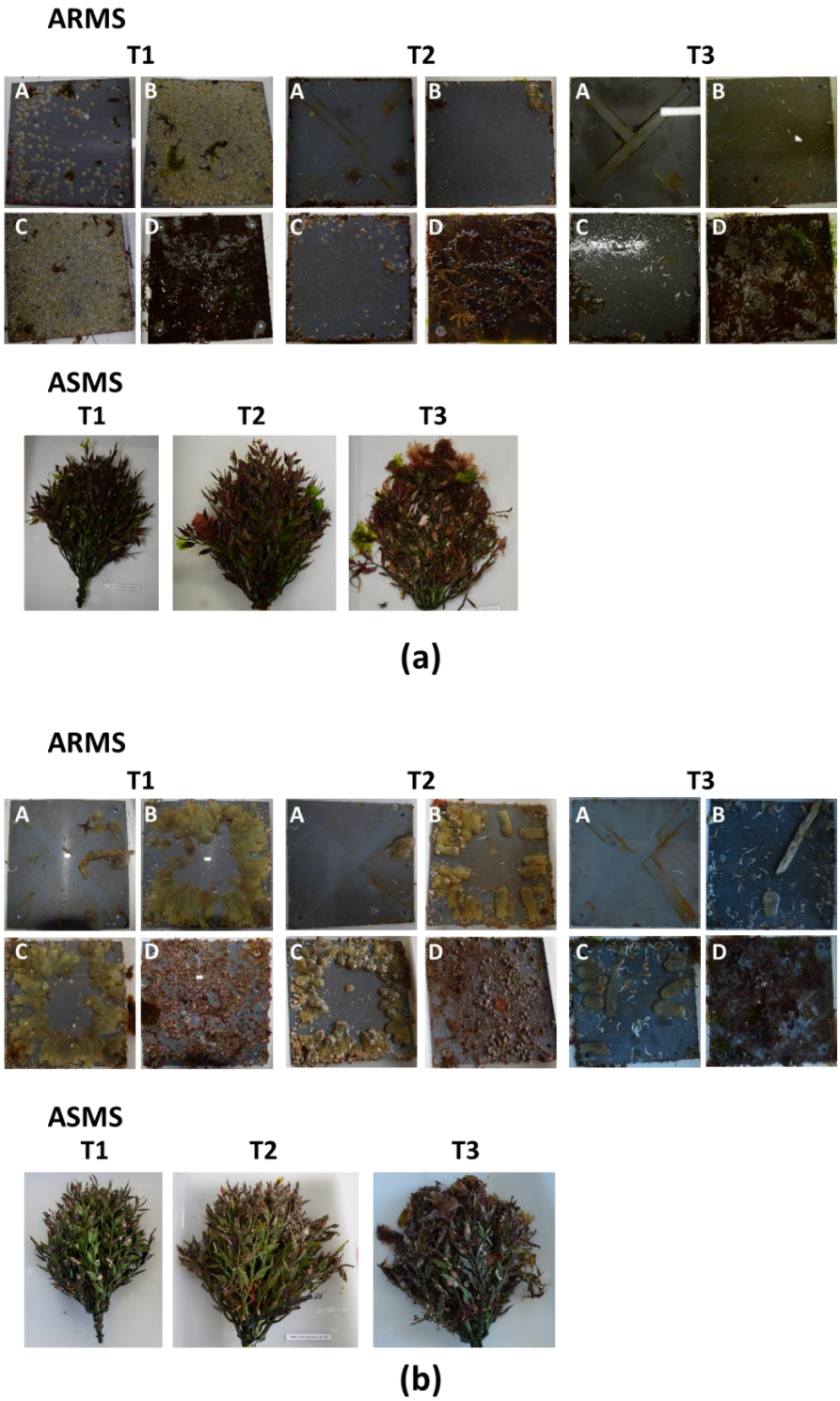

Figure 2. Sampled ARMS face plates and ASMS collected after 6 (T1), 9 (T2), and 12 (T3) months of deployment at (**a**) Ría de Ferrol and (**b**) Ría de Vigo. In ARMS: (**A**) Plate 1 top, (**B**) Plate 5 bottom, (**C**) Plate 9 top, and (**D**) Plate 9 bottom.

High-throughput sequencing from marine macrozoobenthic samples, for both markers and for the total of 24 samples, generated a total of 1,348,329 usable reads for both markers (Table S1) of these, 49% were assigned to marine macrozoobenthos species (30% using mICOIintF/LoboR1 and 19% with TAReuk454FWD1/TAReukREV3).

One-hundred and four species, distributed by 12 high-rank taxa (i.e., phylum, subphylum, and class level) were retrieved from ARMS samples, where 61 species were recovered from Ría de Ferrol and 76 species from Ría de Vigo (Figure 3, Table S5). At both locations, Annelida (15 and 19 species), Crustacea (14 and 12 species), and Echinodermata (10 and 15 species, in Ría de Ferrol and Ría de Vigo, respectively), were the most well represented. In addition, Cnidaria was also among the top rank contributors for the total number of species recovered in Ría de Vigo (15 species) (Figure 3, Table S5). On the other hand, a higher number of species (143) were recovered from ASMS samples, distributed by 14 high-rank taxa, with exclusive detection of Entoprocta and Pycnogonida, where 85 species were recovered from Ría de Ferrol and 100 species from Ría de Vigo (Table S5). The major contributors to ASMS community diversity were Crustacea (21 and 26 species), Annelida (13 species, in both locations), and Cnidaria (9 and 25 species, in Ría de Ferrol and Ría de Vigo, respectively) (Figure 3, Table S5). Furthermore, Gastropoda was also among the top rank contributors for the total number of species in Ría de Ferrol (nine species) (Figure 3, Table S5).

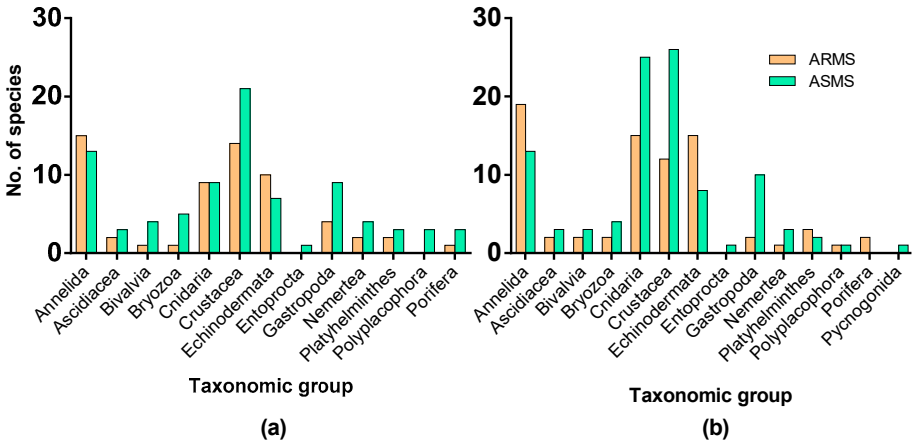

Figure 3. Taxonomic distribution of the cumulative species detected on each substrate and location: along all sampling times since substrate deployment. (**a**) Ría de Ferrol and (**b**) Ría de Vigo.

The lowest number of species was recovered in ARMS deployed in Ría de Ferrol for 6 months (17 species), but since we were not able to produce any amplicons for COI in this particular sampling time and location, this lower number might be the result of having data only for one genetic marker (Figure 4). On the other hand, the highest number of species (71 species) was recorded in ASMS deployed in Ría de Vigo, after 12 months (Figure 4). In general, the highest number of species recovered from ARMS in both locations was found after 12 months of deployment and when data from both genetic markers were combined together (34 and 43 species, for Ría de Ferrol and Ría de Vigo, respectively). On the other hand, in ASMS, the pattern was more variable; while the highest number of species was recovered after 12 months of deployment in Ría de Vigo (71 species), in Ría de Ferrol the highest diversity was attained after 6 months of deployment (51 species).

The contribution of dominant taxonomic groups changed over time of deployment of both substrates at both locations (Figure 4, Table S6). In Ría de Vigo, for ARMS, the contribution of Annelida (6 to 13 species) and Cnidaria (0 to 10 species) increased over time, while for Crustacea (3 to 8 species) and Echinodermata (9 to 13 species), reached the peak after 9 months, but decreased after 12 months. For ASMS, while a similar pattern was found for Cnidaria (9 to 21 species), which increased across time, an opposite pattern was found for Annelida, which decreased over time (10 to 6 species), and Echinodermata (2 to 7 species) and Crustacea (11 to 19 species), which increased over time of deployment (Figure 4).

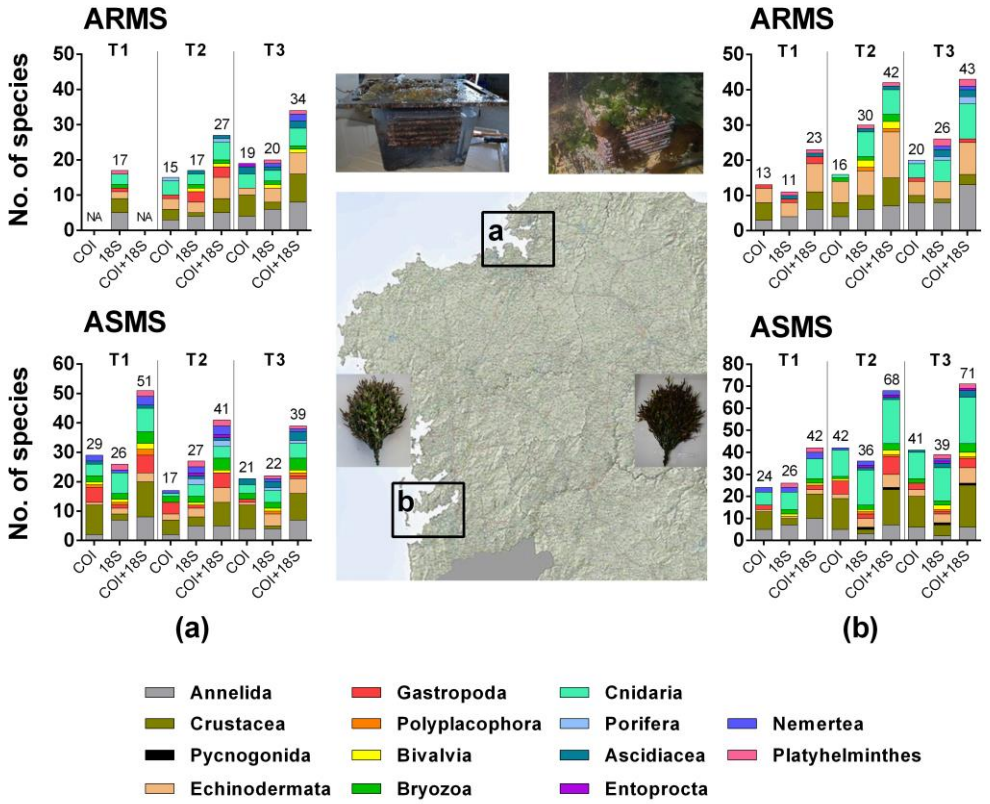

Figure 4. ARMS and ASMS substrates from the two sampling sites: (**a**) Ría de Ferrol and (**b**) Ría de Vigo. Bar charts represent the abundance and taxonomic distribution of species detected on each substrate among sampling times (T1, 6 months; T2, 9 months and T3, 12 months of deployment).

In Ría de Ferrol, a similar pattern was found in ARMS for Annelida (5 to 8 species) and Cnidaria (3 to 5 species), which increased over time, while an opposite trend to the found in Ría de Vígo was observed for Crustacea (4 to 8 species) and Echinodermata (2 to 6 species), which increased with time of deployment. In ASMS, for Annelida, Cnidaria, and Crustacea, the lowest species number was found after 9 months of deployment (5, 4, and 8 species, respectively), while contributions were slightly higher after 6 months of deployment (8, 8, and 12 species, respectively). Other groups displayed an increase in contribution across time, such as Echinodermata (3 to 5 species), while Gastropoda (6 to 1 species) decreased along the time of deployment (Figure 4, Table S6).

Comparing the species composition over time for each substrate individually, and considering the total species detected for each site (combining mobile and sessile fauna, and species recovered with both markers), the similarity of species occurrences was very low, especially for ARMS (5 and 9%, for Ría de Ferrol and Ría de Vigo, respectively), in comparison with ASMS (16 and 20%, for Ría de Ferrol and Ría de Vigo, respectively) (Figure 5, Table S7).

A great percentage of species (45 to 72% in Ría de Ferrol, 48% when joining data from all sampling times, and 53 to 62% in Ría de Vigo, 43% when joining data from all sampling times, respectively), were detected exclusively in ASMS (Figures 6 and S1, Table S8). Only 13 to 21% (25% for all data together) and 8 to 25% (32% for all data together), of the species, were detected on both substrates, in Ría de Ferrol and Ría de Vigo, respectively (Figure 6 and Figure S1, Table S8). Although from a quantitative perspective, ASMS retrieved more taxa in all sampling times and at both sampling sites; qualitatively the set of species observed in each substrate differed considerably (Figure 6, Table S8). When considering all exclusive species retrieved by each substrate in the overall experiment, a clear increase in Crustacea and Gastropoda and a decrease in the contribution of Echinodermata was observed for ASMS, deployed at both locations

(Figure 6, Table S8). Most of these exclusive species retrieved from ASMS at both locations were high mobility species, such as amphipods (e.g., *Ampithoe rubricata* (Montagu, 1808), *Caprella acanthifera* Leach, 1814 and *Jassa herdmani* (Walker, 1893)), but also gastropods such as *Calliostoma zizyphinum* (Linnaeus, 1758) or *Rissoa parva* (da Costa, 1778). In Ría de Vigo, an increase in the number of exclusive Cnidaria species, in particular of hydrozoans (e.g., *Abietinaria filicula* (Ellis and Solander, 1786), *Nemertesia antennina* (Linnaeus, 1758), *Halecium mediterraneum* Weismann, 1883), was also recovered from ASMS (Figure 6b, Table S8). In addition, exclusive groups such as Entoprocta (*Pedicellina cernua* (Pallas, 1774)) and Polyplacophora (e.g., *Acanthochitona fascicularis* (Linnaeus, 1767)) were exclusively detected in ASMS in Ría de Ferrol, while Entoprocta (*P. cernua*) and Pycnogonida (*Achelia echinata* Hodge, 1864) were exclusively detected in ASMS deployed in Ría de Vigo (Figure 6, Table S8). On the other hand, the most exclusive species detected on ARMS were Annelida and Echinodermata (e.g., *Sabellaria spinulosa* (Leuckart, 1849), detected in ARMS deployed at both locations or *Asterias rubens* Linnaeus, 1758, detected exclusively in ARMS deployed in Ría de Vigo) (Figure 6, Table S8).

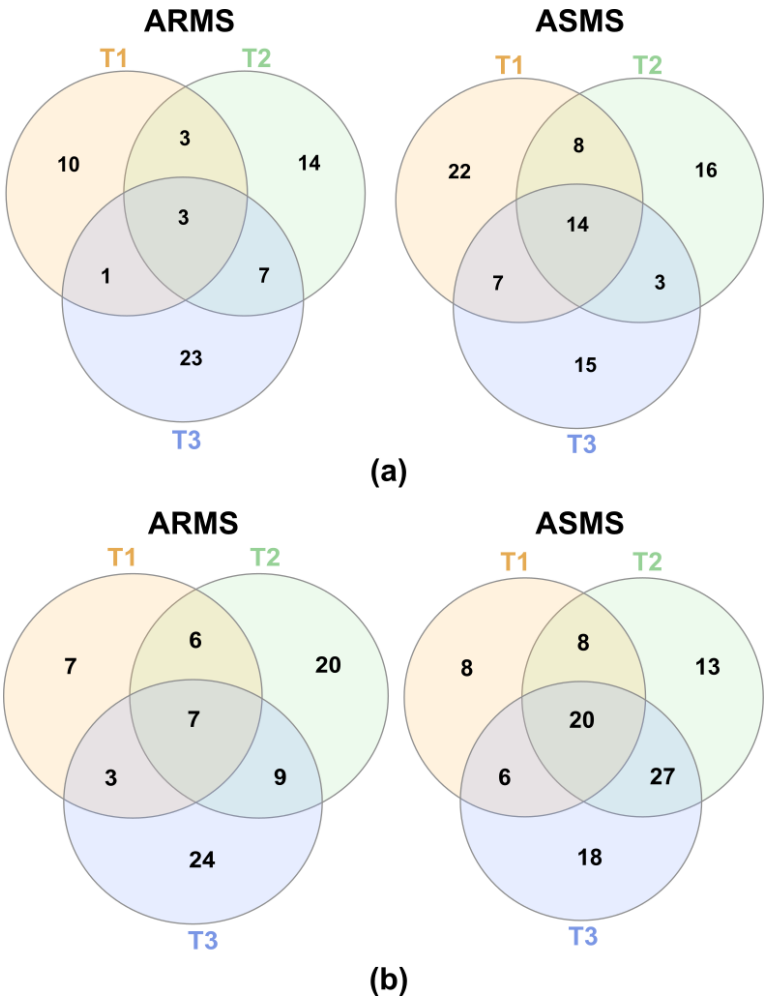

Figure 5. Partitioning of the marine macrozoobenthic species detected exclusively at each time of deployment (T1, 6 months; T2, 9 months and T3, 12 months) and shared by all sampling times (overlapping circles) for each substrate at each location: (**a**) Ría de Ferrol and (**b**) Ría de Vigo.

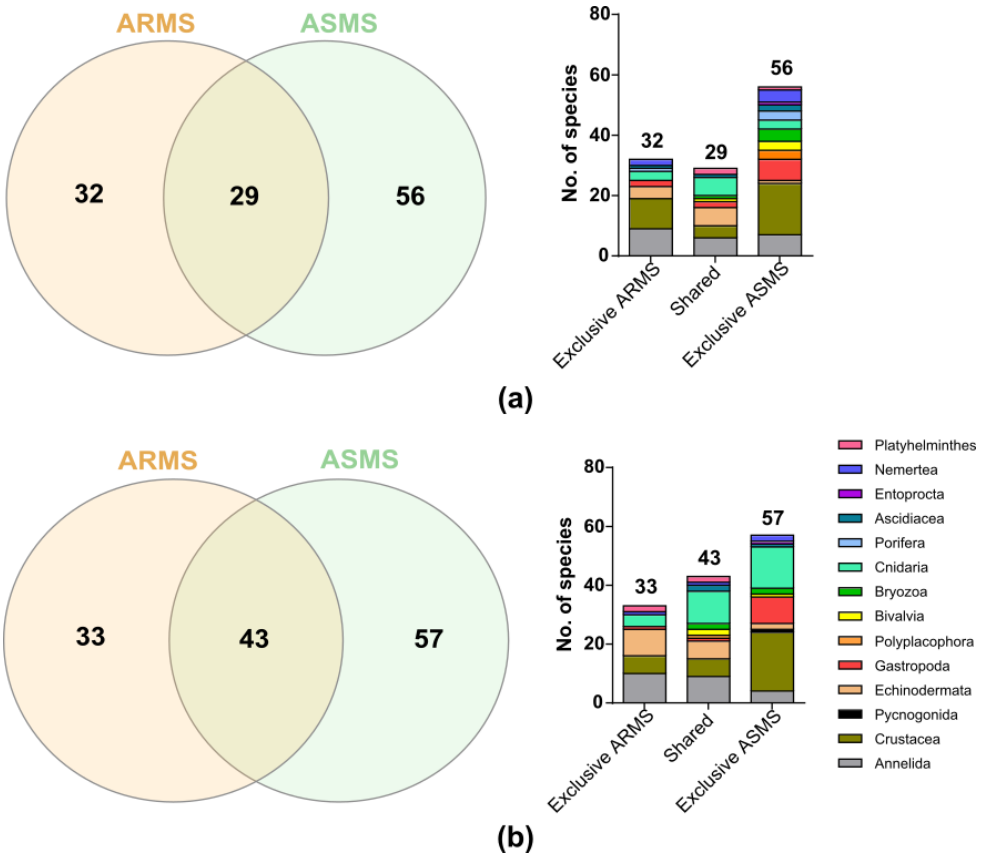

Figure 6. Partitioning of the marine macrozoobenthic species detected exclusively by ARMS, exclusively by ASMS, and shared by both substrates (overlapping circles), through all time of deployment in: (**a**) Ría de Ferrol and (**b**) Ría de Vigo, and respective taxonomic classification of each set of species.

Considering the substrates deployed at Ría de Ferrol (Figure 6a, Table S9), the majority of the species detected in one substrate only (32 in ARMS and 56 in ASMS) were exclusive species (i.e., species detected only in one substrate and one sampling time point; 61%). Furthermore, only 16% of the species in ASMS were pervasive (i.e., detected in all sampling times), while in ARMS only 5% of the species were detected over all sampling times. Among the species detected by both substrates, only two species were detected in all sampling times [the ophiuroid *Ophiothrix* (*Ophiothrix*) *oerstedii* Lütken, 1856, and the bryozoan *Tubulipora liliacea* (Pallas, 1766)].

The same pattern was found in the substrates collected from Ría de Vigo (Figure 6b, Table S9): 33 species were detected only in ARMS while 57 species were exclusively observed in ASMS, with 41% of the species being detected only in one substrate and one sampling time point. The percentage of pervasive species was low in both substrates (9% for ARMS and 20% for ASMS, respectively), whereas no species were detected by both substrates, for all sampling times combinations.

Comparing sampling locations, by combining the detected species by both substrates on each sampling location, a slightly higher number of species was detected in Ría de Vigo (133 species) than in Ría de Ferrol (117 species); Figure 7, Tables S9 and S10). However, differences in taxonomic groups were recorded (e.g., Pycnogonida was only detected in Ría de Vigo). Among the species detected only in Ría de Vigo, the majority were exclusive species (i.e., species detected only in one substrate/sampling time combination, 29%). The same pattern was observed in Ría de Ferrol, where 37% were exclusive species.

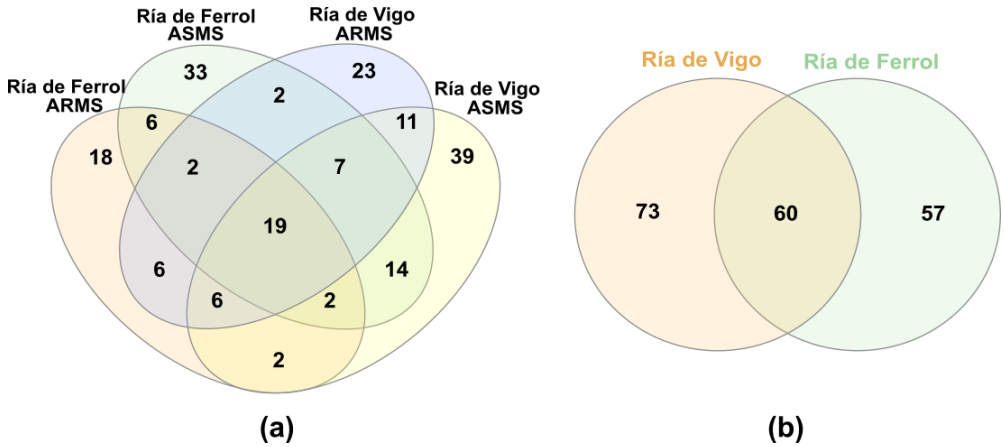

Figure 7. Partitioning of the marine macrozoobenthic species detected exclusively and shared (overlapping circles): (**a**) by each combination of substrate type (ARMS and ASMS) and location (Ría de Vigo and Ría de Ferrol) and (**b**) total number of species detected exclusively and shared (overlapping circles) in both locations.

Regarding the shared species among sampling sites, most of them had a variable occurrence independently of the substrate or sampling times (Table S9). For instance, *Pilumnus hirtellus*, was recovered in Ría de Vigo after 6 months (ARMS) and 9 months (ARMS and ASMS), and after 12 months of deployment in Ría de Ferrol (ARMS). The caprelid *Caprella acanthifera* detected in ASMS on Ría de Ferrol after 6 months of deployment, was then detected in Ría de Vigo at 9 and 12 months of deployment of the same substrate type. Another example was recorded for three decapods (*Eualus cranchii* in ASMS, *E. occultus* in ARMS, and *Hyppolyte varians* in ASMS) that were first detected after 9 months of deployment in Ría de Vigo, and then after 12 months of the same substrate type in Ría de Ferrol (Table S9). Overall, no species was recorded as completely pervasive (detected in all sampling times, substrates, and study sites) (Table S9), but 10% of the species were recovered from both substrates and at both sampled locations (19 species, Figure 7). The prevalence of exclusive species (i.e., species detected only in 1 substrate/sampling time/site combination) was observed for 43.2% of the total species (82 species) (Table S9).

Non-metric multidimensional scaling, based on species detected on each substrate and sampling time, at the two sampling locations and by using data recovered with the 18S marker (for which amplification was successful for all fractions, substrates, and sampling times, at both sites, Figure 8a) and using data from both markers together (18S + COI) (Figure 8b), revealed aggregation of the samples according to the sampling location, and within each sampling location according to the substrate, for both situations (18S alone and both markers together) (Figure 8). However, a higher variation between ARMS is evident by the lower similarity shared among time points, while a high similarity was observed among ASMS samples retrieved at the different time points, for both locations. The differences observed in the nMDS diagrams were supported by two-way PERMANOVA, which indicated that both location and substrate, but not the interaction between both factors, significantly affected the community structure of macrozoobenthic species retrieved either with 18S or with both markers (Table S11).

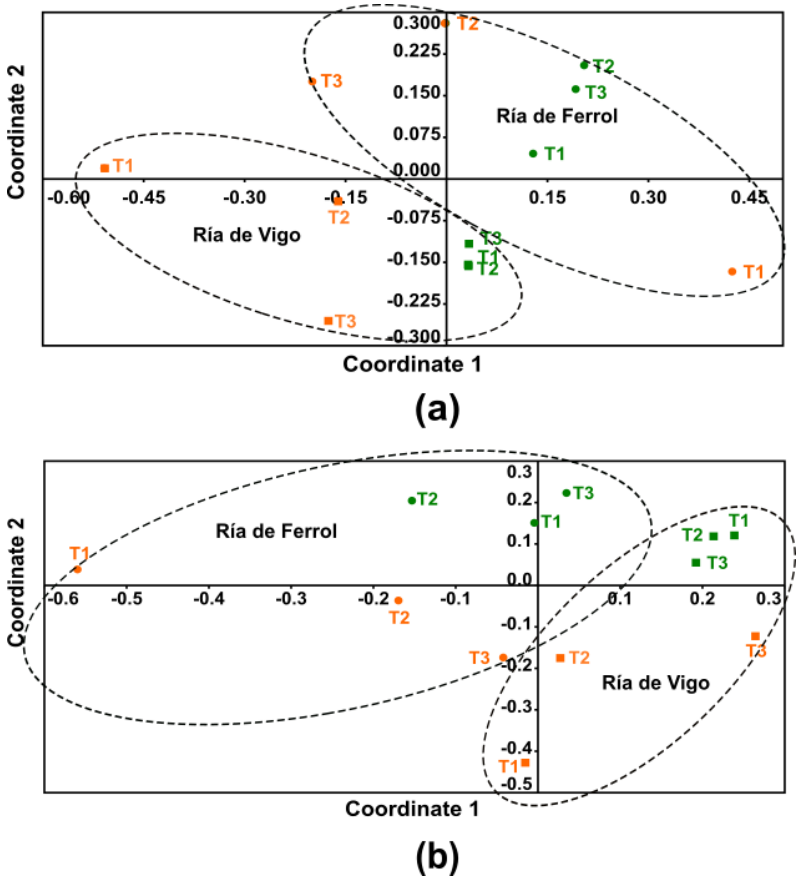

Figure 8. Non-metric multidimensional scaling (nMDS), based on Bray–Curtis similarity index, of the species detected in ASMS (green) and ARMS (orange), for all sampling times (T1, 6 months; T2, 9 months and T3, 12 months of deployment) on each sampled location (Ría de Vigo, squares, and Ría de Ferrol, circles) recovered: (**a**) with the 18S and (**b**) with both markers.

4. Discussion

Over the past decade, the coupling of ARMS with DNA metabarcoding has been increasingly employed and become a customary approach for the monitoring of marine benthic communities [23,27,32,62]. The success of this approach appears to be the combination of the benefits of substrate standardization using ARMS, supplemented with the high-throughput capacity of DNA metabarcoding, thereby providing a very efficient and comparable approach for monitoring these complex and taxonomically challenging communities [63,64]. In spite of the large benefits of substrate standardization, they may come with a cost, particularly if their widespread use tends to lessen, or reduce, the employment of other sampling strategies. In this study, using an alternative substrate with a different structural complexity that resembles macroalgae, we illustrate how a fair fraction of the marine benthic diversity may be missed by employing an ARMS-exclusive sampling strategy. Globally, our results showed: (i) differences in communities' composition and diversity as a result of the duration of the colonization period, in both substrates and sites; (ii) clear differentiation of the communities between Ría de Vigo and Ría de Ferrol, in both substrates; (iii) large and consistent differentiation between ARMS and ASMS communities within each site.

Changes in community composition during colonization periods of increasing duration would be expected as a result of ecological succession and seasonal fluctuation processes, e.g., [40]. In our previous study in Ría de Vigo, using artificial substrates made of different materials, and employing an identical metabarcoding approach [40], we observed similar fluctuations, although differences in the timing and duration of deployment do not allow direct comparisons. In the current study, we confirmed the occurrence of considerable

over-time variations in two separate coastal areas and, importantly, that were also captured by two very distinct types of substrates. The pronounced fluctuations recorded over time indicate that sampling only after 12 months of deployment, which is the minimum deployment period normally used in ARMS [23,26,27,32,34,62,65] may fail to capture a fair diversity of taxa and species. Although more or less long periods of deployment may be required for ecological succession to be completed, and for the colonizing assemblage to reach a point of stability mirroring the natural community in that spot, our data indicates that maximum diversity can be reached under 12 months of deployment. Whilst comparisons with other studies are difficult, mostly due to the employment of different species identification approaches (i.e., morphology-based identifications) and different sampled locations, a previous study suggested that complete colonization of ASMS occurs within 3 months of deployment [66]. However, using ASMS in Ría de Vigo, we detected the maximum number of species after 12 months of deployment and after 6 months in Ría de Ferrol (consistent with Carreira-Flores et al. [29]). Furthermore, higher diversity at intermediate stages of succession has been reported in an earlier study, namely after 7 months of deployment of artificial substrates in Vigo, assessed using both morphology and DNA metabarcoding [40]. The overgrowth of mussels in the substrates that were observed after 10 and 15 months may have contributed to excluding some species and reduced the taxa diversity at later succession stages. The timing of initial deployment will also affect the speed of colonization given that availability of propagules of key species in different stages of succession will vary with season [23,30]. These different results highlighted the importance of seasonal sampling in long-term monitoring to learn when a species is expected to occur, to provide information about communities' changes over temporal scales, and to signal possible faulty detection of pervasive species, which could flag possible changes in the ecosystem. These findings reinforce the importance of considering the time and duration of the deployment of artificial substrates for the monitoring of coastal macrozoobenthic communities. On the other hand, ARMS plain surfaces may require longer colonization periods, particularly due to the lack of refuge and settlement spaces, whereas ASMS canopy may facilitate colonization thereby accelerating the stability of the macrozoobenthic community.

Globally, the variation over time also appeared to be considerably greater in ARMS compared to ASMS, a pattern that, if confirmed, could also have implications for future monitoring considerations. It should be noted that ARMS monitoring was originally developed for tropical reefs [21] where ecological succession may take long but once completed, may be less prone to intense seasonal fluctuations as the ones experienced by temperate communities such as the ones here studied.

In spite of the variations over time, the main differences in community composition were found between locations, with the macrozoobenthos communities from both Rías being clearly distinct from each other, independently of timing and substrate, and each recording a high number of exclusive species. These differences probably reflect particular features of each Ría, and possibly also their geographic location. However, the employment of DNA metabarcoding may have also contributed to more detailed taxonomic profiling, which in turn may have provided greater discrimination ability than using morphological approaches alone. Indeed, most studies to date comparing metabarcoding and morphological approaches have reported the recovery of a higher number of species and diversity with the former [11,15,31,33]. Whereas few ARMS-metabarcoding studies addressed overtime variation in macrozoobenthos, most of them report patterns of spatial variation at local or regional scales [15,23,27,32,34,65]. Interestingly, ARMS and ASMS appear to have consistent patterns of variation over time and between sites, with Ría de Vigo generally showing higher diversity compared to Ría de Ferrol, and comparable overtime variations within the site. Hence, both substrates appear to be similarly effective in capturing beta diversity.

Different communities' composition detected between the two substrates is likely related to the complexity of the shape of the substrates, particularly due to the complex branching pattern in ASMS, in contrast to the ARMS cavities made of flat surface PVC. Additionally, the three-dimensional structure of each substrate has different levels of

exposure to light (even within each ARMS plate [28]), to predators, and differences in water flow. A fair number of studies have employed ARMS as substrates for species colonization [20,23,27] and recommend it as a prime tool for standardized monitoring of macrozoobenthic communities [27]. However, our results demonstrated that a fair portion of the macrozoobenthos diversity may fail to be captured by employing exclusively this artificial substrate, at least in the studied region. Hence, the efficiency of this monitoring tool can be partially compromised if there is the risk of systematic overlooking of fractions of diversity. The results showed that compared to ARMS, in ASMS we frequently detected more species for each taxonomic group (except for Annelida, and Echinodermata), and two additional high-rank taxa (i.e., Entoprocta and Pycnogonida) were exclusively detected. However, both substrates demonstrated to be complementary in their ability to be colonized by macrozoobenthic species, since a low proportion of species were recorded concurrently in both substrates. Although missing taxa may not have a great impact in studies aiming for bioassessments of the ecological status [9], these can be critical for studies aiming for long-term monitoring and assessing global change-induced alterations in species ranges and communities' composition, or detection of non-indigenous species (NIS). Results obtained for ASMS, for the three sampling times and for each location, consistently indicated more species and wider taxonomic diversity, as well as approx. up to 70% of exclusive species. Most of these exclusive species were high mobility species, such as crustaceans, in particular amphipods, which have colonized ASMS, at both locations (e.g., *A. rubricata*, *C. acanthifera* and *J. herdmani*), but also Gastropoda (e.g., *C. zizyphinum* and *R. parva*, were found exclusively in ASMS at both locations). Since the substrates were disposed side-by-side at each location, their tridimensional structure would have been key to favor differently the colonization of species. Contrary to ARMS, which has shaded areas, ASMS allowed greater algal growth, that attracted a particular set of species. Another factor could be the high abundance of hydrozoan species in ASMS (in particular for the ones deployed in Ría de Vigo), that serve as food to several gastropod species (e.g., *C. zizyphinum* feeds on hydrozoans). On the other hand, most exclusive species detected on ARMS were Annelida and Echinodermata (e.g., the annelid *S. spinulosa* was detected in ARMS at both locations), which can be frequently found in more shaded and sheltered areas, such as circalittoral bedrock, boulders, or cobbles, which display a greater resemblance with ARMS. Thus, the complementarity between substrates highlights the need to optimize sampling strategies, where employment of both substrates may provide a broader phylogenetic scope and detect sets of species that otherwise could be invariably overlooked.

The communities from each sampling site were well separated into two groups, where exclusive species were the drivers for those aggregations. No pervasive species were identified (all sampling times, substrate, and locations), and only 2% of the species and no species at all were recorded in all sampling times and substrates, in Ría de Ferrol and Ría de Vigo, respectively. In total, as much as 43.2% of the species recorded were exclusive, i.e., only detected in one substrate or sampling time or location. Differences in species detection in both sites and among sampling times could be a result of species distribution patterns. Habitat specificities and geographical distribution are the drivers for species trends and patterns. The new complexity of the habitat, a consequence of substrate colonization, as well as new spaces for shelter and settlement, can lead to shifts in species abundance and occurrence. In addition to the consequences of natural sampling variation, exclusive species appear to occur more randomly over time and space, suggesting that seasonality may have played a role in the observed patterns. Data from other studies combining ARMS with barcoding revealed patterns similar to ours, with high percentages of exclusive species detected (e.g., 44% of all species [67] and more than 50% of species [32]). Compositional changes in marine macrozoobenthic communities associated with ARMS and ASMS highlighted regional differences and suggest that a substrate with higher complexity (for example a combination between ARMS and ASMS) will result in more space for species settlement and improve colonization capacity.

5. Conclusions

Although the benefits of ARMS employment in marine macrozoobenthic monitoring appear to be well established [27], here we show how its exclusive use could introduce a recurrent bias, with sizeable fractions of the biodiversity being systematically overlooked. The obtained results indicate that no single substrate structure is able to capture comprehensively the diversity of a marine hard-bottom community, and the complementarity recorded between substrates highlighted the necessity to consider implications to sampling design. More intensive sampling strategies are now at reach thanks to the greater throughput provided by DNA metabarcoding. However, comprehensive monitoring of these communities would require ponderation on the use of complementary substrate structures, that encompass the natural structural complexity of coastal marine habitats. Reefs and rocky-bottom shores are structurally very elaborate, and it would be impractical to attempt to fully reproduce such complexity. Recent technological developments in material science and 3D printing systems [68] may offer some standardized solutions that could benefit the comprehensiveness and accuracy of marine benthic monitoring.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15050657/s1, A detailed description of all data used to support results is provided in Supplementary Materials. Figure S1: Partitioning of the marine macrozoobenthic species detected exclusively by ARMS, exclusively by ASMS, and shared by both substrates (overlapping circles), after 6 months (T1), 9 months (T2), and 12 months (T3) of deployment, in Ría de Ferrol (A) and Ría de Vigo (B); Table S1: No. of merged, usable reads (quality-filtered) and taxonomically assigned reads to species level (>97% for COI and >99% for 18S) and with equal or more than 8 reads obtained on each recovered fraction (M: mobile, S: sessile) from each substrate (ARMS and ASMS) and sampling time (6, 6 months; 9, 9 months and 12, 12 months of deployment), on each location (Ría de Ferrol and Ría de Vigo). NA, treatments where no amplicons were produced; Table S2: Taxonomic classification of all species detected in all substrates, sampling times, and locations with COI. RF, Ría de Ferrol; RV, Ría de Vigo; 6, 6 months; 9, 9 months and 12, 12 months of deployment; Table S3: Taxonomic classification of all species detected in all substrates, sampling times, and locations with 18S. RF, Ría de Ferrol; RV, Ría de Vigo; 6, 6 months; 9, 9 months and 12, 12 months of deployment; Table S4: Taxonomic classification of all species detected in all substrates, sampling times and locations with COI + 18S. RF, Ría de Ferrol; RV, Ría de Vigo; 6, 6 months; 9, 9 months and 12, 12 months of deployment; Table S5: Total no. of species, distributed by each taxonomic group, recovered by both markers from each substrate deployed in Ría de Ferrol, Ría de Vigo and in the total experiment; Table S6: No. of species distributed by each taxonomic group (Figures 3 and 4) and % of contribution of each taxonomic group for the total no. of species recovered by both genetic markers (with the exception of ARMS deployed in Ría de Ferrol for 6 months, where only data from 18S was available) for each substrate (ARMS and ASMS) and sampling point (6, 6 months; 9, 9 months and 12, 12 months of deployment), at both locations (Ría de Ferrol and Ría de Vigo); Table S7: No. of species recovered on each sampling time (6, 6 months; 9, 9 months and 12, 12 months of deployment), in the total of all sampling times and no. and % of species shared among all sampling times, for each substrate (ARMS and ASMS), on each location (Ría de Ferrol and Ría de Vigo) (Figure 5); Table S8: No. of species and % detected exclusively and shared between substrates (ARMS and ASMS), on each sampling time (6, 6 months; 9, 9 months and 12, 12 months of deployment) and though all experiment, for each location (Ría de Ferrol and Ría de Vigo), and respective lists and taxonomic classifications of species detected exclusively on each substrate and in both, for each location; Table S9: No. of detections for each substrate (ARMS and ASMS), on each location (RF, Ría de Ferrol; RV, Ría de Vigo) and in the totality of each sampling location and both locations. Additionally, 6, 6 months; 9, 9 months, and 12, 12 months of deployment. Numbers 1 to 12, indicate the number of detections, with 1 being one single combination of location, substrate, and sampling time and 12 the full combination of locations, substrates, and sampling time points; Table S10: No. of detections of each species on each sampling location (Ría de Ferrol and Ría de Vigo) and shared between both locations. *, 1 to 3, indicate the number of sampling time detections, for each location and #, 1, indicates exclusive species of each location, and 2, species shared by both locations; Table S11: Results from two-way PERMANOVA analyses testing the effect of location (Lo) and substrate (Su) and the interaction between both factors (LoxSu) on macrozoobenthos community structure recovered with 18S and with both markers (COI + 18S).

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