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Presence and characterization of the human pathogenic *Vibrio* species in the microbiota of Manila clams using cultural and molecular methods

Angela Zampieri [1], Lisa Carraro [1], Hooriyeh Mohammadpour, Giulia Dalla Rovere, Massimo Milan, Luca Fasolato [*], Barbara Cardazzo

Department of Comparative Biomedicine and Food Science (University of Padova), University of Padua, Viale Università 16, 35020 Legnaro, Pd, Italy

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ABSTRACT

The North Adriatic lagoons and the Po River Delta are important areas for farming Manila clams (*Ruditapes philippinarum*). These areas have been heavily impacted by climate change, reducing livestock numbers and increasing pathogen spread. Shellfish, particularly clams, are primary vectors for *Vibrio* pathogens affecting humans. In this study, the occurrence of human pathogenic *Vibrio* species on Manila clams was investigated using an integrated approach that combined culture-dependent and culture-independent techniques. Samples were collected over three years from farming areas in the northeastern Adriatic lagoons and the Po River Delta, regions seriously impacted by climate change and pollution. In this study, species of the human pathogen *Vibrio* were analyzed in the clam microbiota and characterized using *recA-pyrH* metabarcoding and shotgun metagenomics. Human pathogenic *Vibrio* species were widespread in the clam microbiota, especially in summer, demonstrating that the environmental conditions on the northern Adriatic coasts allowed the growth of these bacteria. *V. parahaemolyticus* and *V. vulnificus* were also quantified using qPCR in <50 % of summer samples Shotgun metagenomics revealed the similarity of *V. parahaemolyticus* strains to other worldwide genomes, enabling improved pathogen identification and tracking. In the future, climate change could cause these conditions to become even more favorable to these bacteria, potentially increasing pathogen spread. Consequently, enhanced monitoring and control of both the environment and seafood products should be planned to ensure food safety.

1. Introduction

The North Adriatic lagoons and the Po River Delta are host to important aquaculture activities, particularly those related to the farming of Manila clams (*Ruditapes philippinarum*), one of the most cultivated mollusks in the world.

Italy is the leading producer of Manila clams in the European market and the second leading producer worldwide, producing 55,000,000 tons annually (Bordignon et al., 2021; Martini et al., 2023; Turolla et al., 2020). However, new challenges to the culture system are emerging due to the climate crisis.

The Mediterranean Sea is a "hot spot" region for climate change, with frequent occurrence of marine heatwaves, which result in prolonged periods of high sea surface temperatures (Darmaraki et al., 2019; Pastor and Khodayar, 2023) with strong effects on the ecosystem (Ferrarin et al., 2024) and on the survival of clams (Rato et al., 2022). In particular, Manila clam production in the northern Adriatic region has experienced a strong reduction in livestock and overall production, as described in Ponti et al. (2017). In addition, a recent study by Peruzza et al. (2023) clearly demonstrated how marine heatwaves continue to impact the physiology of *Ruditapes philippinarum* at multiple levels, including fitness.

Several studies have demonstrated the effects of global warming on the spread and distribution of the human pathogen *Vibrio* (Amato et al., 2022; Fleischmann et al., 2022; Froelich and Daines, 2020; Le Roux and Blokesch, 2023; Vezzulli et al., 2013), with a resulting increase in human infections related to the consumption of seafood products (Mora et al., 2022). Importantly, the *Vibrio* genus is one of the most represented taxa in the clam microbiota (Milan et al., 2018; Pruzzo et al., 2005; Romalde et al., 2014; Gerpe et al., 2023). Several *Vibrio* species are pathogenic to humans (Baker-Austin et al., 2018), and among them, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* are responsible for the

* Corresponding author at: viale dell'Università, 16, Legnaro 350210, Padova, Italy.
E-mail address: luca.fasolato@unipd.it (L. Fasolato).
[1] These authors contributed equally.

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most serious forms of vibriosis in humans and are contracted through the consumption of raw or undercooked seafood products (West, 1989). In particular, the human ingestion of water or raw seafood products contaminated by pathogenic *V. cholerae* and *V. parahaemolyticus* leads to watery diarrhea and gastroenteritis, respectively (Baker-Austin et al., 2018; Morris, 2003). *V. vulnificus* is frequently associated with human ear and superficial wound infections related to exposure to contaminated seawater or shellfish products (Baker-Austin et al., 2018). *V. vulnificus* is responsible for the majority of seafood-associated human deaths worldwide (Heng et al., 2017).

Seafood products are the principal vector for the spread of the main *Vibrio* human pathogens (Baffone et al., 2000; Song et al., 2020; Brauge et al., 2024). In particular, the presence of these species in shellfish products, such as clams, oysters and mussels, is well documented in the literature (Ding et al., 2022; Huehn et al., 2014; Lorenzoni et al., 2021; Robert-Pillot et al., 2014; Yu et al., 2013; Zampieri et al., 2020).

In addition, it has been demonstrated that exposure to chemical pollution affects the microbial community composition of the *R. philippinarum* microbiota (Milan et al., 2018) and the occurrence of *Vibrio* human pathogens in coastal waters (Castello et al., 2022; Goh et al., 2017).

However, despite the frequent detection of *Vibrio* species in the edible tissues of bivalve mollusks, European Legislation still lacks established microbiological safety criteria, and certain diseases associated with *Vibrio* are not classified as notifiable (Amato et al., 2022; Hartnell et al., 2019). Moreover, there is a lack of surveillance programs for monitoring the virulence and distribution of *Vibrio* species, resulting in human illnesses associated with shellfish products and other sources (Amato et al., 2022, Sacheli et al., 2023). Specifically, the Commission Regulation (EC) No 2073/2005 (2005) defines the microbiological criteria that foodstuffs must meet concerning the concentrations of *E. coli* and Salmonella in shellfish tissues, but this does not provide adequate human protection from vibriosis. Moreover, several studies have shown that the common purification cycles used by food business operators to reduce fecal indicator bacteria associated with the edible tissues of bivalve mollusks are often ineffective in decontaminating shellfish from *Vibrio* (Sferlazzo et al., 2018; Vezzulli et al., 2018; Zampieri et al., 2020).

Despite numerous studies conducted over the years to detect *Vibrio* human pathogens associated with Italian shellfish products, there is still a lack of in-depth studies providing detailed information about *Vibrio* species, specifically those associated with *R. philippinarum*, as human pathogens (Caburlotto et al., 2016; Castello et al., 2022; Di Pinto et al., 2011; Lorenzoni et al., 2021).

This study aimed to address these gaps by studying the occurrence of *Vibrio* human pathogenic species on Manila clams using an integrated approach based on both culture-dependent and culture-independent techniques (including DNA metabarcoding, real-time PCR, and metagenomics). NGS technologies, commonly using 16S rRNA, provide a detailed view of microbial communities at the genus level (Zampieri et al., 2020). Here, a new approach was proposed to enhance species-level identification of *Vibrio* in clam samples through an integrated strategy.

The samples were collected over three years from farming areas severely affected by climate change located along the northeastern Adriatic lagoons and the Po River Delta (Milan et al., 2018). One of the major effects of the climate change on the coastal lagoon ecosystems is the rise of the brackish water temperature favored by the higher seasonal atmospheric temperature and to the poor water exchange of the lagoons because of their limited connection to the open sea (Ferrarin et al., 2024). Long-term observations have documented an increase in sea temperature (0.44–0.48 °C per decade) and a rise in sea surface salinity, indicating ongoing salinification in the studied area. Additionally, extreme events such as marine heatwaves in the northern Adriatic Sea are particularly significant during the spring and summer seasons, while floods are predominantly reported in winter (Ferrarin et al., 2024). Consequently, studying *Vibrio* biodiversity associated with clams should account for seasonality to provide accurate insights into the distribution of different *Vibrio* taxa during hot and cold months.

Moreover, in the present study clams were collected from polluted areas of Venice Lagoon, where clam farming is prohibited, in order to assess changes in the *Vibrio* community of clams inhabiting sites affected by severe chemical pollution (Milan et al., 2018).

To conclude, the aim of this study was to detect, characterize, and quantify human pathogenic *Vibrio* species associated with clam samples while considering variables such as seasonality, depuration, and geographic area.

2. Materials and methods

2.1. Sample collection

Batches of *R. philippinarum* specimens were collected in authorized Italian farming areas located in Marano (MA), Chioggia (two sites, 1CH and 2CH), Scardovari (SC) and Goro (GO) for three consecutive years from 2018 to 2020. To investigate the effects of pollution on the presence of the main *Vibrio* human pathogenic species, clams were also collected from the Colmata (CO) and Porto Marghera (PM) sites, where clam farming is prohibited due to high chemical pollution, in accordance with DGR 3366/2004, n.d. All the sites of the Northern Adriatic Sea are shown on the map in Fig. 1S. A *batch* is defined as the amount of animals caught from the same production area on the same day and under the same catching activity (defined as a traceable unit in each supply chain). The number of animals in each *batch* varied according to the common collecting practices performed in each lagoon. The sampling was performed at the seven sites during the summer and the winter seasons of three consecutive years (2018, 2019, and 2020). Each batch of clams was transported to a different depuration plant for depuration treatment. Only the Porto Marghera and Colmata batches were depurated in the same depuration plant (details reported in Table 1S).

To ensure the representativeness of the samples, randomly selected pools of individuals from each batch of depurated and non-depurated clams were used to obtain homogenized clam samples. As suggested by Commission Regulation (EC) No 2073/2005 (2005), a pooled sample consisting of a minimum of 10 individual animals and three replicates were assessed for each batch. For each site were collected depurated and non-depurated batches of live *R. philippinarum* individuals and then processed as described in a previous study (Zampieri et al., 2021).

Briefly, after the shells were cleaned by washing under running potable water and 100 % ethanol, pools of the clams were measured and shucked to collect 25 g of flesh and intervalvular liquid clam tissues in a sterile stomacher bag and then homogenized. The homogenization was performed by adding 225 ml of alkaline peptone water (APW, 2 % NaCl, 1 % peptone, pH 8.5; Microbiol, Macchiareddu, CA) inside the stomacher bags. The pre-depuration clam batches were stored at 4 °C for 24 h before homogenization.

In total, during the three years of the present study, 120 homogenate (HO) clam samples were collected, of which 50 were collected from seven farming sites during summer and 70 during the winter. Moreover, among these samples 60 were pre-depuration homogenate clam samples and 60 were post-depuration ones.

A complete list of the clams samples subdivided according to the origin site, depuration treatments and season/year of collection and analysis performed is included in Table 1.

2.2. Microbiological analysis

The clam homogenate (HO) samples were processed following a tenfold dilution procedure, with 100 µl of each serial dilution being plated on marine agar (MA) media (Condalab, Madrid, Spain) and thiosulfate-citrate-bile salt sucrose (TCBS) media (Biolife, Milano, Italy). MA medium was used to quantify the total marine bacteria, whereas

Table 1
Clam samples subdivided according to the origin site, depuration treatments and the season/year of collection. The analysis applied to each sample is also reported.

SITE	Depuration	Sample type	SUMMER 2018 n. samples	WINTER 2019 n. samples	SUMMER 2019 n. samples	WINTER 2020 n. samples	Total n. samples	Analysis
1CH	PRE	HO	1	2	3	3	9	MB; RT
		ES_MA22	1	2	2	0	5	RT
		ES_TCBS22	1	2	1	0	4	RT
		ES_TCBS37	1	0	1	0	2	MG; RT
	POST	HO	1	2	3	3	9	MC; MB; RT
		ES_MA22	1	2	0	0	3	RT
		ES_TCBS22	1	1	2	0	4	RT
		ES_TCBS37	1	0	0	0	1	MB; MG; RT
2CH	PRE	HO	0	2	3	3	8	MB
		ES_MA22	0	2	1	0	3	RT
		ES_TCBS22	0	2	1	0	3	RT
		ES_TCBS37	0	0	1	0	1	MB; MG; RT
	POST	HO	0	2	3	3	8	MC; MB
		ES_MA22	0	2	1	0	3	RT
		ES_TCBS22	0	2	1	0	3	RT
		ES_TCBS37	0	0	1	0	1	MB; MG; RT
PM	PRE	HO	1	2	3	3	9	MB; RT
		ES_MA22	1	2	1	0	4	RT
		ES_TCBS22	1	2	1	0	4	RT
		ES_TCBS37	1	0	1	0	2	MB; MG; RT
	POST	HO	1	2	3	3	9	MB;RT
		ES_MA22	1	2	1	0	4	RT
		ES_TCBS22	1	2	1	0	4	RT
		ES_TCBS37	1	0	1	0	2	MB; MG; RT
CO	PRE	HO	1	2	3	3	9	MB
		ES_MA22	1	2	1	0	4	RT
		ES_TCBS22	1	2	1	0	4	RT
		ES_TCBS37	1	0	1	0	2	MB, RT
	POST	HO	1	2	3	3	9	MC; MB
		ES_MA22	1	2	1	0	4	RT
		ES_TCBS22	1	2	1	0	4	RT
		ES_TCBS37	1	0	1	0	2	MB; RT
MA	PRE	HO	1	2	3	3	9	MB
		ES_MA22	1	2	1	0	4	RT
		ES_TCBS22	1	2	1	0	4	RT
		ES_TCBS37	1	0	1	0	2	MB; RT
	POST	HO	1	2	3	3	9	MC; MB
		ES_MA22	1	2	1	0	4	RT
		ES_TCBS22	1	2	1	0	4	RT
		ES_TCBS37	1	0	0	0	1	MB; RT
SC	PRE	HO	0	2	3	3	8	MB
		ES_MA22	1	2	1	0	4	RT
		ES_TCBS22	1	2	0	0	3	RT
		ES_TCBS37	1	0	0	0	1	MB; RT
	POST	HO	0	2	3	3	8	MC
		ES_MA22	1	2	1	0	4	RT
		ES_TCBS22	1	2	1	0	4	RT
		ES_TCBS37	1	0	1	0	2	MB; RT
GO	PRE	HO	0	2	3	3	8	MB
		ES_MA22	1	2	1	0	4	RT
		ES_TCBS22	1	2	0	0	3	RT
		ES_TCBS37	1	0	0	0	1	MB; RT
	POST	HO	0	2	3	3	8	MC
		ES_MA22	1	2	1	0	4	RT
		ES_TCBS22	1	2	1	0	4	RT
		ES_TCBS37	1	0	0	0	1	MB; RT

1CH, Chioggia site 1; 2CH, Chioggia site; PM, Porto Marghera; CO, Colmata; MA, Marano, 2; SC, Scardovari; GO, Goro; PRE, pre-depuration homogenate clam samples; POST, post-depuration homogenate clam samples.
MC, Microbiology Counts; MB, MetaBarcoding; MG, MetaGenomics; RT, real-timePCR.

TCBS medium was used to quantify the *Vibrio* load (Zampieri et al., 2020; FDA-BAM, 2004). The MA medium was incubated at 22 °C for 24 h, whereas the TCBS medium was incubated at both 22 °C and 37 °C for 24 h. A temperature of 22 °C was chosen to facilitate the growth of various *Vibrio* species (Vezzulli et al., 2013; Zampieri et al., 2020). The temperature of 37 °C is a selective temperature that promotes the growth and detection of the main *Vibrio* human pathogens (Bonnin-Jusserand et al., 2017). After incubation, microbiological counts were performed, and the results are expressed as log colony-forming units per gram of sample ($\log_{10}$ CFU g^{-1}). From each stomacher bag, a 2-ml aliquot of homogenate was collected and then centrifuged at 10,000 rpm for 1 min to obtain a pellet (Eppendorf centrifuge 5424). In addition, from MA or TCBS first dilution plates, the colonies were scraped after adding 2 ml of PBS to the plate surface to obtain a bacterial community enriched for Marine Bacteria from MA or for Vibrio from TCBS at 22 °C and 37 °C as described in Zampieri et al. (2020). The PBS suspension was collected from the plate surface and centrifuged at 10,000 rpm for 1 min to obtain a pellet (Eppendorf centrifuge 5424). All the pellets were stored at −80 °C until DNA extraction.

The total microbial counts and *Vibrio* counts are depicted in box plots generated using SPSS software (https://www.ibm.com/it-it/analytics/spss-statistics-software). A nonparametric combination (NPC)

test was performed on microbiological counts for both total marine bacteria and *Vibrio* spp. to assess the statistically significant effects of each fixed factor (season and farming site) and their combination (Zampieri et al., 2020).

2.3. DNA extraction, preparation of recA–pyrH metabarcoding libraries and bioinformatics analysis

The bacterial DNA extraction from the clam homogenates (HOs) and the subsequent preparation of the *rec*A–*pyr*H metabarcoding libraries were performed according to the procedure described by Zampieri et al. (2020). Briefly, DNA was extracted from the pellet obtained from 2 ml of homogenate via a DNeasy® PowerSoil® Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Illumina libraries for *rec*A and *pyr*H were prepared by performing two PCR steps of amplification by adapting primers previously used for isolate identification as described in Zampieri et al. (2020). After each PCR step, the amplified products were separated on a 1.8 % agarose gel and then purified via the SPRIselect Reagent Kit (Beckman Coulter Genomics). The final libraries were assembled in an equimolar pool, checked for quality using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and quantified via the Qubit® Assay Kit BR. Libraries were sequenced by the UCDAVIS Genome Center (California) via the MiSeq System, Illumina (300 bp forward and reverse for 15 million reads). The raw sequence data were deposited in the SRA database with accession numbers SRR11318117, SRR11194066 and SRR14425887.

The 120 homogenate clam samples produced a total of 24.575.865 raw reads for *rec*A-*pyr*H library sequencing. The quality check and bioinformatic analyses were performed on the *rec*A–*pyr*H raw reads of the HO samples following the methods of a previous study (Zampieri et al., 2021). After trimming and filtering, the *rec*A and *pyr*H sequences were imported into Kraken2 software (Wood et al., 2019) to perform a full data sequencing analysis against the MiniKraken2_v1_8 GB database. Bracken software was subsequently used to perform Bayesian inference of the abundance of the species detected during data sequencing (Zampieri et al., 2020). A three-way permutational multivariate analysis of variance using distance matrices performed with PRIMER-e software (https://www.primer-e.com/) was performed to evaluate whether the season, site and depuration affected the *Vibrio* community composition. PERMANOVA pairwise comparisons were subsequently performed to test the following contrasts: pre-depuration versus post-depuration, summer versus winter and the origin site. As reported by Zampieri et al. (2020) and (2021), the *rec*A–*pyr*H results were generated according to a qualitative approach (presence/absence) for the species detected. For this reason, the calculations were performed according to the Jaccard index (Hughes and Bohannan, 2004). The frequency of positive homogenate clam samples for the main *Vibrio* human pathogens was subsequently assessed using the NPC test, which considers three fixed factors in the analysis and in the PCoA representation: purification, season, and site. The NPC test was conducted to determine statistically significant differences in *Vibrio* species found in homogenate clam samples according to *recA–pyrH* metabarcoding.

2.4. V. cholerae, V. parahaemolyticus and V. vulnificus qPCR assays

In addition to the DNA extracted from HO for real-time PCR analysis, enriched samples (ES) of marine bacteria (ES_MA) and Vibrio (ES_TCBS) from bacterial communities were extracted after the homogenization of clam samples were plated on marine agar and TCBS media processed as described in Zampieri et al., 2020. Briefly, the total bacterial communities grown on dilution plate −1 of MA (incubated at 22 °C) and TCBS media (incubated at 22 °C and 37 °C) were collected by scrubbing and washing the surface of the plates with 2 ml of phosphate-buffered saline (PBS). Then, the suspended cells were centrifuged at 10,000 rpm for 1 min (Eppendorf centrifuge 5424), and the pellets were stored at −80 °C until DNA extraction was performed. DNA was extracted using the Invisorb® Spin Tissue Mini Kit (Invitek Molecular, GMBH, Berlin, Germany) following the manufacturer's instructions.

The primers used for *Vibrio* spp., *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* are listed in Table 2. To assess amplification efficiency, a range of DNA amounts ranging from 0.02 ng/µl to 40 ng/µl was tested. The sample DNA was diluted 2×, 5×, 10×, 25×, 50×, 100×, 1000×, 10,000×, 20,000× and 40,000×. The reaction volume was 10 µl, containing 5 µl of PowerUp™ SYBR Green Master Mix (ThermoScientific), 250 nM each primer and 2.5 µl of diluted DNA in a LightCycler 480 Roche (Basel, Switzerland). The cycling conditions were as follows: initial incubation at 50 °C for 2 min, followed by 2 min at 95 °C, and 45 cycles at 95 °C for 10 s and 60 °C for 40 s. All reactions were performed in duplicate. qPCR data analysis was performed using LightCycler 480 software version 1.5 (Roche). The specificity of the qPCR products was determined via analysis of melting curves. Standard curves (log genome copy number vs. crossing point, Cp) were obtained using serial dilutions of pure bacterial culture genomic DNA ranging from 5 to 0.003 ng. The copy numbers used in the calibration curves were calculated considering the genome size of the strains used. The Cp was used to calculate the copy number in the samples with the formulas obtained from the standard curves. The final total bacterial concentration was expressed as the log genome copy number in 1 ml. The total *Vibrio* abundance was also calculated, and the cell abundance was obtained by dividing the total 16S rDNA copy number by the average 16S rDNA copy number in Vibrios obtained from the rrnDB database (Vezzulli et al., 2013).

Correlation analysis between the number of *V. parahaemolyticus* and *V. vulnificus* copies determined via qPCR and the number of NGS *rec*A–*pyr*H reads assigned to these species was performed using Spearman method in RStudio software (R version 4.0.0). Data visualization and graph preparation were performed using SRplot (Scientific and Research Plot Tool) (Tang et al., 2023). Additionally, the correlation between the qPCR data for the *Vibrio genus* and the microbiological counts on the TCBS plates was examined.

2.5. Shotgun metagenomics library preparation and bioinformatical analysis

Shotgun metagenomics libraries were prepared from DNA extracted from 8 homogenized clam samples plated on TCBS media and incubated at 37 °C (ES_TCBS37) following the protocol described in Zampieri et al., 2021. DNA quantification was performed using a Qubit dsDNA HS Assay (Invitrogen, Life Technologies, Monza, Italy). Libraries were constructed using the Nextera XT DNA Sample Preparation Kit (Illumina, Inc., San Diego, USA) along with IDT for Illumina Nextera DNA UD Indexes. The final libraries were pooled in equimolar concentrations. The quality of the libraries was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and quantified with a Qubit HS Assay Kit. Sequencing was performed by Biomarker Technologies (BMK) GmbH (Münster, Germany) using a NovaSeq S4 platform with 150 bp paired-end reads. The sequencing of the 8 libraries produced a total of 564,032,284 raw reads. The sequence data have been submitted to the NCBI database under the BioProject ID PRJNA1156359.

Two pipelines (pipelines 1 and 2), each with specific processes, were used to analyze the shotgun metagenomics data, as shown in the diagram in Fig. 1. Specifically, the CZ ID platform was chosen for its advancements in enabling pathogen identification from metagenomic data, as it is an open-source, cloud-based platform designed for effective pathogen detection and monitoring. This dual approach helps validate the findings and increases confidence in the accuracy of the data.

Pipeline 1: The first part involved metagenomic taxonomical profiling. The raw sequences were trimmed on the basis of adapter content and PHRED scores using TrimGalore (https://github.com/FelixKrueger/TrimGalore) with default settings. Quality reports of the raw and trimmed reads were generated using FASTQC (Andrews, 2010) to assess improvements in read quality. Taxonomic profiling was conducted on the trimmed reads using Kraken2 with the MiniKraken2_v1_8

Table 2
Primers used in the study.

Target bacteria	Target gene	Primer	Sequence (5′-3′)	Product length (bp)	Reference
V. cholerae	*gbpA*	Vc gbpA F	CCGCAGCTTCCTTCTACAAC		
		Vc gbpA R	GGCTTTGGTTAGCGTCTCAG	206	Vezzulli et al., 2015
	viuB	Vc viuB F	TCGGTATTGTCTAACGGTAT		
		Vc viuB R	CGATTCGTGAGGGTGATA	77	Nasreen et al., 2022
	ompW	Vc ompW F	TCAATGATAGCTGGTTCCTCAAC		
		Vc ompW R	CGATGATAAATACCCAAGGATTGA	126	Diner et al., 2021
V. parahaemolyticus	*tlh*	Vp tlh F	ACTCAACACAAGAAGAGATCGACAA		
		Vp tlh R	GATGAGCGGTTGATGTCCAA	208	Nordstrom et al., 2007
V. vulnificus	*vvhA*	Vv vvhA F	TGTTTATGGTGAGAACGGTGACA		
		Vv vvhA R	TTCTTTATCTAGGCCCCAAACTTG	100	Campbell and Wright, 2003
Vibrio spp.	16S rRNA	Vib1-Fw	GGCGTAAAGCGCATGCAGGT		
		Vib2-Rev	GAAATTCTACCCCCCTCTACAG	114	Siboni et al., 2016

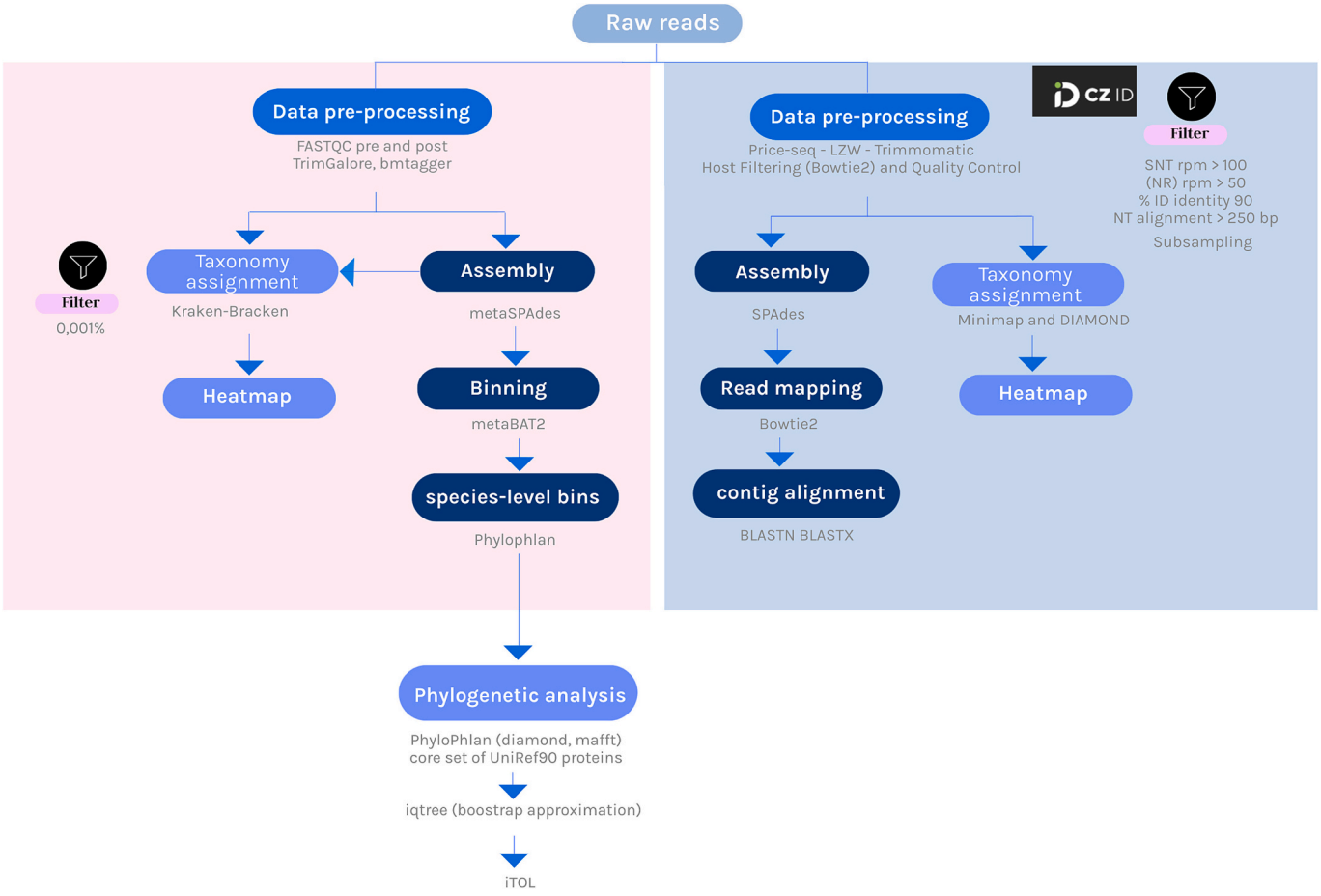

Fig. 1. Diagram of the bioinformatics processes used by the two pipelines employed to analyze the metagenomic data.

GB database. The abundance of bacterial species was estimated with Bracken on the basis of the taxonomy labels assigned by Kraken2. The Bracken report files for the 8 samples were converted into a biom file using the Kraken-biom tool. The microbial community composition was analyzed after abundances lower than 0.001 % were filtered out. The sequences identified by Kraken2 were extracted from the cseqs output data and further validated using BLAST against the NCBI database. The filtered .biom file was uploaded to Microbiome Analyst (Yao et al., 2023), and centered log ratio (CLR) data transformation was applied. The files were then downloaded and analyzed in R (version 4.2.2) using Phyloseq (McMurdie and Holmes, 2013) for both alpha and beta diversity. Alpha diversity was assessed using the ggplot2, dplyr, and ggpubr libraries. Beta diversity was analyzed using the vegan, reshape2, Matrix, ggplot2, dplyr, and ggpubr libraries, and the results were analyzed via the Wilcoxon test. Additionally, PERMANOVA was conducted. Heatmaps were generated with the ComplexHeatmap package in R. The second part of pipeline 1 focused on metagenome-assembled genome (MAG) analysis. For the assembly of cleaned and trimmed reads from ES_TCBS37, metaSPAdes (Nurk et al., 2017) was used. Contigs were sorted by length, and their names were updated to include the contig ID, length, and coverage. Short scaffolds (<1000 bp) were discarded. An assembly report was generated using QUAST (Gurevich et al., 2013). Binning was performed using metaBAT2 (Kang et al., 2019). A PhyloPhlAn (Asnicar et al., 2020) tool option was used to assign metagenome-assembled genomes (MAGs) to species-level genome bins (SGBs) on the basis of the SGB release of January 2019 (Pasolli et al., 2019). Only the closest SGB (−n1) was reported for each MAG in the .tsv output. For *V. cholerae*, *V. parahaemolyticus*, and

V. vulnificus, specific species-level genome bins (SGBs) were sought. A phylogenetic tree was constructed using the bins assigned to *V. parahaemolyticus*. The phylophlan_setup_database script was used to retrieve the core set of UniRef90 proteins for the target species, and the –diversity low option (suitable for phylogenetic analyses of genomes within the same species) was applied as the alignment was built using SGBs and NCBI genomes from a single bacterial species. One hundred genomes of *V. parahaemolyticus* downloaded from NCBI were included in the analysis (Table 10S), of which 56 were derived from clinical cases, 20 from mollusks, 8 from crustaceans, and 14 from other sources. The strains included in the analysis cover the years from 1998 to 2017, 42 of which were submitted to NCBI by the Center for Food Safety and Applied Nutrition (CFSAN) and the Food and Drug Administration (FDA). Additionally, many of the strains are part of the *V. parahaemolyticus* GenomeTrakr database (Allard et al., 2016) maintained by the U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition. The protein alignment obtained from PhyloPhlAn was then used to construct a maximum likelihood phylogenetic tree with IQ-TREE2, with 1000 bootstrap iterations performed (Minh et al., 2020). IQ-TREE2 takes a multiple sequence alignment as input to reconstruct an evolutionary tree that best explains the data, assessing branch support using ultrafast bootstrap approximation. A graphical representation of the phylogenetic tree was generated using iTOL software v6 (Letunic and Bork, 2021).

Pipeline 2 was used to confirm the detection of *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* from the metagenomic data. The first part of pipeline 2 involved metagenomic taxonomical profiling, as in pipeline 1. The raw reads were uploaded to the Chan Zuckerberg ID (CZ ID) portal (Lu et al., 2024), a cloud-based, open-source metagenomics bioinformatics tool designed specifically for pathogen detection. The pipeline (v8.2) processes the data by removing low-quality reads (using Price-seq), low-complexity reads (using LZW), duplicates, and adapters (using Trimmomatic). The cleaned reads were then queried against the NCBI nucleotide (NT) and nonredundant protein (NR) databases using the Minimap and DIAMOND programs (Bolger et al., 2014; Buchfink et al., 2015; Li, 2018; Ruby et al., 2013). Significant microbes were identified on the basis of unique reads per million (Rrpm) mapping to specific species. Rrpm measures the relative abundance of nucleic acids associated with a taxon in the sample. The %ID represents the average percent identity of reads and contigs aligned to taxa in the NCBI NT/NR database, with high %ID indicating greater confidence in taxonomic assignment. High-confidence alignments to taxa were expected to have percent identity matches >90 % to reference sequences. Threshold filters were applied: NT rpm > 100, NR rpm > 50, % ID >90, and average NT alignment >250 bp. A taxon heatmap was generated to display 20 *Vibrio* species for each sample with the highest number of reads per million. In the second part of pipeline 2, the CZ ID mNGS Illumina pipeline used SPAdes for contig assembly. The quality-filtered reads were mapped back to the assembled contigs using Bowtie2 (Langmead and Salzberg, 2012), and the contigs were then blasted against the candidate references from the NT to reassign the corresponding reads to the matched taxa. The assemblies for IDs 666 (*V. cholerae*), 670 (*V. parahaemolyticus*), and 672 (*V. vulnificus*) were downloaded.

Correlation analysis of the *V. parahaemolyticus* and *V. vulnificus* copy numbers and MG reads assigned to these species via qPCR was performed using the Spearman method with RStudio software.

The sequences of primers used in the qPCR were searched in the MAG files obtained with the CZ ID using the grep function (including the reverse complement sequences) in the assembly FASTA files created from the multifasta files using the awk function.

3. Results

3.1. Microbiological counts

In the HO samples, the marine bacteria and *Vibrio* loads were evaluated on MA and TCBS media, respectively, both of which were incubated at 22 °C. In addition, TCBS medium incubated at 37 °C was evaluated for its ability to promote the isolation of *Vibrio* human pathogens. The microbiological counts were analyzed according to the clams' origin site and season of collection. During the refrigeration step, pre-depuration homogenate samples were stored at 4 °C for 24 h. Only the microbiological count results of the post-depuration homogenate clam samples were considered and analyzed. The microbial counts are reported in Table 2S and Fig. 2.

In post-depuration samples, the NPC test revealed significant differences considering both factors: the clams' origin site (p-value = 0.013) and the season of clam sample collection (p-value = 0.0001).

In addition, the clam samples collected in the summer season presented significantly different marine bacterial loads than those collected in the winter season did (p value = 0.02). Specifically, in the summer post-depuration clam samples, the marine bacterial load reached a relatively high value of 5.1 $\log_{10}$ CFU $g^{-1} \pm 0.8$. A statistically significant difference in the *Vibrio* load (p value: 0.002) was observed in terms of the clams' origin site. The PM, CO and GO post-depuration clam samples presented higher *Vibrio* concentrations of 4.4 $\log_{10}$ CFU $g^{-1} \pm$ 0.7, 4.5 $\log_{10}$ CFU $g^{-1} \pm 0.4$ and 4.2 $\log_{10}$ CFU $g^{-1} \pm 0.6$, respectively, in the TCBS media incubated at 22 °C (Table 2S). Furthermore, the analysis of the clams' origin site stratified by season indicated a greater marine bacterial load in samples collected during the summer season at sites 1CH (p value = 0.008), 2CH (p value = 0.02), and SC (p value = 0.03) than in those collected during the winter season (see Fig. 2a). Additionally, as shown in Fig. 2b, among the post-depuration clam samples collected at the 2CH site during the summer season, a higher *Vibrio* concentration was observed than that collected during the winter season (p value = 0.04). No significant differences were observed for the other sites. The *Vibrio* concentration evaluated on TCBS medium incubated at 37 °C revealed no growth from the post-depuration homogenate clam samples collected during the winter season.

3.2. recA–pyrH amplicon-based Vibrio communities of homogenate clam samples

The *Vibrio* community composition of the 120 clam HO samples was studied via *Vibrio*-specific metabarcoding (based on the *rec*A and *pyr*H genes) developed in a previous study (Zampieri et al., 2020).

The complete list of the taxa identified in the clam samples is included in Table 5S (MB Taxa). The list is composed of 49 taxa, of which 28 belong to the *Vibrio genus,* the other three *Vibrionaceae* belong to the *Photobacterium genus,* and the rest are composed of taxa belonging to the order *Alteromonadales*.

The *Vibrio* community composition was investigated according to the clams' origin site, the season of collection, and the depuration effect. PERMANOVA three-way analysis of the *rec*A–*pyr*H metabarcoding data, according to the Jaccard index, revealed that the *Vibrio* community composition of the homogenate clam samples was significantly affected by the clams' origin site (p value = 0.035) and the season of collection (p value =0.00005). In contrast, depuration did not affect the microbiota composition. The interactions among the three factors (site, season, and depuration) were not statistically significant (p value = 0.135). The NPC test was then used to investigate which species were differently distributed between seasons and sites on the basis of the percentage of detection (presence/absence) of the *recA-pyrH* data. The statistical comparison between seasons revealed 14 *Vibrio* species and 3 *Vibrionaceae* that differed significantly between the samples collected during the summer and winter (Table 3S). Most of the taxa that were overrepresented in the summer samples included the human pathogens *Vibrio parahaemolyticus* and *Vibrio vulnificus*. Only *V. splendidus* and *V. tapetis* were more represented in the winter samples. The statistical comparison of the NPC test results among the sites revealed that only 7 taxa (Table 3S) were distributed differently across the sites. While none of these species are human pathogens, some have been reported as

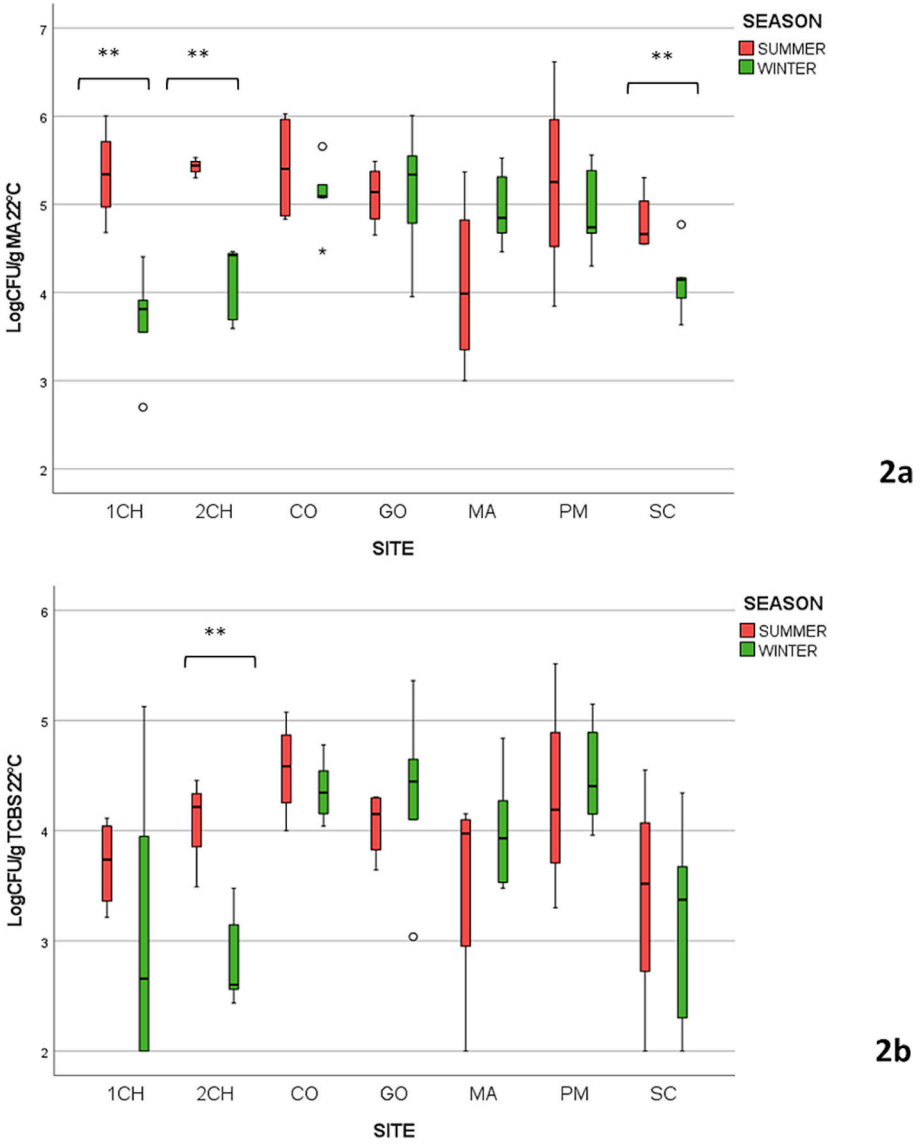

Fig. 2. Total microbial counts and Vibrio spp. counts of the post-depuration clam samples collected in the seven clam farming areas performed on thiosulfate-citrate-bile-salts-sucrose agar (TCBS) (2a) and Marine Agar (MA) (2b) media according to the season of collection. The red and green boxplots correspond to descriptive statistical analysis of the clam samples collected during the summer and winter season, respectively. Thicker black lines in the boxes correspond to the medians. *P*-values of NPC test comparison: ** $P \leq 0.01$. Sites: 1CH, 1Chioggia; 2CH, 2Chioggia; CO,Colmata; GO, Goro; MA,Marano; PM,Porto Marghera; SC, Scardovari. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

pathogens for aquatic organisms (*Photobacterium damselae, Alivibrio wodanis*). In general, all these taxa are common inhabitants of marine and estuarine sediments and waters. Fig. 3 shows the percentages of *Vibrio* human pathogen species (*V. cholerae, V. parahaemolyticus* and *V. vulnificus*) detected in the homogenate clam samples. Specifically, *V. parahaemolyticus* was detected in 82 % and 30 % of the summer and winter samples, respectively (Fig. 3). *V. vulnificus* was present in 92 % of the summer samples and 61.4 % of the winter samples. *Vibrio cholerae* was present in 72 % and 70 % of the summer and winter samples, respectively.

3.3. *V. cholerae, V. parahaemolyticus and V. vulnificus qPCR detection*

The human pathogenic *Vibrio* species were identified via *rec*A–*pyr*H metabarcoding analysis, mostly in the summer samples (Fig. 3). To confirm the presence and quantify the amount of the pathogens (*V. cholerae, V. parahaemolyticus* and *V. vulnificus)* in the clam microbiota, qPCR assays were used to assess the clam HO samples (36, Table 1) and ES_MA, ES_TCBS22 and ES_TCBS37 (54, 52 and 21, respectively, Table 1) via species-specific assays and a *Vibrio* spp. assay. The *Vibrio* spp. assay consistently provided amplification in all the HO samples, with an increase in the absolute amount of *Vibrio* spp. from HO < MA < TCBS22 < TCBS37 (Fig. 4A). The tests specifically targeting the pathogens returned negative results for HO, ES_MA and ES_TCBS22 despite the samples being tested with various amounts of DNA and different dilutions. Successful amplification was achieved from only the ES_TCBS37 samples (with a dilution factor of 10) for *V. parahaemolyticus* (19 samples positive and quantifiable, 1 positive but not quantifiable of a total of 21 samples) and *V. vulnificus* (6 samples positive, 4 positive but not quantifiable of 21), as depicted in Fig. 4C. *V. parahaemolyticus* was present in similar amounts at most of the sites, excluding MA, which was significantly different from PM and GO, as illustrated in Fig. 4B. Conversely, no significant differences in *V. parahaemolyticus* were detected between the pre- and post-depuration samples (data not shown). Despite testing with different primer pairs, the enriched samples consistently showed negative amplification in the presence of

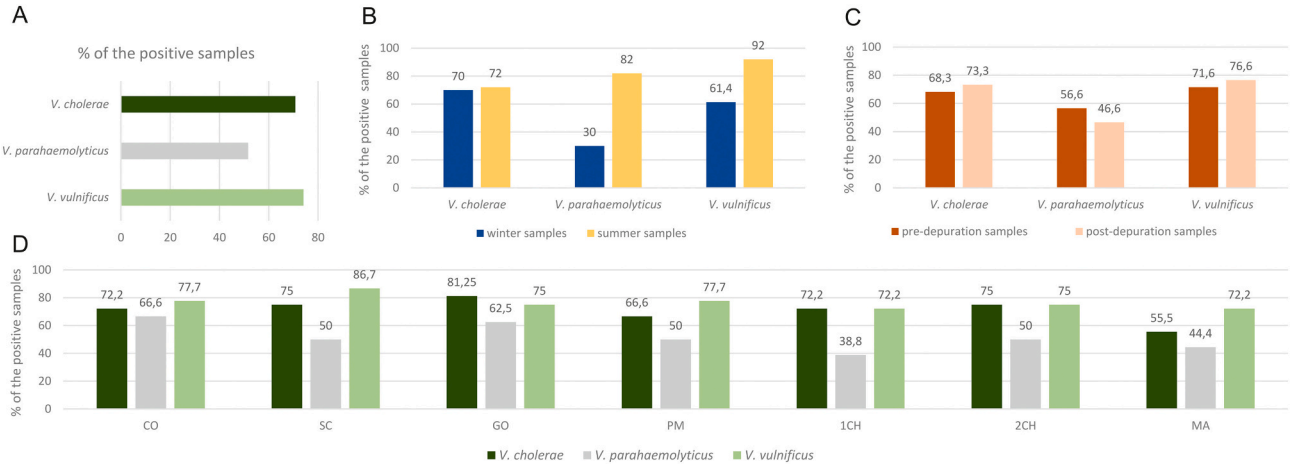

Fig. 3. Histograms showing the percentage of the positive samples for each of the human pathogenic species in clam samples according to *recA–pyrH* metabarcoding. (A) Percentage of positive samples for *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*. (B) Percentage of summer and winter samples positive for *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*. (C) Percentage of pre- and post-depuration samples positive for *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*. (D) Percentage of Chioggia (two sites, 1CH and 2CH), Porto Marghera (PM), Scardovari (SC), Goro (GO) Marano (MA), and Colmata (CO) samples positive for *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*.

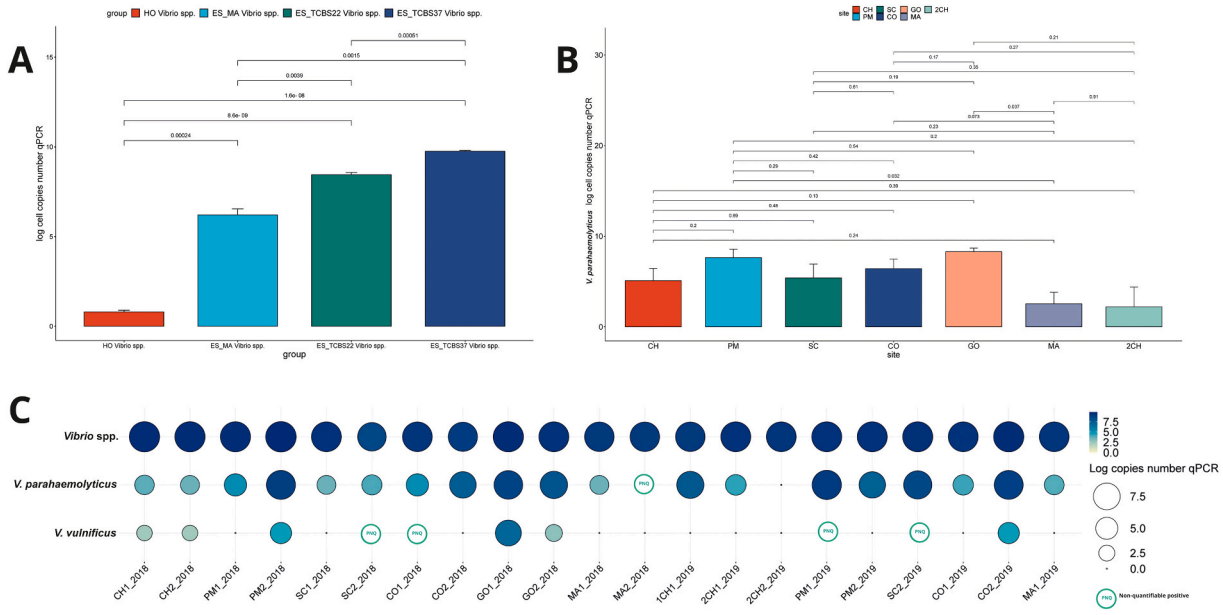

Fig. 4. qPCR data visualization. (A) Absolute amount of *Vibrio* spp. in homogenized samples (HO), marine agar (MA), and on thiosulfate-citrate-bile-salts-sucrose agar (TCBS) incubated at 22 °C and 37 °C with the Y-axis representing the log copies number of cells. (B) Absolute amount of *V. parahaemolyticus* in Chioggia (two sites, 1CH and 2CH), Porto Marghera (PM), Scardovari (SC), Goro (GO) Marano (MA), and Colmata (CO) with the Y-axis representing the log copies number of cells. (C) Bubble chart showing the samples that resulted quantifiable, positive but not quantifiable, and negative using *V. parahaemolyticus*, *V. vulnificus*, and *Vibrio* spp. primers in qPCR.

V. cholerae; therefore, the qPCR results only partially confirmed the results obtained by *recA–pyrH* metabarcoding. The qPCR data obtained for *V. parahaemolyticus* and *V. vulnificus* are listed in Table 7S. The comparison of the *Vibrio* spp. qPCR quantifications with microbiological counts (TCBS37 plates) demonstrated a moderate correlation with a Pearson coefficient of 0.626. However, the *p* value of 0.2225 indicated that this correlation was not statistically significant at the 5 % level. The average number of *Vibrio* spp. cells from the TCBS37 plate was 3.6 ± 0.8, while the average *Vibrio* spp. value from the qPCR was log 9.4 ± 0.3. In contrast, Spearman correlation analysis between MB read numbers and qPCR copy numbers of *V. parahaemolyticus* revealed a strongly positive correlation (rho = 0.799, *p* value = 1.39e-05). No correlation was detected for *V. vulnificus* (rho = 0.408, *p* value = 0.065).

3.4. Shotgun metagenomics of the TCBS37 samples

A subset of 8 TCBS37 samples was investigated using a shotgun metagenomic approach to confirm the presence of pathogenic *Vibrio* species and to verify the results of recA-pyrH metabarcoding and qPCR. For this analysis, summer samples pre- and post-depuration from Chioggia (farming area) and Porto Marghera (polluted area) were included (Table 1). This approach allowed for the comparison of the two developed pipelines for data processing to define species attribution and the feasibility of metagenome-assembled genomes for the *Vibrio* pathogens detected in this study.

A total of 58 species (pipeline 1) or 50 species (pipeline 2) were identified. As expected, most of the species belonged to the genus *Vibrio*

(91 %, pipeline 1; 98 %, pipeline 2). Alpha diversity analysis (using Shannon, observed species, and Chao1 metrics) and beta diversity analysis (using PERMANOVA) comparing the depurated and non-depurated clam samples as well as the Porto Marghera and Chioggia sites did not reveal significant differences.

Fig. 5 shows the heatmap obtained with pipeline 2. The most identified species included *V. diabolicus*, *V. alginolyticus*, *V. antiquarium*, *V. parahaemolyticus*, *V. harveyi*, *V. owensii*, *V. campbellii*, and *V. rotiferianus*. A similar result was obtained with pipeline 1: the same species were identified as the most abundant, except for *V. tubiashii*, which was present at high levels in pipeline 1 but at lower levels in pipeline 2, and *V. antiquarium* was more prominent in pipeline 2 than in pipeline 1. The heatmap obtained with pipeline 1 is shown in Fig. 2S.

Among the three pathogens we focused on in this study, *V. parahaemolyticus* was the most abundant and was present in all 8 samples. *V. vulnificus* was present in 7 samples (with both pipelines), whereas *V. cholerae* was present in all samples with pipeline 1 and in 7 samples with pipeline 2.

Spearman correlation analysis between MG read numbers and qPCR copy numbers revealed a strongly positive correlation for

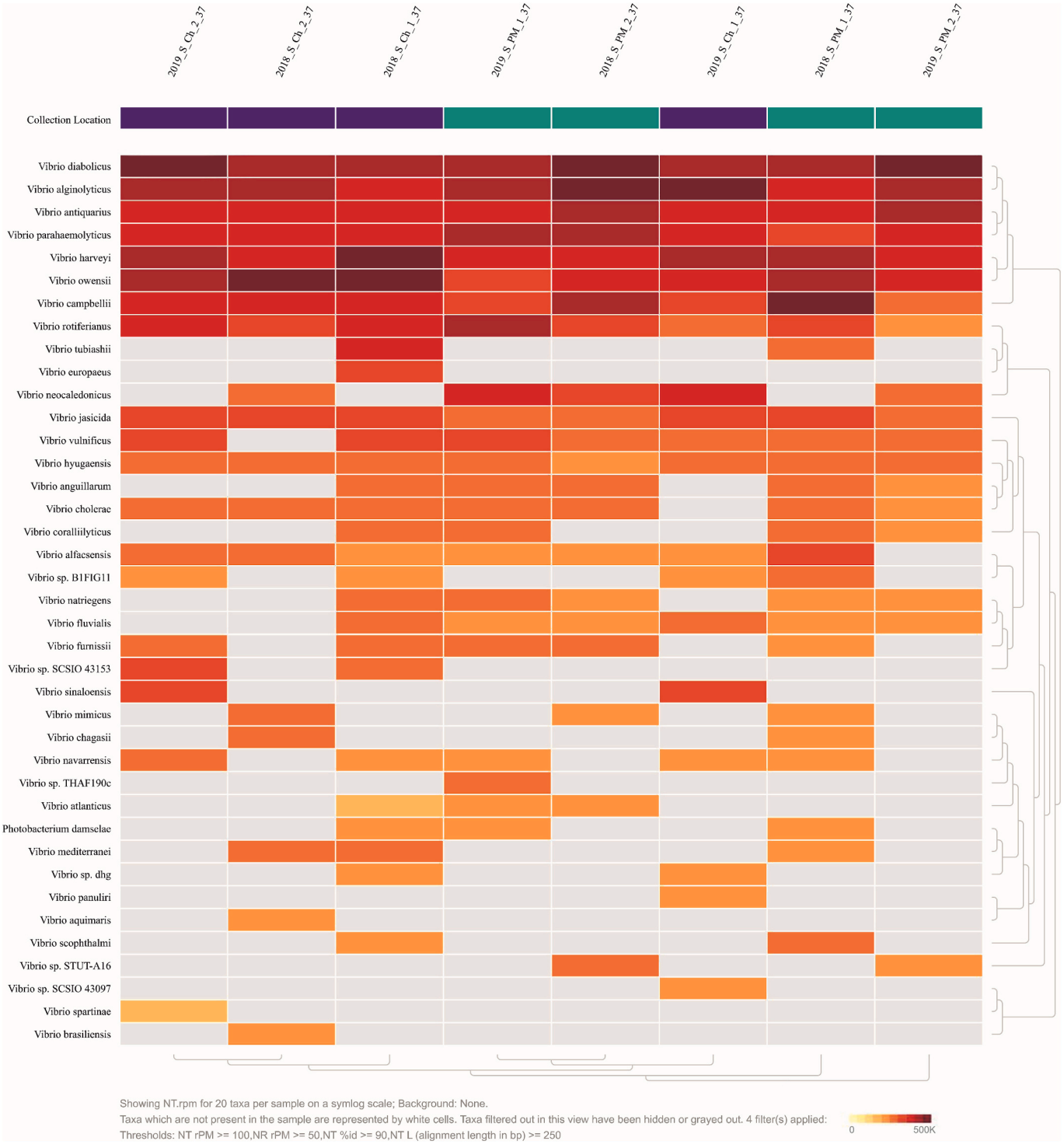

Fig. 5. *Vibrio* species heatmap displaying 20 taxa for each sample with the highest reads per million, created by using the CZ ID mNGS Illumina pipeline (Pipeline 2).

V. parahaemolyticus (rho = 0.893, *p* value = 0.01). For *V. vulnificus,* no correlation was detected. In light of the negative result of the qPCR amplification analysis and to corroborate the species identification, the sequences identified as *Vibrio cholerae* by Kraken2 with both the MB and MG approaches were extracted from the cseqs metabarcoding and metagenomic data and cross-checked using BLAST on NCBI.

With pipeline 1, only 5 bins were assigned to *V. parahaemolyticus* after the SGB assignment (bin2_PM2_2018, bin3_PM1_2019, bin6_PM1_2019, bin7_PM1_2019, bin11_PM1_2019). No bins were assigned to *V. cholerae* or *V. vulnificus*. With pipeline 2, 8 assembly files were generated for *V. parahaemolyticus*, with 7 each for *V. cholerae* and *V. vulnificus*. The downloaded assemblies assigned to *V. cholerae* for the 8 samples had an average length of 59,365 bp ± 24,760. For *V. parahaemolyticus*, the average length was 2,148,032 bp ± 1,739,202. For *V. vulnificus*, the average length was 170,101 bp ± 59,090. The sequence primers used in the qPCR were searched in the assembly FASTA files, and only the primers for *V. parahaemolyticus* were found in the assembly files of 3 samples (PM1_2019, PM2_2018, and PM1_2018). With respect to the assembly process for identifying metagenome-assembled genomes (MAGs), pipeline 1 proved to be more stringent, identifying MAGs for *V. parahaemolyticus* in only 2 samples. Specifically, MAGs were assigned to specific pathogenic kSGBs by PhyloPhlAn only in PM1_2019 and PM2_2018. In these samples, a high quantity of *V. parahaemolyticus* was also detected via qPCR, with the highest quantification levels among the samples (log cell/ml of 8,9 and 9,1, respectively).

Among the 5 MAGs assigned to *V. parahaemolyticus* by PhyloPhlAn, which were also confirmed by CZ ID, all had the appropriate length to be used for constructing a phylogenetic tree with PhyloPhlAn (Fig. 6).

Our 5 MAGs in the phylogenetic tree clustered with a group of 7 other genomes, including 3 from Malaysia, 1 from Mozambique, 1 from Canada, and 2 from the USA. Of the latter, 1 is isolated from feces, and

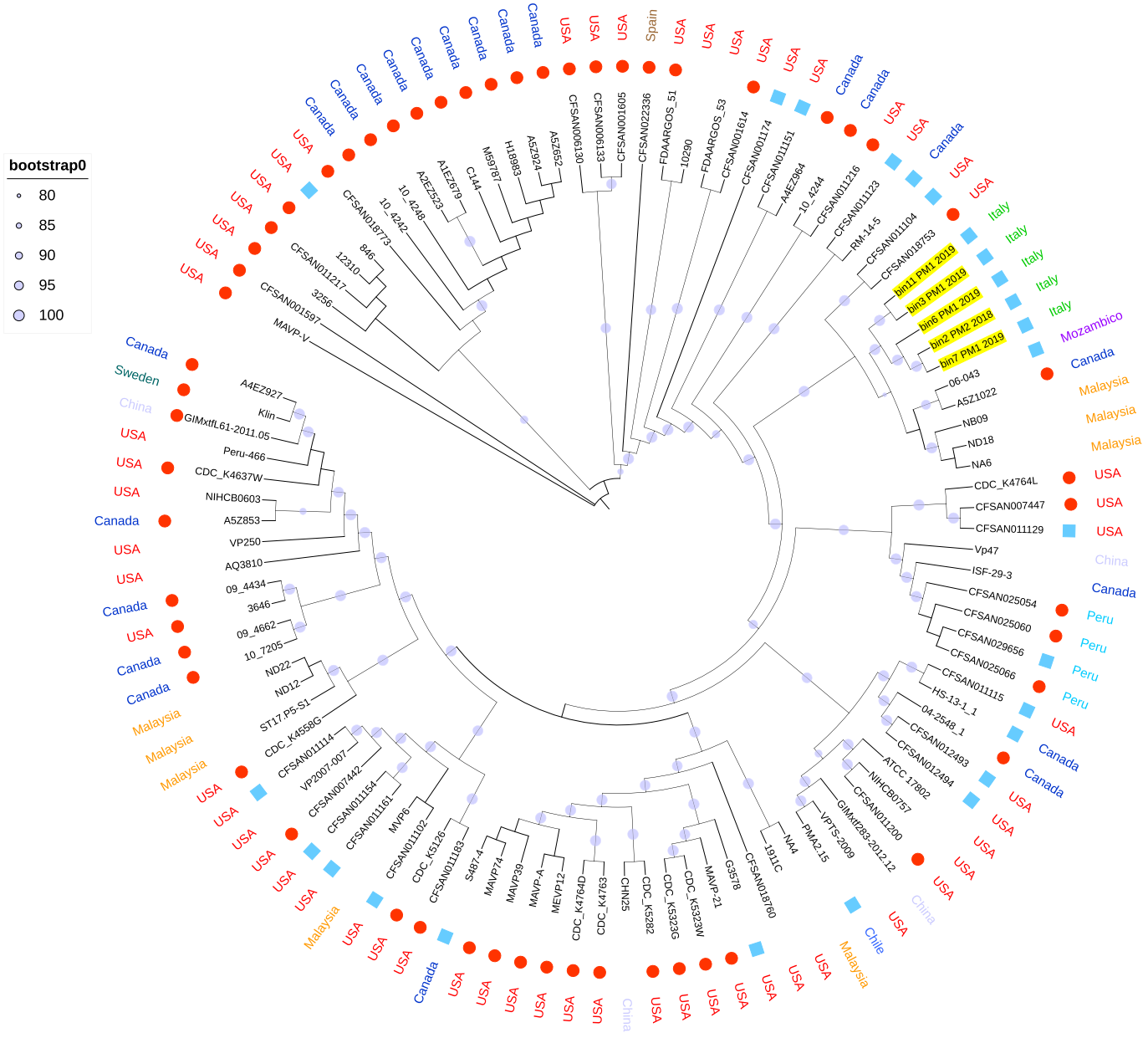

Fig. 6. Maximum likelihood phylogenetic tree generated using IQ-TREE2. The tree was constructed using the 5 bins assigned to *V. parahaemolyticus* by PhyloPhlAn and 100 *V. parahaemolyticus* genomes downloaded from NCBI. Red circles represent clinical samples, while blue squares represent samples derived from mollusks. iTOL was used solely for graphical visualization. Table 10S_Genomes contains all accession numbers and additional metadata for the public genomes used to construct the phylogenetic tree. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the other is from a reported case of gastroenteritis.

4. Discussion

In light of the worldwide distribution of *Vibrio* spp. and the significant human health risk posed by *Vibrio* human pathogens detected in several seafood products, clam microbiota collected in operating shellfish farming areas and polluted areas along the northeastern Adriatic coast were analyzed to investigate the incidence of *Vibrio* human pathogens. The clam microbiota was analyzed using both culture-dependent and culture-independent methods, and the effects of depuration, the clams' origin site, and the season of collection were evaluated. The determination of the microbial content of post-depuration clam samples provides crucial insights into the safety of depurated shellfish products intended for human consumption in accordance with Commission Implementing Regulation (EU) 2019/627 (2019).

The presence of the main *Vibrio* human pathogen species *V. cholerae, V. vulnificus* and *V. parahaemolyticus* was demonstrated by *rec*A-*pyr*H metabarcoding and shotgun metagenomics. The application of a *Vibrio*-specific metabarcoding and shot-gun metagenomics allowed a comprehensive description of the microbiota at the species level, information not available from the most common 16S amplicon sequencing. The metagenomic data were analyzed using two different bioinformatics pipelines to ensure the reliability and robustness of the results. While both pipelines confirmed the presence of the pathogens, only a few differences were found in the identified species (Tables 8S and 9S). These differences may be attributed to variations in the sensitivity and specificity of the detection methods used by each pipeline.

qPCR analysis of HO and ES samples (MA, TCBS22, TCBS37) showed increasing *Vibrio* quantities from homogenate to MA and TCBS plates, as expected due to medium specificity. A moderate correlation was observed between TCBS37 plate counts and qPCR quantifications, with qPCR detecting higher *Vibrio* levels. This discrepancy may result from limitations in common media, leading to underestimation in culture-based approaches. Quantification using qPCR may differ from plate counting for various reasons, including differences in colony size and growth conditions. Larger colonies may contain more cells, leading to higher DNA detection by qPCR. Additionally, incubation conditions may favor the growth of certain colonies, affecting the amount of DNA available for amplification. The TCBS-selective *Vibrio* medium incubated at 37 °C promoted the growth of potential human pathogenic *Vibrio* species, which explains the detection of *V. parahaemolyticus* and *V. vulnificus* by species-specific qPCR assays only in the TCBS37. This result confirmed the presence of these two species in the clams' microbiota, albeit in varying amounts. However, it is important to note that qPCR quantification may not accurately reflect the pathogen load in the clam samples. Pathogens constitute a small fraction of the microbiota and may not be directly amplifiable, falling below the method's limit of detection (Kralik and Ricchi, 2017). The limit of detection of qPCR is significantly reduced when the target is part of a highly complex and diverse community (Saingam et al., 2018). Therefore, it cannot be ruled out that the pathogens may be present in quantities surpassing the infection threshold in cases where clams are not thoroughly cooked.

The negative qPCR results for *V. cholerae*, despite its presence in metabarcoding and metagenomics data, could be attributed to the absence of its DNA fragments rather than an amplification limitation. Three distinct species-specific assays were used, all of which have been validated in prior studies (Campbell and Wright, 2003; Diner et al., 2021; Nasreen et al., 2022; Vezzulli et al., 2015). The reduced growth of this pathogen in TCBS37, possibly due to competition from other *Vibrio* species, could result in too little DNA in the TCBS37 samples to be amplified by qPCR.

The sequences of the primers used in the qPCR were searched in the assembly files, but they were not found at all for *V. cholerae* and *V. vulnificus*. Importantly, the lengths of the obtained bins were very short. This suggests that fragments of DNA specific to *V. cholerae* and *V. vulnificus* may be present in samples too short or degraded, which could also explain the lack of detection via qPCR for *V. cholerae*. However, during taxonomic assignment, the short reads were mapped to the *V. cholerae* genome in both metabarcoding and metagenomic analyses. For *V. parahaemolyticus*, the sequences of the qPCR primers were found in only 3 assembly files, with the 3 samples with better-resolved MAGs showing the highest quantities of *V. parahaemolyticus* in the qPCR analysis.

A phylogenetic tree was constructed using the 5 bins assigned to *V. parahaemolyticus* and 100 genomes of this species downloaded from NCBI. The clustering of samples PM1_2019 and PM2_2018 in the phylogenetic tree with clinical cases and tropical samples from Malaysia and Mozambique underscores significant implications for our study. The presence of foreign genetic variants, correlated with an increase in warming conditions, was identified as a cause of emerging *V. parahaemolyticus* outbreaks linked to shellfish consumption (Martinez-Urtaza et al., 2018). As climate change alters environmental conditions, tropical pathogens may spread to new regions, increasing global health risks (Harrison et al., 2022; Sheahan et al., 2022; Vezzulli, 2023; Baker-Austin et al., 2024). The presence of these pathogens in clinical cases highlights the potential for increased the number of outbreaks (Martinez-Urtaza et al., 2018) and the need for ongoing surveillance to address the impacts of climate change on pathogen distribution and virulence. Furthermore, the metagenomic approach, combined with genome analysis, can help identify the spread and introduction of these new variants (Martinez-Urtaza et al., 2018).

Metabarcoding data demonstrated that the season of collection significantly affects the distribution of pathogenic Vibrios. The difference in the *Vibrio* community composition related to the season factor is in line with several studies in which researchers reported different *Vibrio* biodiversities present in the free-living state or associated with seafood products according to the season of collection (Zarei et al., 2012; Chen et al., 2020; Suzzi et al., 2023; Zhao et al., 2023). Moreover, 14 *Vibrio* and 3 *Vibrionaceae* species were detected at different percentages in samples collected during the summer and winter (Table 3S).

The different composition of the *Vibrio* community in the summer and winter clam microbiotas could explain the lack of difference in the microbial load (TCBS) between most summer and winter clam samples (except for the 2CH2 samples, Fig. 2B). Different species can dominate the final microbiota in winter compared with summer while maintaining a similar overall load of *Vibrio* spp. (log CFU/g) in HO samples at 22 °C.

The detection of *V. parahaemolyticus* and *V. vulnificus* in more than half of the homogenate clam samples collected during the summer compared with those collected during the winter confirms the temperature-dependent nature of these two species: outbreaks tend to occur in warmer months (Baker-Austin et al., 2010; Horseman and Surani, 2011). The high percentage of samples positive for *V. vulnificus* (92 %) in summer is in line with other studies based on culture dependent method, in which this pathogen was found only in seafood products collected during the hot season (Huehn et al., 2014; Strom and Paranjpye, 2000). The high percentage of HO samples collected during the summer that were positive for *Vibrio* human pathogens highlights the high risk of human vibriosis during the warmer season. In the literature, the link between the rise in sea temperature and the incidence of human vibriosis is becoming clearer (Kim and Chun, 2021; Vezzulli et al., 2015). On the basis of these data, the lack of European legislation for the establishment of microbiological safety criteria and the notification of certain diseases associated with *Vibrio* require urgent revision. In the phylogenetic tree of *V. parahaemolyticus* strains, many strains that were included were derived from those tracked by the FDA's *Vibrio parahaemolyticus* Tracker Program, which plays a crucial role in monitoring and sequencing these pathogens. This program utilizes advanced genomic technologies, such as whole-genome sequencing, to analyze and characterize *V. parahaemolyticus* strains from both food and environmental samples. By identifying genetic markers associated with pathogenicity and tracking strain diversity, the program significantly

enhances outbreak investigations and informs public health responses. Aligning European safety standards with established monitoring programs such as the FDA's Tracker Program is essential for more effectively managing and mitigating risks associated with *Vibrio* species, improving seafood safety, and safeguarding public health (Stevens et al., 2022).

Although the sites where clams were collected showed variation in *Vibrio* load, the differences in Vibrio biodiversity were only marginally significant. The study included different sites, such as Lagoons (Venice and Marano) and the Po River Delta (Goro, Scardovari). Some of these sites were farming areas (Chioggia, Scardovari), whereas others were polluted sites where clam collection is prohibited (Colmata, Porto Marghera). Previous studies reported marked differences in the total microbiota of clams in polluted areas of Venice Lagoon compared with those in the farming site (Milan et al., 2018), whereas in this study, despite the differences in the environment, the *Vibrio* community remained fairly consistent, with variation in concentration. The higher *Vibrio* loads exhibited by the PM and CO clams (Fig. 2b) could be explained by their geographical proximity, shared depuration plants and higher temperatures in these areas. The summer-harvested clams from 1CH and 2CH presented significantly greater marine bacterial loads and *Vibrio* loads (only 2CH, Fig. 2a and Fig. 2b). The greater proximity of the 1CH and 2CH sites to the Chioggia inlet, a major gateway to Venice Lagoon with substantial maritime traffic, likely contributed to the elevated marine bacteria concentrations. The movement of ships facilitates the introduction of various marine bacteria through ballast water discharge, a potential source of microbial spread (Hess-Erga et al., 2019; Fykse et al., 2012; Soleimani et al., 2021). In addition, this area experiences significant hydrodynamics, leading to sediment resuspension. As sediments serve as potential reservoirs for *Vibrio* species, their resuspension can directly impact *Vibrio* spread (Takemura et al., 2014; Vezzulli et al., 2009).

The depuration treatment did not affect *Vibrio* biodiversity and had no effect on the percentage of samples positive for *Vibrio* human pathogens. Several studies have highlighted the lack of efficiency of depuration treatment in removing *Vibrio* spp. from shellfish products (Sferlazzo et al., 2018; Vezzulli et al., 2018; Zampieri et al., 2020). Nevertheless, the persistence of *Vibrio* human pathogens after depuration treatment could be related to the formation of biofilms associated with the surface of the depuration facilities. Several *Vibrio* species, including the human pathogens *V. cholerae* and *V. parahaemolyticus*, form biofilm (Han et al., 2016; Silva and Benitez, 2016). Researchers have found *Vibrio* spp. associated with compartments such as tanks and biofilters of the recirculation aquaculture system (RAS) used in aquaculture farming (Martins et al., 2013; Schreier et al., 2010). Considering that the shellfish depuration plants are equipped with a recirculation system of seawater and biological filters (Table 1S), *Vibrio* species associated with these components may be similar to the contamination found in the facilities used to farm freshwater and marine fish. Further work is necessary to explore these facilities in the future to better understand the potential for *Vibrio* contamination in these environments.

In conclusion, the results of the present study show that the human pathogenic *Vibrio* species are widespread in clams on the northern Adriatic coast of Italy, in both the lagoon and delta of the Po, especially in the summer months. The analysis of the shotgun metagenomics data revealed that *V. parahaemolyticus* strains are very similar to those isolated in different parts of the world, both tropical and temperate, highlighting the spread of potentially pathogenic strains. Like other temperate regions, the northern Adriatic coasts already present environmental conditions favorable to *V. cholerae* and *V. vulnificus* strains. In the future, such conditions could become even more favorable due to climate change for the spread of these pathogens, including more pathogenic and pandemic strains. These data demonstrate the necessity of monitoring the environment and controlling seafood products to manage risks and ensure food safety and demonstrate that shotgun metagenomics could provide comprehensive description of the microbiota at the species level favoring the identification and tracking of the pathogens.

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CRediT authorship contribution statement

Angela Zampieri: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Lisa Carraro:** Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Formal analysis, Data curation. **Hooriyeh Mohammadpour:** Writing – review & editing, Formal analysis, Data curation. **Giulia Dalla Rovere:** Writing – review & editing, Formal analysis, Data curation. **Massimo Milan:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Luca Fasolato:** Writing – review & editing, Writing – original draft, Software, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Barbara Cardazzo:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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Data availability

Data will be made available on request.

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