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# High-throughput sequencing as a tool for monitoring prokaryote communities in a wastewater treatment plant

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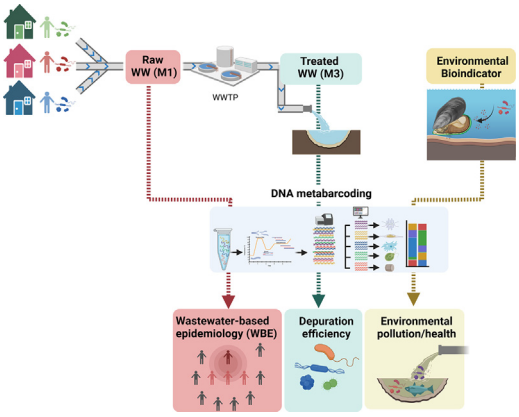
## HIGHLIGHTS

- Different prokaryote communities were obtained from mussels, raw and treated wastewater samples.
- Genetic material of potential human pathogens was detected in raw wastewater.
- Abiotic factors could be involved in the seasonal prevalence of pathogens in raw water.
- The depuration efficiency was confirmed after wastewater treatment with chlorine.

## GRAPHICAL ABSTRACT

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## ABSTRACT

In this study, the DNA metabarcoding technique was used to explore the prokaryote diversity and community structure in wastewater collected in spring and winter 2020–2021 as well as the efficiency of the treatment in a wastewater treatment plant (WWTP) in Ría de Vigo (NW Spain). The samplings included raw wastewater from the inlet stream (M1), the discharge water after the disinfection treatment (M3) and mussels used as bioindicators of possible contamination of the marine environment. Significant differences were discovered in the microbiome of each type of sample (M1, M3 and mussels), with 92 %, 45 % and 44 % of exclusive OTUs found in mussel, M3 and M1 samples respectively. Seasonal differences were also detected in wastewater samples, with which abiotic parameters (temperature, pH) could be strongly involved. Bacteria present in raw wastewater (M1) were associated with the human gut microbiome, and therefore, potential pathogens that could be circulating in the population in specific periods were detected (e.g., *Arcobacter* sp. and *Clostridium* sp.). A considerable decrease in putative pathogenic organisms from the M1 to

*Abbreviations:* WWTP, wastewater treatment plant; WBE, wastewater based epidemiology; CFUs, colony-forming units; ARGs, antibiotic resistance genes; AOB, ammonia oxidizing bacteria; NOB, aerobic nitrite bacteria; EBPR, enhanced biological removal organisms; PAOs, polyphosphate accumulating organisms; BOD, biochemical oxygen demand; COD, chemical oxygen demand; TKN, total Kjeldahl nitrogen; TC, total coliforms; FC, faecal coliforms; TE, total Streptococcus; CTAB, cetyl trimethyl ammonium bromide; SRA, sequence read archive; OTU, operational taxonomic units; NMDS, nonmetric multidimensional scaling; RDA, redundancy analysis; GLM, generalized linear model; CPL, characteristic path length; NGS, next generation sequencing; CLR, centred log-ratio; ANOSIM, analysis of similarities.

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M3 wastewater fractions and the scarce presence in mussels (<0.5 % total reads) confirmed the effectiveness of pathogen removal in the wastewater treatment plant.
Our results showed the potential of the DNA metabarcoding technique for monitoring studies and confirmed its application in wastewater-based epidemiology (WBE) and environmental contamination studies. Although this technique cannot determine if the infective pathogens are present, it can characterize the microbial communities and the putative pathogens that are circulating through the population (microbiome of M1) and also confirm the efficacy of depuration treatment, which can directly affect the aquaculture sector and even human and veterinary health.

## 1. Introduction

The major challenge of wastewater treatment is the removal of contaminants and faecal microorganisms from discharge water (Liu et al., 2020). The main contaminants present in wastewater include organic compounds (Liu et al., 2017), heavy metals (Elzwayie et al., 2017), suspended nutrients (e.g., nitrogen and phosphorus) (Gross and Hagy, 2017) and microorganisms (e.g., viruses, bacteria, prokaryotes), including pathogens and multidrug-resistant bacteria of anthropogenic origin. The inefficient treatment and disinfection of wastewater can discharge high loads of these contaminants and pathogens into the environment and therefore cause severe ecological impacts (e.g., eutrophication and habitat destruction) (Liu et al., 2020; Preisner et al., 2021) and global health risks (Templar et al., 2016). Hence, the most critical task for wastewater treatment is to monitor the human sewage microbiome to document its fate and removal (Cai et al., 2014).

The microbial community of wastewater treatment plants (WWTPs) is dominated by bacteria (Wagner et al., 2002), which have been studied for several years by culture-dependent methods and molecular tools based on polymerase chain reaction, fluorescent in situ hybridization and cloning (Muyzer et al., 1993; Schuppler et al., 1995; Erhart et al., 1997; Sánchez et al., 2011; Ye and Zhang, 2013; Ramo et al., 2017; Hortelano et al., 2020). Routine wastewater monitoring for public health is based on the detection of cultivable pathogenic bacteria of interest, such as faecal coliform counts in colony-forming units (CFUs) per volume of filtered water (Cai and Zhang, 2014; Chan et al., 2019). However, wastewater samples are characterized by their high genetic diversity, with almost 99 % of uncultivable bacteria (Garrido-Cardenas et al., 2017). This limitation has been overcome by next-generation sequencing (NGS) techniques, such as DNA metabarcoding approaches (Taberlet et al., 2012), which benefit water quality monitoring in the public health field because they can be used to simultaneously test all microbial pathogens, including uncultivable microorganisms. This information also allows tracking possible microbial contamination in the environment (e.g., rivers, lakes, estuaries, etc.), but also optimizing wastewater treatment systems that promote the growth of the microbial population responsible for the effective degradation of contaminants in wastewaters (Chan et al., 2019). The use of high-throughput sequencing-based methods in the study of prokaryote communities associated with activated sludge (Saunders et al., 2016; Wu et al., 2019; Nierychlo et al., 2020; Yu and Zhang, 2020), as well as the detection of bacteria carrying antibiotic resistance genes (ARGs), are among the most interesting topics of study in recent years in WWTPs (Kang et al., 2022; Nguyen et al., 2021). However, only a few studies have addressed the use of amplicon sequencing techniques in wastewater samples to determine prokaryote abundance and diversity differences at each treatment step (Marti et al., 2013; Ye and Zhang, 2013; Da Silva et al., 2015; Ahmed et al., 2017; Zhang et al., 2019; Martínez-Santos et al., 2018), the efficiency of depuration of pathogens (Cai et al., 2014; Lu et al., 2015; Numberger et al., 2019; Kumaraswamy et al., 2014; Santiso-Bellón et al., 2020) and seasonal and short-term diversity fluctuations (Ju et al., 2014; Guo et al., 2019; Soares-Castro et al., 2019; de Celis et al., 2020).

The activated sludge in WWTPs is in general mainly composed of aerobic and anaerobic microorganisms that serve effective roles in the removal of organic pollutants and nutrients such as nitrogen and phosphorus (Saunders et al., 2016; Wu et al., 2019; Nierychlo et al., 2020; Yu and Zhang, 2020) and generate useful compounds of interest, such as fertilizers and biofuels (Sharma and Arivalagan, 2021). The most dominant bacteria in this process are usually from the Proteobacteria phylum, followed by Bacteroidetes, Chloroflexi, Actinobacteria, Planctomycetes and Firmicutes (Ferrera and Sánchez, 2016). Prokaryotes of the activated sludge metabolize toxic compounds released in wastewater, such as ammonia and nitrite, that exert harmful effects on aquatic life and contribute to eutrophication. Ammonia oxidation is performed by bacterial ammonia oxidizers (AOB) (e.g., the Betaproteobacteria *Nitrosomonas* sp. and *Nitrosospira* sp. and the Gammaproteobacteria *Nitrosococcus* sp.), ammonia oxidizer archaea (AOA) and anaerobic ammonia oxidizers (anammox) (e.g., Planctomycetes). After the first oxidative step, the AOB group produces nitrite, which is transformed to nitrate by aerobic nitrite bacteria (NOB) (e.g., *Nitrobacter* sp., *Nitrococcus* sp. and *Nitrospira* sp.). The anammox removes, under anoxic conditions, nitrogen from ammonia to produce nitrogen gas ($N_2$). Denitrification (nitrate or nitrite transformation into gaseous forms $N_2$ and $N_2O$) is performed by Archaea, Fungi and different bacterial lineages, including the genera *Bacillus, Pseudomonas, Methylobacterium,* and *Paracoccus*. Microorganisms also eliminate or accumulate phosphorus in WWTPs, and they are known as enhanced biological removal organisms (EBPR) and polyphosphate accumulating organisms (PAOs), respectively (Ferrera and Sánchez, 2016).

The implementation of molecular-based methods as wastewater surveillance tools for the detection of potential pathogens has been growing in the context of One Health in last decades, especially since the emergence of COVID-19 pandemic (Ahmed et al., 2017; Medema et al., 2020; Randazzo et al., 2020; Gallardo-Escárate et al., 2021; Novoa et al., 2022).Therefore, the implementation of high-throughput sequencing methodologies in wastewater epidemiology could serve as a useful tool to track potential pathogens of global health concern by providing insights into the whole population in a specific area instead of testing individual people. Furthermore, the value of wastewater as an early indicator of circulating disease through the population has been demonstrated, providing preventive measures for disease mitigation (Diamond et al., 2022; Pájaro et al., 2022).

Due to the potential of high-throughput sequencing methodologies, the main objectives of this work were to explore, by high-throughput sequencing, the microbiome in raw water and the discharge effluent after treatment and disinfection of a wastewater treatment plant. Additionally, since the effluent is discharged to Ría de Vigo (NW Spain), a very productive aquaculture area, we also analysed the microbiome of wild mussels located near the effluent discharge. Mussels, due to their filtering-feeding activity, could accumulate microbial organisms as an indicator of putative pathogenic organisms that could resist disinfection treatment and be introduced in the marine environment.

## 2. Methodology

### 2.1. Sampling and description of the WWTP

The study was carried out in the WWTP of Baiona (NW Spain), a medium-sized municipality with a population of 12,090 inhabitants located on the Atlantic Ocean side of the Ría de Vigo (Supplementary Fig. 1), an area characterized by its high productivity in the aquaculture sector (especially mussel culture by rafts) and its exposure to several anthropogenic factors derived from industrial and domestic wastes (Guerra et al., 2002). The WWTP is characterized by a designed flow of 7314 $m^3$/day and a peak flow of 690 $m^3$/h. The treatment process consists of water pre-treatment (screening and grit removal), followed by a biological secondary treatment and a disinfection process. In the pretreatment stage, larger and smaller suspended solids are

removed by using two rotary screens. Then, the water flows to a grit removal-degreasing tank where sand and grease are removed, thus completing the pretreatment phase. The biological treatment (activated sludge) consists of two carousel reactors with a surface aeration system using rotors, where the nitrification-denitrification process is conducted for the elimination of nitrogen and organic matter. Oxygenation needs are regulated by automatic control of the venting systems using oxygen probes installed in the bioreactors. The bioreactor outlet stream is clarified in two secondary settling tanks. Clarified water is used for tertiary treatment, which is disinfection with sodium hypochlorite. All waste generated in the treatment process is externally managed by an authorized waste manager. The final treated effluent is discharged into the sea through a marine outfall (Geseco Aguas, 2022).

Sampling was performed in two seasonal periods (May–June 2020 and December 2020–January 2021), and a total of 10 samples from each sampling point were collected in the WWTP as 24 h composite samples using an automatic sampling system (Teledyne ISCO, model 3700 full size, USA). One litre of each wastewater sample was collected and stored in amber glass bottles and kept at 4 °C. Samples from the raw wastewater in the inlet stream (named M1) were collected, and they represented water samples with anthropogenic and domestic origins. Samples from the discharge effluent after disinfection treatment (named M3) were taken to examine the efficiency of wastewater treatment. In the marine environment wild mussels (named BIOIND) were sampled as bioindicators in a distance of 175 linear meters away from the discharge effluent point to evaluate the possibility of faecal bacteria from WWTPs growing in their tissues. A summary of the samplings is presented in Table 1. Monthly physicochemical parameters were measured in wastewater before and after treatment in the sampling period (2020 − 2021): pH, temperature (°C), conductivity (S/m), biochemical oxygen demand (BOD [mg/L $O_2$]), chemical oxygen demand (COD [mg/L $O_2$]), suspended solids (SS [mg/L]), total Kjeldahl nitrogen (TKN) and total phosphorus (TP). Faecal microbial indicators were also measured before and after the wastewater treatment: total coliforms (TC, [CFU/100 mL]), faecal coliforms (FC, [CFU/100 mL]) and total *Streptococcus* (TE, [CFU/100 mL]) according to the European and Spanish wastewater regulations (Dir 91/271/EEC 21 May 1991; RDL 2116/1998 October 2nd). All environmental factors were normalized (z score) for redundancy analysis (RDA). Abiotic factors of the marine environment at Baiona station (B1) were downloaded from INTECMAR (Intecmar (Instituto Tecnológico para el Control del Medio Marino de Galicia), Xunta de Galicia, 2010) to detect whether possible environmental changes could affect the results in the mussel microbiome, which was used as an environmental bioindicator.

### 2.2. DNA isolation, amplification and sequencing

A total of 29 samples, including M1, M3 and mussels, were processed. Water samples were pretreated by filtration (20–25 μm cellulose filter) followed by precipitation with aluminium chloride following the procedure described by Randazzo et al., 2019. Concentrated water samples were then processed for DNA extraction using a Maxwell RSC Pure Food GMO and Authentication kit (Promega, Madison, USA) with a slight modification. Briefly, 300 μL of concentrated sewage water was mixed vigorously with 400 μL of cetyl trimethyl ammonium bromide (CTAB), 40 μL of proteinase K and 20 μL of RNase A, and the mix was incubated for 10 min at 60 °C. After centrifugation at 16,000 ×*g* for 10 min, the supernatant was mixed with 300 μL of lysis buffer and transferred to the Maxwell® Instrument Cartridge, where DNA was automatically isolated in 50 μL of elution buffer. Mussels were carefully extracted from the shell. Small fragments from the gill, mantle, gonad and digestive gland of 3 individuals of each sampling were pooled in a single sample for DNA isolation using the Maxwell RSC Blood DNA Kit (Promega, Madison, USA) according to the manufacturer's instructions. The concentration and purity of the isolated DNA were determined with a Qubit (Thermo Fisher Scientific, Waltham, USA) fluorometer, and DNA was kept at −20 °C until further use.

The V3-V4 region of the 16S rRNA gene (~460 bp long) was amplified using the universal prokaryotic-specific primers (16S amplicon PCR forward primer = 5′-CCTACGGGNGGCWGCAG-3′ and 16S amplicon PCR reverse primer 5′GACTACHVGGGTATCTAATCC-3′) described in Klindworth et al. (2013). After amplicon purification with AMPure XP beads, a library for 16S rRNA gene metabarcoding sequencing was prepared using the Herculase II Fusion DNA Polymerase Nextera XT Index Kit V2 (Illumina), and paired-end sequencing (2 × 300) was performed on an Illumina MiSeq platform (Macrogen, Korea).

The raw read sequences obtained were deposited in the Sequence Read Archive (SRA) (http://www.ncbi.nlm.nih.gov/sra) under the BioProject accession number PRJNA874871.

### 2.3. Bioinformatic analysis

The Microbial Genomics Module of Qiagen CLC Workbench (version 21) was used for sequencing data analysis. Paired-end reads were trimmed using the sequences of the primers, and adapters were also removed. Low-quality reads were also trimmed by quality scores (limit 0.05 = minimum average quality score 20) and by the number of ambiguous nucleotides (maximal 2 ambiguous nucleotides). The OTU clustering tool was used for merging the paired reads followed by the open-reference OTU picking. Briefly, reads were clustered at a 97 % level of similarity into operational taxonomic units (OTUs) against the 16S SSU SILVA 99 % reference database (release 132). Next, reads not associated with the reference database were de novo clustered to create new OTUs with 90 % similarity. Singletons and chimaeras were excluded from the analysis.

### 2.4. Microbial community composition

One of the objectives of this study was to determine the number of common and exclusive OTUs between both wastewater fractions and mussels. Therefore, to avoid overrepresentation of a given compartment, the OTU table of prokaryotes was subsampled to the sample with the lowest number of reads, which was 21,246 reads.

The OTU richness, Shannon–Wiener and Pielou alpha diversity indices were estimated (Vegan R package) from the subsampled OTU table, and the statistical Kruskal–Wallis test was performed to evaluate differences in alpha diversity present for each type of sample. The UpsetR tool (Conway et al., 2017) was used with the subsampled OTU table to estimate the number of common and exclusive prokaryote OTUs from the inlet raw water (M1), the discharge water after disinfection (M3) and mussels.

The community composition of prokaryotes was represented as relative abundances calculated from the subsampled abundance OTU table. Taxonomy was represented according to the following criteria. Overall, all the orders representing >1 % of the total reads were represented. When the sum of the relative abundance of all OTUs within an order was between 0.5 % and 1 %, these OTUs were classified at the class or phylum level. Finally, orders with abundances lower than 0.5 % were grouped as “Other” because

**Table 1**
Date and type of sample in this study. Mussel samples (denoted as BIOIND) were not obtained on December 29, 2020 due to weather conditions.

| Spring samplings | | | Winter samplings | | |
|---|---|---|---|---|---|
| Date | Month | Sample | Date | Month | Sample |
| 13/05/2020 | May | M1 | 14/12/2020 | Dec | M1 |
| | May | M3 | | Dec | M3 |
| | May | Bioind | | Dec | Bioind |
| 20/05/2020 | May | M1 | 21/12/2020 | Dec | M1 |
| | May | M3 | | Dec | M3 |
| | May | Bioind | | Dec | Bioind |
| 27/05/2020 | May | M1 | 29/12/2020 | Dec | M1 |
| | May | M3 | | Dec | M3 |
| | May | Bioind | | – | – |
| 03/06/2020 | Jun | M1 | 04/01/2021 | Jan | M1 |
| | Jun | M3 | | Jan | M3 |
| | Jun | Bioind | | Jan | Bioind |
| 10/06/2020 | Jun | M1 | 09/01/2021 | Jan | M1 |
| | Jun | M3 | | Jan | M3 |
| | Jun | Bioind | | Jan | Bioind |

of their low representativeness in the prokaryote composition. The efficiency of depuration treatment of the WWTP was also evaluated by searching for human-animal pathogenic organisms, as well as organisms from the digestive human-animal microbiota detected in M1 samples in the discharge water (M3) and mussels representing >1 % of the total reads.

### 2.5. Statistical analysis

To account for the limitations of working with compositional data, the abundances of the subsampled OTU tables were transformed using the centred log-ratio (CLR). First, zero values were replaced by the minimum abundance value larger than 0 divided by 2 (Gloor et al., 2017), and then, the Vegan R package (Oksanen et al., 2020) was used to perform the CLR transformation.

Nonmetric multidimensional scaling (NMDS) analysis (R Vegan package) based on a Euclidean distance matrix was performed from CLR-transformed data to study differences in prokaryote composition among each type of sample (M1, M3 and mussel). The differences between each type of sample and between seasons (spring, winter) were statistically tested with the analysis of similarity (ANOSIM). Redundancy analysis (RDA) was performed from CLR transformed data and z score normalized physicochemical parameters to analyse the association among the abiotic factors and the community composition for each environmental fraction.

Differential abundance analyses were performed with the Microbial Genomic Module of the CLC workbench (v.21) to statistically detect which OTUs were differentially abundant between each type of sample (M1, M3, mussel) and between spring and winter samplings from each sample type. Briefly, the tool modelled each OTU as a separate generalized linear model (GLM), and then the Wald test was used to determine significance between group pairs. For each fraction or season, the 20 highest $\log_2$ fold changes and significant OTUs (Wald test, FDR, $p < 0.05$) were represented. All the differentially abundant OTUs are included in Supplementary Table 1.

Network analyses were conducted to detect potential keystone interactions within prokaryotes in M1, M3 and mussels. The 30 most abundant and frequent OTUs among all samples were selected to perform network analyses. Spearman correlations with the Benjamini–Hochberg $p$ value adjusted procedure (Benjamini and Hochberg, 1995) at a false discovery rate of 5 % were conducted to minimize false-positive associations. Three network analyses were conducted (M1, M3 and mussels), including all significant correlations (Spearman p value $\leq 0.05$). The Igraph R package was used to obtain the network topology of correlations from 25 nodes and 45 significant edges in M1 and from 16 nodes and 20 significant edges in M3. The network obtained was compared against 100 randomized networks generated using the Erdos–Rényi model. Finally, the significance of the networks was estimated by the two-sample z-test means comparing the random and real values of characteristic path length (CPL) and clustering coefficient (CL).

## 3. Results

### 3.1. Sequencing results

A total of 29 samples for the V3-V4 region of the 16S rRNA sequencing were successfully amplified. After sequencing and trimming, a total of 8,530,690 reads were obtained, with an average of 328,103 reads per sample. The OTU-clustering process generated 6339 OTUs using a total of 616,134 filtered reads (Table 2). A detailed description of the number of reads in each step of the analysis (including trimming and OTU-clustering procedure) for individual samples is specified in Supplementary Table 2, and the subsampled OTU table is included in Supplementary Table 3.

### 3.2. Physicochemical parameters and faecal microbial indicators

The physicochemical parameters of the Baiona WWTP before and after wastewater treatment are represented in Fig. 1 and Supplementary Table 4. The temperature was similar in both raw and treated wastewater, and it was lower in cold periods (14.2 °C on average) than in warm periods (19.6 °C on average). The pH oscillated from 6.7 to 7.5. The strongest differences in pH between raw and treated wastewater were detected from August 2020 to February 2021 (Fig. 1A). Higher values of $BOD_5$, COD, nutrients (N, P), coliforms and *Streptococcus* were obtained in raw wastewater than in treated wastewater (Fig. 1B-D). All of the environmental factors registered after the wastewater treatment were below the maximum limits allowed according to the European and Spanish wastewater regulations (Council directive EU, 1991; Ley 9/2010, 2010) (Fig. 1B-C). Normal values of physicochemical parameters of the marine environment were detected in data downloaded from Intecmar (Instituto Tecnológico para el Control del Medio Marino de Galicia), Xunta de Galicia, 2010, and they are included in Supplementary Table 4.

### 3.3. Alpha and beta diversity of prokaryote communities

Significant differences in prokaryote richness (Kruskal–Wallis, $p = 0.0001$) and Shannon index (Kruskal–Wallis, $p = 0.0026$) were obtained among each wastewater sampling and mussels, but not between the M1 and M3 water samples (on average 1093 OTUs in M1, 985 OTUs in M3 and 230 OTUs in mussels) (Fig. 2 A-B). Nevertheless, similar results were obtained for the Pielou index, which showed a similar evenness of the prokaryote community in each type of sample (Kruskal–Wallis, $p = 0.1088$). Furthermore, higher diversity indices (richness, Shannon and Pielou) were obtained in winter and spring in both the M1 and M3 samples, whereas this pattern was not observed in mussels (Fig. 2C).

Considering the number of total OTUs in each compartment, almost 92.6 % of the total OTUs present in mussels were exclusive, followed by 45.13 % in M3 and 43.87 % in M1 water samples (Fig. 3A). Furthermore, 62 shared OTUs were obtained among the M1, M3 and mussel samplings, and they represented 8.6 %, 2.6 % and 3.2 % of the total reads, respectively (Supplementary Table 5).

In the NMDS analysis, samples from the raw influent water (M1), the discharge effluent (M3) and mussels were clustered according to the type of sample, and in the case of the M1 and M3 samples, they were also seasonally clustered (Fig. 3B). In general, significant differences were observed between M1, M3 and mussel samples (ANOSIM, $R = 0.74$, $p = 0.0001$) and after pairwise comparisons of interest (M1-M3 and M3-mussel): M1-M3 (ANOSIM, $R = 0.55$, $p = 0.0003$) and M3-mussel (ANOSIM, $R = 0.68$, $p = 0.0003$). Significant differences were also observed between seasons (winter, spring) in both the M1 and M3 samples (ANOSIM, $R = 0.5$, $p = 0.0001$).

The RDA based on Euclidean distances showed that pH (ANOVA $F = 4.8$, $p = 0.001$), temperature (ANOVA, $F = 6.7$ $p = 0.001$), SS (ANOVA, $F = 2.8$, $p = 0.004$) and $BOD_5$ (ANOVA, $F = 6.5$, $p = 0.001$) significantly influenced the microbial composition in wastewater samples, with a total variance of 75.74 % explained by these factors. In the M1 samples, 45.13 % of the variance could be influenced by them: temperature could

**Table 2**
Number of reads and OTUs obtained through the analysis of data using Illumina. The numbers of reads before and after the trimming and clustering procedure are specified. The numbers of OTUs based on the database and de novo OTUs obtained after the clustering procedure were also specified. The number of OTUs and reads after subsampling was indicated and used in further analysis.

| Sequence description | Prokaryote sequencing ($N = 29$) |
|---|---|
| Raw reads | 8,589,273 |
| Reads after trimming | 8,530,690 |
| Number of merged reads | 4,531,792 |
| Filtered or chimaeric reads | 32,973 |
| Reads in OTUs | 1,127,171 |
| OTUs based on database | 5457 |
| De novo OTUs | 1312 |
| Total predicted OTUs | 6769 |
| **OTUs after subsampling** | **6339** |
| **Reads after subsampling** | **616.134** |

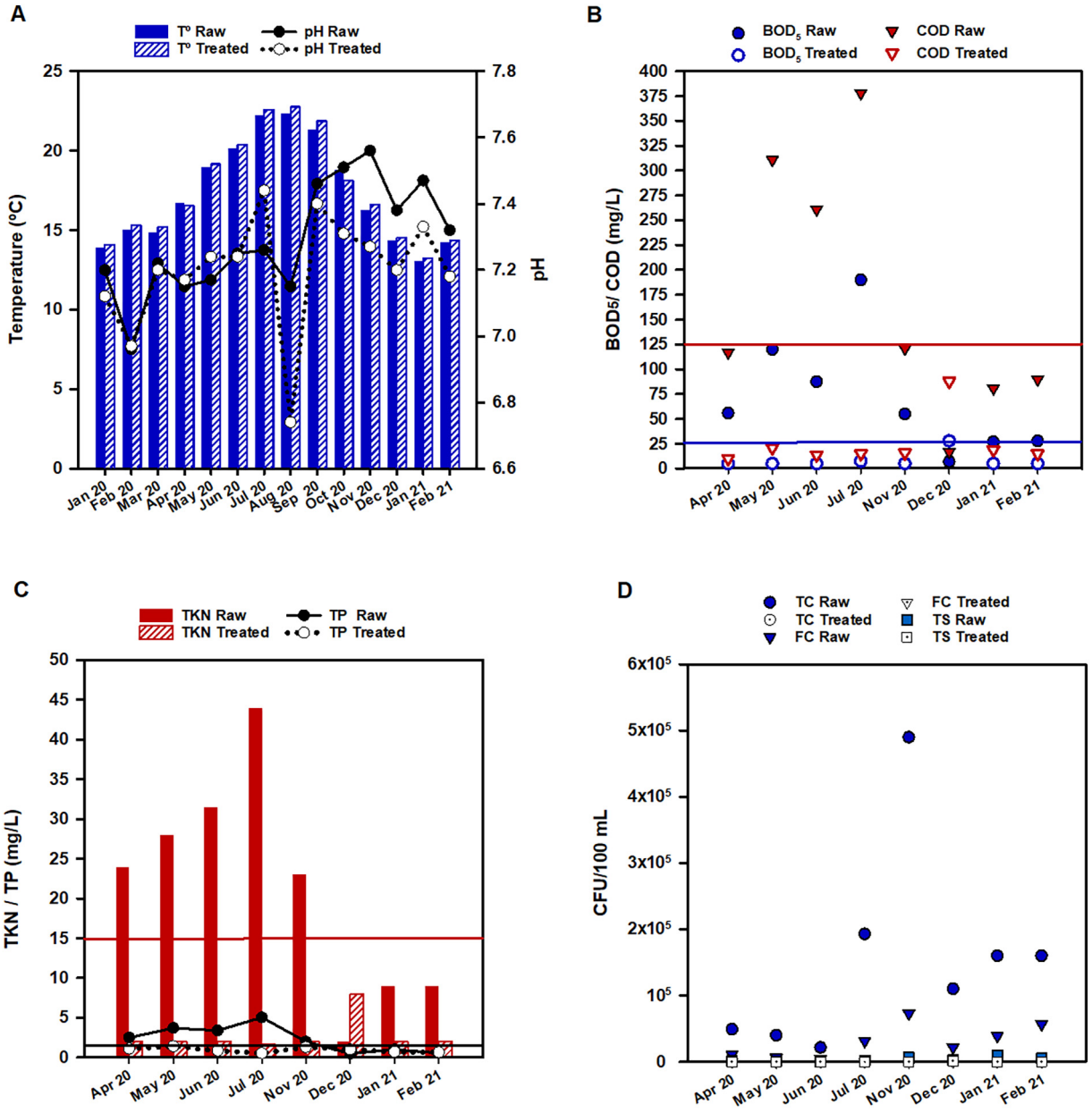


**Fig. 1.** A) Temperature and pH, B) biological oxygen demand ($BOD_5$) and chemical oxygen demand (COD), C) total Kjeldahl nitrogen (TKN) and total phosphorus (TP) measured in raw and treated wastewater. D) Total coliforms (TC), faecal coliforms (FC) and total Streptococcus (TS) in raw and treated wastewater. The legal limits of $BOD_5$ (blue horizontal line in Fig. B), COD (red horizontal line in Fig. B), TKN (red horizontal line in Fig. C) and TP (black horizontal line in Fig. C) were represented as per the European (91/271/EEC) and Spanish (BOE-A-1998-24,166) regulations for urban wastewater treatment (Council directive EU, 1991).

contribute to changes in the microbial composition in spring periods, whereas pH could influence the microbial community in the winter samples of raw wastewater. Additionally, significant changes in the microbial community in raw wastewater could be influenced by $BOD_5$ and SS (Fig. 3C).

### 3.4. Prokaryote community composition

Most of the prokaryote composition was from the Bacteria domain (99.9 %), and the remaining organisms were classified as members of the Archaea kingdom. Different patterns of prokaryote repertoires were observed in M1, M3 and mussels (Fig. 4), with differentially abundant OTUs associated with each type of sample and with each season between the same type of samples (Supplementary Fig. 2).

The **Campylobacterales** order was mainly found in water fractions, especially in M1 samples, and it represented only 0.14 % of the total reads in mussels (Fig. 4, Supplementary Fig. 2A). OTUs from this order were differentially associated with spring periods in M1 and winter periods in M3 samples (Fig. 4A-B, Supplementary Fig. 2B-C). The phylum **Bacteroidetes** was detected in all types of samples (Fig. 4); however, it was mainly associated with both wastewater fractions (Fig. 4A-B). In general, the Flavobacteriales order was the most representative group of this phylum (Fig. 4), and it was differentially abundant in winter samplings from M1 wastewater (Fig. 4A, Supplementary Fig. 2B). Despite the low representativeness of the Flavobacteriales order in mussels, the genus *Aquimarina* contributed to 40 % of the total reads in the sampling of May 20th, 2020. The **Gammaproteobacteria** class represented 60 % of the total number of reads in mussels, followed by 26 % of the total reads in both wastewater fractions. However, different orders of this class have been found to be representative depending on the type of sample (Fig. 4A-C). While the order Pseudomonadales was mainly found in raw

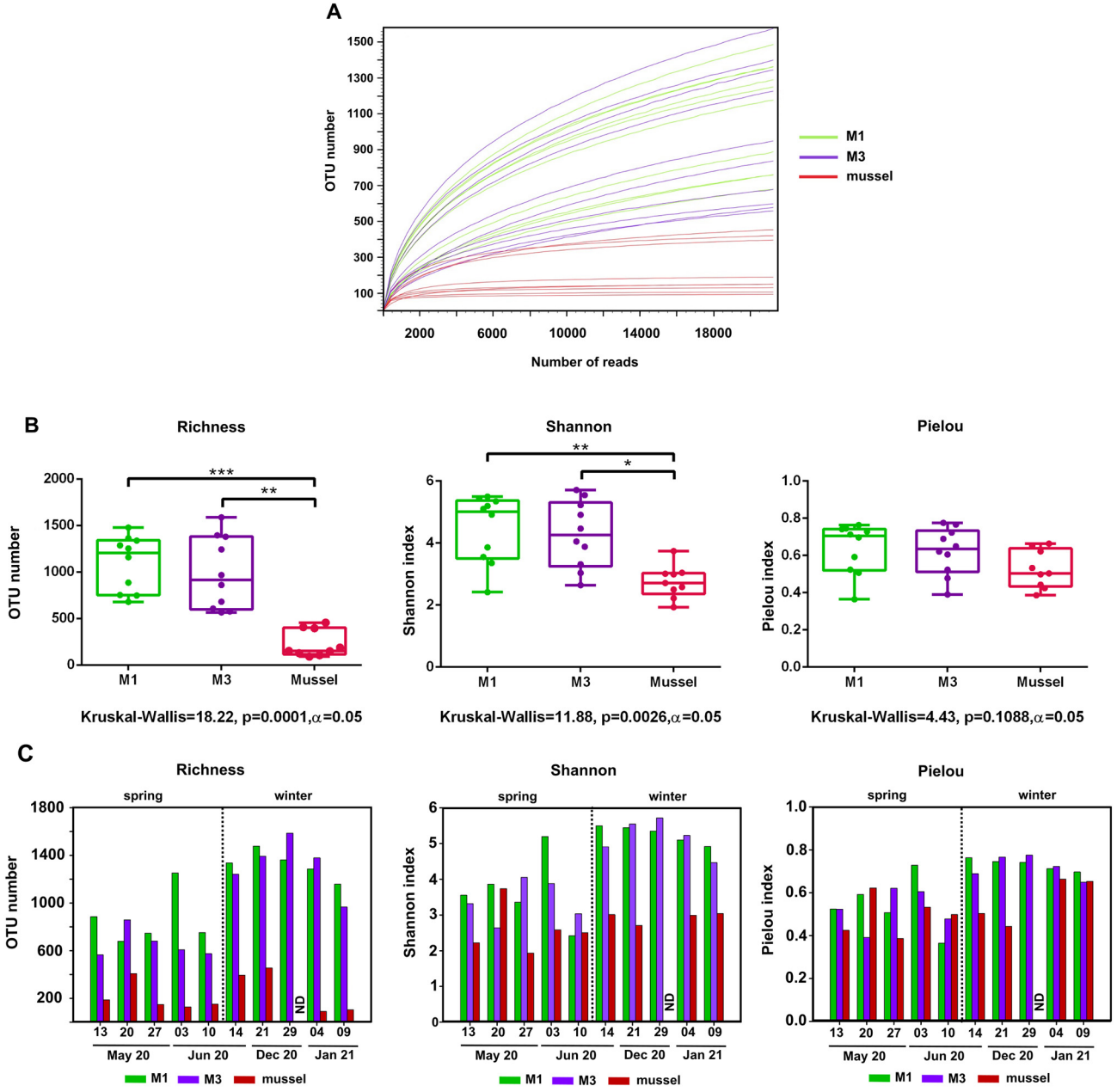


**Fig. 2.** Alpha diversity indices obtained from the M1, M3 and mussel samples. A) Rarefaction curve obtained for each type of sample. Box plot representing the richness, Shannon and Pielou indices obtained from A) each type of sample (M1, M3 and mussel) and C) from individual samplings of each compartment.

wastewater (M1), the order Betaproteobacteriales was more representative in treated wastewater (M3), especially on May 20th, 2020 (51 % of total reads). In mussels, the order Oceanospirillales, particularly *Endozoicomonas* sp., was the most representative bacteria in all mussel samples (from 40 to 60 % total reads) (Fig. 4C). The phylum **Firmicutes** was mainly represented by the orders Bacillales (mainly in spring M3 samplings (Fig. 4B, Supplementary Fig. 2C)) and Clostridiales (mainly in M1 samplings). The order Clostridiales punctually contributed to the majority of the bacterial composition in both M1 (71% total reads) and M3 (40% total reads) wastewater sampling on June 10th, 2020 (Fig. 4A-B). Furthermore, some Clostridiales OTUs were differentially abundant in the spring periods of the M1 samplings (Supplementary Fig. 2B). The **superphylum Patescibacteria** was exclusively detected in M3 wastewater samples (Fig. 4B), and it was mostly representative in winter periods, with several OTUs, such as Candidatus Nomurabacteria, Saccharimonadales and Parcubacteria, being differentially abundant (Supplementary Fig. 2C). The **class Alphaproteobacteria** and the order **Mycoplasmatales** were mainly representative in mussels (Fig. 4C), and this order was differentially abundant in spring mussel samplings (Supplementary Fig. 2D).

### 3.5. Pathogens and depuration efficiency

Potential pathogens previously described in wastewater (Cai et al., 2014; Vadde et al., 2019; Boukerb et al., 2021) were detected in our samples (Fig. 5). As expected, the pathogen profile differed between wastewater fractions (M1 and M3) and mussels (Fig. 5A, Supplementary Fig. 3A). In general, the fate of some potential pathogens was followed from M1 and M3. However, some pathogens were mainly detected in M3 after the disinfection treatment and in mussels, suggesting a different origin than the raw wastewater (Fig. 5A).

The highest number of potential human pathogenic genera was detected in raw wastewater (M1) (almost 60 % total reads), and a considerable

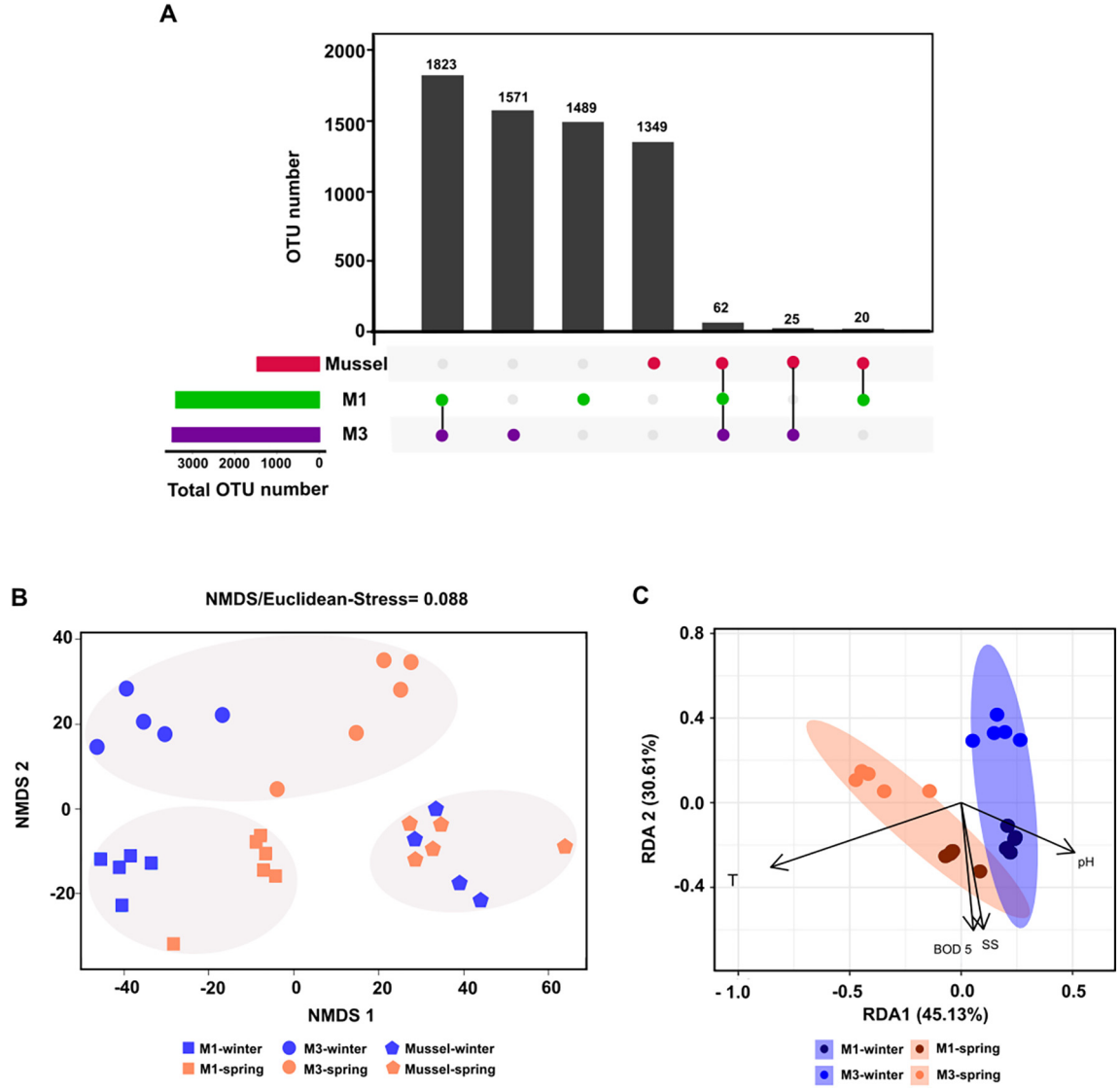


**Fig. 3.** Beta diversity analysis obtained from each type of sample. A) Exclusive and common OTUs obtained from each wastewater sample (M1 and M3) and mussel. B) NMDS based on Euclidean distances. C) RDA obtained from the M1 and M3 wastewater samples. Samples were grouped according to their season and fraction. The total number of OTUs in each compartment was 3396 in M1, 3483 in M3 and 1457 in mussels.

reduction in their abundance was found in treated wastewater (M3) (almost 30 % of total reads) (Supplementary Fig. 3A). The most abundant potential pathogens were *Arcobacter* sp. (Campylobacterales) and *Acinetobacter* sp. (Pseudomonadales), but in general, they were highly reduced after the wastewater treatment (<6 % total reads) and even more so in mussels (*Arcobacter* sp. <0.2 % of total reads) (Supplementary Fig. 3A). The reduction of *Arcobacter* sp. abundance from raw to treated wastewater was more evident in spring than in winter (Fig. 5B). Interestingly, a higher percentage of *Arcobacter* sp. was found in M3 than in M1 in the sampling of December 14th, 2020: after comparing common OTUs between M1 and M3 with relative abundances >1 %, only *Arcobacter* sp. 4 and *Arcobacter* sp. 10 were detected in both wastewater fractions with <10 % total reads on December 14th, 2020, suggesting a different origin of *Arcobacter* sp. genera from M1 (Supplementary Fig. 2B). The genus *Acinetobacter* was mainly detected in winter samplings, with a considerable reduction from the M1 to M3 samplings (Fig. 5B). *Clostridium* sp. (Clostridiales), in particular *Clostridium* sensu stricto *15*, was exclusively shared in the M1 and M3 samples, and it was punctually and highly detected in June 2020 in both wastewater samples (Fig. 5B, Supplementary Fig. 2B). Despite the reduction of this OTU from raw to treated wastewater, the percentage obtained after disinfection was still representative. In contrast, *Clostridium* sp. was barely present in mussels (>1.12 % total reads) (Fig. 5C). The genus *Pseudomonas* represented <5 % in both the M1 and M3 water samples (Supplementary Fig. 3A), with only one common OTU with a relative abundance >1 % in both the M1 and M3 samples. Exclusive *Pseudomonas* sp. OTUs were detected in the punctual M3 samplings (data not shown), explaining their higher abundance than in M1 (Supplementary Fig. 3B). Enterobacteriales were detected in all samples, although the highest percentage (M1 from June 3rd, 2020) only contributed 1.3 % of the total reads (Fig. 6). In general, faecal coliforms such as *Klebsiella*, *Citrobacter* and *Enterobacter* decreased from M1 to M3 and disappeared in mussels (Figs. 5A, 6B). However, *Escherichia-Shigella* was mostly associated with mussels and M1 samples (Figs. 5A, 6).

Some potential pathogens (*Bacillus* sp., *Legionella* sp., *Massilia* sp., *Gordonia* sp.) were mainly detected in M3, but not in M1 and mussels (Fig. 5A). The most representative was *Bacillus* sp., mainly detected in spring samplings. The rest were punctually found but at very low abundances (1–3 % total reads) (Fig. 5B).

Other potential pathogenic genera, such as *Staphylococcus*, *Francisella*, *Brevundimonas*, *Vibrio*, *Corynebacterium* and *Mycoplasma*, have been associated with mussels (Fig. 5A). The most representative pathogens observed in

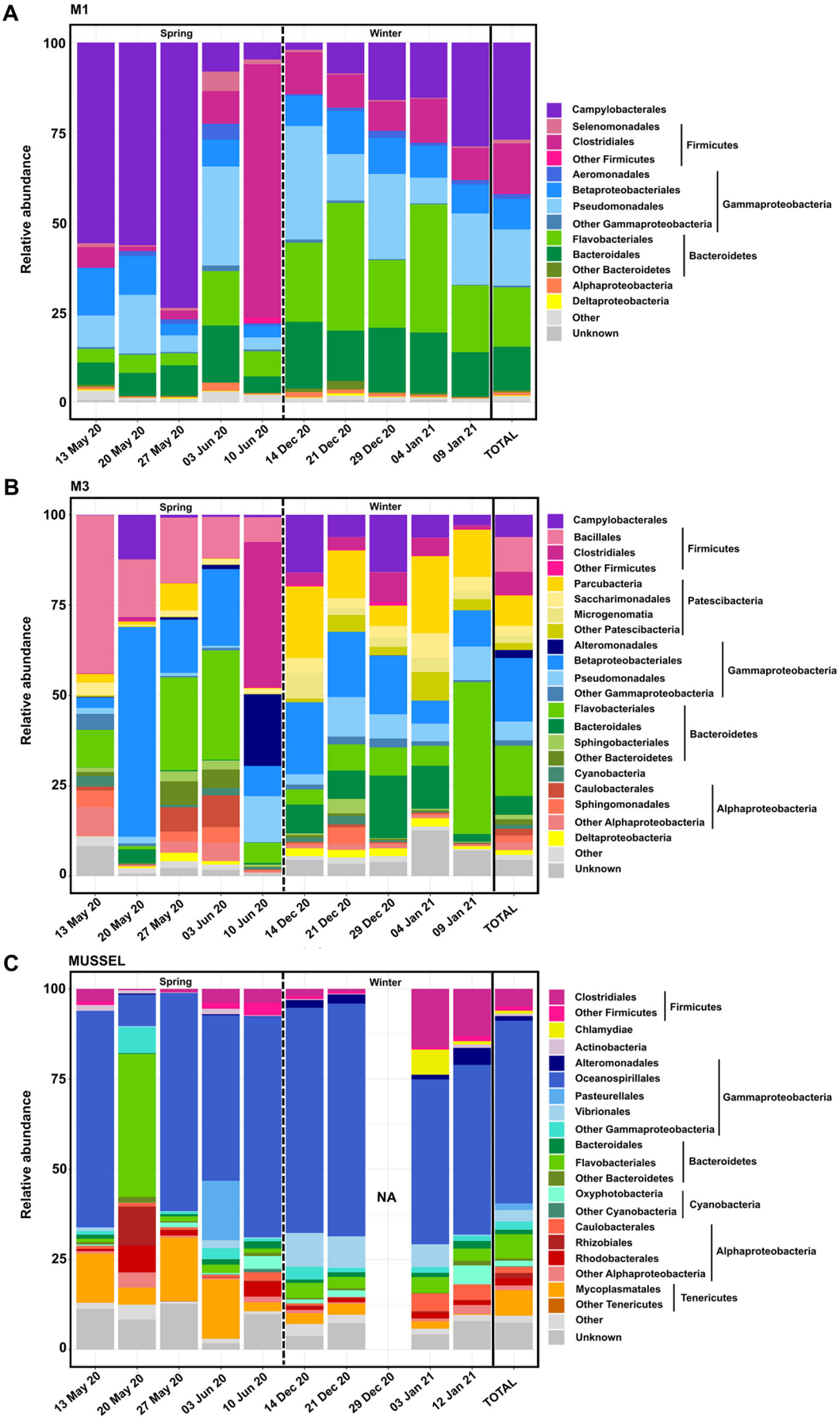


**Fig. 4.** Prokaryote composition obtained from A) M1, B) M3 and C) mussel samples. The relative abundance of each taxonomic group was represented.

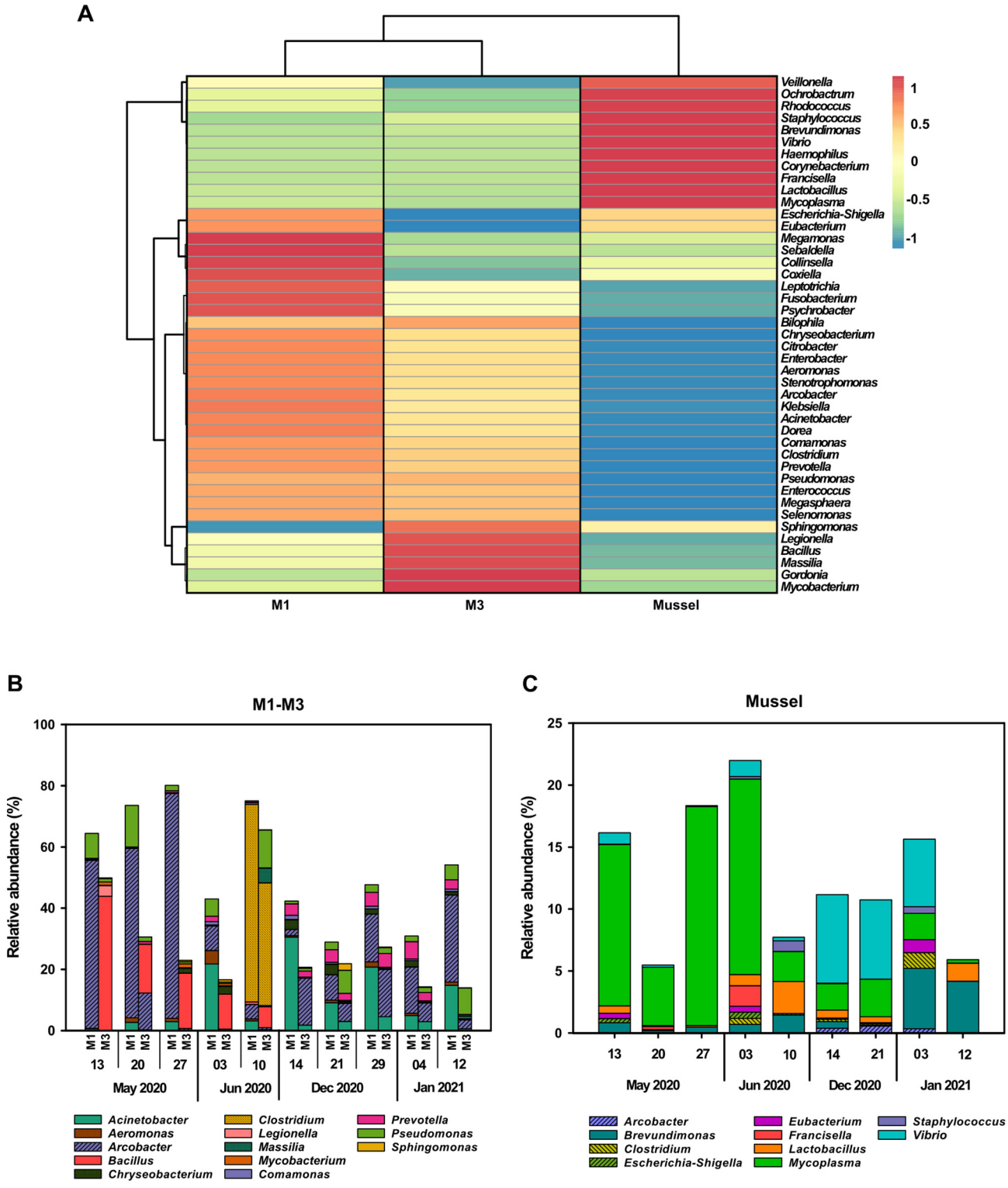


**Fig. 5.** A) Heatmap of potential pathogens detected in M1, M3 and mussels at the genus level. B) Relative abundance of pathogens at the genus level of each sampling. C) Relative abundance of pathogens found in mussels at each sampling.

mussels were *Mycoplasma* sp. and *Vibrio* sp., which were mainly detected in spring and winter samplings, respectively (Fig. 5C).

### 3.6. Co-occurrence networks

Co-occurrence networks (Spearman $p$ value > 0.05) are represented in M1 and M3 (Fig. 7). Significant differences were observed in both networks (M1 and M3) after their comparison against randomized networks (M1, $Z$-test, $p = 1.21852E^{-06}$; M3, Z-test, $p = 0.00016$). The detailed characteristics of both networks are included in Table 3.

A higher number of significant correlations was detected in M1 than in M3, and in both networks, positive correlations prevailed (co-occurrences) over negative correlations (co-exclusions) (Fig. 7). In M1, OTUs of the genus *Arcobacter* sp. (Campylobacteriales) and *Acidovorax*

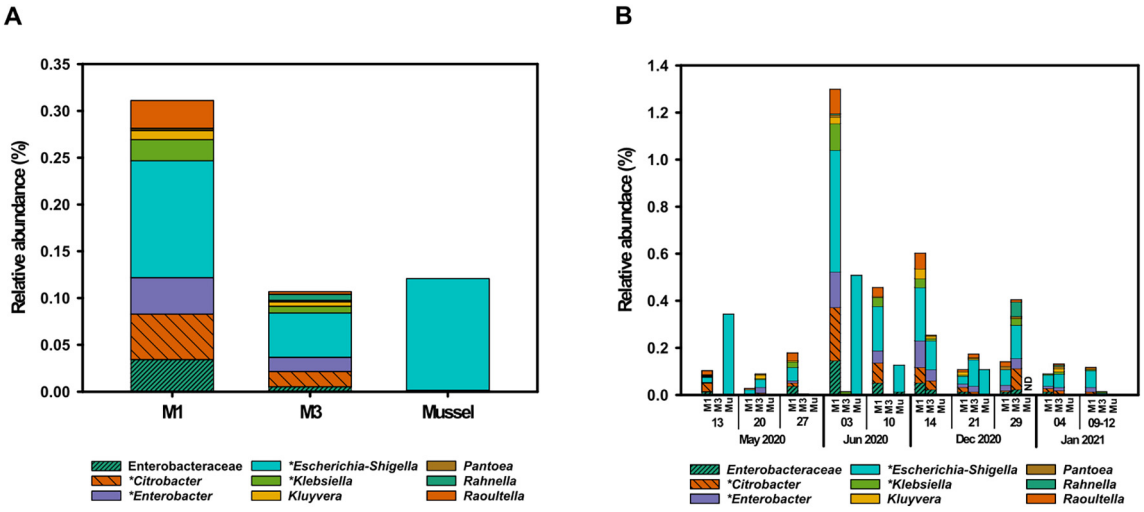


**Fig. 6.** Relative abundance (% total reads) of the order Enterobacteriales detected in A) all M1, M3 and mussel samplings and B) in each M1, M3 and mussel sample. Relative abundance was represented at the genus level except for the family Enterobacteriaceae. *Faecal coliforms that are usually monitored in WWTPs by culturing methods.

sp. (Betaproteobacteriales) were involved in the majority of correlations. The strongest positive correlations were mainly obtained among several OTUs of *Arcobacter* sp. (Campylobacterales), but also between *Bacteroides* sp. (Bacteroidales) and *Acinetobacter* sp. (Pseudomonadales). OTUs from the genus *Arcobacter* sp. were also involved in the majority of co-exclusions with *Cloacibacterium* sp. (Flavobacteriales), *Acinetobacter* sp. and *Acidovorax* sp. (Fig. 7A). In the M3 co-occurrence network, the strongest positive correlation was observed between two *Arcobacter* sp. OTUs, but also between two OTUs of the Patescibacteria group. Moreover, positive correlations were also detected between several genera of the Betaproteobacteriales order (*Aquaspirillum* sp., *Aquabacterium* sp., *Chitinivorax* sp. and *Simplicispira* sp. 1) with *Arcobacter* sp. OTUs. Negative

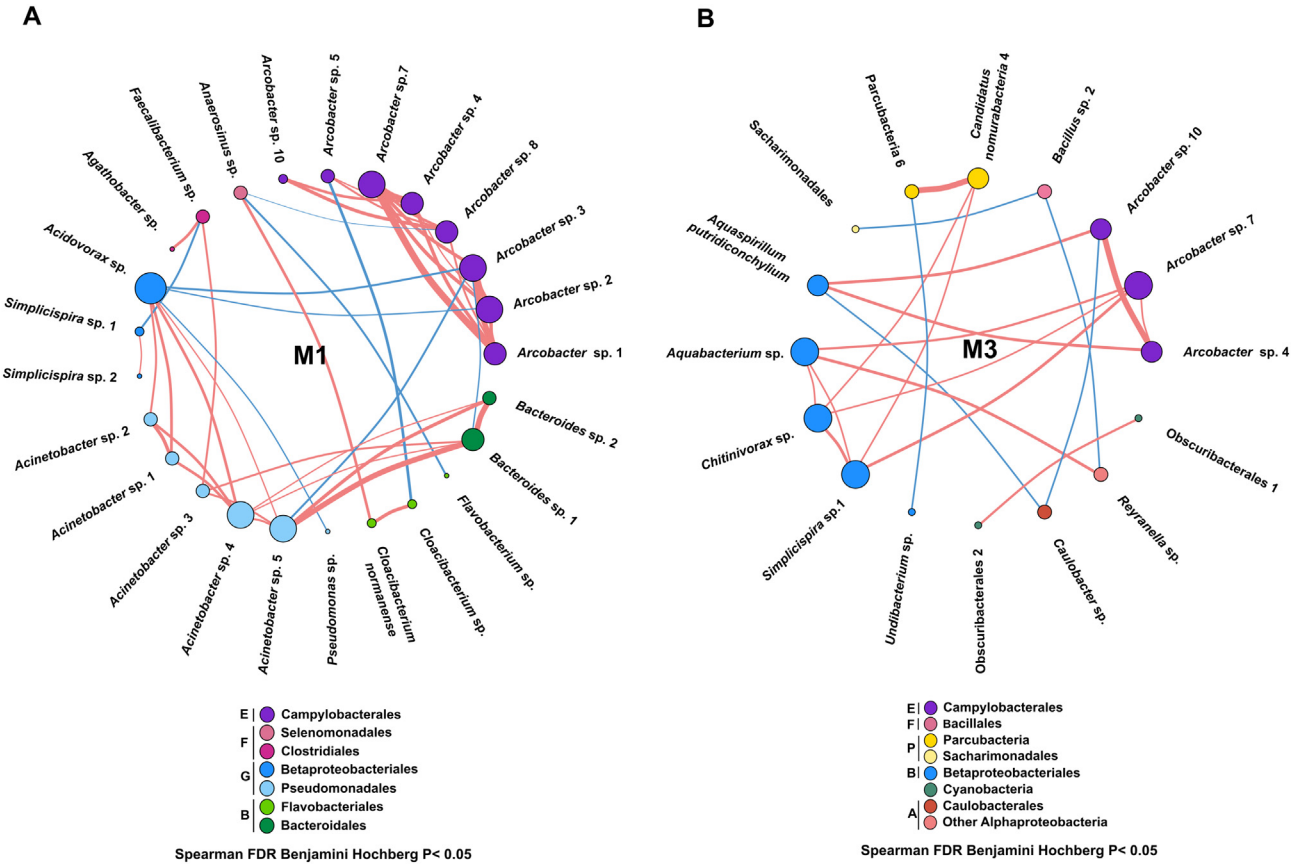


**Fig. 7.** Undirected network that represents all the links among prokaryotes in A) M1 and B) M3 samples. Significant co-occurrences or coexclusions (Spearman *p* value <0.05) were represented. Red edges represent positive correlations (co-occurrences), whereas blue edges represent negatives (coexclusions). The size and colour of the nodes represent the degree and the taxonomic group, respectively.

**Table 3**
Topology of the co-occurrence networks from the M1 and M3 wastewater samples.

| Network topology | M1 | M3 |
|---|---|---|
| Number of nodes | 25 | 16 |
| Number of edges | 45 | 20 |
| Connected components | 1 | 2 |
| Isolated nodes | 0 | 0 |
| Number of self-loops | 0 | 0 |
| Average number of neighbors | 3.520 | 2.500 |
| Characteristic path lenght (CPL) | 3.547 | 3.195 |
| Density | 0.150 | 0.166 |
| Network diameter | 9 | 7 |
| Cluster coefficient (CL) | 0.417 | 0.525 |

correlations were observed between Sacharimonadales and *Reyranella* sp. (Alphaproteobacteria) with *Bacillus* sp. 2 and between *Caulobacter* sp. with *Arcobacter* sp. and *Aquaspirillum* sp. (Fig. 7B).

## 4. Discussion

One important concern in the field of global health is wastewater discharge to the environment and possible water reuse after disinfection in WWTPs. This is due to the increase in human and animal infections and illnesses associated with contamination of drinking, coastal and irrigation water (Randazzo et al., 2019). However, the monitoring of prokaryotes, including pathogens, in both influent and discharge wastewaters has been poorly studied by high-throughput sequencing (Bai et al., 2014; Cai et al., 2014; Numberger et al., 2019). Furthermore, the main studies in Galicia are focused on the specific detection of human-derived pathogens (e.g., microsporidia, sapovirus, hepatitis E virus) in WWTPs (Izquierdo et al., 2011) and in commercial shellfish (Mesquita et al., 2016; Varela et al., 2015) by PCR and RT–qPCR, and recently, detection of ecological responses and chemical contamination of urban wastewater outfalls in an estuarine area by toxicity assays, chemical composition analysis and changes in benthic communities (morphological taxa identification) have been studied in Ría de Vigo (Viana et al., 2021). One of the main interests of the present study was the use of the eDNA metabarcoding tool to provide a detailed description of the microbiome diversity associated with raw wastewater, discharge effluent water after the treatment and mussels as bioindicators located near the effluent area to evaluate the depuration efficiency of the WWTP.

Changes in bacterial communities in wastewater can be highly influenced by fluctuations of abiotic parameters in seasonal periods (Wells et al., 2011; Wang et al., 2012; Gao et al., 2016; Numberger et al., 2019; Wu et al., 2019) and in this study, significant seasonal changes of bacteria composition were obtained within the same type of wastewater sample in both raw and treated wastewater. Our results show that variations in pH could significantly change the community in raw wastewater in winter periods, whereas temperature could affect community changes in both M1 and M3 samples.

The implementation of wastewater-based epidemiology (WBE) regarding the emergence of new diseases and the re-emergence of existing diseases is a current global concern of public health, but also for the environmental monitoring of potential hazards (Sims and Kasprzyk-Hordern, 2020; Mao et al., 2020). The raw influent wastewater microbiome has been strongly related to the human gut microbiome, which is composed of harmless and even beneficial microorganisms to human health, but also of opportunistic pathogens and antibiotic resistance gene carriers (Cai and Zhang, 2014; Newton et al., 2015; Numberger et al., 2019). DNA metabarcoding allows the multiple detection of potential foodborne and waterborne enteric pathogens (*Arcobacter* sp., *Clostridium* sp. and Enterobacteriales) known for being causative agents of diarrhoea (Shen et al., 2019; Igwaran and Okoh, 2019; Numberger et al., 2019). Interestingly, faecal coliforms, which are the most routinely tested pathogens in WWTPs for monitoring faecal contamination (Cai and Zhang., 2014, Chan et al., 2019), represented a low percentage of pathogens detected in raw wastewater. Therefore, it was possible to highlight the potential of DNA metabarcoding for the simultaneous detection of several potential pathogenic organisms, including those that are not currently routinely tested due to being difficult to culture. Furthermore, DNA sequencing provided information about the seasonal and punctual prevalence of these pathogens and suggested possible abiotic factors that could also be involved in the seasonal prevalence of pathogens. For example, *Arcobacter* sp. was the most abundant bacteria in raw wastewater of late spring (55–73 % total reads). This fact has been previously confirmed by other studies that compare the abundance of this genus in warm (spring-summer) and cold (winter-autumn) seasonal periods (Stampi et al., 1999; Fisher et al., 2014). Hence, the prevalence in wastewater could be associated with an increase in water temperature, one of the abiotic factors with a major influence on the bacterial community. The punctual detection of *Clostridium* sensu stricto 15 in June 2020 could suggest a high prevalence of this bacteria throughout the population in that period.

Given the increasing public risk associated with contamination of coastal waters, the fate of prokaryotes with an anthropogenic origin, especially potential pathogens, has been followed in WWTPs to validate the efficiency of the depuration system. It is important to highlight that in this study we have only detected prokaryote communities, so it was not possible to confirm the pathogen removal of other groups (e.g., eukaryotes). A considerable reduction in potential human and animal pathogens detected in the influent wastewater was observed after secondary and tertiary wastewater treatment with chlorine. Chlorine is the most widely used disinfectant for the inactivation of pathogens in water treatment, and the working conditions and execution of this treatment are simple in comparison with other disinfection procedures (e.g., ozone, UV radiation). Chlorine treatment modifies the chemical structure of the enzymes serving as the basis of the nutrition and metabolism mechanisms of pathogenic bacteria (Collivignarelli et al., 2017). However, some bacteria are known for resistance to chlorination treatment and are carriers of antibiotic resistance genes and biofilm producers (Luo et al., 2021). This fact could explain the presence of potential pathogens in treated wastewater (M3) which were previously detected in raw wastewater (M1). However, despite the detection of potential pathogens after the chlorine disinfection procedure, the DNA metabarcoding technique did not allow us to distinguish whether these pathogens are active (Pawlowski et al., 2016) or, by contrast, if they actually represent traces of genetic material from inactivated and degraded microorganisms. Hence, the use of eRNA (metatranscriptomics) or the combination of DNA metabarcoding with several diagnostic techniques, could serve as potential tools to confirm the active or inactive state of the pathogen.

Biological wastewater treatment plants (BWWTPs) based on activated sludge constitute an essential tool in environmental protection to achieve optimal sewage depuration by bioremediation, where bacteria, fungi and protists perform different roles, such as the removal of organic carbon, nitrogen, sulphate and phosphate (bacteria) and the reduction of suspended solids and turbidity (protists) in the final discharged water (Garrido-Cardenas et al., 2017; Stoeck et al., 2018). In fact, the majority of NGS-based studies are focused on activated sludge. Our results revealed bacteria that may be involved in bioremediation processes and mainly associated with discharge wastewater (M3), such as the recently proposed Patescibacteria superphylum (Parks et al., 2018) and the genus *Bacillus* sp. Previous studies revealed that both taxonomic groups are involved in nitrogen removal (Kim et al., 2005; Yang et al., 2011; Zhang et al., 2012; Saleem et al., 2013; Rout et al., 2017; Hosokawa et al., 2021; Yan et al., 2021; Zhao et al., 2021) and that bacteria from the Patescibacteria superphylum inhabit groundwater and oligotrophic environments (Luef et al., 2015; Frey et al., 2016; Herrmann et al., 2019; Chaudhari et al., 2021). The exclusive detection of members of the Patescibacteria superphylum and the differential abundance of *Bacillus* sp. in the discharge water (M3) as well as the associations found between OTUs from both taxonomic groups with other denitrifiers and chitinolytics could reinforce the role of these bacteria in bioremediation. For example, co-occurrences found between *Candidatus nomurabacteria* (Patescibacteria) and the chitinolytic bacterium *Chitinivorax* sp. (Betaproteobacteria) (Chen et al., 2012) and with the denitrifying

bacterium *Simplicispira* sp. (Feng et al., 2017) could suggest cooperative processes in the degradation of nitrogen compounds, whereas the coexclusions observed between *Bacillus* sp. with Shacharimonadales and *Reyranella* sp. could be associated with the competence of resources in nitrogen removal.

Although we suggest the possible role of *Bacillus* sp. in bioremediation, some species of the *Bacillus* genus are considered medically relevant (e.g., *Bacillus cereus* and *Bacillus anthracis*) (Celandroni et al., 2016) and resistant to chlorine disinfection (Paes et al., 2012; Luo et al., 2021), so further investigations should be considered to confirm the pathogenicity of bacteria from this genus.

The release of microbial organisms derived from sewage could introduce perturbations (e.g., eutrophication, pathogen release) that might produce impacts in the marine environment, such as changes in community composition and functional processes (Nogales et al., 2011). The low amount of shared microbiome between M3 and mussels and the insignificant abundance (<0.5 % of total reads) of possible potential pathogens from the influent water detected in wild mussels (bioindicators) could suggest a low risk of prokaryote pathogen release through the effluent to the marine environment. However, it could be also interesting to include in future studies seawater samples to obtain more conclusive results about the incorporation of wastewater derived microorganisms into the marine environment. Interestingly, the presence of *Escherichia-Shigella* mainly in raw wastewater and mussels could suggest uncontrolled domestic discharges, although this genus was generally found in very low abundance. In general, the bacterial community associated with different tissues of mussels was mostly exclusive to mussels (96 %) and represented by the phyla Proteobacteria, Firmicutes and Bacteroidetes, coinciding with previous studies (Vezzulli et al., 2018; Musella et al., 2020). However, the most representative bacteria in mussels were *Endozoicomonas* sp. (Oceanospirillales), a ubiquitous symbiotic marine organism associated with a wide variety of marine organisms, such as cnidarians, molluscs, fish, poriferans and tunicates (Neave et al., 2016; Schill et al., 2017).

In conclusion, the use of the metabarcoding methodology based on the sequencing of the V3-V4 region of the 16S rRNA gene has provided different prokaryote composition associated with raw wastewater (M1), discharge effluent (M3) from WWTPs and wild mussels from the marine coastal environment and the influences of environmental factors on seasonal differences of wastewater composition obtained in each type of sample. Since the WBE postulates that the detection of human biomarkers in wastewater can reflect the health of the population, DNA metabarcoding could therefore be used for WBE. Furthermore, the effectiveness of prokaryotic pathogen removal in the wastewater treatment plant could be confirmed with DNA metabarcoding due to the decrease in possible pathogenic organisms from the M1 to the M3 wastewater fraction and the scarce presence of pathogens found in wastewater in mussels. This technique only detects genetic material, therefore, it is not possible to determine if the active pathogens are present and the risk they carry for human and animal health. However, although the sequencing of short amplicons provides little taxonomic resolution at the species level of potential pathogenic genera without information about the viability of pathogens found, our results show that DNA metabarcoding is a valuable tool for surveillance under the "One Health" concept.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.160531.

## CRediT authorship contribution statement

**Raquel Ríos-Castro**: Methodology, Formal analysis, Investigation, Data Curation Writing-Original draft, and Visualization. **Adrián Cabo**: Methodology, Writing-Review. **Eva Teira**: Formal analysis, Writing-Review & Editing, and Supervision. **Claudio Cameselle:** Methodology, Writing-Review, Resources, Funding acquisition**, Susana Gouveia**: Methodology, Writing-Review, **Pedro Payo:** Conceptualization, Resources, Funding acquisition. **Beatriz Novoa:** Conceptualization, Resources, Methodology, Writing-Review & Editing, Supervision, and Funding acquisition. **Antonio Figueras**: Conceptualization, Resources, Methodology, Formal analysis, Writing-Review & Editing, Supervision, and Funding acquisition.

## Data availability

The data presented in the study of prokaryote sequencing in wastewater of Baiona were deposited in the Sequence Read Archive (SRA) (http://www.ncbi.nlm.nih.gov/sra), accession number PRJNA874871.

## Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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