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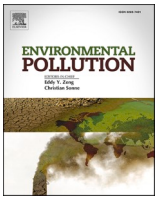

Integrating culture-based and molecular methods provides an improved assessment of microbial quality in a coastal lagoon☆

Marco Basili [a], Laura Perini [b], Luca Zaggia [c], Gian Marco Luna [a], Grazia Marina Quero [a,*]

[a] CNR IRBIM, National Research Council – Institute of Marine Biological Resources and Biotechnologies, Largo Fiera della Pesca, 60125, Ancona, Italy
[b] Department of Environmental Science, Aarhus University, 4000, Roskilde, Denmark
[c] CNR IGG, National Research Council – Institute of Geosciences and Earth Resources, Via G. Gradenigo 6, 35131, Padova, Italy

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ABSTRACT

Faecal pollution in aquatic environments is a worldwide public health concern, yet the reliability and comprehensiveness of the methods used to assess faecal contamination are still debated. We compared three approaches, namely a culture-based method to enumerate Faecal Indicator Bacteria (FIB), a FIB-targeting qPCR assay, and High-Throughput Sequencing (HTS) to detect faeces- and sewage-associated taxa in water and sediment samples of an impacted model lagoon and its adjacent sea across one year. Despite at different levels, all approaches agreed in showing a higher contamination in the lagoon than in the sea, and higher in sediments than water. FIB significantly correlated when considering separately sediment and water, and when using both cultivation and qPCR. Similarly, FIB correlated between cultivation and qPCR, but qPCR provided consistently higher estimates of FIB. Faeces-associated bacteria positively correlated with cultivated FIB in both compartments, whereas sewage-associated bacteria did only in water. Considering their benefits and limitations, we conclude that, in our study site, improved quali-quantitative information on contamination is provided when at least two approaches are combined (e.g., cultivation and qPCR or HTS data). Our results provide insights to move beyond the use of FIB to improve faecal pollution management in aquatic environments and to incorporate HTS analysis into routine monitoring.

1. Introduction

The microbiological quality assessment of aquatic environments is based on the use of the Faecal Indicator Bacteria (FIB) *E. coli* and enterococci as proxies to indicate the presence of microbial pollutants of faecal origin from humans and other warm-blooded animals (Byappanahalli et al., 2012; Luna et al., 2016, 2019; Holcomb and Stewart, 2020). However, since several studies have shown that FIB can persist and adapt to the environment (Luo et al., 2011; Byappanahalli et al., 2012), questions on their reliability as indicators of faecal contamination have emerged (Wilkes et al., 2011; Yang et al., 2020). Moreover, considering cultivation-based methods, despite being relatively inexpensive, the time needed to obtain results (18–48 h) often fails to provide on-time responses to prevent potential health risks connected to pollution conditions (Holcomb and Stewart, 2020); in addition, traditional methods are non-specific and possibly underestimating the level of contamination due to their weak efficiency in detecting slow-growing or stressed cells (i.e., "viable-but-not-culturable" or VBNC) (Rodrigues and Cunha, 2017). Based on this, alternative methods and approaches have been tested and proved to be more efficient in terms of rapidity, sensitivity, and taxonomic coverage for detecting and quantifying faecal microbiota in water (reviewed in McLellan and Eren, 2014). These include quantitative PCR (qPCR) to quantify traditional FIB and several other faeces-associated taxa in the frame of microbial source tracking (MST) specific to an environmental source of interest, such as bacteria specific to the gastrointestinal tracts of certain animals (dogs, ruminants or birds) or sewage and human faeces (Hughes et al., 2017; Luna et al., 2019; Ahmed et al., 2020; McClary-Gutierrez et al., 2021), as well as methods based on High-Throughput Sequencing (HTS) data to simultaneously identify several potential microbes of concern (Newton et al., 2013; Luna et al., 2016; Basili et al., 2021, 2022). However, each approach presents pros and cons, leading to a still open debate on which of them represents the most efficient choice. qPCR allows to bypass culturing, with analysis time limited to a few hours (Dorevitch et al.,

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* Corresponding author.
E-mail address: grazia.quero@irbim.cnr.it (G.M. Quero).

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2017), a higher sensitivity and with the opportunity for multiplexing (Liu et al., 2015; Riedel et al., 2014); however, given the possible occurrence of free DNA resulting from damaged or dead cells, signals from non-viable organisms may be detected (Emerson et al., 2017) and, likewise, qPCR may suffer from inhibition from other substances common in environmental samples (Nappier et al., 2019). HTS of 16S rRNA genes combines the advantage of testing for the presence of multiple microbial targets at the same time as well as of determining the source of contamination (e.g., human from non-human) (McLellan et al., 2013; Newton et al., 2011, 2013); moreover, the recent introduction of more affordable and portable long-read sequencing platforms, promises to accelerate the use of sequencing to characterise faecal contamination (Hu et al., 2018; Acharya et al., 2019). HTS bacterial community analysis has thus a large potential to supplement current water quality monitoring and to better understand interactions between water quality and human health indicators (McClary-Gutierrez et al., 2021). On the other hand, 16S rRNA gene HTS is still far from providing taxonomic resolution at the species level as well as quantitative estimates. Such limitations still represent a major constraint to be addressed before expanding their use in the future and highlight the urgent need for comparative studies.

To date, most of the bathing water quality regulations involve the analysis of faecal pollution in water samples only (e.g., Bathing Water Directive, BWD, 2006/7/EC). However, increasing evidence have shown that sediments can represent a reservoir of both chemical and biological contaminants (Ridgway and Shimmield, 2002), including faecal pollutants (Luna et al., 2010; Perini et al., 2015; Ahmed et al., 2018; Saingam et al., 2020; O'Mullan et al., 2019). Microbial contaminants may reach sediments as a result of settlement of either planktonic bacterial cells or particle-attached bacteria (Saingam et al., 2020); once in the sediments, microorganisms may survive better due to the increased nutrient availability and protection (Garzio-Hadzick et al., 2010; Haller et al., 2009). In relatively shallow environments such as lagoons, sediment disturbance, including, among others, frequent resuspension events, may lead to the remobilization of FIB from sediments to water (Yakirevich et al., 2013; Boehm et al., 2014; Perini et al., 2015; Saingam et al., 2020), thus leading to water quality impairment and requiring to routinely extend the assessment of faecal pollution also to the benthic compartment.

Due to the large fluxes of tourists, the unique architecture of the historical centre, determining a poor efficiency of sewage treatment (Zaggia et al., 2007; Sfriso and Facca, 2013; Vecchiato et al., 2016), as well as the presence of a variety of human pressures on the environment, the Venice Lagoon (Northern Adriatic Sea, Italy) represents a highly impacted ecosystem, where faecal pollution is increasingly being recognized as a threat to its health (Perini et al., 2015; Basili et al., 2021). Based on this, we used the Venice Lagoon as a model environment to investigate, by comparing different methods, the occurrence, distribution and temporal variations of faecal pollutants in both the pelagic and benthic compartments. To this aim, we applied a conventional, culture-based method for FIB enumeration, as well as two molecular methods, including a FIB-targeting qPCR assay and HTS 16S rRNA gene sequencing to assess the presence and relative abundance of alternative faecal-associated and sewage-associated bacterial taxa, with the objective of assessing their comparability, strengths and weaknesses. This study represents a further contribution to the ongoing debate at the science–policy interface on water quality assessment for the identification of the best methods, the use of alternative approaches to assess the quality in aquatic environments, and the creation of a robust scientific basis to inform decision-makers for a proper management.

2. Material and methods

2.1. Study site and data

Water and sediment samples were collected at 9 stations, 5 in the Venice Lagoon and 4 in the adjacent Adriatic Sea, as reported in Quero et al. (2017) (Supplementary Figure 1). Briefly, water samples were collected using a Niskin bottle (capacity 5 L) and sediment samples were collected using a Van Veen grab sampler (capacity ca. 3 L) onboard the research boat Litus (ISMAR-CNR, Venice). Water samples were stored at 4 °C in the dark until returning to the laboratory, where they were filtered onto sterile 0.22 μm cellulose nitrate membrane filters (Sartorius). Each filter was stored at −20 °C until DNA extraction. For sediment samples, the uppermost 0–2 cm layer was aseptically collected and transported at 4 °C in the dark to the laboratory, where the samples were stored at −20 °C until DNA extraction. The depths of the sampling stations ranged from 5 to 16 m. Sampling activities were performed on March 25, 2014 (hereafter referred to as "spring"), 20th–21st May 2014 (hereafter referred to as "summer"), 21st–22nd October 2014 (hereafter referred to as "autumn"), and 11th–12th February 2015 (hereafter referred to as "winter").

2.2. Environmental variables

Environmental variables were measured as described by Quero et al. (2017). Briefly, water temperature (°C), salinity (PSU), dissolved oxygen (%) and turbidity (NTU) were measured by a CTD probe (Seabird 911, Sea-Bird Electronics, Washington, USA). The concentration of dissolved inorganic nutrients (DIN, the sum of nitrates, nitrites and ammonia), phosphates (PO_4) and silicates (SiO_2) was determined by standard colorimetric methods (Grasshoff et al., 1999), using an ALPKEM Flow Solution III (OI Analytical, Texas, USA) autoanalyzer. Chlorophyll-a concentration was determined spectrofluorometrically as described by Bernardi-Aubry et al. (2013).

2.3. FIB enumeration by cultivation methods

E. coli (EC) and enterococci (ENT) were analysed in water and sediment samples of lagoon and open sea areas using the Membrane Filtration (MF) culture-based method. For lagoon and water, an appropriate volume of water (1–200 ml) was vacuum-filtered (pore size, 0.22 μm diameter, 47 mm; Sartorius) in triplicate and the filters were placed on m-FC agar plates. EC abundance in sediment was estimated by the MF technique. For MF, triplicate aliquots (1–5 g) of wet sediment were mixed (vol/vol 1:5 or 1:10) with sterile water or sterile physiological solution (0.8% NaCl), vigorously shaken, and sonicated to dislodge bacteria from sediment particles (Luna et al., 2010, references therein). After sonication, aliquots (1 ml) of undiluted and 10-fold serial dilutions of the supernatant were filtered as described above and filters were placed on m-FC agar plates. Plates were incubated at 44.5 °C for 24 h. Blue colonies were considered as presumptive EC and randomly isolated for identity confirmation as described in Perini et al. (2015). Their abundance was reported as CFU (colony-forming units) 100 ml^{-1} of water or CFU 1 g^{-1} (dry weight) of sediment.

2.4. qPCR for FIB quantification

Water samples (1 L) were filtered through 0.22 μm cellulose nitrate membrane filters (Sartorius) and stored at −20 °C until processing. Microbial DNA was extracted from each filter using the PowerWater DNA Isolation Kit (MoBio Laboratories). For sediment analysis, DNA was extracted from 1 g of each sediment sample previously stored at −20 °C using the PowerSoil DNA Isolation Kit (MoBio Laboratories). DNA extraction from water and sediment was performed according to the manufacturer's instructions, with slight modifications made to increase DNA yield and quality as described in Quero et al. (2017). For each sampling event, a known volume of sterile milliQ water sample was also filtered and processed for DNA extraction and amplification as a blank. DNA concentrations were determined by spectrophotometry and DNA was stored at −80 °C until use. Quantitative PCR reactions were performed using the primer sets F395 and R490, targeting the 16S rDNA of

E. coli (Penders et al., 2005), and ECST748F and ENC854R, targeting the 23S rDNA of *Enterococcus* spp. (Haugland et al., 2005), as described in Luna et al. (2019). FIB abundance was expressed as cell equivalents (CE) 100 ml^{-1} of water or CE g^{-1} of sediment. For calculation of cell equivalents from gene copies, we considered 7 copies of 16S rDNA gene per genome for EC, and 4 copies of 23S rDNA gene per genome for ENT (Luna et al., 2019; Di Cesare et al., 2013 and references therein).

2.5. Cultivation-independent microbial community analysis by High-Throughput Sequencing (HTS) of 16S rRNA amplicons

HTS library preparation, amplification and sequencing were performed following the Illumina Nextera protocol as reported in Quero et al. (2017). In brief, amplifications were run to obtain amplicon libraries of the V3–V4 regions of the 16S rRNA gene, which were sequenced on the Illumina MiSeq platform using the 341F and 785R universal bacterial primers (Eiler et al., 2012). Raw sequences are stored in the SRA Sequence Read Archive (BioProject Accession PRJNA342950).

The raw reads were analysed with CUTADAPT (Martin, 2011) to remove the primer and adapter sequences; paired-end reads were then imported and analysed in RStudio (RStudio Team, 2020) using the DADA2 package (Callahan et al., 2016). Quality check and trimming of the reads at 280 and 210 bp for the forward and reverse reads, respectively, were performed (maximum estimated error per 100 bp, >2 and 2 for the forward and reverse reads, respectively) according to the package instructions. Oligotype inference was performed on the dereplicated sequences after pooling samples, to minimise any bias due to low sampling depth. Then, paired-end reads were merged in Amplicon Sequence Variants (ASVs), the chimeric sequences were identified and prokaryotic taxonomy was assigned using a native implementation of the naive Bayesian classifier method against the *silva* database (v132; https://www.arb-silva.de/documentation/release-132/). ASVs were defined as clusters sharing 100% sequence identity. The reads sum for each sample were checked and then the sample INS_WAT_OCT was removed, due to the extremely low number of reads. The ASV table from DADA2 was then normalised to the median number of reads present among the samples (n = 36,297) as reported in Fullerton et al. (2019) using the *vegan* package (Oksanen et al., 2018). The occurrence and distribution of faecal contamination was analysed, we sought for ASVs identified as potential faecal contaminant bacterial taxa, including (i) traditional faecal bacteria (i.e., the Enterobacteriaceae family, including the genera *Escherichia*, and *Enterococcus*) and (ii) alternative faeces-associated bacteria from human and non-human sources (i.e., Bacteroidaceae, Clostridiaceae, Lachnospiraceae, Porphyromonadaceae, Prevotellaceae, Rikenellaceae and Ruminococcaceae families). We then looked for sewage-associated bacteria, including the genera *Acinetobacter*, *Arcobacter* and *Trichococcus* genera as previously reported (Newton et al., 2013; Luna et al., 2016; Buccheri et al., 2019; McLellan and Roguet, 2019; Basili et al., 2020; Basili et al., 2021).

2.6. Data analysis

Statistical analyses were performed in Rstudio. To this aim, INS, PORT1, WAT2, WAT3 and 7M were considered as "lagoon" samples, whereas WAT4, SEA1, SEA2 and PTF as "open sea" samples. We assessed the presence of significant differences in the abundance of the aforementioned microbial pollutants between: i) sediment and water samples, ii) open sea and lagoon samples, iii) seasons; to this aim, we used the Kruskal-Wallis test (function *kruskal.test* in the *stats* R package) and *pairwise.wilcox.test* to calculate a non parametric pairwise comparisons between group levels with corrections for multiple testing. Moreover, the same comparisons were used to test the presence of significant differences at the compositional level considering the only potential pathogenic community (ASVs identified within the aforementioned microbial pollutants); to this aim, we used the *anosim* function (*vegan* R package). Spearman correlations (*cor.test* function, use = "pairwise.complete.obs") (*stats* R package) were calculated to: (i) assess the presence of correlations between faecal contaminants (i.e., log-transformed cultivated and qPCR-derived EC and ENT abundances, and faeces- and sewage-associated taxa relative abundances) and (ii) assess the relationships of environmental variables with each individual potentially pathogenic taxon previously described and with each total potential pathogenic signature (the sum of the ASVs identified as taxa belonging to conventional and alternative faecal taxa, sewage taxa). Spearman correlations were calculated between individual potentially pathogenic taxon and environmental variables using the *cor* function (*stats* R package) and a correlogram was plotted in Rstudio (*corrplot* function, *corrplot* package) (Wei and Simko, 2017).

3. Results

3.1. Environmental variables

A detailed description of environmental conditions in the different sampling sites and seasons is reported in Quero et al. (2017).Temperature was typically higher within the lagoon (avg., 16.9 ± 6.7 and 15.4 ± 6.3 °C in lagoon and open sea, respectively), except in winter, when an opposite trend was observed. Salinity was higher in the open sea (33.52 ± 2.5 psμ) than in the lagoon (30.3 ± 1.9 psμ). Turbidity was rather homogenous across seasons, with an increase in winter within the lagoon (up to 156.11 NTU). In the lagoon, an increase in DO was observed from spring to summer (>100%), while decreased from winter to spring (80%); DO concentration in the open sea was overall constant from spring to autumn and decreased during winter, reaching values of 75.5%. Nutrients were typically higher in the lagoon; DIN values reached their maxima in summer both in lagoon and open sea sampling sites. Silicates were rather homogeneous in open sea stations across all seasons, but higher lagoon stations. Higher concentrations of phosphates were observed in the lagoon than in the open sea, with autumn peaks in the lagoon. Again, in the lagoon, chlorophyll-a had highest values in summer and lowest in winter. In the open sea, chlorophyll-a was highest in autumn.

3.2. FIB abundance assessment by culture-based method

The FIB abundance data are summarised in Fig. 1, panel A. In general, we observed that ENT were more abundant ($p < 0.001$) in sediments (avg. 1049.38 ± 1952.84 CFU/g) than in water (avg. 33.77 ± 57.95 CFU/100 mL), while there wasn't a clear difference in *E. coli* abundances. As regards water, EC abundance (Fig. 1, panel A, Supplementary Figure 2) ranged from undetectable to 363.3 CFU 100 ml−1 (Spring, WAT2), whereas ENT abundance (Fig. 1, lower panel A, Supplementary Figure 2) ranged from undetectable to 297.5 CFU 100 ml−1 (Spring, 7M). In sediments, EC abundance ranged from undetectable to 330.7 CFU 1 g−1 (winter, 7M) and ENT from undetectable to 8803.42 CFU 1 g−1 (winter, WAT2). Significant differences were found in both ENT and EC abundances between lagoon and open sea ($p < 0.001$). A positive significant correlation was found between FIB in water (rho = 0.73); similarly, in sediments, EC and ENT were significantly correlated with each other (rho = 0.85). No correlation was found between EC in water and sediment, nor between ENT in water and sediment.

In lagoon samples, an overall decreasing trend of FIB was observed from spring to winter in water (Fig. 1, panel A), whereas this trend was not detected in the open sea. In sediments, higher FIB concentrations were observed in lagoon than in the open sea samples, with an overall increasing trend from spring to winter (Fig. 1, panel A); however, it must be pointed out that, for open sea, data were only available for PTF station, were both EC and ENT were undetectable (Supplementary Figure 2).

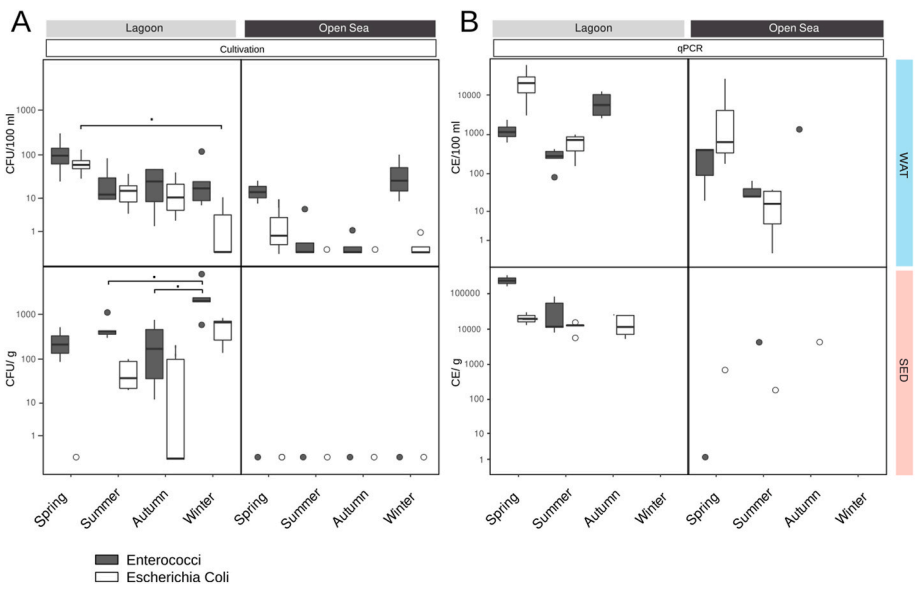

Fig. 1. Comparison of FIB abundance (ENT and EC) in water (upper panels) and sediment (lower panels) in lagoon and open sea, as determined in our study site by culture-based (panel A) and qPCR approaches (Panel B). The boxplots show FIB abundance in the different seasons; the abundance values were log-transformed. Please note that, in panel A, the open sea sediment data are represented by values measured at PTF only (values = 0). Significant differences between pairs of samples are reported (. = $p < 0.05$); statistical differences were calculated considering absolute numbers and not log-transformed data.

3.3. qPCR quantification of FIB

EC and ENT abundance data as measured with qPCR are summarised in Fig. 1 (panel B) and Supplementary Figure 2. Compared with the culture-based method, the qPCR approach provided consistently higher estimates of FIB abundance in both water and sediments (Kruskal-Wallis $p < 0.001$); in water, EC abundance was 22- to 25,000-fold higher and ENT abundance was from 2- to 3800-fold higher than those measured with the traditional approach. In general (as seen in cultivation-based data), we observed that FIB abundances were higher in sediments than in water samples, and in addition EC and ENT displayed comparable positive trends (total rho = 0.69, sediment rho = 0.82 and water rho = 0.71, Fig. 3). Considering qPCR data from Spring and Summer only (where FIB data are available for both water and sediment), both EC and ENT were more abundant ($p < 0.001$) in sediments (avg. 11,067 ± 8992 and 64,605 ± 95,260 CE/g for EC and ENT, respectively) than in water (avg. 9929 ± 17,117 and 560 ± 640 CE/100 mL). Significant differences were found in both ENT and EC abundances between lagoon and open sea ($p < 0.001$). Notably, in contrast to what observed on the cultivation data, PTF displayed values of both FIB higher than zero.

3.4. HTS analysis of faecal pollutants

Out of a total number of 16,374 total ASVs identified using the 16S rRNA gene HTS, 126 were identified as faecal bacteria (i.e., 5 traditional and 121 alternative faeces-associated bacteria) and 91 as sewage-associated bacteria. Within the faecal bacteria, 5 ASVs were identified as belonging to the family Enterobacteriaceae, which included only 2 ASVs identified at the genus level as belonging to the traditional faecal indicators (FIB) *Escherichia/Shigella* genus. *Escherichia/Shigella* ASVs were only found in few of the water samples, either in the lagoon and in the open sea, and at different levels according to the season (Supplementary Figure 3), with relative abundance ranging from 0 to 0.1% of the total prokaryotic community. Moreover, no ASVs were identified as *Enterococcus* nor Enterococcaceae. For this reason, traditional faecal indicator bacteria as depicted by the HTS approach were not considered for further analyses.

Overall, the cumulative relative abundance of alternative faecal bacteria and sewage-associated varied widely across samples, and displayed significantly higher relative abundances in the lagoon than in the open sea (Kruskal-Wallis, $p < 0.001$), as well as higher abundance in water than in sediment samples (Kruskal-Wallis, $p < 0.1$) (Supplementary Table 1).

Alternative faeces-associated taxa abundances, when considering water and sediment together, highlighted a significant difference between lagoon and open sea (Kruskal-Wallis, $p < 0.001$) and between water and sediment samples (Kruskal-Wallis, $p < 0.001$). Across all samples, a total of 7 ASVs were identified as alternative faeces-associated taxa. In the open sea, the relative abundance of faeces-associated taxa accounted for 0.03 ± 0.044% whereas, in the lagoon, they represented up to 0.32 ± 0.30%. It is noteworthy mentioning that, in the lagoon, faeces-associated taxa were significantly higher in water samples (0–1.24% of the total community) than in sediments (0–0.91%) (Kruskal-Wallis, $p < 0.001$). The most abundant taxa within this group in water lagoon samples were represented by Bacteroidaceae (0.10 ± 0.14%, with peaks of 0.45), Ruminococcaceae 0.05 ± 0.12%) and Lachnospiraceae (0.01 ± 0.03%) that equally contribute to the highest values. Alternative faeces-associated taxa in sediments were mainly represented by Ruminococcaceae (avg. in lagoon 0.28 ± 0.17%) and Lachnospiraceae (avg. in lagoon 0.11 ± 0.18%) (Supplementary Figure 2, panel B), that were both present in almost all of the lagoon samples.

Sewage-associated bacteria showed differences, although not significant, in their relative abundances between lagoon and open sea (Fig. 2, panel C). Across all samples, a total of 3 ASVs were identified as sewage-associated taxa. In opposition to what has been observed for faeces-associated taxa, for sewage-associated taxa within the lagoon, water samples (avg. 1.73 ± 2.06%) displayed significantly higher relative abundances than sediments (avg. 0.17 ± 0.32%) ($p < 0.001$). Overall, sewage indicators were dominated by *Arcobacter* (about 91% of the total sewage-associated taxa) in both sediment and water samples, with a peak in relative abundance of 6.49% in the water collected at 7M in spring, and 2.34% in the sediments collected at PTF in autumn.

It is noteworthy that, in water, the proportion of sewage-associated taxa was strongly correlated to that of faeces-associated bacteria ($p < 0.001$ and rho = 0.69), something that was not observed when considering the sediment samples only (Fig. 3). In water, we found that, overall, INS, PORT1 and 7M in spring, as well as WAT2 in autumn and WAT2 and 7M in winter were the most contaminated stations.

We then evaluated the seasonal variations of microbial contaminants in our dataset, by focusing on differences between sediment and water in the lagoon site only. When using HTS, no significant differences were found in seasonal abundances between the open sea sites, while in lagoon water samples, faecal contamination (i.e., alternative faeces-

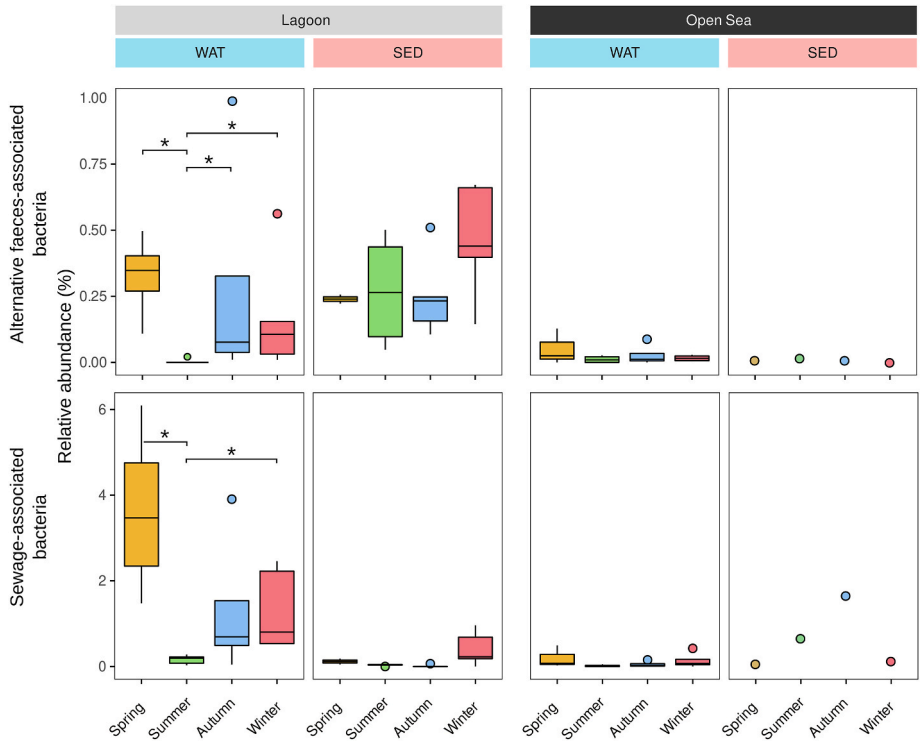

Fig. 2. Box plots showing the relative abundance (%) of sequences belonging to alternative faeces- (upper panels) and sewage-associated taxa (lower panels) in water and sediment samples as assessed by HTS (* = p < 0.01).

Fig. 3. Correlation analyses performed to compare data of faecal pollution obtained by the different methods herein analysed (i.e., cultivation, qPCR, HTS). Cultivation and qPCR data are log-transformed. For each panel, Spearman rho is reported, and coloured according to the matrix for which has been calculated (i.e., cyan = water, red = sediment). Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1). CFU = Colony Forming Units per gram or 100 mL in sediment and water samples, respectively; CE = Cell Equivalents per gram or 100 mL in sediment and water samples, respectively; % = relative abundance. Alternative = alternative faeces-associated taxa; Sewage = sewage-associated taxa. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

associated bacteria) was generally highest during spring (avg., 0.36 ± 0.17%) and lowest during summer (avg., 0.09 ± 0.08%) (Fig. 2). In sediment, the highest values were reached in winter (avg., 0.56 ± 0.24%), although no significant differences were observed among seasons. In lagoon sites, sewage-associated indicators exhibited highest relative abundance in spring (avg. 3.06 ± 2.01%), with an increasing trend from summer (avg. 0.16 ± 0.10%) to winter (avg., 1.3 ± 0.9%), while in sediment samples the highest value was reached in winter (avg., 0.41 ± 0.39%), with no significant differences between each season. With regard to the average value observed in the three indicator groups (excluding the traditional faecal indicators, in which we had very small abundances in just a few samples), the typical trend remains clear in the water samples, with an increasing relative abundance from summer to spring.

3.5. Relationships between cultivation and HTS approaches

Spearman correlations were performed to test the occurrence of correlations between the abundances of faecal pollutants as assessed by the different approaches herein used. Considering the entire dataset of FIB enumerated by cultivation method and qPCR data (including both water and sediment samples), we found that ENT abundances calculated using the two approaches were positively and significantly correlated (Spearman rho = 0.7), whereas a weak, although positive, relationship between cultivated and qPCR-derived EC abundances was observed (rho = 0.39).

The comparison between cultivation data of FIB and HTS showed that strong, positive correlations occurred in both sediment and water samples between cultured FIB and faeces-associated bacteria abundances (ENT *vs.* faecal bacteria, rho = 0.73; EC *vs.* faecal bacteria, rho = 0.54) (Fig. 3). Similarly, statistical correlations were displayed when comparing cultivated FIB data and sewage-associated bacterial abundances, but only in water samples (ENT, rho = 0.75; EC, rho = 0.66).

4. Discussion

FIB are currently used worldwide to determine faecal contamination of water and the associated health risks, providing a simple and reasonably reliable tool to assess water quality (Holcomb and Stewart, 2020). However, challenges and limitations to the use of FIB, with particular reference to the recommended use of cultivation-based approaches have been identified, and including the lack of relationships between FIB abundances and health risks (Fewtrell and Kay, 2015; Sclar et al., 2016; Korajkic et al., 2019), the recognition that FIB may persist and grow in the environment (Byappanahalli et al., 2003, 2004; Ishii and Sadowsky, 2009) and the relatively long times needed to obtain results with culture-based methods (Dorevitch et al., 2017). In addition, regardless of the technical approach applied to assess FIB concentrations in the environment, the failure to identify pollution sources has been frequently reported as a main drawback to their use (Stewart et al., 2013; Fewtrell and Kay, 2015). To overcome such limitations, in recent years, alternative approaches and microbial targets have been optimised and tested (Stoeckel and Harwood, 2007; Harwood et al., 2014; Unno et al., 2018). Nevertheless, the debate on which of such approaches comprehensively addresses time and informational needs in water quality assessment is still open. To further advance on this topic, we thus compared the data on faecal contamination from three different approaches, namely the traditional (culture-based) FIB method, a FIB-targeting qPCR assay and HTS of 16S rRNA gene to clarify whether they provide comparable findings on the microbial quality in the anthropogenically-impacted lagoon of Venice (Italy).

4.1. Fecal contamination in the Venice Lagoon and the adjacent sea

It is well known that faecal microorganisms severely and chronically contaminate the Venice Lagoon (Perini et al., 2015; Luna et al., 2019; Basili et al., 2021). Despite detecting pollutants at different orders of magnitude, all approaches herein used agreed in showing that, as expected, faecal contamination was higher within the lagoon than in open sea samples, in both water and sediment. It is noteworthy to mention the presence of *Arcobacter* representing up 6.49% of total community; *Arcobacter* species are highly abundant in sewage where they often comprise approximately 5–11% of the bacterial community and represent emerging pathogens whose risks for humans need to be better studied (Fisher et al., 2014; Ghaju Shrestha et al., 2022). Faecal microorganisms can enter water bodies in diverse ways, including runoff, sewage discharge, and direct faecal deposition (Korajkic et al., 2019). In our case, these high pollution levels in the lagoon are likely the result from the continuous faecal inputs from the mainland to the lagoon area (highly evident at WAT2), coupled with the direct release of untreated, or only partially treated, domestic waste into the canals from the historic city centre that receives millions of tourists across the year (Rose et al., 2006; Coraci et al., 2007; Perini et al., 2015; Quero et al., 2015; Basili et al., 2022). The increasing distance from the potential sources and the high dispersion rates in the open sea contribute to the lower levels of faecal pollution observed when moving seaward (Luna et al., 2016; Basili et al., 2022). The high abundances of sewage-associated taxa at the PTF marine site determined by HTS analysis are unclear. Notably, a submarine urban wastewater outfall has been moved from the inner lagoon to 10 km outside the outer coasts of the Venice Lagoon after 2013 (Ostoich et al., 2018), and this may be linked to such results, but more investigations are needed.

4.2. Faecal contamination patterns in water and sediments

We consistently found evidence, when using the FIB-targeting approaches (both cultivation and qPCR, and mostly evident for ENT than EC), for a higher average faecal contamination in sediments than in water, as previously reported in several studies (Anderson et al., 2005; Perini et al., 2015; O'Mullan et al., 2019). This pattern was however not consistently observed with HTS data, where only alternative faeces-associated indicators showed highest values of relative abundance in sediments. Taken together, our results confirm that aquatic sediments act as a favourable environment for faecal pollutants accumulation, likely due to their protective role toward predators and the organic and inorganic nutrient-rich characteristics favouring faecal microbiota survival or even re-growth (O'Mullan et al., 2019; Garzio-Hadzick et al., 2010; Haller et al., 2009; Smith et al., 2019). For these reasons, sediments, where longer FIB persistence compared to the water occurs typically, have been often referred to as potential long-term sinks or reservoirs for potential pathogenic microorganisms, including faecal contaminants (Vignaroli et al., 2013; Cupit et al., 2019; Saingam et al., 2020). It is also increasingly recognized that FIB prefer a particle-associated rather than a free-living lifestyle (Fries et al., 2006; Suter et al., 2011; Mote et al., 2012; Basili et al., 2022), which promotes their settlement and accumulation on the seabed (Solo-Gabriele et al., 2016; O'Mullan et al., 2019). Our results corroborate the need, as previously suggested (Ducklow et al., 1982; Garcia-Armisen and Servais, 2009), to start routinely incorporating sediments in microbial quality assessments in addition to water samples, especially in highly-contaminated lagoon and semi-enclosed systems characterized by shallow depths like the one investigated in our study. Conversely to FIB and faeces-associated indicators, sewage indicators (*Acinetobacter*, *Arcobacter* and *Trichococcus*) showed a lower relative average abundance in lagoon sediments than in water. This finding may suggest, among others, that lagoon sediments are not a favourable environment for these bacteria, however the ecological factors that affect the survival, growth and behaviour of these sewage indicators in aquatic sediments still remains unclear, deserving more studies (Fisher et al., 2014; Carney et al., 2020).

4.3. Correlations of faecal contamination using different approaches

EC and ENT abundances were significantly and positively correlated with each other when considering separately sediments and waters, as emerged from both conventional and qPCR approaches. Contrasting results are reported in literature regarding this aspect, with several studies supporting this finding (Luna et al., 2019; Nappier et al., 2019; O'Mullan et al., 2019; Saingam et al., 2020), and others showing the lack of such a correlation, at least in sediment samples (Saingam et al., 2020). However, the absence of significant correlations between EC and ENT when considering both matrices together suggests that, once released in the environment, FIB face different behaviour and fate in the two compartments, with sediments acting more as a sink for faecal bacteria and other pathogens (Luna et al., 2019; Saingam et al., 2020) while water being more dynamic and affected by hydrological processes, including circulation and tidal flushing (about 1 m excursion during spring tides in the Venice Lagoon) (Ferrarin et al., 2010, 2014), that are able to influence bacterial survival, distribution and fate. Additionally, several studies have suggested that FIB contamination in water reflects recent contamination events (Mattioli et al., 2017), especially in highly dynamic environment like coastal lagoons, whereas sediments typically represent mid-to long-term collectors of faecal contaminants and recorders of pollution events (Devane et al., 2020 and references therein).

We observed that EC and ENT abundance data obtained by FIB-targeting approaches (i.e. cultivation *versus* qPCR) were significantly correlated, both in water and sediments, indicating an overall comparability of the two methodologies for microbial quality assessments in large datasets. Although multiple studies have assessed the relationship between FIB culture and qPCR results, only a few of them focused on comparisons on paired water samples (Noble et al., 2010; Shrestha and Dorevitch, 2019). The qPCR approach has the advantage of being settable for multiplexed assays to detect several microbial targets simultaneously, and its analytical costs are becoming increasingly affordable. Moreover, qPCR-based procedures can yield same-day results that should improve public health protection to recreational users (Haugland et al., 2021). While showing correlating results, our study indicate that qPCR-based FIB estimates were higher than cultivation, likely due to the ability of qPCR to detect also recently non-viable cells, dead cells and detrital DNA persisting in the environment (Luna et al., 2010; Di Cesare et al., 2013; Luna et al., 2019) and leading to an overestimation of FIB abundance. This qPCR overestimation has been already reported in other studies in the US (Haugland et al., 2005; Gonzalez and Noble, 2014; Shrestha and Dorevitch, 2019), despite others have sometimes reported underestimations by qPCR likely resulting from inhibition of DNA amplification, to the fact that primers may be more specific to the target species than to the wider range of ENT species enumerated using cultivation, or to the growth of non-target species on culture media (Noble et al., 2010). Our results stress the need to carry on more comparative studies in microbially impaired environments to better evaluate the performance of the two methods.

On the other hand, the proportion of alternative faeces-associated bacteria data positively correlated with cultivation-related FIB abundances in both sediment and water samples, whereas sewage-associated bacteria abundances showed correlation with cultivated EC and ENT only in water samples. This finding, in addition to the observed positive correlation between the proportion of sewage- and faeces-associated taxa in water samples, let hypothesize that, in our study site, a common source of contamination contributed to the occurrence of these microbial pollutants.

Lastly, no tests for correlations between EC and ENT (assessed by cultivation and qPCR) FIB and HTS were performed, given the low abundance, or even the absence, of ASVs identified as belonging to traditional FIB in our HTS datasets. This supports the mounting evidence that FIB are typically poorly represented with respect to the faecal taxa in HTS datasets (Newton et., al 2011), also confirmed in successive studies (McLellan et al., 2013; Newton et al., 2013; Luna et al., 2016; Basili et al., 2021). This may suggest, among others, the need to design and test more specific primer pairs for FIB for microbial quality monitoring purposes. HTS is extremely promising and rapidly advancing in water quality assessment, as it allows the simultaneous assessment of multiple bacterial taxa, thus providing information on the overall quality in the area, as well predicting the source of microbial pollution (human *vs.* non-human) by means of novel bioinformatics pipelines, or detecting specific microbes that may be associated with transient or continuous human health-relevant pollution sources (McClary-Gutierrez et al., 2021). Despite being still time-demanding and relatively expensive, the recent introduction of portable sequencing platforms and the declining costs of HTS analyses will certainly help to overcome these issues. While being able to provide only relative abundance data within the community, studies are increasingly showing that patterns of faecal and sewage taxa detected by qPCR very closely resemble shifts in HTS data (VandeWalle et al., 2012), suggesting the potential of HTS to be a useful tool to assess pollution and human health risks, as sequencing technologies develop and become more appropriate for routine use (McClary-Gutierrez et al., 2021).

4.4. Seasonal trends of pollutants assessed using different approaches

We observed similar seasonal trends in faecal contamination according to the different approaches used, suggesting the overall comparability of data obtained by the different analytical methods. Seasonal variation in faecal contamination has been reported to vary greatly according to the site-specific characteristics (Enns et al., 2012; Wiegner et al., 2016; Buccheri et al., 2019), being the result of different processes and drivers, that include variability in point and non-point source inputs, seasonally-driven variations in water chemical-physical variables (e.g., temperature, salinity), river flow intensity, rainfall, solar radiation and tidal height (Boehm et al., 2003; Wymer et al., 2004; Enns et al., 2012; Perini et al., 2015; Mattioli et al., 2017; Wiegner et al., 2016). In this study, faecal contamination levels in lagoon water showed typically highest values in spring, corroborating previous findings on the same site (Perini et al., 2015; Quero et al., 2015). We hypothesize that the higher levels of faecal bacteria in spring may depend upon higher inputs from the densely populated, historical city centre (receiving millions of tourists yearly, with peaks in spring and summer; data from Venice Municipality, https://www.comune.venezia.it/en) and from the tributaries of the lagoon drainage basin, coupled with more favourable spring conditions for FIB survival and growth in the aquatic environment. The lack of clear trends in open sea samples underlines how different processes drive the occurrence of faecal contaminants in the coastal sea, depending on the higher distance from point sources, the extreme dilution as well as the higher impact of salinity (Manini et al., 2022). On the other hand, sediments displayed a different pattern of temporal changes in faecal contamination, with peaks observed in winter. This finding, again, highlights the different behaviour exerted by the benthic and pelagic compartments on faecal microbiota, which likely respond to the different drivers to which they are subjected.

5. Conclusions

In summary, through the use of independent methods applied in this research, we found consistent evidence for a high level of faecal pollution in the Venice Lagoon across the year. However, despite yielding overall comparable results, our results highlighted the limitation of traditional FIB cultivation in assessing microbiological quality in the lagoon environment and in evaluating the type of pollutants (faecal, sewage), stressing the need for more comprehensive microbial risk assessment approaches. Given that none of the approaches herein used alone provided comprehensive results, we conclude that the combination of at least two of such approaches (e.g., cultivation and qPCR data or cultivation and HTS data) should be used in the future, to improve quantitative and qualitative assessments of microbial quality in water

and sediments. While further technological advancements are needed before HTS community analysis can be incorporated into routine microbial monitoring, including more work needed to identify thresholds for future development of HTS-based monitoring techniques (McClary-Gutierrez et al., 2021), our study confirms how HTS can provide extremely useful information on the occurrence and proportion of faecal and sewage-associated pollutants, useful to complement FIB data. Finally, our data also bolsters the need to incorporate sediments into routine pollution assessments, to improve our understanding of contamination processes in response to environmental stressors, to address management practices and to better define the risks posed to human health.

Author statement

GMQ, GML: Conceptualization; GMQ, GML, MB, LP: Methodology; LP, MB: Formal analysis; GMQ, GML, LP: Investigation; GML: Resources; GMQ, MB, GML: Writing - Original Draft; GMQ, MB, GML, LP, LZ: Writing - Review & Editing; MB, GMQ: Visualization; GML: Funding acquisition.

Declaration of competing interest

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.

Data availability

The SRA database accession number for the sequence file are provided in the text.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.122140.

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