



Contrasting resistance of prokaryotic plankton biomass and community composition to experimental nutrient inputs in a coastal upwelling system (NW Spain)

A. Gutiérrez-Barral · E. Fernández ·
M. Hernández-Ruiz · E. Teira

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Abstract Increasing nutrient inputs driven by global environmental pressures may lead to changes in prokaryotic plankton biomass and community composition in coastal environments. Nutrient addition experiments (inorganic, organic, and mixed) were performed at the continental shelf outside the Ría de Vigo, in spring, summer and autumn 2014, and the results contrasted with those from similar previous experiments carried out inside the ría in 2013. The mixed nutrient additions caused the greatest changes in community composition, mostly consisting of blooms of Vibrionales. Inorganic nutrients yielded increased proportions of Oceanospirillales and Alteromonadales. Euclidean distances among samples were used to estimate compositional resistance to disturbances derived from nutrient additions. Changes in prokaryotic biomass were used as an indicator of biomass resistance. Generally, prokaryotic communities were more resistant to inorganic than organic nutrient

additions. Communities sampled inside the ría, presumably exposed to greater perturbations, showed milder variability in the non-amended control than those from outside the ría. By contrast, shelf communities, with higher ambient organic matter concentrations, were more resistant to organic and mixed nutrient additions than those from the ría. Our data suggest that the perturbation history is related to the resistance of microbial communities to changes in nutrient inputs into the coastal ocean.

Keywords Nutrient inputs · Prokaryotic response · Biomass · Community composition · Upwelling · Ría de Vigo

Introduction

The composition and function of prokaryotic plankton can change after being affected by disturbances, which have been defined as “causal events that either (1) alter the immediate environment and have possible repercussions for a community or (2) directly alter a community” (Glasby & Underwood, 1995; Shade et al., 2012). These disturbances may include nutrient additions. Resistance has been defined as “the degree to which a community withstands change in the face of disturbance” (Allison & Martiny, 2008; Shade et al., 2012), and different communities show variable resistance to the disturbance, often to the extent of not changing under perturbed conditions (Allison &

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A. Gutiérrez-Barral (✉) · E. Fernández ·
M. Hernández-Ruiz · E. Teira
Centro de Investigación Mariña da Universidade de Vigo,
Departamento de Ecoloxía e Bioloxía Animal, Facultade
de Ciencias do Mar, Universidade de Vigo, Vigo, Galicia,
Spain
e-mail: alberto.gutierrez@uvigo.gal

Martiny, 2008; Lindh & Pinhassi, 2018; Shade et al., 2012).

Prokaryotic community resistance can be assessed in terms of function (hereafter referred to as functional resistance) based on functional parameters such as biomass, enzyme activity, and bacterial production or taxonomic composition (hereafter referred to as compositional resistance) (Allison & Martiny, 2008; Székely & Langenheder, 2017; Awasthi et al., 2014; Hillebrand et al., 2018; Sjöstedt et al., 2018). Functional resistance might partly result from functional redundancy, and compositional resistance might be related to metabolic plasticity, although in most cases microbial communities are compositionally sensitive, or prone to changes (Allison & Martiny, 2008; Lindh & Pinhassi, 2018; Comte et al., 2013). Microbial communities previously exposed to disturbances may show increasing resistance to similar or different perturbations, and the historical environmental conditions may have an impact on functional but not compositional resistance (Sjöstedt et al., 2018; Renes et al., 2020). Changes in prokaryotic composition and function have been tested after different types of stress, such as solar radiation (Manrique et al., 2012; Durán-Romero et al., 2017; Teira et al., 2019), oxidative stress (Glaeser et al., 2010; Andrade-Linares et al., 2016; Santos et al., 2021), changes in pH (Sjöstedt et al., 2018; de Scally et al., 2020; Renes et al., 2020) and temperature (Xiong et al., 2016; Allen et al., 2020; Renes et al., 2020), nutrient addition (Shade et al., 2011; Teira et al., 2016; Joglar et al., 2020), or CO₂ levels (Oliver et al., 2014; Allen et al., 2020), among other perturbations (Bonilla-Findji et al., 2010; Awasthi et al., 2014). Nevertheless, most previous research on microbial resistance to environmental change was carried out on soil communities, and on lakes (Griffiths & Philippot, 2013; Awasthi et al., 2014; Sjöstedt et al., 2018; Renes et al., 2020), but in comparison, fewer studies have been conducted on marine prokaryotes (Oliver et al., 2014; Lindh & Pinhassi, 2018; de Scally et al., 2020).

In upwelling regions, inorganic nutrients are intermittently pumped from deep waters into the photic layer, enhancing phytoplankton growth (Messié & Chavez, 2015), with the associated release of organic matter readily available for prokaryotes (Teira et al., 2015; Wear et al., 2015). Some bays have special topographic and bathymetric features which enhance the effects of upwelling (Largier, 2019; Broullón

et al., 2023). In addition, in some upwelling systems, coastal waters are also frequently affected by anthropogenic organic and inorganic nutrient inputs (Fernández et al., 2016).

Within this context, we hypothesize that the resistance of prokaryotic communities to nutrient inputs may be of different magnitude in coastal embayments affected by upwelling and anthropogenic nutrient inputs compared to the adjacent shelf. Since the embayment is likely to be more frequently impacted by nutrient enrichment than the adjacent shelf, the same nutrient additions would be expected to have weaker impacts on the prokaryotic plankton inside the embayment as compared with the microbial communities outside the embayment. To test this hypothesis, we analyzed prokaryotic DNA and biomass samples obtained from addition experiments consisting of inorganic, organic, and a mix of inorganic and organic nutrients.

Materials and methods

This study was carried out at the embayment Ría de Vigo and its adjacent shelf, which are located at the North West (NW) Iberian Peninsula, an area affected by intermittent upwelling pulses dominating in spring and summer, and downwelling pulses taking place mostly during the rest of the year (Barton et al., 2015); Fernández et al., 2016). It is an area with extensive aquaculture activity, especially mussel mariculture, which is known for releasing high quantities of fecal material which is re-mineralized into ammonium (Alonso-Pérez et al., 2010).

Experimental procedures

Natural surface water samples were taken using Niskin bottles from the central area of the Ría de Vigo (42.23° N, 8.79° W) in May, July, and October 2013, and from the adjacent shelf (42.14° N, 8.96° W) in April, June, and October 2014 (Fig. 1). Assessment of water column conditions, such as temperature and in situ fluorescence, were conducted to a maximum depth of 25 m, using an SBE (Sea Bird Electronics) 9/11 CTD (Conductivity, Temperature, and Depth) probe, along with a fluorometer attached to a rosette sampler. The water was prefiltered through 200-µm pore size to remove large zooplankton. Nutrients were

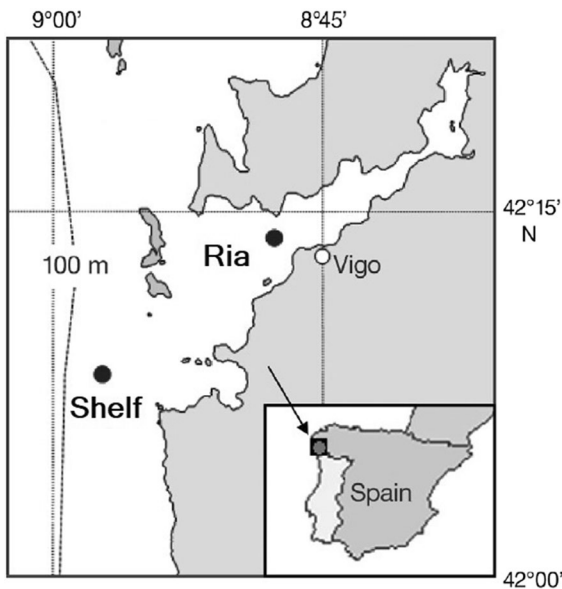


Fig. 1 Map showing the stations where the water for the experiments was sampled. Modified from Gago et al. (2011)

then added to each 2-L-volume triplicate. The additions consisted of inorganic ($5 \mu\text{M NO}_3^-$, $5 \mu\text{M NH}_4^+$, $1 \mu\text{M HPO}_4^{2-}$), organic ($5 \mu\text{M}$ glucose and a $5 \mu\text{M}$ equimolar mix of amino acids), and mixed (a mix of inorganic and organic) nutrients. The nutrients were selected based on the chemical composition of atmospheric and continental inputs (Gago et al., 2005; Jickells et al., 2017; Traving et al., 2017). Samples were incubated in ultraviolet-transparent Whirl-pak® bags at in situ light and temperature conditions, which is a common procedure in microbial plankton experiments (Liao et al., 2019; Justel-Díez et al., 2023). The bags were tightly sealed and submerged in open tanks which were constantly fed with water from the Ría de Vigo (to keep in situ temperature) and were located at ECIMAT (Toralla Island Marine Science Station). Incubations lasted 48 h in the ría experiments, and 72 h in the shelf experiments, in both cases, within the range proposed by (Downing et al., 1999), and were sampled every 24 h. At the end of each experiment, triplicates were pooled into 2-L volumes, which were used for filtering DNA. Budgetary constraints were the main reason for pooling the samples. Furthermore, previous literature on 16S rRNA amplicon sequencing did not show much variability among replicates (Kennedy et al., 2014; Marotz et al., 2019). Subsequently, prokaryotic DNA was obtained by

filtering through 20 μm , 3 μm , and 0.2 μm , to obtain only picoplankton.

Seawater samples for analyzing inorganic nutrients were obtained from the Niskin bottles and poured into acid-washed polyethylene bottles. Afterwards, they were frozen and stored at -20°C until further analysis, which involved segmented flow analysis (SFA), using an Alliance Futura autoanalyzer. Colorimetric methods were used following Grasshof et al. (1999) for nitrite, nitrate, phosphate, and silicate; and following K erouel & Aminot (1997) for ammonium.

For analyzing dissolved organic carbon (DOC) and dissolved organic nitrogen (DON), all-glass 250 ml flasks were employed. Each sample was subsequently filtered through pre-combusted Glass Fiber/Fine (GF/F) filters (450°C , 4 h) in mild N_2 positive pressure conditions. Thereafter, the filtrates were stored at -20°C in 20 ml glass flasks, until characterization. Following this, they were analyzed using the high temperature catalytic oxidation method using a Shimadzu Total Organic Carbon (TOC)-V analyzer, attached to a nitrogen measuring unit Total Nitrogen Measurement (TNM)-1 by chemiluminescence ( lvarez-Salgado & Miller, 1998), which resulted in the obtention of the total dissolved nitrogen (TDN) value. Finally, DON was obtained from the extraction of nitrite, nitrate, and ammonium from TDN.

The 16S rRNA gene was Illumina-sequenced using the following primers: 515F-Y (5'-GTGYCA GCMGCCGCGTAA-3') and 926R (5'-CCGYCA ATTYMTTTRAGTTT-3') (Parada et al., 2016). The sequences are publicly available at the European Nucleotide Archive, <https://www.ebi.ac.uk/ena> (PRJEB47591). These DNA samples were processed together as described by Guti errez-Barral et al. (2021). Sequenced reads went into the Logares (2017) pipeline. First, the Bayesian clustering technique BayesHammer was used for raw read correction (Nikolenko et al., 2013; Schirmer et al., 2015). Afterwards, the paired-end reads, after being corrected, were merged using Pair-End Read Merger (PEAR), selecting for lengths of at least 200 bp (Zhang et al., 2014). Errors were checked and sequences were dereplicated using VSEARCH-2.14.1 (Rognes et al., 2016).

The abundance of each operational taxonomic unit (OTU) was assessed by clustering at 99% similarity, where the average OTU read length was 411.3659 bp. The SILVA reference database was used for Chimera

check and removal (Quast et al., 2013). The taxonomic assignment was achieved using Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) against the SILVA 119.1 database. Subsequently, OTUs with <200 bp, <60% coverage, <90% similarity and >0.00001 e-values were removed. Furthermore, OTUs which represented chloroplasts, mitochondria, or eukaryotes were also removed. The OTU table was then subsampled (rarefied) to the lowest read number, 5018.

Bacterial abundance, sampled every 24 h, was assessed using a Beckton Dickinson (BD) FACSCalibur flow cytometer with a 488-nm-laser beam, after staining the cells with SybrGreen DNA fluorochrome. Subsequently, the empirical calculations described in Calvo-Díaz and Morán (Calvo-Díaz & Morán, 2006), which use side scatter and mean cell diameter, were used to estimate biovolume (BV). Finally, biomass was obtained from biovolume using Norland's allometric relationship (Norland, 1993): $\text{fg C cell}^{-1} = 120 \times \text{BV}^{0.72}$.

Statistical analyses

The subsampled (rarefied) OTU table was used for calculating OTU richness (S), Shannon index (H), and Pielou's (J), using the R Vegan package (Oksanen et al., 2020). To assess statistical differences in the medians of the diversity indices, compared to the control, a Kruskal–Wallis test ($\alpha < 0.05$) was applied.

The relative read abundances of the subsampled table were centered log-ratio (CLR) transformed (Gloor et al., 2017), after the substitution of zeros by the lowest number of reads (1) divided by two. The CLR abundances were used for computing the Euclidean distance matrix. Environmental variables were normalized, i.e., each value was subtracted the variable's mean, and divided by standard deviation.

In order to identify which specific taxonomic groups (biological factors) or nutritional resources (abiotic factors) better explained the changes in prokaryotic community composition, a distance-based redundancy analysis (dbRDA), combined with a distance-based linear model (DistLM), and using a step-wise procedure and adjusted R^2 as selection criteria, was conducted using PRIMER6 software (Clarke & Gorley, 2009). For reducing the risk of overfitting the data, which was contained in 24 samples, the 20 OTUs showing the greatest relative

contributions were selected as explicative biological variables for DistLM, but only the most explicative ones were displayed (up to 95% adjusted R^2 , sequential tests). DON, DOC, nitrate, ammonium, and phosphate concentrations were included as environmental explicative factors for the DistLM, but only the significant variables were plotted ($P \leq 0.05$).

Principal Coordinate Analysis (PCoA), using Euclidean distances, was used to visualize the similarities in the prokaryotic composition of the different samples using PRIMER6 software. Analysis of similarity (ANOSIM, 999 permutations) was used to test for significant differences among samples from different locations, seasons, and treatments.

Baert et al. (2016) calculated compositional resistance using Bray–Curtis similarity, with 0–1 values. That calculation can be used with counts, although it cannot be done using CLR-transformed data, which is more robust for compositional data analysis. Therefore, the current study measured dissimilarity using Euclidean distance within CLR-transformed compositional data, and its inverse to obtain greater values for greater resistance, and vice versa. Even though communities change throughout incubations, the changes might differ across samples. Consequently, the compositional resistance of the non-amended control (natural change) was estimated as the inverse of the Euclidean distance between T0 and the control at the end of the experiment. This measured the resistance to change resulting from manipulation in the laboratory, as well as the natural evolution of the communities. Resistance to a given nutrient addition treatment (inorganic, organic, or mixed) was estimated as the inverse of the Euclidean distance between the control and the different nutrient treatments, both measured at the end of the experiment.

Orwin & Wardle (2004) proposed an equation which could not be used with compositional resistance in the current studies due to the lack of univariate properties with high or low values. However, there were biomass units, and that equation would fit biomass resistance. Nevertheless, for keeping calculations consistent and comparable across both types of resistance, biomass resistance (used as a proxy for functional resistance) was assessed as the biomass value at a control divided by the biomass value at a nutrient-amended sample, after 48 h, or by dividing biomass at T0 by biomass at the 48 h control, in the case of resistance to natural change. Alternatively,

Bray–Curtis similarity (using counts instead of CLR-transformed values) as compositional resistance; and the Orwin and Wardle equation for calculating biomass resistance have also been used (Fig. S2).

OTU nomenclature

Many OTUs in the SILVA database have not been described as species, so correlative numbers were given to OTUs assigned to the same genus. For example, *Vibrio* 1, *Planktomarina* 3, *Oleispira* 2.

Results

Changes in prokaryotic diversity on shelf waters off the Ría de Vigo

Initial (T0) values of diversity, evenness, and richness were different in the three sampled seasons. In spring, Shannon diversity (H) and evenness (J) showed their highest values ($H=4.6$, $J=0.8$). In summer, richness (S) had its lowest value ($S=304$). In autumn, H and J had their lowest values ($H=4.14$, $J=0.718$), and S showed its highest value ($S=322$) (Table 1). Table S2 shows data on these variables corresponding to the Ria samples.

These indices showed lower values in nutrient addition treatments as compared to the control, except in April, when inorganic and organic nutrient additions slightly increased richness. Including controls and treatments, S ranged from 205 to 341, H from 3.36 to 4.53, and J from 0.63 to 0.78. The difference in H index between controls and mixed additions was significant (Kruskal–Wallis, $P \leq 0.05$). Median Pielou's J values were significantly different from the control in inorganic, organic, and mixed additions (Kruskal–Wallis, $P \leq 0.05$) (Table 1).

Table 1 Prokaryote richness (S), Shannon index (H), and Pielou's J, within each amendment and month

	April			June			October			Median		
	S	H	J	S	H	J	S	H	J	S	H	J
T0	315	4.60	0.80	304	4.35	0.76	322	4.14	0.72	315	4.35	0.76
Control	215	4.04	0.75	230	4.24	0.78	341	4.53	0.78	230	4.24	0.78
Inorganic	220	3.96	0.73	217	3.83	0.71	326	4.28	0.74	220	3.96	0.73*
Organic	224	3.83	0.71	217	3.87	0.72	327	4.13	0.71	224	3.87	0.71*
Mix	205	3.49	0.66	207	3.36	0.63	305	3.8	0.66	207	3.49*	0.66*

Asterisks indicate $P \leq 0.05$ (Kruskal–Wallis)

Changes in prokaryotic community composition on-shelf waters off the Ría de Vigo

Major prokaryotic taxa

The prokaryotic community at the shelf station was dominated by Flavobacteriales and Archaea at the beginning of the incubation (T0) in spring. After 72 h incubations, the contributions of Alteromonadales and Rhodobacterales increased in the control and in the inorganic nutrient treatments. By contrast, the amendment of organic or mixed additions resulted in increased proportions of Vibrionales with respect to the control (Fig. 2).

In summer, the initial water was dominated by the SAR11 clade, turning into dominance of Flavobacteriales, Alteromonadales, and to a lesser extent, Rhodobacterales after incubations. The addition of inorganic nutrients caused a mild increase in the proportions of Oceanospirillales and Alteromonadales compared with the control, whereas both organic and mixed nutrients caused an important increase in the contribution of Vibrionales, like in spring (Fig. 2).

In autumn, at the beginning of the experiment, the greatest contributions to prokaryotic composition were those of Archaea, and the SAR11 clade. After incubations, the communities were very similar to those of spring and summer, with dominance of Alteromonadales and Flavobacteriales, and to a lesser extent, Rhodobacterales and Oceanospirillales. The addition of inorganic nutrients resulted in a considerable increase in the relative abundance of Oceanospirillales, which represented ca. 40% of the total reads. As in the spring and summer experiments, the additions of organic and mixed nutrients greatly increased the proportions of Vibrionales (Fig. 2).

At higher taxonomic resolution (Fig. 3), changes were mostly related to the seasonal variability of the prokaryotic communities, from the initial water (T0)

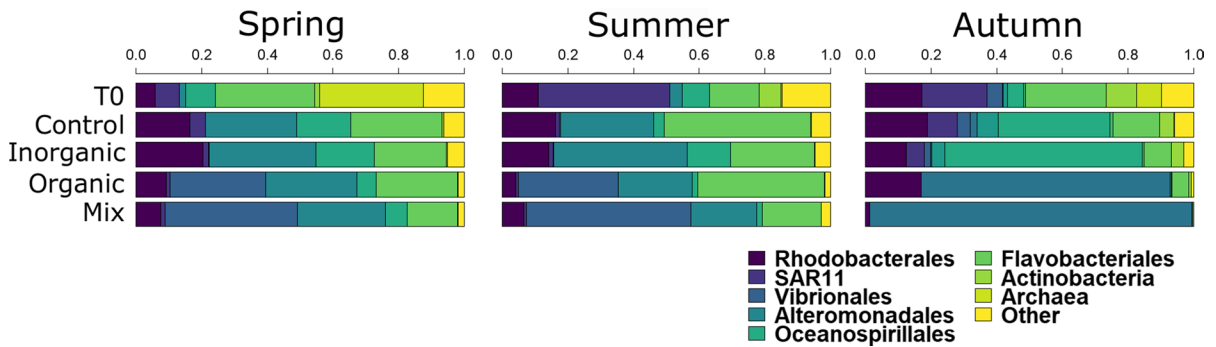


Fig. 2 Relative read abundances of the major prokaryotic taxa in all treatments, throughout the 3 sampling seasons. The samples were obtained either at the beginning of the experiments (T0), or after 72 h-incubation (Control, Inorganic, Organic, and Mix). Control: no addition; Inorganic: addition of 5 μmol

l^{-1} nitrate, 5 μmol l^{-1} ammonium, and 1 μmol l^{-1} phosphorus; Organic: 5 μmol l^{-1} glucose and 5 μmol l^{-1} equimolar mix of 18 amino acids (excluding tyrosine and cysteine); Mix: mixture of Inorganic and Organic

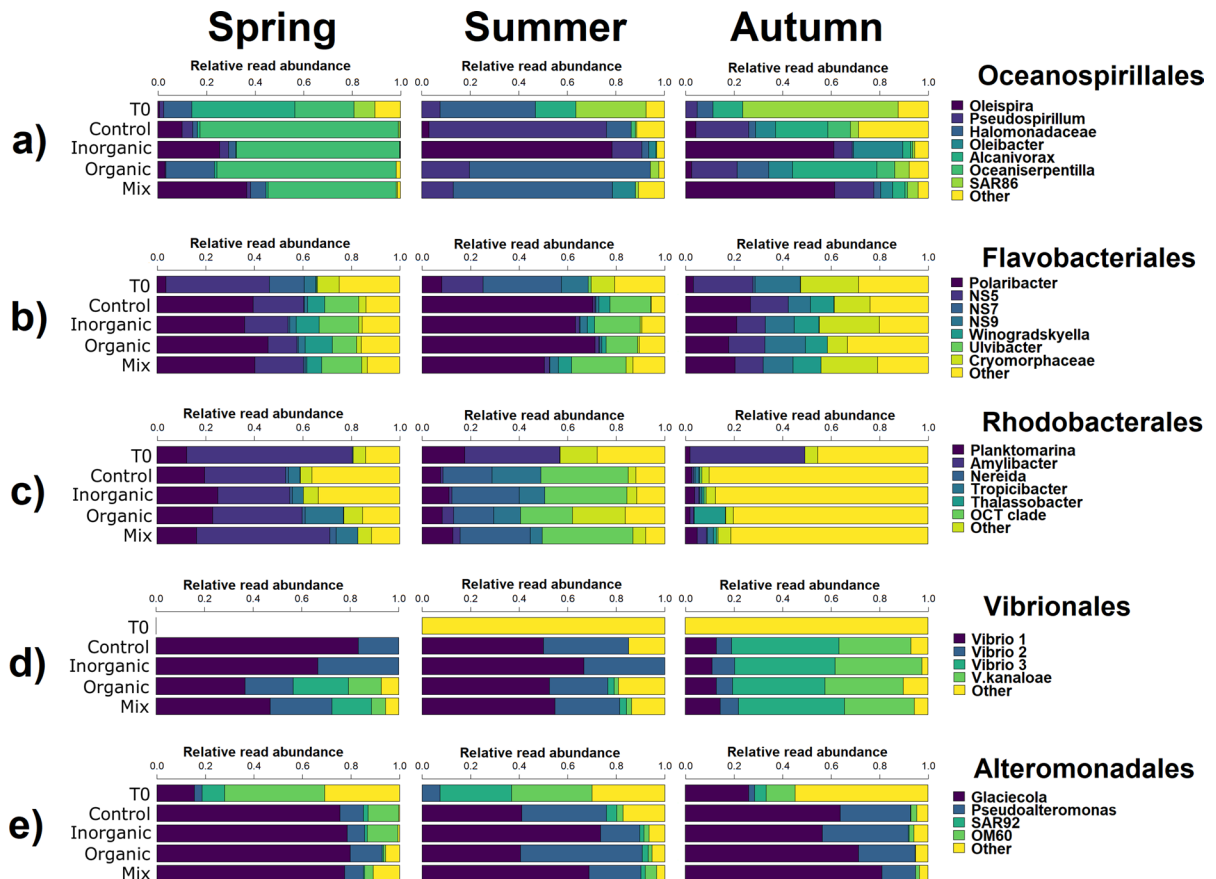


Fig. 3 Relative OTU abundance within the orders Oceanospirillales, Flavobacteriales, Rhodobacterales, Vibrionales, and Alteromonadales in all treatments, throughout the 3 sampling seasons. The samples were obtained either at the beginning of the experiments (T0), or after 72 h-incubation (Control, Inorganic, Organic, and Mix). Control: no addition; Inor-

ganic: addition of 5 μmol l^{-1} nitrate, 5 μmol l^{-1} ammonium, and 1 μmol l^{-1} phosphorus; Organic: 5 μmol l^{-1} glucose and 5 μmol l^{-1} equimolar mix of 18 amino acids (excluding tyrosine and cysteine); Mix: mixture of Inorganic and Organic. Blank bars did not have any read assigned to the order

to the unamended control, as described below for the major orders detected in this study.

Oceanospirillales

In spring, except T0, all samples were greatly dominated by *Oceaniserpentilla* (Fig. 3a). In summer, the inorganic addition greatly increased the proportion of the *Oleispira* genus, while other samples were dominated by the Halomonadaceae family or *Pseudospirillum* (Figs. 2, 3a). In autumn, the Oceanospirillales were dominated by the SAR86 clade, in the initial waters, but the composition notably changed after incubations. In the control and the organic addition, the taxa which contributed the most were *Pseudospirillum* and *Alcanivorax*, with increased relative abundance of Halomonadaceae in the organic treatment; while after both the inorganic and mixed addition, the order was dominated by *Oleispira* (Fig. 3a).

Flavobacteriales

Overall, the nutrient additions did not change the composition of the Flavobacteriales order with respect to the control (Fig. 3b). In spring, the composition of the order Flavobacteriales at T0 was dominated by the NS5 clade, but it developed into a diverse community, in which *Polaribacter* was dominant. In summer, the composition of Flavobacteriales at the beginning of the experiment was dominated by uncultured clades, such as NS5, NS7, and NS9. After incubations, there was a strong dominance of *Polaribacter* in all samples (Fig. 3b). In autumn, the Flavobacteriales order in the initial water was dominated by the NS5 and NS9 clades, along with the Cryomorphaceae family. After incubations, the composition barely changed, except for an increase in the proportion of reads assigned to *Polaribacter* in all samples, except in the control. The Cryomorphaceae family showed greater proportions after inorganic and mixed additions, than in the control (Fig. 3c).

Rhodobacterales

In spring, the Rhodobacterales were mostly dominated by *Amylibacter*, which increased after mixed additions. (Fig. 3c). In summer, the composition of the order showed a high proportion of *Amylibacter*, which changed after incubations, leading to a

Rhodobacterales order dominated by *Nereida* and *Thalassobacter*, with the OCT clade, *Roseovarius*, and *Amylibacter* increasing their proportions after organic additions (Fig. 3c). In autumn, at T0, the Rhodobacterales were, again, dominated by *Amylibacter*, and other taxa, but the proportion of *Amylibacter* was greatly reduced after incubations in the control and nutrient addition treatments. Organic additions increased the proportions of *Tropicibacter*, with respect to the control (Fig. 3c).

Vibrionales

Sequences belonging to Vibrionales were not detected at T0 in spring. The composition changed substantially after incubations in all sampling seasons. In fact, in spring, *Vibrio* 3, which was almost absent in the control and the inorganic samples, became one of the main taxa after organic and mixed additions, along with *Vibrio* 1 and 2. Organic and mixed additions increased the contributions of *Vibrio* 3 and *V. kanaloae*. In summer and autumn, the composition did not change among treatments; in the case of summer, *Vibrio* 1 and 2 dominated, whereas *Vibrio* 3 and *V. kanaloae* were important contributors in the autumn composition. (Fig. 3d).

Alteromonadales

In the initial (T0) water in spring, around 40% of Alteromonadales was dominated by the OM60(NOR5) clade, with lower proportions of other taxa (Fig. 3e). After incubations, the order evolved into dominance of *Glaciecola*. In summer, the initial community within the order involved virtually even contributions of the SAR92 and OM60(NOR5) clades, and other taxa. After incubations, the composition evolved into high proportions of *Glaciecola*, and *Pseudoalteromonas*. After inorganic and mixed additions, *Glaciecola* dominated the composition of Alteromonadales. In autumn, the initial composition of the order involved approximately 25% *Glaciecola*, and lower percentages of the OM60(NOR5) clade, and nearly 40% was assigned to other taxa. After incubations, the order was dominated by *Glaciecola*, with smaller proportions of *Pseudoalteromonas*, and slight changes across samples. (Fig. 3e).

Biotic and abiotic factors driving changes in prokaryotic community composition in the Ría de Vigo and the adjacent shelf

We combined the datasets obtained in the nutrient addition experiments conducted in the Ría de Vigo and described in Gutiérrez-Barral et al. (Gutiérrez-Barral et al., 2021), with those obtained on the shelf adjacent to the Ría de Vigo and described here, with the aim of detecting which OTUs or nutritional resources drove the changes in prokaryotic community composition associated with the experimental nutrient enrichment (Fig. 4). Combination of datasets implied that samples from both sites were processed in the same clustering pipeline. The distance-based linear model (DistLM) performed using, as explicative variables, the CLR abundance of the 20 OTUs with the

greatest relative contribution, explained 63.1% of the fitted variation, and the significant OTUs were plotted on a distance-based redundancy analysis (dbRDA) (Fig. 4a). Among the OTUs explaining the largest fraction of variation, *Amylibacter* 2 (9.9%, sequential test), Rhodobacteraceae 4 (2.2%), and the archaeon Marine Group II 2 (4.2%) positively correlated with dbRDA1 and dbRDA2 axes, and were mostly associated with controls, organic, and inorganic treatments from spring and summer samples. On the other hand, *Vibrio kanaloae* 4 (33.7%); associated with organic and mixed samples mostly in autumn, negatively correlated with both axes; *Oleispira lenta* 1 (21.3%), *Glaciecola* 1 (13%), SAR11 Surface 1_2 (7.7%), and *Oceaniserpentilla* 1 (1.8%), showed positive correlations with the dbRDA1 axis, and negative correlations with the dbRDA2 axis, being associated with control and inorganic treatments in the three seasons, both at the ría and on the shelf (Fig. 4a).

The distance-based linear model (DistLM), using as explicative variables the concentrations of inorganic nutrients, dissolved organic carbon, and nitrogen explained 78.4% of the fitted variation and included 5 variables, although only DON, DOC, and ammonium were statistically significant ($P \leq 0.05$) (Fig. 4b). DON (14.3%, sequential test) and DOC (24.7%) grouped near organic and mixed samples in autumn, both inside the ría and on the shelf; whereas ammonium (0.15%) was closer to organic and mixed additions outside the ría in spring (Fig. 4b).

Compositional and biomass resistance to nutrient amendments inside the Ría de Vigo and in the adjacent shelf

In the PCoA plot (Fig. 5), the initial water communities (T0) located far from the rest of the samples in all the experiments. The distance between T0 and the control tended to be shorter in the ría than on the shelf, whereas the distance between controls and organic or mixed samples was longer in the ría. In spring, both ría and shelf samples grouped together, and close to the summer samples, which also grouped together, independently of their origin. The autumn samples from inside or outside the ría grouped together, but clearly apart from the spring and summer samples (Fig. 5). The observed differences among seasons were moderate (ANOSIM, $R=0.5052$, $P=0.001$), low among treatments (ANOSIM, $R=0.2916$, $P=0.001$), and very

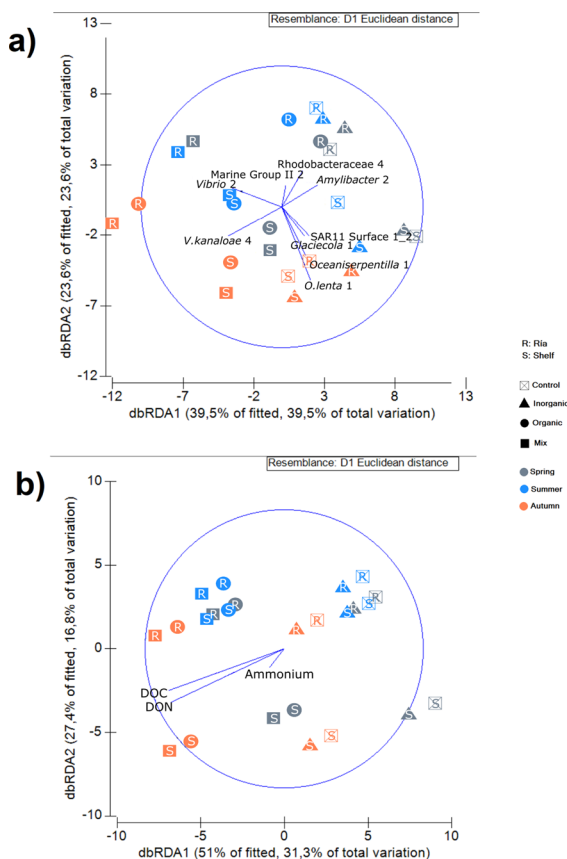


Fig. 4 dbRDA using the most abundant significant (DISTLM, $P < 0.05$) CLR-transformed OTUs as response variables (a), and the main nutrients (b). R: Ría de Vigo. S: Shelf. *O.lenta*: *Oleispira lenta*, *V.kanaloae*: *Vibrio kanaloae*

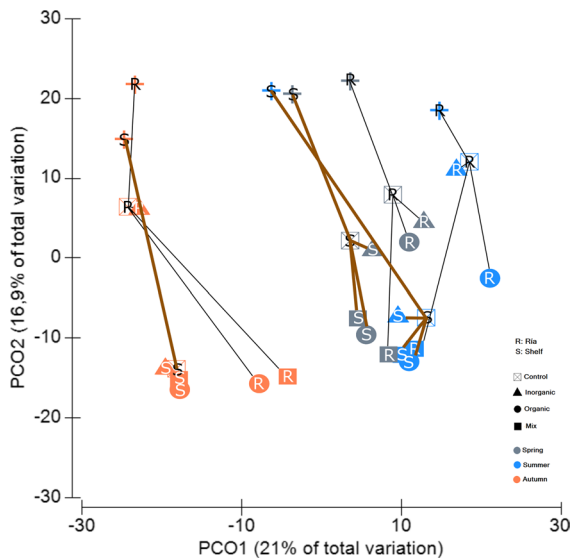


Fig. 5 PCoA on a CLR-transformed OTU table (Euclidean distance) showing the T0, control, inorganics, organic, and mixed additions in the Ría de Vigo (R), and the adjacent shelf (S), in 2013 and in 2014, respectively. OTUs were CLR-transformed

low between sites (ANOSIM, $R=0.1795$, $P=0.002$) although all of them were statistically different.

The differences in prokaryotic community composition or biomass observed between T0 and the controls were associated with the effect of incubation, as well as the natural temporal dynamics of the initial community. The compositional resistance to natural change (t -test, $P=0.00852$), and the biomass resistance to inorganic nutrients (t -test, $P=0.0245$) were significantly greater at the ría samples, than at on-shelf samples (Fig. 6). By contrast, biomass and compositional resistance to mixed nutrient additions were significantly greater on-shelf than inside the ría. Across experiments, compositional resistance was significantly higher in spring than in autumn, whereas biomass resistance showed higher values in spring than in summer, (t -test, $P=0.0365$).

Discussion

Response of prokaryotic diversity and community composition to nutrient additions

The addition of inorganic, organic, and mixed nutrients resulted in significant reductions in prokaryotic

equitability and diversity, which contrasts with the lack of changes in diversity indices observed in the eukaryote community in the same experiments (Hernández-Ruiz et al., 2020; Table 1). Gutiérrez-Barral et al. (2021) showed the results of the experiments carried out inside the Ría de Vigo describing reductions in diversity, richness, and evenness after mixed additions; in diversity and evenness after organic additions, and only in diversity after inorganic additions in experiments.

The most substantial changes in prokaryotic plankton composition happened after the addition of mixed and organic additions (Fig. 2), probably because those additions included labile organic compounds readily available for uptake by prokaryotes. These results are consistent with previous research carried out using the same experimental approach in the same sampling area, together with the results using water from the middle Ría de Vigo (Teira et al., 2011; von Scheibner et al., 2017; Hernández-Ruiz et al., 2020).

Within Gammaproteobacteria, the response of Oceanospirillales to inorganic additions coincided with a great increase in the proportion of the OTUs affiliated to the genus *Oleispira* (Fig. 2, 3a), a significant increase in bacterial biomass (Fig. S3), and a decrease in Pielou's evenness (Table 1). Gutiérrez-Barral et al. (2021) also described an increase in the proportion of *Oleispira* after inorganic nutrient additions inside the ría. In fact, the CLR abundance of *Oleispira lenta* 1, described in the literature as obligately hydrocarbonoclastic, explained a significant portion of the variation in the response of the prokaryotic community to nutrient amendments, and was closely associated with the inorganic treatments in autumn (Golyshin et al., 2010) (Fig. 4a). In these cases, the prokaryotic community might be initially limited by organic and inorganic nutrients, and once inorganic nutrients were added, the Oceanospirillales order could be one of the few taxa able to use sources of carbon present in the water, in an area affected by oil spills (Bôto et al., 2021).

Among the Vibrionales, the compositions were very similar to those described in Gutiérrez-Barral et al. (2021) inside the ría, where two OTUs dominated in spring and summer, and *Vibrio kanaloae* was one of the main contributors to the order in autumn, along with *Vibrio* 3 (Fig. 3d). The response of these *Vibrio* OTUs was linked to the availability

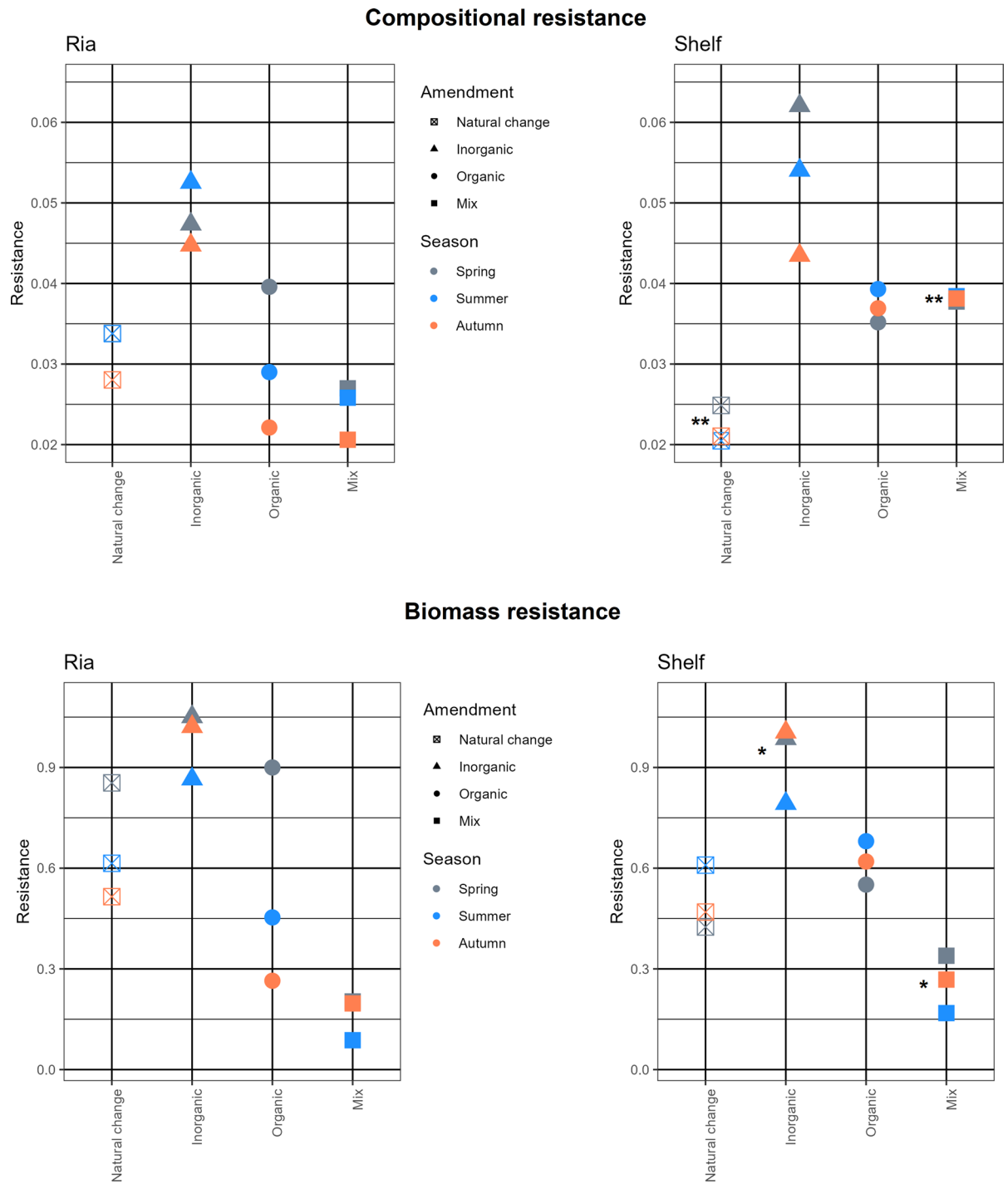


Fig. 6 Inverse of Euclidean distances between CLR-transformed values at T0 and control, and control and treatment; as well as the result of dividing biomass in T0 by biomass in the

control sample, and value in the control by that in a treatment. Asterisks indicate significant difference between resistance across sampling sites (* $P < 0.05$; ** $P < 0.01$)

of dissolved organic matter (Fig. 4b), as described by Gutiérrez-Barral et al. (2021).

Lastly, the order Alteromonadales was mainly composed of the genus *Glaciecola* (Fig. 3e), which has been associated with early phases of phytoplankton blooms (von Scheibner et al., 2017), that could partly explain the greater proportions of this genus after inorganic additions than in control samples in summer (Fig. 2), when the addition of inorganic nutrients caused a threefold increase in chlorophyll *a* concentration compared to the control (Hernández-Ruiz et al., 2020).

Prokaryotic composition (compositional) and biomass resistance on the shelf and inside the Ría de Vigo

The prokaryotic resistance to inorganic nutrient inputs both in terms of biomass and composition was the highest, which likely reflects the adaptation of these communities to the frequent and intermittent pulses of inorganic nutrients associated with the upwelling dynamics and freshwater runoff in this coastal area (Ríos et al., 1992; Gago et al., 2005; Barton et al., 2015). A non-excluding explanation would be that prokaryotic communities in this coastal system are mostly limited by organic carbon, and thus barely respond to inorganic nutrient amendments (Martínez-García et al., 2010; Teira et al., 2016).

The resistance estimates from the dataset of this study were compared with those from a previous one, where equal inorganic, organic, and mixed nutrients were added to microcosms from the central section of the Ría de Vigo in May, July, and October 2013 (Figs. 4, 5, 6; Gutiérrez-Barral et al., 2021). Compositional resistance to natural change and biomass resistance to the inorganic treatment were significantly greater inside the ría than outside (Fig. 6). It has been suggested that resistance in prokaryotic communities might be related to the history of disturbance, thus causing prokaryotic communities to become resistant to the same or even a different type of perturbation (Renes et al., 2020). In this case, the ría is characterized by higher primary production rates than the shelf, is affected by a few small rivers which contribute inorganic nutrients, and is more influenced by anthropogenic pollution, oil spills, or incompletely treated sewage water discharge, which altogether could contribute to an increase in the resistance of

prokaryotic communities to natural change and the inorganic treatment (Gago et al., 2005; Lorenzo et al., 2005; Fernández et al., 2016; Román et al., 2019; Bôto et al., 2021). The greater biomass resistance in the ría associated with inorganic amendments could also be related to the fact that the sampled communities were more diverse in the ría (Table S2) than on-shelf waters (Table 1), which could lead to functional redundancy. This might be related to the notion that in diverse communities, different taxa may occupy the same niches, creating an “insurance” against perturbations (Allison & Martiny, 2008; Awasthi et al., 2014; Louca et al., 2018). However, the concept of functional redundancy in marine microbial communities has been questioned, since Galand et al. (2018) found strong correlations between the compositions of microbial communities and their functions, which could mean that different communities carry out different functions. When considering the whole dataset, we found that compositional resistance is pronouncedly linked to biomass resistance (Fig. 6), supporting the idea of a reduced functional redundancy in this coastal ecosystem. In fact, Fuhrman et al. (2006) demonstrated that the distribution and abundance of certain prokaryotic plankton taxa can have strong predictability based on the conditions of the water, which suggests little functional redundancy.

Unexpectedly, both compositional and biomass resistance to mixed additions were significantly greater on the shelf than inside the ría (Figs. 6, S2), which suggests that the shelf station may be more exposed to intermittent inputs of both inorganic and utilizable organic matter than the ría. Consistently, higher DOC and DON concentrations were measured in the initial water outside than inside the ría (Table S1). Prego (1993) described greater remineralization and sedimentation of organic matter at the mouth of the ría than at its inner part, which suggests a higher concentration of labile organic matter at the outer section of the embayment, resulting from surface currents which carry the newly created organic nutrients out of the ría (Fraga, 1981; Prego, 1993; Álvarez-Salgado et al., 1999). Furthermore, the shelf can receive nutrient plumes from the river Miño, 30 km south of the ría (Des et al., 2019).

There were noticeable differences in nutrient and chlorophyll *a* concentrations (greater concentrations in the middle ría than on the shelf) (Table S1), along with the community compositions of prokaryotic

plankton between the ría and the shelf (Fig. S1). This might explain, at least partially, the differences in resistance. For instance, the Rhodobacterales order showed greater proportions in the ría than on the shelf (Fig. S1), whereas the SAR11 clade had greater proportions outside the ría [Fig. S1, which could explain the higher resistance to natural change in the ría than on the shelf (Fig. 6)]. The Rhodobacterales order can include copiotrophic taxa, so it might be better adapted to perturbations; whereas the SAR11 clade is oligotrophic, which could make it sensitive to environmental changes (Giovannoni, 2017; Teira et al., 2019; Gutiérrez-Barral et al., 2021). While in most studies resistance is reported to be caused by environmental factors which trigger a response in physiological attributes, such as cell plasticity, stress tolerance, gene expression, etc., the results obtained in this investigation show that the presence of specific taxa in the initial community may also contribute to compositional resistance (Shade et al., 2012; Sjöstedt et al., 2018; Renes et al., 2020).

Despite our results pointing to nutrient availability as a likely cause of resistance, other stressors not addressed here could foster the prokaryotic community resistance to the experimental nutrient perturbations (Renes et al., 2020). In this context, future experiments aimed at assessing resilience, besides resistance, to both pulse (short-term) and press (long-term) nutrient enrichment disturbances would be of particular interest.

Conclusions

Both compositional, and biomass resistance to nutrient additions in prokaryotic plankton communities may be of different magnitude depending on the inputs received by the two mesotrophic coastal areas. Prokaryotes inhabiting the embayment showed greater responses to combined organic and inorganic nutrient amendments than those from the adjacent shelf, and presented greater responses to organic and mixed additions than to inorganic amendments alone. These results are of particular relevance in the current global change scenario, where anthropogenic organic nutrient inputs into coastal systems are expected to increase and nutrient limitations might switch, which suggests that further research is needed on this topic

to infer the impact of these changes on prokaryotic communities.

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Author contributions AG: Writing—Original draft, Writing—Review and Editing, Formal Analysis, Visualization. EF: Writing—Review and Editing, Methodology, Resources, Conceptualization, Project Administration, Funding Acquisition. MH: Investigation. ET: Writing—Review and Editing, Methodology, Resources, Conceptualization, Project Administration, Funding Acquisition.

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Data availability The prokaryotic DNA sequences are publicly available at the European Nucleotide Archive, <https://www.ebi.ac.uk/ena> (PRJEB47591).

Declarations

Competing interests The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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