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New methodologies to investigate factors affecting the *Vibrio* species community composition on *Ruditapes philippinarum* microbiota and their application on food safety in shellfish production

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ABSTRACT

As filter-feeders, bivalve molluscs accumulate several *Vibrio* species in their edible tissues. Among these species, *Vibrio alginolyticus*, *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* represent a risk to human health. Given the worldwide human consumption of bivalve molluscs, an accurate and reliable characterisation of the *Vibrio* community associated with these seafood products represents a key issue to ensure food safety in shellfish production. The goal of this study was to provide new insights about the *Vibrio* species biodiversity, with a particular concern to the detection of the main *Vibrio* human pathogens, associated to a shellfish product of high commercial interest, namely the Manila clam (*Ruditapes philippinarum*). To achieve this goal, a new metabarcoding approach and a culture-dependent metagenomics were developed to characterise the *Vibrio* biodiversity down to the species level, with a specific focus on detecting the main *Vibrio* human pathogens. First, the *rec*A–*pyr*H metabarcoding let possible to study changes in *Vibrio* biodiversity in the *R. philippinarum* microbiota in response to depuration. In particular, *Vibrio* spp. and human pathogens were found in the depurated clams. Moreover, *rec*A–*pyr*H metabarcoding allowed the evaluation of culture-dependent and culture-independent methods to characterise the *Vibrio* community composition, with a focus on *Vibrio* human pathogens. The application of *recA*–*pyr*H metabarcoding directly to homogenate clam samples as well as plating samples on Marine Agar medium could be a useful screening approach to detect *Vibrio* human pathogens in seafood products. Moreover, the developed culture-dependent metagenomics resulted a valid approach for an accurate characterisation of the living fraction of the *Vibrio* community with a higher sensitivity respect to the *rec*A-*pyr*H metabarcoding.

The characterisation of the *Vibrio* community composition of all samples collected during the 3-year PhD project revealed a high frequency of detection of *Vibrio* pathogens in clams collected during the summer. *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* were found in homogenate clam samples from seven sampled shellfish farming areas operating along the North-east Adriatic coast. Taken together, the results highlight the importance to include the detection of *Vibrio* species in the official microbiological seafood control measures. This endeavour could be doubly useful: it would allow the acquisition of *Vibrio* surveillance data – which is necessary to maintain updated knowledge about the real *Vibrio* risk associated with seafood consumption – and it would contribute to prevent the spread of the human vibriosis.

1. GENERAL INTRODUCTION

1.1 A group of marine heterotrophic bacteria: the *Vibrio* genus

The *Vibrio* genus belongs to the *Vibrionaceae* family, a group of *Gammaproteobacteria* currently represented by 147 *Vibrio* species and 4 subspecies throughout the world spread in brackish and marine ecosystems (Sampaio *et al.*, 2022). Vibrios are gram-negative, comma-shaped bacteria 0.5–0.8 μm in width and 2–3 μm in length.

The etymology of *Vibrio*, recalls the vibration with tremulous motion performed by the bacterial cells during their motion and was given to these bacteria in 1854 by Pacini (Sampaio *et al.*, 2022). These marine bacteria in fact show active motility in aquatic environments thanks to the presence of 1–3 polar flagella. Given this active motility, some *Vibrio* spp. perform chemotaxis along a nutrient gradient to find suitable colonisation sites (Stocker *et al.*, 2012).

Among marine bacterial biodiversity, the *Vibrio* genus represents one of the most culturable fractions of the aquatic microbial community (Destoumieux-Garzón *et al.*, 2020). These marine bacteria present chemoorganotrophic and mesophilic metabolism, which requires an organic substrate and the presence of sodium for growth on culture media (Thompson *et al.*, 2005). Given their obligate heterotrophic growth, *Vibrio* spp. play an active role in carbon, phosphorus and nitrogen cycling through the uptake and degradation of organic matter (Jesser *et al.*, 2018; Zhang *et al.*, 2018).

Moreover, among marine bacteria, vibrios present great genome plasticity, which allows them to adapt to new ecological niches such as the ones defined by the constantly changing marine environmental conditions. In marine ecosystems, *Vibrio* spp. encounters with other bacteria could require coexistence in the same ecological niche and, consequently, coordination of biological activities. This coexistence is possible because of the quorum sensing mechanism, by which vibrios respond to variable aquatic conditions and maintain associations with animal hosts (Milton, 2006). This signalling mechanism is adopted in many situations: to regulate the cascade of virulence gene expression in species such as *Vibrio cholerae* and *Vibrio anguillarum* and to commence the chemiluminescence process in species such as *Vibrio fischeri* and *Vibrio harveyi* (Milton, 2006).

Vibrios also present great flexibility in lifestyle. Marine bacteria can be found in a free-living state or attached to biotic and abiotic surfaces. Specifically, the attachment to different substrates represents an important step for the growth strategy of marine bacteria. By

adhering to the body surface of animals and marine plants, such as crustaceans and algae, vibrios can metabolise biopolymers such as chitin and algal polysaccharides (Takemura *et al.*, 2014). Moreover, attachment to substrates offers the possibility to avoid predation by protozoans, small heterotrophic flagellates present in the water column (Matz *et al.*, 2005). Among the substrates colonised by *Vibrio* spp. are marine plastic debris (Laverty *et al.*, 2020). Specifically, the biofilm-forming community that attaches to plastic substrates includes the main potential human pathogens *V. cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* (Silva *et al.*, 2019). Consequently, given the wide distribution of the plastic debris across marine and estuarine ecosystems, this human-generated artificial substrate might represent an efficient 'driver' for the spread of potential virulent *Vibrio* spp. all over the world.

While vibrios exploit biofilm formation to avoid their planktonic predators, the viable but not-culturable (VBNC) state represents a persistence and survival strategy of these marine bacteria to face changes in marine aquatic environments. Specifically, the VBNC condition offers the possibility to several *Vibrio* spp., including the main human pathogens, to overcome adverse environmental conditions such as low nutrient availability, reduced temperature, high salinity or excessive radiation. During the VBNC state, *Vibrio* spp. reduce their metabolic rate and lose the ability to grow on culture media but remain viable (Orruño *et al.*, 2017). Interestingly, VBNC is a reversible state: if environmental conditions return to a suitable level, the bacteria come back to their initial physiological state through the resuscitation process (Zhang *et al.*, 2020).

1.2 Vibrio–bivalve interactions

Vibrio spp. represent a part of marine microbial communities easily associated with the body of several aquatic animals. Indeed, the high colonisation and lifestyle versatility of vibrios allow to them to establish mutualistic, commensal or pathogenic relationships with their hosts (Le Roux *et al.*, 2018). Among the *Vibrio* hosts are bivalve molluscs. The filter-feeding habit of these animals, performed to uptake food particles from the water column, enhances the association of *Vibrio* spp. with their edible tissues. Hence, the concentration of vibrios in both healthy and diseased bivalves could easily be higher with respect to the concentration of vibrios in the water column (Destoumieux-Garzón *et al.*, 2020). In the literature, there are controversial results about the stability or transitory nature of *Vibrio*–bivalve associations. Specifically, some researchers have found a correspondence between the frequency and

diversity of *Vibrio* spp. in the water column and the species detected in the shellfish microbiota (Destoumieux-Garzón *et al.*, 2020). Moreover, Musella *et al.* (2020) described the contribution of shellfish in the maintenance and spread of *Vibrionaceae* in *Mytilus* spp., where these bivalves release bacteria, probably related to the gill microbiota, into the seawater surrounding mussel farms. Other researchers have reported an unequal distribution of vibrios between oyster tissues and the water column (Le Roux *et al.*, 2016; Bruto *et al.*, 2017). In addition, researchers have reported that *Vibrio* biodiversity differs according to which bivalve tissues are colonised (Lokmer *et al.*, 2016; Destoumieux-Garzón *et al.*, 2020). This finding suggests that *Vibrio* biodiversity is unpredictable and depends on the adaptation of these marine bacteria to both environmental and host-body conditions.

Several authors have revealed that *Vibrio* colonisation of the shellfish microbiota leads to the establishment of a complex population structure of these marine bacteria (Romalde *et al.*, 2014; Chen *et al.*, 2016; Bruto *et al.*, 2017). During the colonisation process, the biodiversity of marine bacteria also depends on the shellfish provenance. Species such as *Vibrio splendidus*, *Vibrio alginolyticus* and *Vibrio harveyi*, for example, predominate in samples collected from Canada, Brazil, Spain or Italy (Beaz-Hidalgo *et al.*, 2010).

Vibrio–bivalve associations can also be neutral. In a recent study, Wegner *et al.* (2019) suggested that the natural extended contact of bivalves with these marine bacteria over several generations has led vibrios to be part of the natural oyster microbiota. Vibrios, in fact, are frequently found in the haemolymph of healthy oysters (Lokmer *et al.*, 2016). However, several *Vibrio* species represent a health risk to the growth of bivalves during harvesting management. For this reason, researchers have raised concerns regarding *Vibrio*–bivalve pathogenic interactions (Travers *et al.*, 2015; Dubert *et al.*, 2017). These species are subdivided into primary and opportunistic pathogens. The former cause outbreak of disease(s) that kill healthy bivalves. The latter induce the insurgence of disease(s) when the protective barriers of the host are already compromised by a co-infection or by environmental stressors (Destoumieux-Garzón *et al.*, 2020).

The genetic determinants for bivalve colonisation and disease expression identified in vibrios represent the main factors that determine the establishment and development of *Vibrio*–bivalve pathogenic interactions (Lemire *et al.*, 2015; Bruto *et al.*, 2017; Rubio *et al.*, 2019). In addition, sea-surface warming, caused by climate change, favours the temperature-dependent expression of the virulence factors present in several *Vibrio* species (Destoumieux-Garzón *et*

al., 2020). Moreover, changes in water temperature modify the host physiology – in particular, the immune status – and make bivalves more vulnerable to opportunistic *Vibrio* pathogens (Le Roux *et al.*, 2016). The pathogenic *Vibrio* species can lead to disease during the larval, juvenile and adult stages of bivalves (Beaz-Hidalgo *et al.*, 2010). Specifically, species such as *V. alginolyticus*, *V. anguillarum*, *Vibrio bivalvicida*, *Vibrio coralliilyticus*, *Vibrio ostreicida*, *Vibrio neptunius* and *Vibrio tubiashii*/*Vibrio europaeus* affect oyster spat and/or larval development (Destoumieux-Garzón *et al.*, 2020). Despite the major resistance of mussels to vibriosis, *V. splendidus* strains are the main cause of disease and mortality of adult *Mytilus edulis* (Ben Cheikh *et al.*, 2016). Regarding adult clams and juvenile oysters, well-known *Vibrio*-related infections lead to brown ring disease (BRD) and 'summer mortality"' (Beaz-Hidalgo *et al.*, 2010).

BRD is caused by *Vibrio tapetis*; it affects the juveniles and adults of clam species such as the Manila clam (*Ruditapes philippinarum*) and *Ruditapes decussatus* (Paillard, 2004). The disease's name recalls the brown-coloured conchiolin deposited in the inner shell of the infected clams. In this disease, the host not develops correctly and its reproduction is arrested. In addition, the proliferation of *V. tapetis* in the clam tissues could led to mortality (Rodrigues *et al.*, 2015). Moreover, salinity and temperature of the seawater play a role on the development of BRD, which tends to be more frequent during the spring and winter (Paillard, 2004).

'Summer mortality', which affects the Pacific oyster *Crassostrea gigas*, represents an example of an infection caused by interaction among several opportunistic *Vibrio* species. In this disease, species such as *V. splendidus*, *V. aestuarianus* and *V. harveyi* contribute to the mortality of already debilitated oysters (Lacoste *et al.*, 2001; Garnier *et al.*, 2008; Renault *et al.*, 2009). As suggest by the name of the disease, episodes take place mainly during the summer. In addition, in the summer the higher temperature of the seawater (> 18°C), combined with low dissolved oxygen and toxic substances in the sediment, contribute to the debilitation of the oysters (Beaz-Hidalgo *et al.*, 2010).

1.3 Vibrio and human health hazard

The *Vibrio* genus comprises a dozen species that are pathogenic to humans (Baker-Austin *et al.*, 2018). Among these species, *V. alginolyticus*, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* are responsible for the most serious forms of vibriosis in humans (West, 1989). In

the literature, human vibriosis is subdivided into two main groups: cholera and non-cholera infections (Baker-Austin *et al.*, 2018). The cholera infections are generally caused by the ingestion of contaminated seafood and water. In cholera infections, *V. cholerae* is the main aetiological agent; it causes watery diarrhoea that, if untreated, could lead to death (Morris, 2003). *V. cholerae* is classified by the O group (O1, O139), the biotype (Classical, El Tor) and the serotype (Ogawa, Inaba). In the literature, the El Tor biotype represents the major causative agent of cholera throughout the world (Baker-Austin *et al.*, 2018). The symptoms such as profuse diarrhoea are related to the presence and expression of the cholera toxin (CT) in the *V. cholera* strains. Over the years, non-O1 and non-O139 *V. cholerae* strains have been found; compared with *V. cholerae* O1 and O139, these agents lead to sporadic gastrointestinal infections (Deshayes *et al.*, 2015).

The non-cholera infections are transmitted by species such as *V. parahaemolyticus* and *V. vulnificus*; they cause mild gastroenteritis and primary septicaemia, respectively, as clinical manifestations (Baker-Austin *et al.*, 2018). *V. parahaemolyticus* spreads mainly during the warmer months through the consumption of raw contaminated seafood (Baker-Austin *et al.*, 2017). The virulence of *V. parahaemolyticus* strains is related to the expression of thermostable direct haemolysis (TDH) and TDH-related haemolysin (TRH) toxins. *V. vulnificus* is an opportunistic human pathogen that express the virulence in case of underlying disease such as liver disease, diabetes and malignant tumours. *V. vulnificus* infections in humans mainly originate from the exposure of open wounds to contaminated shellfish products and seawater; the condition quickly degenerates into a deadly septicaemia (Baker-Austin *et al.*, 2018). Around the world, this species is responsible for the majority of seafood-associated human deaths (Heng *et al.*, 2017).

Among the *Vibrio* human pathogens, *V. alginolyticus* is frequently associated with human ear and superficial wound infections related to exposure to contaminated seawater (Baker-Austin *et al.*, 2018). Gastroenteritis is a rare symptom on *V. alginolyticus* infection in humans (Uh *et al.*, 2001). Moreover, this species is pathogenic to several marine animals such as fish, shellfish and echinoids. Specifically, for cultured fish such as *Sparus aurata* and *Dicentrarchus labrax*, this species leads to notable economic losses (Zorrilla *et al.*, 2003).

Beyond the four aforementioned *Vibrio* species, *Vibrio mimicus*, *Vibrio cincinnatiensis*, *Vibrio hollisae*, *Vibrio furnissi*, *Vibrio fluvialis* and *Vibrio metschnikovii* are also associated with

human diseases. Fortunately, human infections related to these species are relatively rare (Baker-Austin *et al.*, 2018).

Seafood products represent the principal vector for the spread of the main *Vibrio* human pathogens (Passalacqua *et al.*, 2016; Song *et al.*, 2020). In particular, the filter feeding habit of bivalve molluscs favours the accumulation of several marine bacteria, including vibrios and allochthonous bacteria derived from faecal contamination of water, into the edible tissues of these animals. Hence, bivalve mollusc farming areas are subjected to microbiological risk control. Precisely, in Europe, European Regulation (UE) 2019/627 classifies bivalve mollusc farming areas as zone A, B or C, according to the *Escherichia coli* concentration reached in the flesh and intra-valvular liquid of the molluscs. Specifically, bivalve molluscs harvested in zone A could be collected for direct human consumption only if they contain < 230 *E. coli* per 100 g of pulp and intra-valvular liquid. Bivalve molluscs harvested in zone B must not exceed 4,600 *E. coli*/100 g of flesh and intra-valvular liquid in 90% of samples. In addition, a depuration treatment of these animals is required before their sale as products for human consumption. Bivalve molluscs collected in zone C must not exceed 46,000 *E. coli* per 100 g of pulp and intra-valvular liquid in 90% of samples. In this case, the animals require a relay in a zone A area for at least 2 months to achieve the microbiological requirements; this action ensures their suitability for human consumption. In addition, these molluscs can only be consumed as cooked seafood products.

The Commission Regulation (EC) No 2073/2005 establishes the microbiological criteria that foodstuffs must meet during the official controls. Specifically for bivalve molluscs, this regulation provides the microbiological criteria concerning the concentrations of *E. coli* and *Salmonella* in shellfish tissues. Nevertheless, the evaluation of the *E. coli* concentration in edible tissues of bivalves does not provide a measure of human protection from vibriosis. In fact, there is a lack of correlation between the concentration of faecal indicators and *Vibrio* spp. inside edible tissues of bivalve molluscs (Oliveira *et al.*, 2011). Consequently, the European legislation of microbiological criteria still lacks criteria regarding punctual monitoring of *Vibrio* contamination in shellfish products. Fortunately, in Italy there has been major concern given to *Vibrio* contamination. In 2016, the guidelines according to (EC) No 882/2004 and 854/2004 also include details on the detection of *V. cholerae* O1, *V. cholerae* 0139, *V. cholerae* non-O1, *V. cholerae* non-O139 (potentially enteropathogenic) and *V. parahaemolyticus* in edible shellfish tissues. Although (EC) No 882/2004 and 854/2004 were repealed by (EC) No

2017/625, the guidelines are still valid in Italy according to the note of the Ministero della Salute DI.GI.SAN 0069887 of 18/12/2019.

1.4 Vibrio and Ocean warming

Vibrios are temperature-dependent marine bacteria that grow preferentially in warm seawater and brackish waters with a temperature > 18°C, commonly achieved during the warmer seasons (Vezzulli *et al.*, 2013). The heating of the sea surface temperature (SST), related to global warming, directly affects the spread and the distribution of *Vibri*o spp. around the world. Vezzulli *et al.* (2016) showed that the SST warming trend has acted as the main factor of the long-term increase in *Vibrio* concentrations over the past 54 years. Moreover, the direct link between the SST increase and the incidence in *Vibrio*-related diseases has become clearer (Vezzulli *et al.*, 2019; Froelich et al., 2020). Researchers have noted that the arrival of warmer water in cold and temperate regions of the world has corresponded with outbreaks of *V. alginolyticus, V. cholerae* non O1/O139 and *V. parahaemolyticus* infections (Andersson et al., 2006; Frank *et al.*, 2006; Schets et al., 2006). Moreover, the heating of seawater has caused an increase in cholera cases in cholera-endemic areas such as the Bay of Bengal and sub-Saharan Africa (Pascual *et al.*, 2000; Luque Fernández *et al.*, 2009). In addition, during the warmer seasons the spread of *V. cholerae* is promoted by zooplankton blooms. Indeed, zooplankton populations represent an important environmental reservoir of *V. cholerae*: this species is associated with the exoskeleton of zooplankton and grows and multiplies over this chitin surfaces (Pruzzo *et al.*, 2008; Martinelli Filho *et al.*, 2020; Islam *et al.*, 2020). Consequently, it appears clear that, as global warming continues, *Vibrio* infections in humans are likely to increase in frequency and intensity.

Seawater heating is also threating the health of several marine animals. Species such as *Vibrio shiloi* and *V. harveyi* are promoting bleaching of the coral *Oculina patagonica* in the Mediterranean Sea and leading to mortality outbreaks of marine gastropods, respectively (Rosenberg *et al.*, 2004; Fukui *et al.*, 2010). Among summer mortalities of bivalves, species such as *V. aestuarianus, V. harveyi, V. splendidus* and *V. tapetis* represent the main causes of deadly infections (Paillard *et al.*, 2004; Beaz-Hidalgo *et al.*, 2010; Barbosa Solomieu *et al.*, 2015). The higher seawater temperature not only influence the *Vibrio* species abundance but also their pathogenicity. Several studies have revealed that the increased temperature upregulates the virulence factors of species such as *Vibrio corallyticus, V. vulnificus* and *V.*

parahaemolyticus (Mahoney *et al.*, 2009; Kimes *et al.*, 2012; Oh *et al.*, 2012). Moreover, in *V. cholera* strains, the higher temperature promotes the conversion of *V. cholerae* from non-O1 to the human pathogenic O1 serogroup (Vezzulli *et al.*, 2013)

In addition to the rise in seawater temperature, global warming is increasing the incidence and strength of hurricanes and tropical storms around the world. The abundant rainfalls associated with these climatic events reduce the salinity of the seawater and thus promote the proliferation of non-cholerae vibrios, which have a minimal salinity requirement (Froelich *et al.*, 2020). Moreover, Esteves *et al.* (2015) reported that the decrease in salinity caused by heavy rainfalls has corresponded to an increase in the concentration of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* species in seawater samples collected in French Mediterranean coastal lagoons. Consequently, given the effect of the climate change on the distribution and pathogenicity of *Vibrio* species, it is more important than ever to use reliable methods to characterise these marine bacteria to prevent vibriosis, which could affect human and aquatic animals of commercial interest.

1.5 Methodologies for the characterisation of the *Vibrio* biodiversity

Over the years, the characterisation of *Vibrio* biodiversity has relied on culture-dependent and culture-independent methods (Figure 1).

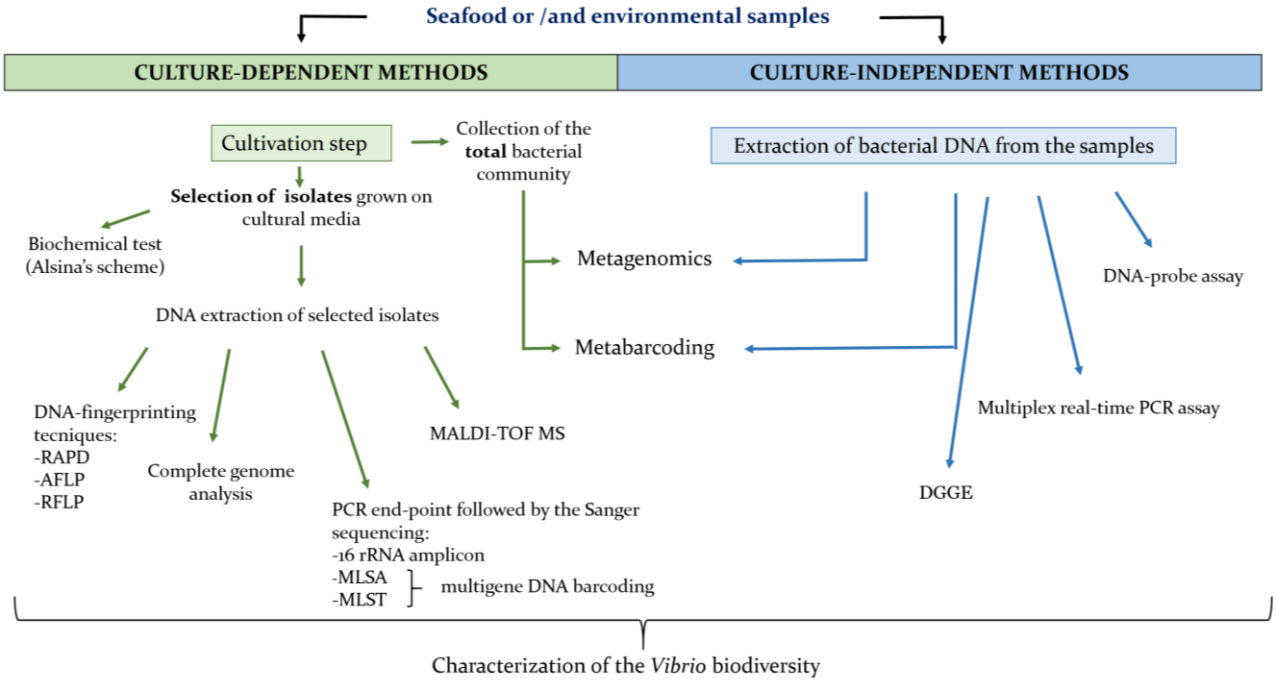

Figure 1. Overview of culture-dependent and culture-independent methods for the characterisation of *Vibrio* biodiversity.

The culture-dependent methods are based on the isolation of the fraction of bacteria for which the medium satisfies the metabolic and physiological requirements of the organisms. Among *Vibrio* biodiversity, the main target species of culture-dependent methods are pathogens of public health concern such as *V. alginolyticus*, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. To detect these species, selective media are used: thiosulfate-citrate-bile-salts-sucrose agar (TCBS) and CHROMagar Vibrio (CV). In particular, the use of TCBS medium is recommended by the standard procedure of the Food and Drug Administration's Bacteriological Analytical Manual (FDA-BAM, 2004) and ISO 21872-1:2017 for the isolation and characterisation of *V. cholerae* and *V. parahaemolyticus*. In this medium, sucrose-positive *V. cholerae* appear as yellow colonies, while sucrose-negative *Vibrio* species such as *V. parahaemolyticus* form green colonies. Unfortunately, the TCBS medium cannot discriminate between *V. parahaemolyticus* and *V. vulnificus* and between *V. cholerae* and *V. alginolyticus*. Nevertheless, the TCBS medium is useful for the recovery of several other *Vibrio* and non-*Vibrio* species (Nigro *et al.*, 2015). An easier discrimination of pathogenic *Vibrio* spp. is obtained by exploiting the chromogenic CV medium (Messelhäusser *et al.*, 2010; Di Pinto *et al.*, 2011; Lee *et al.*, 2020). In CV medium, *V. alginolyticus* colonies are colourless, *V. parahaemolyticus* colonies are purple and *V. vulnificus*/*V. cholerae* colonies are green blue to turquoise blue (Kriem *et al.*, 2015). Moreover, a combined use of CV and TCBS media is suggested to reduce the number of false positives for the detection of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* (Nigro *et al*, 2015). Then, Marine Agar (MA), a generic medium for the isolation of several marine bacteria, is widely used to perform a first cultivation step, which provides the recovery of several *Vibrio* spp., which are then investigated according to the focus of the study (Haldar *et al.*, 2010; Preetha *et al.*, 2010; Shiva Krishna *et al.*, 2019).

Once isolated, the colonies are characterised by choosing single pure isolates of particular interest, or by collecting the total bacterial communities grown on the plates. Single isolates are selected according to the dimension and colour assumed by the colonies on the medium used for their growth. After selection, the isolates are screened by using biochemical tests such as Gram staining, oxidase and motility. In particular, the standard protocol of the FDA-BAM (2004) recommends the application of polymerase chain reaction (PCR) after biochemical identification because this approach provides more accurate characterisation of the isolate's pathogenicity (Bonnin-Jusserand *et al.*, 2017). Researchers also use the practical

set of biochemical keys developed by Alsina and Blanch in the 1990s; they represent a useful method for rapid biochemical identification of a large number of environmental *Vibrio* isolates (Noguerola and Blanch, 2008).

Moreover, a rapid preliminary identification of a large number of isolated colonies is achieved by performing DNA fingerprinting techniques such as amplified fragment length polymorphism (AFLP), randomly amplified DNA (RAPD) and restriction fragment length polymorphism (RFLP). AFLP and RAPD are mainly applied for gene profiling and genetic fingerprinting of the main human pathogenic species such as *V. cholerae* and *V. parahaemolyticus* (Mishra *et al.*, 2012*;* Hu *et al.*, 2020; Taneja *et al.*, 2020). RFLP is used for rapid clustering and characterisation of the collected isolates (Maeda *et al.*, 2003). In addition, to characterise several isolates collected from environmental and seafood samples, matrix-assisted laser-desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) is used. This technique exploits the fingerprint spectra from bacterial cells to identify the *Vibrio* species and to distinguish them from closely related bacterial species such as *Photobacterium* spp. (Dieckmann *et al*., 2010; Cho *et al*., 2017).

Among the culture-dependent methods, multilocus sequence analysis (MLSA) represents a multigene DNA barcoding method to characterise isolates. MLSA developed for housekeeping genes such as *rec*A, *pyr*H, *rpo*A and *atp*A is used widely to identify several *Vibrio* species and to generate phylogenetic trees (Preheim *et al.*, 2011; Rahman *et al.*, 2014; Thompson *et al.*, 2005). A similar approach is multilocus sequence typing (MLST), a multigene DNA barcoding technique in which the variation in housekeeping genes as well as internal gene fragments is exploited to characterise strains (Glaeser and Kämpfer, 2015). In the *Vibrio* research field, this technique is mainly applied to investigate the main human pathogens such as *V. parahaemolyticus* and *V. cholerae* (Jiang *et al.*, 2019; Kanampalliwar and Singh, 2020)

The pathogenicity and identification of the isolates is achieved by PCR end-point analysis followed by Sanger sequencing. Specifically, PCR end-point analyses developed using 16S ribosomal RNA (rRNA) amplicons reveals whether the isolates belong to the *Vibrio* genus. Moreover, as reported in several studies, PCR end-point analysis can identify *Vibrio* species if developed using the housekeeping genes of the MLSA scheme (Thompson *et al.*, 2004; Machado and Gram, 2015). In addition, when this approach is carried by using the toxin genes of the isolates, it can provide information about their pathogenicity (Shirai *et al.*, 1992; Federici *et al.*, 2018). Then, complete genome sequencing is performed for the isolates of

particular concern for human and aquatic farmed animal health, to obtain information concerning the taxonomy, pathogenicity, genetic and metabolic mechanisms of these strains (Gao *et al.*, 2020; Zhang *et al.*, 2020; Weng *et al.*, 2021; Zheng *et al.*, 2021).

The cultivation step also provides the possibility to study the living fraction of a microbial community by collecting all the grown colonies from the surface of the plates. In this case, metabarcoding and metagenomic analyses provide a suitable approach to characterise viable microbial communities. In the literature, there is a lack of studies in which the total *Vibrio* community is recovered from the plates and then characterised down to the species level. The culture-dependent method is most frequently used to collect single isolates and to focus on the detection on the main *Vibrio* human pathogens or to identify the *Vibrio* species that affect farmed fish and bivalves (Jakšić *et al.*, 2002; Abdelaziz *et al.*, 2017; Bonnin-Jusserand *et al.*, 2017).

In the literature, metabarcoding is a frequently used culture-independent method. With this technique, the microbial community is characterised based on the bacterial DNA directly extracted from the samples. Researchers have performed 16s rRNA metabarcoding using next-generation sequencing (NGS) to profile *Vibrio* community down to the genus level (Rubiolo *et al.*, 2018; Vezzulli *et al.*, 2018; Lasa *et al.*, 2019). Only a few studies have combined sequencing of 16s rRNA and the heat shock protein 60 (*hsp60*) housekeeping gene to characterise *Vibrio* species biodiversity in water and oyster samples (Jesser and Noble, 2018; King *et al.*, 2019). Moreover, from the total amount of extracted DNA, multiplex real-time PCR targeted to the toxin genes can be performed for the simultaneous detection of the main human pathogens. This information could be useful to prevent the spread of human vibriosis through contaminated seafood (Garrido-Maestu *et al.*, 2014). Other culture-independent methods include the DNA-probe assay, which is applied mainly to detect the primary *Vibrio* human pathogens (Teng *et al.*, 2017; Ali *et al.*, 2021). Denaturing gradient gel electrophoresis (DGGE) is useful for the detection and quantification of *Vibrio* populations in marine water samples (Eiler *et al.*, 2006).

One of the most recent culture-independent methods is shotgun metagenomics, which involves sequencing all DNA extracted from samples. Compared with metabarcoding, shotgun metagenomics reaches a higher resolution of taxonomic annotation thanks to the great amount of sequencing data obtained from the samples (Liu *et al.*, 2021). For these reasons, this technique is used to obtain a detailed overview of the microbial biodiversity

associated with several substrates such as the human gut, soil and food (Rampelli *et al.*, 2019; Abraham *et al.*, 2020; McHugh *et al.*, 2020). In the marine research field, shotgun metagenomics has been applied extensively to characterise microbial communities associated with deep-sea hydrothermal vent to communities as well as the gut of fish and shrimp (Tyagi *et al.*, 2019; Tepaamorndech *et al.*, 2020). However, there is still a lack of studies in which shotgun metagenomics has been applied to characterise *Vibrio* species present as part of the Manila clam (*R. philippinarum*) microbiota.

2. OBJECTIVES

Vibrio spp. are distributed worldwide in marine and brackish ecosystems. Several species, including the human pathogens *V. alginolyticus*, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, are frequently isolated from seafood products. To date, few studies have characterised the total *Vibrio* community composition down to the species level. This gap needs to be filled to obtain crucial information regarding the epidemiology, public health relevance and ecology of these marine bacteria associated with seafood products.

To address this gap, the objective of the present PhD project was to characterize the *Vibrio* community composition associated to the bivalve *Ruditapes philippinarum* microbiota by reaching the species taxonomic level.

In order to achieve this accurate *Vibrio* biodiversity description, for the first time were developed and applied 1) a metabarcoding analysis carried out on two housekeeping genes: *rec*A and *pyr*H and 2) a culture-dependent metagenomics analysis.

Once developed, the sensitivity and specificity of the new metabarcoding approach were used to describe the *Vibrio* community changes in response to the depuration treatment to provide information about the risk of *Vibrio* human pathogens contamination in *R. philippinarum* microbiota. Subsequently, the robustness of the *rec*A–*pyr*H metabarcoding was applied to evaluate the culture-dependent and culture-independent methods used to characterise the *Vibrio* species associated with the *R. philippinarum* microbiota. In addition, in this part the culture-dependent metagenomics approach was developed and the results was compared to the *rec*A–*pyr*H metabarcoding. These two applications of the new metabarcoding approach and the comparison with culture-dependent metagenomics are described in the Chapter 1 and 2 of the present thesis, respectively. Then as reported in the Chapter 3, a broad range application of the *rec*A–*pyr*H metabarcoding was carried out to characterise the *Vibrio* community composition and to detect the main *Vibrio* human pathogens of all the clam samples collected during a specific campaign along the three years of the PhD project. In particular, the effects of three fixed factors were evaluated, namely the depuration effect, the clams' origin site and the season of collection, on the *Vibrio* community composition and on the detection of *Vibrio* human pathogens (Figure 2).

AIM

Characterization of the *Vibrio* species community composition associated to the bivalve *Ruditapes philippinarum* microbiota

to reach this

Development and applications of *rec*A-*pyr*H metabarcoding & culture-dependent metagenomics

	CONTENT	STUDY	OUTCOMES
Chapter 1	The **Specificity** and **Sensitivity** of *rec*A-*pyr*H metabarcoding: Development and on field application	The *rec*A-*pyr*H metabarcoding applied to describe the *Vibrio* community changes in Manila clam microbiota in response to the depuration treatment	*Published article*
Chapter 2	**Robustness** and **Performance** of *rec*A-*pyr*H metabarcoding and development of culture-dependent metagenomics: Comparison of the methods	The *rec*A-*pyr*H metabarcoding applied to evaluate the culture-dependent and -independent methods on the *Vibrio* species characterization associated with the *R. philippinarum* microbiota, and compared to the developed culture-dependent metagenomics	*Published article*
Chapter 3	Broad-range application	Investigation on all the homogenate clam samples collected in the PhD and evaluation of three fixed factors	*Draft of results*

Figure 2. Workflow of the PhD thesis.

Chapter 1. The Specificity and Sensitivity of the *rec*A-*pyr*H metabarcoding: Development and on field application

In the present PhD project, the goal to characterize the *Vibrio* community composition associated with the *R. philippinarum* microbiota was reached by the development and application of a new metabarcoding approach based on the *rec*A and *pyr*H housekeeping genes. These genes belong to the panel of molecular markers commonly used in MLSA for the identification of *Vibrionaceae* species (Thompson *et al.*, 2004; Machado and Gram, 2015). Specifically, Rahman *et al.* (2014) showed that MLSA based on *gyr*B, *pyr*H, *rec*A and *atp*A molecular markers was useful for the characterisation of several *Vibrionaceae* isolates collected from shellfish harvested in the Venetian Lagoon (https://pubmlst.org/organisms/vibrio-spp). Given these results, two of the four genes of that MLSA scheme were adapted to NGS to characterise the *Vibrio* community composition. In particular, among the four available molecular markers of that MLSA scheme, *rec*A and *pyr*H genes were the most informative in terms of the level of variability to classify *Vibrio* to the species level (Rahman *et al.*, 2014). In addition, with respect to the single-marker metabarcoding approach proposed by Jesser and Noble (2018) and King *et al.* (2019), the metabarcoding technique developed in this PhD project exploited the genetic information of two phylogenetic markers to obtain more accurate resolution of *Vibrio* species identification, which present high genome plasticity.

Once chosen the metabarcoding molecular markers, the first on field application verified its sensitivity and specificity on *Vibrio* species detection and community characterization. More specifically, the preparation of a mock community, by mixing nineteen *Vibrio* species and one Vibrionaceae, and its sequencing on a technical replicate tested the sensitivity and specificity of *rec*A-*pyr*H metabarcoding approach on the characterization of the *Vibrio* community biodiversity. Moreover, the qualitative application of the new metabarcoding achieved a reliable sensitivity taxonomic attribution for a minimum of 10 reads. In addition, the specificity of *rec*A-*pyr*H metabarcoding resulted in the successfully identification of 12 out of 19 *Vibrio* species forming the mock community. Among these 12 *Vibrio* species were correctly identified the main *Vibrio* human pathogens such as *V. alginolyticus*, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. In addition, the specificity on *Vibrio* human pathogens

characterization was achieved within the *V. cholerae* and *V. parahaemolyticus* species. The new metabarcoding in fact correctly discriminated the four *V. cholerae* and two *V. parahaemolyticus* strains present in the mock community. These results highlighted the value of *rec*A-*pyr*H metabarcoding on the detection and identification of the main human *Vibrio* species, which could contaminate shellfish product and consequently affect the human health. Going on with the usefulness of the new metabarcoding approach, other important results were achieved by its on-field application.

More specifically, the on field application consisted in a depuration trial, in which the new metabarcoding approach was used to describe the *Vibrio* biodiversity changes in Manila clam microbiota in response to the depuration treatment. During the depuration trial, carried out in May 2019, were obtained 50 homogenate clam samples. Among these homogenates, 10 belong to non-depurated clams and 40 to the depurated ones. In addition, during the depuration trial were collected 4 water samples, one from each of the depuration facilities selected for the study. In this study, the marine bacteria and the *Vibrio* concentration were quantified through the microbiological counts performed on the Marine agar (MA) and Thiosulfate-citrate-bile salts sucrose agar (TCBS) media, respectively. Then, the *Vibrio* species community composition associated to the Manila clam homogenates was characterized by using the *rec*A-*pyr*H metabarcoding, in order to implement the knowledge about the risk of *Vibrio* human pathogens contamination during the shellfish depuration process. The *rec*A-*pyr*H metabarcoding application on this study highlighted a persistence of the *Vibrio* species to the depuration treatment and the presence at low concentration of the *Vibrio* human pathogens into the depurated clams. In particular, the new metabarcoding resulted with higher sensitivity than qPCR. Moreover, the new metabarcoding offered new knowledge about the modification of *Vibrio* community composition based on the type of depuration plant. In each depuration plant, in fact, occurred a *Vibrio* species community variation according to the different depuration process. In particular, the *V. cholerae* was detected in all of the four depuration plants investigated in the study. Moreover, in one of the four depuration plants were found all the main human *Vibrio* pathogenic species such as *V. alginolyticus, V. cholerae, V parahaemolyticus* and *V. vulnificus*. To conclude the on field application of *rec*A-*pyr*H in the depuration trial highlighted the importance to evaluate the *Vibrio* community composition specific to each plant during the risk assessment to guarantee a food safe shellfish-product for the consumer. The complete methodology, scientific results

and discussion, which describe the on-field application of *rec*A-*pyr*H metabarcoding, are provided by the original full-text publication, which is attached below. :

- **Zampieri, A**.; Carraro, L.; Cardazzo, B.; Milan, M.; Babbucci, M.; Smits, M.; Boffo, L.; Fasolato, L. Depuration processes affect the Vibrio community in the microbiota of the Manila clam, Ruditapes philippinarum. *Environ Microbiol*. 2020; 22(10): 4456–4472. doi.org/10.1111/1462-2920.15196.
  Supporting Information available online at:
  https://sfamjournals.onlinelibrary.wiley.com/doi/10.1111/1462-2920.15196

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Depuration processes affect the *Vibrio* community in the microbiota of the Manila clam, *Ruditapes philippinarum*

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Summary

As filter-feeders, bivalve molluscs accumulate *Vibrio* into edible tissues. Consequently, an accurate assessment of depuration procedures and the characterization of the persistent *Vibrio* community in depurated shellfish represent a key issue to guarantee food safety in shellfish products. The present study investigated changes in the natural *Vibrio* community composition of the *Ruditapes philippinarum* microbiota with specific focus on human pathogenic species. For this purpose, the study proposed a MLSA-NGS approach (rRNA 16S, *rec*A and *pyr*H) for the detection and identification of *Vibrio* species. Clam microbiota were analysed before and after depuration procedures performed in four depuration plants, using culture-dependent and independent approaches. Microbiological counts and NGS data revealed differences in terms of both contamination load and *Vibrio* community between depuration plants. The novel MLSA-NGS approach allowed for a clear definition of the *Vibrio* species specific to each depuration plant. Specifically, depurated clam microbiota showed presence of human pathogenic species. Ozone treatments and the density of clams in the depuration tank probably influenced the level of contamination and the *Vibrio* community composition. The composition of *Vibrio* community specific to each plant should be carefully evaluated during the risk assessment to guarantee a food-safe shellfish-product for the consumer.

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Introduction

Vibrios are a well-known and large genus of Gram-negative marine bacteria, of which a number of species are pathogenic to marine organisms and humans. Vibrios easily come into contact with a wide range of marine organisms, interacting in pathogenic or mutualistic ways (Le Roux and Blokesch, 2018). Due to their filter-feeding habits, bivalves can accumulate large quantities of *Vibrio* species in their tissues (Pruzzo *et al*., 2005). The genus *Vibrio* is an important member of the microbial community associated with the haemolymph and the digestive gland of several bivalve molluscs (Milan *et al*., 2018; Vezzulli *et al*., 2018; Milan *et al*., 2019). The diversity of *Vibrio* species associated with bivalves includes non-pathogenic species as well as human and mollusc pathogens.

This biodiversity distribution is directly influenced by environmental parameters, such as water temperature, which play a key role in the spread of pathogenic *Vibrio* species (Romalde *et al*., 2014). In addition, the opportunistic nature of Vibrios combined with heat stress events for the host contribute to bivalve mass mortality episodes and losses in aquaculture production (Alfaro *et al*., 2018; Green *et al*., 2018).

Among bivalves, the Manila clam is a species with a worldwide coastal distribution that inhabits sandy-mud bottoms. While this species was originally from Asia, it was introduced to other continents both accidentally and intentionally, due to its rapid growth rate and resistance to harsh environmental conditions (Cordero *et al*., 2017). Today, the Manila clam represents one of the major cultured species worldwide (4 228 594 tons in 2016; FAO data, n.d.) and the most important species for commercial clam landings in Europe. China is by far the leading producer on the global scale (97.4% of total annual production), while Italy has a smaller but notable production of over 65 000 tons per year, mainly derived by harvesting activities performed in the Venetian region.

In the European Union (EU), the classification of shellfish production areas (zones A, B and C) is carried out according to *Escherichia coli* concentrations as a faecal indicator of bacteria in flesh and intra-valvular liquid (Rubiolo *et al*., 2018; De Souza *et al*., 2019). Given the dynamic and changing nature of marine bacteria, the European Regulation (EC) No. 853 and 854/2004 stipulates that clams collected from zone B areas need to be treated through a depuration process to ensure a safe seafood product for human consumption. Shellfish collected from zone C areas require displacement to a zone A area for at least 2 months prior to human consumption exclusively as a cooked sea-product. Despite increasing incidences of *Vibrio*-associated human illness (Martinez-Urtaza *et al*., 2010; Ellis *et al*., 2012), European legislation is still lacking in detailed monitoring of *Vibrio* contamination in shellfish products. In Italy, major concern regarding *Vibrio* contaminations resulted in the guidelines (EC) No 882/2004 and 854/2004 in 2016, which included the detection of *V. cholerae* non-O1, *V. cholerae* non-O139 and *V. parahaemolyticus*. Depuration procedures applied to bivalve molluscs were shown to be effective in removing faecal bacteria such as *E. coli* (Sferlazzo *et al*., 2018). Unfortunately, the concentration of faecal indicators does not correlate with the presence of *Vibrio* spp. (Oliveira *et al*., 2011) and many studies report that the efficiency of depuration processes in *Vibrio* de-contamination is not as successful as for coliform bacteria (Sferlazzo *et al*., 2018; Vezzulli *et al*., 2018).

In recent studies, most concern is on the effectiveness of a depuration treatment in removing *Vibrio* species pathogenic to humans from the edible tissues of shellfish products. Specifically, depuration performance is investigated for its ability to reduce the *Vibrio* load in mollusc tissues (Ciulli *et al*., 2017; Rubiolo *et al*., 2018).

For this purpose, several studies have conducted an artificial contamination of shellfish product with human pathogenic *Vibrio* species. The efficiency of the treatment is evaluated in terms of duration of the depuration cycle, water flow rate and density of shellfish per tank to remove the contamination of *Vibrio* pathogenic species (Barile *et al*., 2018; Ming *et al*., 2018; Shen *et al*., 2019). Other studies investigated the impact of depuration on shellfish products naturally contaminated by Vibrios mainly describing the presence of human pathogenic *Vibrio* species such as *V. cholerae* 01, *V. parahaemolyticus* and *V. vulnificus* (Croci *et al*., 2002; Sferlazzo *et al*., 2018; Tokarskyy *et al*., 2019). The study on the impact of depuration process on seafood bacterial biodiversity also included the investigation on the shift in bivalve microbiota composition by means of 16S rRNA gene-based analyses through next generation sequencing technologies (Rubiolo *et al*., 2018; Vezzulli *et al*., 2018). Unfortunately,16S RNA gene sequencing applied to Vibrionaceae provides an accurate identification of the bacterial community only up to the genus level (Thompson *et al*., 2005).

Despite a large number of studies focused on the detection of *Vibrio* human pathogenic species in depurated shellfish product, information is still lacking on defining, in parallel, the changes in *Vibrio* species community composition in response to depuration practices. It follows that an accurate *Vibrio* species delineation specifically on depurated shellfish products represents an important knowledge gap to cover. To do so, a more comprehensive picture of *Vibrio* species dynamics during depuration treatments could be provided by Next Generation Sequencing (NGS).

Recent studies have combined 16S rRNA analyses of several new targets, such as heat shock protein 60 (*hsp60*) amplicon sequencing, to improve the detection and identification of *Vibrio* species, successfully identifying human pathogenic *Vibrio* species in water and oyster samples (Jesser and Noble, 2018; King *et al*., 2019). Following these promising results, the present study goes a step further and applies NGS technologies 16S rRNA, as well as to *rec*A and *pyr*H, two housekeeping genes with suitable taxonomic resolution as demonstrated in several studies for *Vibrio* isolate identification (Sawabe *et al*., 2013; Rahman *et al*., 2014; Fang *et al*., 2018; Pacual *et al*., 2010). Combining this approach with bacterial culture techniques, four depuration facilities were investigated. Differences related to each plant–environment were described in light of the detection of human pathogenic *Vibrio* species, offering a more in-depth description of the *Vibrio* species community composition in the Manila clam microbiota.

Results

Microbiological counts

The microbial load in homogenates was evaluated for marine bacteria (MA) and *Vibrio* (TCBS). Specifically, the median of microbiological counts for homogenates depurated into the four depuration plants revealed an increase in bacterial contamination with respect to non-depurated homogenates (*p*-value 0.002, Fig. S1). In details, the bacterial load in depurated homogenates reached a value of 5 and 4 $\log_{10}$ CFU g^{-1} in MA and TCBS media respectively. Non-depurated homogenates showed levels of 4 $\log_{10}$ CFU g^{-1} in MA and 2 $\log_{10}$ CFU g^{-1} in TCBS media. In addition, plant-specific depuration conditions and environmental

factors affected the quantity of total microbial counts and *Vibrio* (p-value 0.0002) respectively. Pairwise comparisons indicated that homogenates collected in PLANT A had the highest level of contamination, while PLANT B and PLANT C homogenates showed comparable bacterial loads. PRE-DEPURATION and EXPERIMENTAL PLANT homogenates presented the lowest median load of total microbial counts (4 $\log_{10}$ CFU g^{-1}) and 2 $\log_{10}$ CFU g^{-1} for *Vibrio* contamination. Boxplots showed that data was dispersed in all depurated groups (Fig. 1). Pairwise comparisons according to PERMANOVA analysis revealed statistically significant differences between depuration plants. Particularly, PLANT A (post-depuration) was significantly different in comparison to the other three depuration plants in the study (p-value 0.0002). PRE-DEPURATION total microbial counts were significantly different between PLANT A (p-value 0.0002), PLANT B (p-value 0.0002) and PLANT C (p-value 0.0002), but similar to EXPERIMENTAL PLANT. When considering all the microbial feature profiles, there appears to be a clear segregation of PLANT A if compared to the other plants, as indicated by the Non-metric multidimensional scaling (NMDS) reported in supplementary materials (Fig. S2). No statistical differences were detected between technical replicates performed during sample processing (p-value 0.8). Microbial load of the four water samples taken from the depuration plants also showed a higher contamination in PLANT A than in the water samples from the other three depuration plants. Specifically, PLANT A water sample had a bacterial load of 5.7 and 2.6 $\log_{10}$ CFU g^{-1} for MA and TCBS respectively. PLANT B and EXPERIMENTAL PLANT water samples revealed similar levels of contamination in MA media, reaching 1.8 and 1.7 $\log_{10}$ CFU g^{-1} respectively. In the PLANT C water sample, bacteria load was 2.7 $\log_{10}$ CFU g^{-1} in MA media. No colonies grew on TCBS media from water samples of PLANT B, PLANT C or EXPERIMENTAL PLANT (Table S1). *Vibrio* colonies were selected based on morphology and collected from the plates, then confirmed as belonging to the *Vibrio genus* by Kraken software. More specifically, Kraken attributed 87% of isolates to the Splendidus clade, while a *Vibrio* species assessment was not possible for 10% of the isolates.

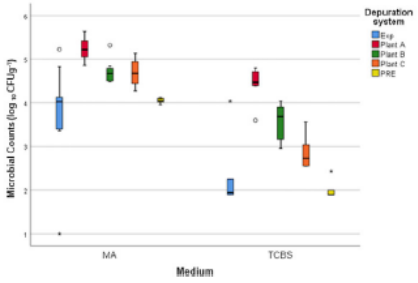

Fig 1. Total microbial counts and *Vibrio* spp. counts of depurated and non-depurated homogenates performed on MA and TCBS media respectively. Each boxplot corresponds to a statistical analysis of 10 samples; thicker black lines in the boxes correspond to the medians. (MA: Marine Agar; TCBS: Thiosulfate-citrate-bile salts-sucrose agar; Exp: EXPERIMENTAL PLANT).

16S amplicon based microbial communities of clams

The microbial community of homogenates was reconstructed using 16S rRNA amplicon sequencing. A rarefaction curve of filtered reads indicated that sequencing depth was sufficient for a good representation of the microbial community (Fig. S3).

According to biomolecular analyses of the 16S amplicon sequences, the depuration processes affected both the biodiversity rate and the composition of the microbial community. The biodiversity Richness (defined as the number of different taxa represented in an ecological community, landscape or region) in depurated homogenates, with exception of EXPERIMENTAL PLANT ones, was higher when compared to PRE-DEPURATION homogenates. In PLANT C homogenates, the highest level of taxa was detected with variability between biological replicates. Homogenates from PLANT A, PLANT B and PRE-DEPURATION showed comparable Richness among biological replicates (Fig. 2A). The variation of microbial community between depuration plants (beta diversity) was investigated with NMDS representation (Fig. 2B). The stress value obtained indicated a good fit between the NMDS representation and the 16S sequencing data (Stress ≤0.15) (Fig. 2B). NMDS plots showed a clear clustering of PLANT A homogenates separated from all the other depurated and non-depurated homogenates. Homogenates from the other depuration plants also showed individual segregation; however, homogenates from PLANT B and PLANT C revealed partial overlapping of microbial composition, and homogenates from the EXPERIMENTAL PLANT were close to PLANT B, PLANT C and to PRE-DEPURATION ones. Homogenates from PLANT B, PLANT C and PRE-DEPURATION showed a low level of dispersion between biological replicates. Pairwise comparisons were performed by PERMANOVA to evaluate the statistical differences between depuration plants. The results corroborate the NMDS representation in that clams showed a microbial composition specific to each depuration plant (p-value < 0.001), and all depurated homogenates differed from non-depurated homogenates, regardless of the depuration plant. In addition, the NMDS representation including the four water samples revealed a clear clustering of water samples separated from depurated and non-depurated homogenates (Fig. S4).

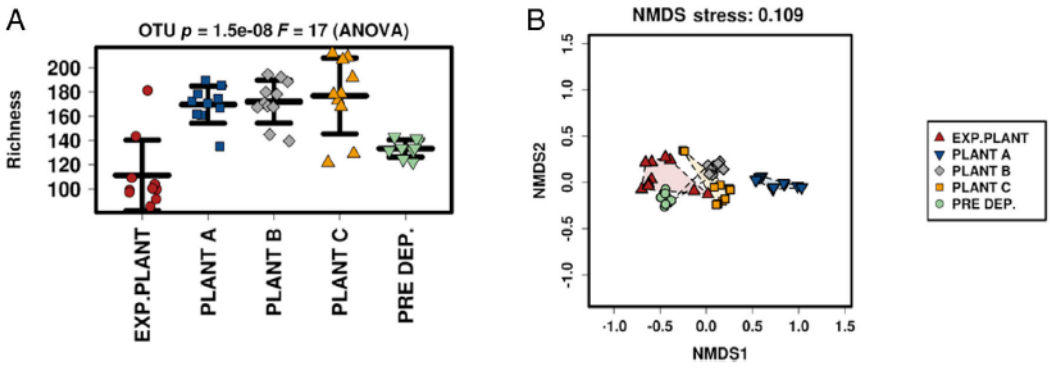

Fig 2. 16S amplicon based microbial analysis of homogenates.
A. Alpha-diversity tested with ANOVA according to Richness Index.
B. 16S amplicon based microbial beta-diversity representation with NMDS (PERMANOVA *p*-value: 0.001).

An ANOVA analysis was carried out on 16s sequence data to determine which genera significantly differ, in terms of abundance, considering depurated and non-depurated homogenates (Fig. 3). Specifically, PLANT A, PLANT B and PLANT C showed a higher abundance of *Colwellia*, *Vibrio*, *Pseudoalteromonas* and *Shewanella* when compared to PRE-DEPURATION and EXPERIMENTAL PLANT homogenates. Specifically, homogenates from PLANT A had the highest abundance of the genera *Colwellia*, *Vibrio* and *Pseudoalteromonas* compared to depurated and non-depurated homogenates. PRE-DEPURATION and EXPERIMENTAL PLANT homogenates showed comparable abundances for all the seven taxa distinguished from the ANOVA analysis. *Endozoicomonas* genus was abundant in all depurated homogenates from the four depuration plants as well as in non-depurated ones.

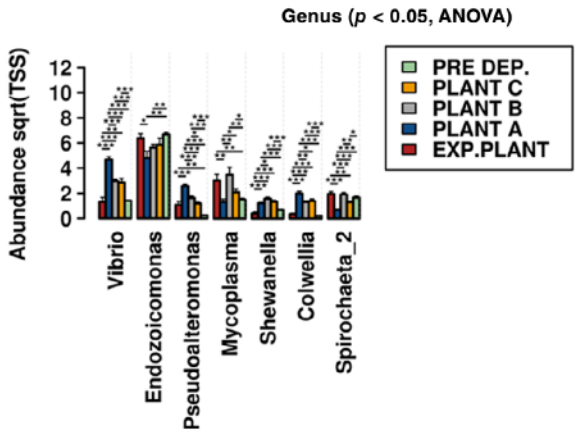

Fig 3. The seven taxa statistically different in terms of abundance among depurated and non-depurated homogenates according to ANOVA analysis on 16s data sequencing.

Mock community characterization by MLSA-NGS approach

Nineteen *Vibrio* species and one Vibrionacea, spanning nine clades and including the main dangerous species for human health and clam farming, were included in the mock community (Table 1). Subsequently, the *rec*A and *pyr*H amplicons were amplified from the DNA extracted from the mock community and sequenced in technical triplicate producing an average of 395008.3 raw reads.

Subsequently, raw reads were elaborated by Kraken software and a minimum number of 10 reads were considered in order to reach a reliable taxonomic attribution. Different threshold cut-offs were tested in Kraken and the value of 0.1 improved the sensitivity of the approach, reducing the identification of non-target species. This compromise increased the stringency of *Vibrio* species identification, although three mock *Vibrio* species were lost (*V. campbelli*, *V. diabolicus*, *V. owensii*). The level of species identification increased when the two markers were combined by Kraken software since species not identified by *rec*A were recovered by the *pyr*H gene (Table S3). As shown in Table 1, identification using the combination of *rec*A and *pyr*H in each technical replicate identified the same species, and 12 out of 19 *Vibrio* species were included in the mock community. According to these markers, the assessment of *Vibrio* species presented different levels of resolution depending on the clade of origin. Specifically, we were able to detect all *Vibrio* species belonging to the Cholerae clade. Sixty percent of the *Vibrio* species belonging to the Harveyi clade were also successfully identified, including the main species pathogenic to humans: *V. algynoliticus* and *V. parahaemolyticus*. The Splendidus clade presented a lower level of resolution, allowing only the identification of *V. splendidus*.

Table 1. Species identification of the three technical replicates (V1, V2, V3) of the mock community.

Mock composition	V1	V2	V3
Photobacterium damselae ATCC 35083 ■			
Vibrio aestuarianus LMG 7909 □			
Vibrio algynoliticus LMG 17749▲			
Vibrio anguillarum LMG 10861 □			
Vibrio anguillarum 5934/83[a]			
Vibrio campbelli CECT 523▲			
Vibrio chagasii LMG 21353▼			
Vibrio cholerae ATCC 9458 ○			
Vibrio cholerae ATCC 9459○			
Vibrio cholerae ATCC 25872 ○			
Vibrio cholerae ISS17b ○[b]			
Vibrio diabolicus LMG 23867▲			
Vibrio fluvialis LMG 7894 ○			
Vibrio furnissi CECT 4203 ○			
Vibrio harveyi CECT 4215▲			
Vibrio harveyi LMG 4044			
Vibrio mediterranei CECT 621 ●			
Vibrio orientalis CECT 629►			
Vibrio owensii LMG 25443▲			
Vibrio parahaemolyticus ATCC 17802▲			
Vibrio parahaemolyticus ATCC 43996▲			
Vibrio rotiferianus LMG 21460▲			
Vibrio shilonii LMG 19703 ●			
Vibrio splendidus LMG 19031▼			
Vibrio tapetis DSMZ 21475◄			
Vibrio vulnificus ATCC 27562 ♦			

Positive (white) and negative (grey) identification of species was obtained by *rec*A-*pyr*H combined data sequencing. *Clades identified*: ■ Photobacterium clade; □ Anguillarum clade; ▲ Harveyi clade; ▼ Splendidus clade; ○ Cholerae clade; ● Mediterranei clade; ►Orientalis clade; ◄ Tapetis clade; ♦ Vulnificus clade.
a. From Department of Comparative Biomedicine and Food Science (BCA), Legnaro (PD) field collection.
b. From Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe), Legnaro (PD) field collection.

recA and pyrH amplicon-based Vibrio communities on clam samples

After testing a mock community to validate the effectiveness of *rec*A and *pyr*H sequencing for the identification of *Vibrio* species, the assay was applied to the depurated and non-depurated homogenates and to water samples.

In order to reduce PCR bias for variable efficiency of amplification in the different *Vibrio* species (Acinas *et al*., 2005), *rec*A and *pyr*H NGS data were elaborated with a qualitative approach based on the presence/absence of species identified by the two markers.

Fifteen different *Vibrio* species were identified considering all 54 samples collected during the trial (10 non-depurated homogenates, 40 depurated homogenates and 4 water samples). More specifically, 10 *Vibrio* species were identified in water samples and 12 in

homogenates, of which *V. broeganii*, *V. splendidus* and *V. tapetis* represent the only three species detected in non-depurated homogenates. Moreover, human pathogenic *Vibrio* species such as *V. cholerae* and *V. parahaemolyticus* were identified in PLANT C and PLANT A water samples respectively. *V. algynoliticus* *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* were identified only in depurated homogenates. Specifically, *V. algynoliticus* was found in depurated homogenates from PLANT A, and *V. cholerae* was found in all depurated homogenates, while *V. vulnificus* was found only in homogenates from PLANT C. With regard to *Vibrio* species pathogenic to clams, *V. splendidus* was found in all homogenates and water samples collected. *V. tapetis* was present in depurated and non-depurated homogenates and in PLANT A and EXPERIMENTAL water samples. In addition, the merging approach that was applied allowed the identification of 16 taxa up to *Vibrio* at the species level. *Aliivibrio fischeri*, *Aliivibrio wodanis* and *Photobacterium damselae* were among the *Vibrionacea* detected in water and homogenates from PLANT A (Table 2A and B).

Variation in *rec*A/*pyr*H community species composition (beta diversity) was visualized by NMDS, with a stress value < 0.15 providing an excellent representation in reduced dimensions (Fig. 4). More specifically, homogenates from PLANT B, PLANT C and EXPERIMENTAL PLANT were closely represented in the two-dimensional configuration, suggesting a similar *Vibrio* community composition. Depurated homogenates from PLANT A, however, revealed a major dissimilarity with respect to those from other depuration plants. PRE-DEPURATION homogenates clustered together with EXPERIMENTAL PLANT ones, indicating a similarity in *Vibrio* species biodiversity.

PERMANOVA pairwise comparisons confirmed the NMDS results, showing species community variations occurred according to the different depuration processes and other specific factors related to each plant such as water quality, pH, oxygenation and temperature, and revealing significant differences among depurated and non-depurated homogenates.

More specifically, *Vibrio* communities associated with PRE-DEPURATION homogenates were significantly different (p-value < 0.05) compared to depurated homogenates from PLANT A (p-value 0.0001), PLANT B (p-value 0.0001) and PLANT C (p-value 0.03). Statistical differences were also observed between PLANT C and PLANT A (p-value 0.04), between PLANT B and PLANT A (p-value 0.003), and between EXPERIMENTAL PLANT and both PLANT B (p-value 0.008) and PLANT A (p-value 0.0002). In addition, an ANOVA analysis applied to species abundance according to the *rec*A/*pyr*H data showed six species of bacteria, which were significantly different between depurated (from the four plant) and the non-depurated homogenates (Fig. 5). More specifically, depurated homogenates from PLANT A had a higher average abundance of *Aliivibrio wodanis*, *Alteromonas mediterranea* and *Vibrio scophthalmi* compared to homogenates from the other three depuration plants, as well as compared to the non-depurated homogenates. *Aliivibrio fischeri* had the highest abundance in homogenates from PLANT A and PLANT C. Homogenates from PLANT A had a smaller abundance of *Vibrio splendidus* compared to the other depurated and non-depurated homogenates sample. *Vibrio tapetis* had the highest statistically significant level of abundance in PLANT B homogenates. In addition, *Vibrio tapetis* abundance showed a higher level of dispersion between biological replicates from each of the plants and from non-depurated homogenates.

qPCR evaluation of V. cholera, V. parahaemolyticus, V. vulnificus *and* E. coli

The MLSA-NGS identified the three human-pathogenic *Vibrio* species (*V. cholera*, *V. parahaemolyticus*, *V. vulnificus)* in the depurated-homogenates and water samples (Table 2). In order to quantify the three species and evaluate the presence of *E. coli*, a qPCR analysis was applied. Total bacteria and the *Vibrio* spp. qPCR assays were also included in the analysis. The qPCR did not amplify the three pathogenic *Vibrio* species and *E. coli* from all the samples including homogenates and water. Total bacteria concentrations were $\log_{10}$ 4.4 to $\log_{10}$ 6.9 and *Vibrio* spp. concentrations were from $\log_{10}$ 1.1 to $\log_{10}$ 5.4 bacterial cells in 1 ml. Regarding the water samples, PLANT A water showed the highest total bacterial ($\log_{10}$ 6.6) and *Vibrio* spp. ($\log_{10}$ 5.4) concentration, calculated as the cells in 1 ml. The results are reported in Fig. S6 and Table S5.

To confirm the quantification obtained with qPCR, correlation was measured between *Vibrio* spp. copy number and NGS 16S reads identified as belonging to the *Vibrio* genus, demonstrating a positive correlation (Spearman test: $R = 0.85$, p-value < 0.05) between the two series of values.

In order to define the limit of detection of the qPCR assay in the homogenate extracts, spike-in trials were carried out including a defined amount of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* or *E. coli* in homogenate extracts. The results demonstrated a sensitivity of 4 copies/2.5 µl, 3 copies/2.5 µl and 37 copies/2.5 µl for *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* respectively. The sensitivity of qPCR detection for *E. coli* was 3 copies /2.5 µl copies.

Table 2. List of marine bacteria and *Vibrio* species detected into the four water samples (**A**) and in depurated and non-depurated homogenates (**B**) by *rec*A and *pyr*H data merged according to Kraken attribution.

A

			PLANT A WATER	PLANT B WATER	PLANT C WATER	EXP. PLANT WATER
Marine bacteria	1	*Aliivibrio fischeri*	X			
	2	*Alteromonas australica*	X			
	3	*Alteromonas mediterranea*	X			
	4	*Alteromonas naphthalenivorans*	X			
	5	*Haemophilus parainfluenzae*				X
	6	*Photobacterium damselae*	X			
	7	*Pseudoalteromonas donghaensis*	X			
	8	*Pseudoalteromonas spongiae*	X			
	9	*Psychrobacter alimentarius*	X			
	10	*Psychrobacter* sp. *AntiMn-1*	X			
	11	*Psychrobacter* sp. *P11F6*	X			
	12	*Shewanella baltica*	X			
	13	*Shewanella japonica*	X			
Vibrio species	1	*Vibrio breoganii*	X			
	2	*Vibrio diabolicus*	X			
	3	*Vibrio cholerae*			X	
	4	*Vibrio fluvialis*				X
	5	*Vibrio parahaemolyticus*	X			
	6	*Vibrio scophthalmi*	X			
	7	*Vibrio shilonii*	X			
	8	*Vibrio splendidus*	X	X	X	X
	9	*Vibrio tapetis*	X			X
	10	*Vibrio tubiashii*	X			

B

			HOMOGENATES				
			PRE-DEPURATION	PLANT A	PLANT B	PLANT C	EXP. PLANT
Marine bacteria	1	*Aggregatibacter aphrophilus*			X	X	
	2	*Aliivibrio fischeri*		X		X	X
	3	*Aliivibrio wodanis*		X			
	4	*Alteromonas mediterranea*		X			
	5	*Haemophilus parainfluenzae*	X				
	6	*Natrialba magadii*			X		
	7	*Photobacterium damselae*		X			
	8	*Psychrobacter alimentarius*	X				
	9	*Shewanella japonica*	X	X			
Vibrio species	1	*Vibrio alginolyticus*		X			
	2	*Vibrio anguillarum*		X			
	3	*Vibrio breoganii*	X				
	4	*Vibrio cholerae*		X	X	X	X
	5	*Vibrio fluvialis*		X	X	X	X
	6	*Vibrio furnissii*		X		X	
	7	*Vibrio parahaemolyticus*		X			
	8	*Vibrio rotiferianus*		X			
	9	*Vibrio scophthalmi*		X			
	10	*Vibrio splendidus*	X	X	X	X	X
	11	*Vibrio tapetis*	X	X	X	X	X
	12	*Vibrio vulnificus*				X	

Discussion

A number of human and marine animal diseases are caused by *Vibrio* species which inhabit marine and estuarine ecosystems, and can contaminate seafood destined for human consumption (Iwamoto *et al*., 2010; Siboni *et al*., 2016). As currently practised, the depuration processes that are applied to reduce microbial contamination in marine molluscs is inadequate for removing Vibrios from edible bivalve tissues (Sferlazzo *et al*., 2018; Vezzulli *et al*., 2018), including human pathogenic *Vibrio* species such as *V. parahaemolyticus* and *V. cholerae* 01 (Croci *et al*., 2002).

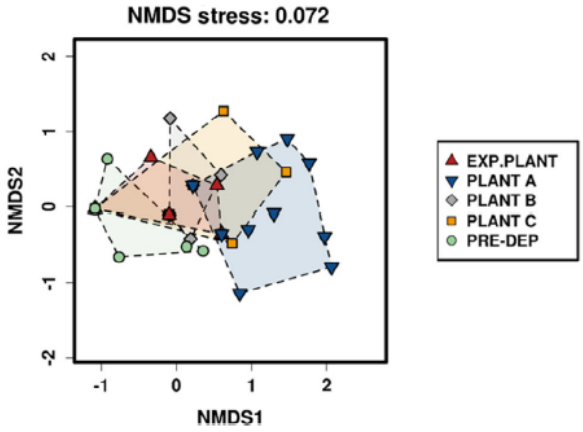

Fig 4. *rec*A-*pyr*H amplicon based microbial beta-diversity representation of depurated and non-depurated homogenates with NMDS representation (presence/absence of species).

The Manila clam, *R. philippinarum*, reared in Mediterranean sea, is a popular seafood product in which there have been several incidences of contamination by human-pathogenic *Vibrio* species such as *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* (Passalacqua *et al*., 2016; Serratore *et al*., 2016).

Consequently, the aim of this study was to evaluate the changes in *Vibrio* community in Manila clam specimens following treatment in different depuration plants. To do so, a new NGS-Multi Locus Analysis (NGS-MLA) approach able to define *Vibrio* species and to highlight pathogenic species for humans was proposed.

First, in the present study the level of *Vibrio* contamination was evaluated through microbial counts. These confirmed a persistence of *Vibrio* bacteria in the bivalve tissues after depuration treatment performed at each of the four plants selected for the investigation.

More specifically, samples from each depuration plant presented different bacterial loads. Microbial counts of depurated homogenates highlighted PLANT A as the most contaminated plant in that it had the highest *Vibrio* load (Fig. 1). In addition, microbial counts of the water sample collected from PLANT A were in accordance with the results from the homogenates (Table S1). Moreover, the qPCR results for total bacteria and *Vibrio* spp. corroborated the higher contamination of the PLANT A water sample with respect to all the other water and homogenates from the three other depuration plants (Fig. S6). These findings suggest that the higher Vibrios contamination of clams depurated in PLANT A could be mainly related to the Vibrios load present in the marine water used during the depuration cycle, as well as to the absence of ozone treatment, which is not applied in this plant as opposed to others. In consequence, it appears that the depuration process carried out in PLANT A is ineffective in reducing the proliferation of bacteria already present in the marine water in the plant environment, and consequently in the Manila clam tissues. In particular, Blogoslawski and Stewart (2011) demonstrated that ozone treatment was efficient in reducing *Vibrio* contamination affecting a shrimp hatchery. However, the inclusion of the ozone treatment in PLANT B and PLANT C did not completely eliminate Vibrios. The reduced *Vibrio* contamination achieved in EXPERIMENTAL PLANT could be explained by the smaller size of the plant and the reduced density of clams in the depuration tanks (Table S2). Moreover, the higher *Vibrio* contamination in PLANT A, PLANT B and PLANT C could be justified by the higher density of clams during depuration and by the age of the facilities, which are considerably older than the more recently built EXPERIMENTAL PLANT. These two conditions could have prompted the formation of bacterial biofilms on tank and filter surfaces of the three long-established depuration plants that were considered in this study (A, B and C). The persistence of *Vibrio* in depurated homogenates could also be related to interactions between the bacteria and the invertebrate host. More specifically, Pruzzo and colleagues (2005) justified the persistence of certain *Vibrio* species in bivalves tissues to their resistance to the bivalve haemolymph bactericidal activity of. Moreover, the inability of depuration treatments to completely remove *Vibrio* could also be associated with the long co-evolutionary history that these bacteria often have with their hosts. In oysters, Vibrios are important components of the haemolymph microbiota and, for some strains, have previously been used as probiotics (Gatesoupe, 1999), suggesting a mutualistic interaction with the invertebrate host (Wegner *et al*., 2019).

In the present study, a biomolecular method was applied for the detection and identification of *Vibrio* species which naturally contaminate the microbiota of Manila clam. For this purpose, the novel NGS-MLSA was applied to enhance the detection and identification of *Vibrio* species using two alternative markers. According to the mock results, eight *Vibrio* strains belonging to the four main human pathogenic species (*V. algynoliticus*, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*) were detected. Sequences of *rec*A and *pyr*H were merged using Kraken software to exploit the complementary capacity of detection-identification of the two genes. Based on these markers, main human pathogenic species such as *V. algynoliticus*, *V. cholerae*, *V. parahaemolyitucs*, *V. vulnificus* and bivalve pathogenic species, such as *V. tapetis* and *V. splendidus*, were successfully identified on the mock community (Table 1). In the recent study of King and colleagues (2019), the power of discrimination of *hsp60* gene resulted on the identification of all the *Vibrio*

species defining the mock (five clades) including the main human pathogenic species. The mock sample used in this study included nine clades comprising four strains of *V. cholerae* and two strains of *V. parahaemolyticus*, which were correctly identified by the *rec*A-*pyr*H genes. Consequently, the validation of the primers showed a powerful level of discrimination of the two genes within *V. cholerae* and *V. parahaemolyticus* species with respect to the mock sample tested by King and colleagues (2019). This result highlights the value of the combined use of *rec*A and *pyr*H to correctly detect and identify human-pathogenic *Vibrio* species, which represent a risk for human consumption and for the management of clam aquaculture.

In the mock sample, in addition to the three clades of the human pathogenic species, *rec*A and *pyr*H genes provided the identification of six *Vibrio* clades, including the molluscan pathogens *V. tapetis* and *V. splendidus*. Given this result, the development of this tool allows for the interpretation of *Vibrio* dynamics inside each depuration plant and provides a better understanding of the modifications occurring through depuration treatments for human pathogenic *Vibrio* species.

The investigation on the bacterial community, which is naturally present in Manila clam tissues, started with the rRNA 16s sequencing analysis to achieve a global vision of the bacterial community. Interestingly, Alfa-diversity analysis demonstrated an increase in Richness after depuration treatment, with the exception of the homogenates from the EXPERIMENTAL PLANT (Fig. 2A). This result was not in accordance with some recent studies (Rubiolo *et al*., 2018; Vezzulli *et al*., 2018). Such findings suggest that depuration tank environment and the water used during the treatment in PLANT A, PLANT B and PLANT C could be among the main causes of higher marine bacterial load and specifically of *Vibrio* contamination in mollusc samples. As previously suggested, the depuration procedure may have favoured the formation of biofilms, namely due to the recirculation of marine water through tanks and filter surfaces, as conducted during the depuration process in PLANT A, PLANT B and PLANT C. Moreover, biofilm formation is a common phenomenon and is notoriously difficult to avoid in facilities with tank water recirculation. A recent study by Roalkvam and colleagues (2019) reported finding *Vibrio* in biofilms attached to different surface types and elements that were part of a flow-through fish farm. Biofilm formations and different technologies applied during the depuration cycle may also have induced the shift of community composition of Manila clam microbiota observed in our results. Specifically, ANOVA analysis on 16s sequencing data showed the presence of seven bacterial genera, which significantly differ between depurated and non-depurated homogenates (Fig. 3). Among these seven genera, *Vibrio* was most abundant in samples from PLANT A, possibly related to the absence of Ozone treatment in this plant, as mentioned above. The low abundance in EXPERIMENTAL PLANT of four out of the seven bacterial genera could be again related to the reduced size and time of utilization of this plant with respect to the facilities of PLANT A, PLANT B and PLANT C. In addition, the abundance of *Endozoicomonas* genus obtained in depurated and non-depurated homogenates may be explained by the associations of this marine bacteria with a wide variety of marine animal hosts, including bivalves (Neave *et al*., 2016).

The efficacy of depuration was also monitored by applying a qPCR assay for *E. coli* as a faecal indicator bacteria (Walker *et al*., 2017; Vezzulli *et al*., 2018). The spike-in test results suggested a suitable detection and quantification of this target (3 copies numbers/2.5 μl) which was not amplified in any of the depurated and non-depurated homogenates. On the other hand, the 16S rRNA-NGS results suggested the presence of *Enterobacteriaceae* in some homogenates and water samples, perhaps due to the presence of other related genera inside this family or to the presence of *E. coli* under the limit of detection of the qPCR assay.

The beta-diversity analysis suggested that microbial communities were specifically associated to each depuration facility (Fig. 2B). In addition, the beta-diversity analysis including water samples indicated differences in the microbial composition of water samples compared to the homogenates (Fig. S4). This result suggests a possible host-specific microbiota which differs significantly from the microbial community present in the surrounding water, as reported in the study of Vezzulli and colleagues (2018).

Regarding the *Vibrio* species identified in depurated homogenates, NGS-MLSA revealed the presence of human pathogenic species, such as *V. algynoliticus*, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. Particularly, *V. cholerae* was detected in all of the four depuration plants investigated in this study, but not in non-depurated homogenates (Table 2B). PLANT A homogenates showed a higher abundance of *V. algynoliticus*, *V. cholerae* and *V. parahaemolyticus* with respect to the other depurated homogenates (Fig. S5). These findings could be again related to the absence of ozone treatment as previously described for PLANT A. In addition, *V. algynoliticus*, *V. parahaemolyticus* and *V. vulnificus* were also absent in non-depurated homogenates. Despite the detection of *Vibrio* human pathogenic species obtained with NGS approach, the qPCR analysis performed on homogenates and water samples was negative, possibly due to concentrations of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* that fell below the level of detection of the qPCR analysis, defined by the spike-in trial. In addition, while there was no amplification of human pathogenic *Vibrio*

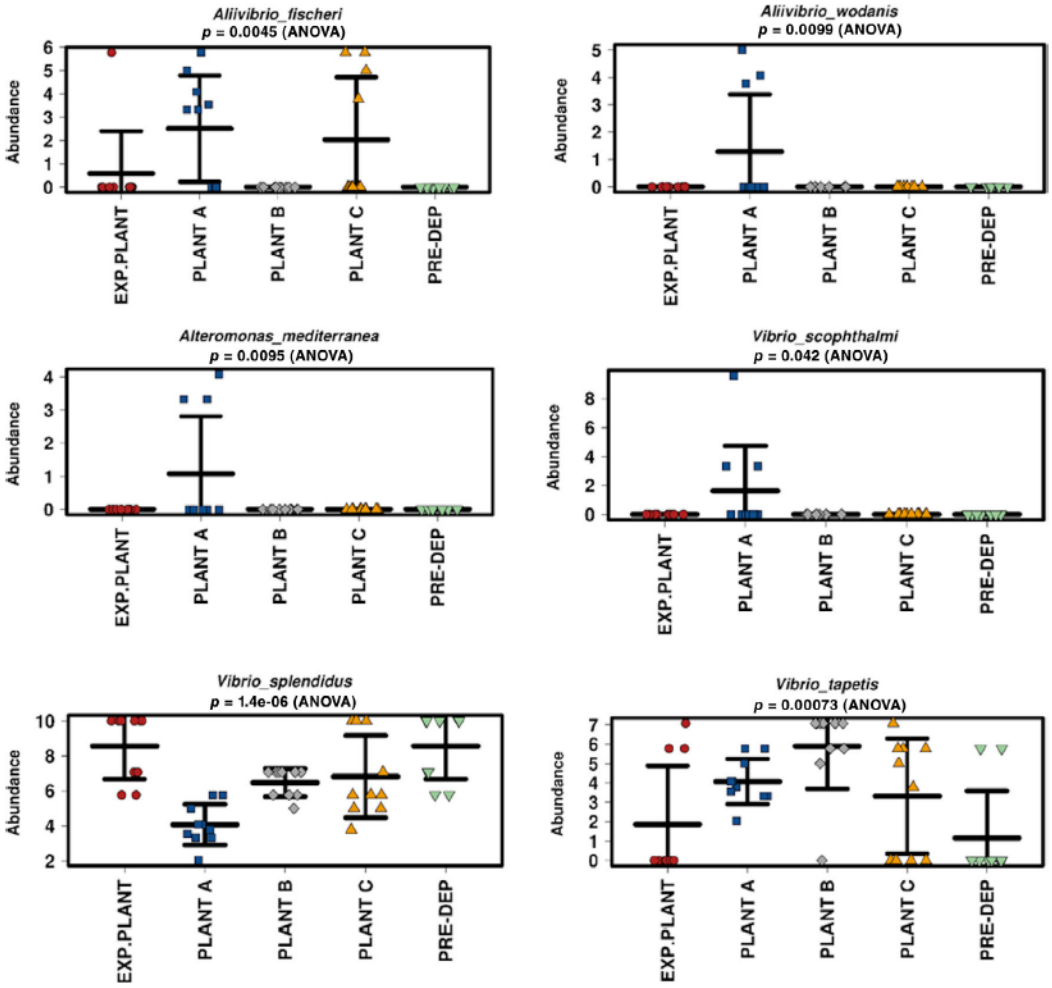

Fig 5. The six species statistically different in terms of abundance among depurated and non-depurated homogenates according to ANOVA analysis on *rec*A-*pyr*H merged data sequencing. [Color figure can be viewed at wileyonlinelibrary.com]

species in the qPCR-time assay, amplification was obtained for *Vibrio* genus in all homogenates. Moreover, the significant positive correlation (Spearman test: $R = 0.85$, p-value < 0.05) between qPCR *Vibrio* genus copy number and the NGS 16S reads corroborated the *Vibrio* contamination in homogenates. Consequently, there may well be a contamination of *Vibrio* spp. in homogenates with low concentrations of the main human pathogenic species. This result demonstrates the higher sensibility of the NGS-MLSA approach for the detection of human pathogenic *Vibrio* species, and was largely expected as the NGS protocol includes two steps of PCR amplification combined with the application of NGS for a more specific definition of the microbial community.

Human pathogenic *Vibrio* species identified in depurated homogenates might be again associated with the biofilm formation on tank surfaces. Biofilm production by *Vibrio* spp. is well documented in the existing literature, particularly for human pathogenic species (Yildiz and Visick, 2009; Liu *et al*., 2017). While the present study did not include the investigation of biofilms, merged *rec*A and *pyr*H may be a suitable technique to study biofilm community composition, providing valuable information to both shellfish farmers and authorities involved in food safety controls. As reported in the literature, biofilm samples can be harvested with swabs directly from the suspected biofilm surfaces for subsequent DNA extraction (Maukonen and Wirtanen, 2000).

The two genes applied in this study also provided a description of the *Vibrio* community by identifying several mollusc pathogens in addition to the human pathogenic species. Specifically, the application of NGS-MLSA with

MiniKraken2_v1_8GB database correctly assigned 90% of the obtained reads to *Vibrio* species. MiniKraken2_v1_8GB database offered 603 available refseq *Vibrio* genomes and 75 Vibrionacea genomes (Supporting Information). If compared to the database used in King and colleagues (2019), containing 106 different *Vibrio* species, MiniKraken2_v1_8GB database offered a wider representation of the *Vibrio* community thanks to the number of available genomes. Despite this, it is possible there remains a certain level of underestimation of *Vibrio* species detected in both homogenates and water samples since the number of identified *Vibrio* species is constantly increasing and changing (Romalde *et al*., 2014).

Similar to the findings of King and colleagues (2019), 15 different *Vibrio* species were identified in the collected samples. More specifically, 10 *Vibrio* species were identified in water samples and 12 in homogenates (Table 2). *V. broeganii*, *V. splendidus* and *V. tapetis*, which largely represent the *Vibrio* community of non-depurated homogenates, were also present in water and depurated homogenates. Moreover, six species contributed in significantly differentiating the community composition by depuration plant (Fig. 5). Specifically, the bivalve-pathogenic species *V. splendidus* and *V. tapetis* were particularly abundant in all homogenates both depurated and non-depurated ones. The persistence of *V. tapetis* in clam tissues could be explained by reduced phagocytic capacity of *R. philippinarum* haemocytes in the presence of the bacteria (Allam *et al*., 2001). In addition, the temperature range of depuration water tanks (13.5 ± 0.9) was within the optimum range for *V. tapetis* (Borrego *et al*., 1996), which may have played a role in facilitating the proliferation and persistence of *V. tapetis* inside the plant facilities as well as in Manila clam extra-pallial compartment. The abundance of *V. splendidus* in both depurated and non-depurated homogenates, collected and processed in the spring (May 2019), may be explained by the predominance of this species in seawater during the warmer seasons, and consequently their presence in bivalves tissues (Lacoste *et al*., 2001). Previously, Rahman and colleagues (2014) also indicated the presence of *V. splendidus* in *R. philippinarum* and *Mytilus galloprovincialis* samples collected in the Venice lagoon. These results suggest that the Venice lagoon environment may be an ideal niche for *V. splendidus*, especially during the spring season.

Subsequently, *Vibrio* community species results were compared to *Vibrio* species identified by single gene Sanger sequencing (*pyr*H) of isolates collected by plating the homogenates on TCBS. Kraken software identified 87% of the isolates as *V. splendidus*, leaving 10%, which did not achieve a correct *Vibrio* species attribution. *V. splendidus* may have been particularly abundant in the marine environment during the period of our experiment, and persisted in the facilities and bivalve tissues throughout the depuration procedures. In addition, the single gene sequencing allowed for the detection of *V. jasicida* and *V. antiquarius* (Harveyi clade) species, which were not seen in the NGS analysis. This is likely due to the stringent threshold cut-off applied to the NGS reads to avoid non-specific identification. More specifically, *V. jasicida* and *V. antiquarius* were present in the samples but their concentration may have been too low for a significant number of reads to be maintained after the filtering.

Conclusion

In conclusion, the NGS approach allowed for the interpretation of *Vibrio* dynamics, including the main human pathogenic species, inside four depuration plants and a better understanding of the modifications to *Vibrio* community composition based on the type of depuration treatment. The major outcomes could be summarized as: (i) MLSA-NGS analysis of *rec*A and *pyr*H appears successful in detecting and identifying the main human and molluscan pathogenic *Vibrio* species present within the Manila clam microbiota with higher sensitivity than qPCR, (ii) depurated homogenates revealed an increase in *Vibrio* species richness when compared to non-depurated homogenates, (iii) human pathogenic *Vibrio* species were found mainly into depurated homogenates, suggesting that depuration plants may act as a niche for the proliferation of and contamination due to these species. Fortunately, as expected for a bivalve farming area, the pathogenic species occurred at low frequency. In addition, qPCR analysis in spike-in samples suggested a low concentration of human pathogenic *Vibrio* species in the homogenates. In conclusion, application of MLSA-NGS based on *rec*A and *pyr*H as screening techniques could be useful for the management of mollusc hatcheries and for the prevention of bivalve mortalities, as well as for the prevention of human vibriosis caused by *V. alginolyiticus*, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. Finally, given the wide distribution of different *Vibrio* spp. in a great variety of aquatic environments, this sequenced-based approach could also represent a tool for seafood traceability and safety.

Experimental procedures

Sample collection and experimental design

A batch of clams was collected from the harvesting area of Chioggia 14L008 (N45°14′12″ E12°16′49″) during the spring season (May 2019). *R. philippinarum* individuals of commercial size (weight: 14.0 g ± 4.7, shell size: 35.9 mm ± 6.3, age: 20 months) were analysed before

and after depuration treatment. In details, an aliquot was analysed before depuration procedure; the other one was split to be analysed in four depuration facilities. For this experimental trial four depuration plants operating in Chioggia (Venezia, Italy) were selected. Technical information regarding depuration facilities and the technologies applied were recorded during the trial period (Table S2). Specifically, PLANT A, PLANT B, PLANT C with semi-closed recirculating system and the EXPERIMENTAL PLANT, with small-scale closed system. Biological and mechanical filter were applied in all four plants with also UV-treatment conducted on depuration water before and after the depuration process. The major differences among plants were represented by the absence of Ozone treatment on PLANT A and by the small-size and consequently density of clams depurated on EXPERIMENTAL PLANT. Five biological replicates were investigated according to each depuration plant. Each biological replicate consisted in a pool of flesh and intervalvular fluid to reach 25 g then collected in a sterile stomacher bag. In addition, samples processing also two technical replicates performed by two different operators. In total, 50 homogenates were obtained of which 10 non-depurated and 40 depurated. Analysis included also four water samples.

Microbiological analysis and colony identification

Live pre-depuration clams were stored at 4 °C for 24 h. Quantitative methods for *Vibrio* spp. and total microbial counts were performed and adjusted to design a suitable protocol for growth of *Vibrio* species (Caburlotto *et al*., 2016; EN ISO 21872-1:2017, 2017). Briefly, molluscs were scrubbed under running potable water and the shell cleaned with ethanol 100%. Molluscs were weighed, measured and shucked to obtain 25 g of flesh and intervalvular liquid then homogenized. The homogenization was performed, for 1 min, using a VWR Laboratory Blender, by adding 225 ml of Alcaline Peptone Water (APW, 2% NaCl, 1% Peptone, pH 8.5, Microbiol, Macchiareddu, CA) inside sterile bags. From each homogenate an aliquot of 3 ml was collected, of which 1 ml was used for serial dilutions performed on APW and the subsequently plating of 100 µl of each serial dilution on Marine Agar (MA) (Condalab, Cagliari, CA) and Thiosulfate-citrate-bile salts-sucrose agar (TCBS) (Biolife, Monza, MI) plates. The plates were incubated at 22 °C for 24–48 h (Brenner *et al*., 2005). The remaining homogenate aliquot of 2 ml was centrifuged at 10 000 rpm for 1 min to obtain a pellet (The Eppendorf centrifuge 5424). In addition, from each depuration centre 50 ml of spray bars water was collected, while from the experimental closed system only water from the depuration bin was collected. The water samples were processed as previously described for microbiological analysis. For the determination of microbial community, samples were centrifuged at 4000 rpm for 20 min (The Eppendorf centrifuge 5810). After discarding the supernatant, the pellet was re-suspended with 1 ml of Phosphate-buffered saline (PBS) and re-centrifuged for 1 min at 12 000 rpm (The Eppendorf centrifuge 5424). The obtained pellet samples were stored at − 80 °C until performing DNA extraction for culture independent analysis. Total microbial counts were expressed as log colony-forming units per gram of sample ($\log_{10}$ CFU g^{-1}) for total viable counts. Suspected colonies were selected and purified for the subsequent species identification. Based on their morphology a total of 60 suspected green and yellow *Vibrio* colonies grown on TCBS were transferred to fresh TSA + 2% NaCl plates and then incubated at 22 °C for 24 h.

Subsequently, to confirm *Vibrio* colonies, selected isolates were prepared for *pyr*H gene sequencing following the procedure described in Rahman and colleagues (2014) with some modifications. In details, each single pure colony was dissolved in 1 ml of PBS and then centrifuged for 1 min at 10 000 rpm (The Eppendorf centrifuge 5424). The supernatant was removed and the pellet re-suspended on 100 µl of sterile DNAse free water. Finally, DNA was extracted by boiling at 98 °C for 10 min in a 2720 Thermal Cycler Applied Biosystems. The purity and concentration of DNA were assessed using a Nanodrop ND-1000 (Thermo Scientific). Diluted DNA of isolates (1:50) was amplified in a final volume of 20 µl containing 2 µl of Dream Taq Buffer 10X (ThermoFischer, Massachuttes, USA), 0.2 µl of dNTPs 25 mM, 0.25 µl of *pyr*H primer (10 µM) (Rahman *et al*., 2014) and 0,1 µl of Taq (5 U μl^{-1}) (ThermoFischer). Thermal profile applied started with a denaturation at 94 °C for 2 min followed by 35 cycles of 94 °C for 20 s, 54 °C for 30 s, 72 °C for 30 s and a final extension at 72 °C for 7 min. PCR reaction was conducted in a 2720 thermal cycler (Applied Biosystem). Post-PCR products were purified with ExoSAP-IT™(ThermoFischer) and sent to MACROGEN (Amsterdam) for Sanger sequencing.

Mock community for culture independent analysis

A mock community was assembled with 25 *Vibrio* strains, belonging to 19 different species and one Vibrionaceae strain (Table S3) previously revitalized on TSA + 2% NaCl at 22 °C for 24 h. After incubation, one colony from each strain was re-suspended in 100 µl of sterile DNAse free water and incubated at 98 °C for 10 min in 2720 Thermal Cycler Applied Biosystems to perform the DNA extraction. Subsequently, DNA was centrifuged at 4000 rpm for 4 min (The Eppendorf centrifuge 5810) and the supernatant transferred to a fresh tube. DNA of all *Vibrio* strains was quantified by using a Nanodrop ND-

1000 (Thermo Scientific), then the final mock community was assembled by adding 10 µl of each DNA strain. Finally, the mock was diluted 1:100 for library construction.

DNA extraction and libraries preparation

Microbial community DNA of homogenate and water samples was extracted from the pellet obtained from 2 ml of homogenate and from 50 ml of water by using DNeasy® PowerSoil® Kit (Qiagen, Hilden, Germany) following the manufacturer's instruction. Illumina libraries for 16S rRNA, *rec*A and *pyr*H were prepared by performing two PCR steps of amplification. During the first PCR step, the amplification reaction prepares the amplicons for the subsequent insertion of sample specific barcode, which will take place at the second PCR step. In addition, second PCR step is required to prepare the amplicons to the binding to Illumina flow-cell for a successful sequencing process.

*rec*A and *pyr*H amplicons for NGS sequencing were obtained by adapting primers previously used for isolates identification (Rahman *et al*., 2014). Each DNA homogenate was diluted 1:5 and amplified in 20 µl in each of the three PCR replicates reaction per step. The thermal profile and PCR composition was conducted as described in Milan and colleagues (2018), with exception of the temperature used in the first PCR step for specific annealing of *rec*A and *pyr*H primers (Table S4), which was performed at 55 °C. The mock community was included in technical triplicate in the libraries as an internal control to verify the efficiency of *rec*A and *pyr*H genes on *Vibrio* species assessment. After each PCR step, amplified products were checked on an Agarose gel 1.8% and then purified using SPRIselect reagent Kit (Beckman Coulter Genomics). Briefly, purified samples were quantified using Qubit™ dsDNA BR Assay Kit as an end point fluorimetric detection. Final libraries were assembled in an equimolar pool, checked for quality using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and quantified using Qubit® Assay Kit BR. Libraries were sequenced by UCDAVIS Genome Center (California) using 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 PRJNA606686 and PRJNA612880.

Bioinformatic and statistical analyses

Before proceeding to the bioinformatic analyses, the quality of raw reads was checked and visualized by using FastQC software. After quality control, 16S rRNA raw sequence data were trimmed and merged with DADA2 and full analysis were conducted by using QIIME2 version platform (https://qiime2.org/). Subsequently, after trimming (with Trimmomatic) and filtering of *rec*A and *pyr*H, raw reads were imported to Kraken 2 software (https://ccb.jhu.edu/software/kraken2/) to perform full data sequencing analysis against MiniKraken2_v1_8GB database (Wood *et al*., 2019). Kraken 2 performs a mapping of input sequencing data against a reference database built from the refseq bacteria, archaea and viral libraries, to match and classify the *Vibrio* species present in sequencing data (https://ccb.jhu.edu/software/kraken2/index.shtml?t=downloads). Braken software was then used to carry out a Bayesian inference of abundance of species detected in data sequencing (https://ccb.jhu.edu/software/bracken/). Specifically, Braken estimates the number of reads originating from each species present in a sample by using the taxonomy labels assigned by Kraken 2 (Lu *et al*., 2017). Confidence cut-off was set at 0.1, as suggested by Kraken authors, and used to describe species attribution inside the bacterial community. In addition, a filter was applied for a minimum number of reads (10) reliable for *Vibrio* species detection.

Microbiological features such as total microbial counts and *Vibrio* counts were investigated by adopting different multivariate approaches. Hierarchical clustering was applied as an agglomerative approach based on the full linkage method using the PRIMER-e software (https://www.primer-e.com/). Non-metric multidimensional scaling (NMDS) plots were used for visualizing samples variability, i.e. dissimilarity between pairs of objects in a two-dimensional space. One-way PERMANOVA was adopted to estimate the effects of different factors as the comparison between operators, the depuration treatments and the statistically significant differences observed between production plants.

Output files of QIIME2 for 16S rRNA gene and Braken output for the *rec*A and *pyr*H combined reads were imported in CALYPSO software to carry out ecological and exploratory analysis. In the CALYPSO platform, the characterization of microbial communities was conducted according to the two main factors considered in the study: depuration treatment (treated vs. not-treated clams) and depuration plant (considering the four different systems). 16S Illumina sequencing yielded a total of 6.487.279 raw reads. Homogenates produced a total of 10.869.501 raw reads from *rec*A and *pyr*H genes merged. First, for 16S rRNA, *rec*A and *pyr*H sequences analysis, a rarefaction curve was done to estimate the representation of microbial community by the sequence data. Subsequently, for data sequencing data an evaluation of the microbial alpha diversity was carried out through an ANOVA analysis (p-value < 0.05) performed at the feature level according to Richness Index. For a first explorative analysis of microbial community data, Beta-diversity was visualized with a NMDS. Specifically,

for the *pyr*H/*rec*A the merged results (bracken matrix) were analysed according a qualitative approach (presence/absence of species). Taxonomic features were than analysed by PERMANOVA. One-way PERMANOVA were performed to highlight the effects of the fixed factors (depurated vs. not-depurated or to test differences according to depuration plants). In the case of significant effects, a posteriori pairwise comparisons were carried out. Finally, an ANOVA analysis (p-value < 0.05) was carried out to evaluate the abundance of genus and species for 16S and *rec*A/*pyr*H sequence data respectively, detected in the depurated and non-depurated samples.

qPCR and spike-in trial with V. cholerae, V. parahaemolyticus, V. vulnificus *and* E. coli *DNA*

The qPCR was performed on clam, water and mock samples to define total bacteria, *Vibrio* spp, *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus* and *E. coli* using the assays described in Milan and colleagues (2019) (16rRNA total bacteria and 16srRNA *Vibrio* spp.); Vezzulli and colleagues (2015) (*V. cholerae*); Nordstrom and colleagues (2007) (*V. parahaemolyticus*); Campbell and Wright (2003) (*V. vulnificus*); Walker and colleagues (2017) (*E. coli*). The reaction volume was 10 µl containing 5 µl Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, California), 10 µM each primer and 2.5 µl of template DNA. The LightCycler 480 System instrument (Roche, Basilea) was used for the amplification reaction. The cycling conditions were conducted as described in Carraro and colleagues (2018). Calibration curves (Log gene copy number vs. the cycle number at which the fluorescence intensity reaches a set cycle threshold value) were obtained using serial dilutions of pure bacterial culture genomic DNA ranging 5–0.003 ng. *E. coli* K12 was used for '16rRNA total bacteria' assay and *V. parahaemolyticus* for '16srRNA *Vibrio* spp.' assay. Copy numbers used in the calibration curves were calculated considering the genome size of the used strains (4.64 Mbp for *E. coli* and 5.09 Mbp for *V. parahaemolyticus*, www.ncbi.nlm.nih.gov/genome) and their 16S rDNA copy number (7 for *E. coli* K12 and 11 *V. parahaemolyticus*) (Stoddard *et al*., 2015).

The abundance of *Vibrio* spp. and the total bacteria spp. cells were calculated on the basis of qPCR results. The Cp (Crossing point) were used to calculate copy number with the formulas obtained from the calibration curves: $Cp = -36\,303 * \log_{10}(\text{copy number}) + 33\,852$ for 16S rRNA assay and $Cp = -35\,167 * \log_{10}(\text{copy number}) + 3233$ for *Vibrio* spp. Assay. The cell abundance were obtained dividing the total 16S rDNA copy number by the average of 16SrDNA copy number in Phyla bacteria ($n = 5$) and Vibrios ($n = 10$) obtained from rrnDB database (Vezzulli *et al*., 2012). Final total bacterial concentration was expressed as number of cells in 1 ml.

Subsequently, a spike-in trial was performed to define the level of sensitivity of qPCR assay on detection of *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus* and *E. coli* in DNA extracted from homogenates. In details, homogenate genomic DNA extract of samples 15 and 43 (same quantity used in library preparation), negative to the three *Vibrio* species and of samples 18 and 19 negative to *Enterobacteriaceae* (according to *rec*A-*pyr*H or 16S NGS sequencing), were spiked with *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus* and *E. coli* DNA ranging from 0.005 ng μl^{-1} (10^3 copies) to 0.00002 ng μl^{-1} (3 copies) and analysed with qPCR assays.

The correlation analysis to compare *Vibrio* contamination obtained by the two culture independent methods applied (qPCR *Vibrio* genus copies number and NGS 16S reads) was performed between by using Rstudio software (R version 4.0.0).

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Authors have no conflicts of interest to declare.

Data Availability Statement

The dataset analysed during the study is included in Supplementary information (Table S6). MiniKraken2_v1_8GB database list of available bacteria genomes is included in Supplementary information.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1: Supporting information

Fig. S1. Depuration effect (*p*-value: 0.002). Total microbial counts performed in MA and TCBS media (*Vibrio* spp.) of homogenates sample. Red boxplot correspond to a statistical analysis of 10 non-depurated homogenates, blue boxplot

corresponds to statistical analysis of 40 depurated homogenates. Thicker black lines in the boxes correspond to the medians.

Fig. S2. NMSD of microbial features according to Gower Index, *p*-value 0.0002; Vector TMC: Total Microbial counts performed on MA, Vector *Vibrio* spp.: colonies counted on TCBS.

Fig. S3. Rarefaction curve of depurated, non-depurated and water samples reads obtained by 16S sequencing.

Fig. S4. 16S amplicon based microbial beta-diversity of homogenates and water samples representation with NMDS.

Fig. S5. Abundance with ANOVA of the main human dangerous *Vibrio* species detected on depurated and non-depurated homogenates, according to *rec*A-*pyr*H merged data sequencing.

Fig. S6. qPCR total bacteria and *Vibrio* spp. evaluation in homogenate and water samples.

Table S1. Microbiological counts (log_{10} CFU g^{-1}) of the four water samples collected from each of the four depuration plants.

Table S2. Technical information of depuration facilities and environmental parameters of harvesting area.

Table S3. Strains list of mock composition.

Table S4. Primer used for amplification and sequencing of *Vibrio* species.

Table S5. qPCR counts of total bacteria (16rRNA). Vibrio genus, *V. cholera*, *V. parahaemolyticus*, *V. vulnificus* and of faecal indicators *E. coli*. (Cp = Crossing point detected by qPCR. Nd = Not detected).

Table S6. Supporting information.

Chapter 2. Robustness and Performance of the *rec*A-*pyr*H metabarcoding and development of culture-dependent metagenomics: Comparison of the methods

Once evaluated the sensitivity and specificity of the *rec*A-*py*rH metabarcoding approach and its usefulness on the characterization of the *Vibrio* species associated to the shellfish product, the new metabarcoding was used to perform a methodological application. Specifically, the methodological application consisted in a study in which the robustness of the new metabarcoding was used to compare the *Vibrio* microbial communities composition characterized by the cultural-independent and –dependent methods. Specifically, the *rec*A-*pyr*H metabarcoding was applied to analyse and compare the *Vibrio* community composition of total bacterial communities grown on the plate with the first serial dilution of each medium, called plated clam samples (culture-dependent method), with the counterparts provided by the clam homogenate samples (culture-independent method). The culture-dependent method adopted three different growth media such as Marine agar (MA), Thiosulfate-citrate-bile salts sucrose agar (TCBS) and CHROMagar Vibrio media (CV) to define marine bacteria, vibrios and potential human pathogenic *Vibrio* species such as *V. alginolyticus*, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, respectively. The investigation on the *Vibrio* community was performed on both clam homogenate and plated clam samples through 16S rRNA and *rec*A-*pyr*H metabarcoding. In addition, the total bacterial communities grown and collected on MA and TCBS plates from 16 samples was investigated using shotgun metagenomics. This methodological application of the new metabarcoding implemented the knowledge of the Manila clam *Vibrio* community composition and highlighted the usefulness of the combined use of culture dependent and -independent method to achieve a more complete *Vibrio* biodiversity description in order to prevent human vibriosis related to the contaminated seafood products. Specifically, the culture-dependent method coupled with the culture-independent ones resulted a valid tool to detect the main *Vibrio* human pathogenic species such as *V. alginolyticus*, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. In particular, the study reassessed the MA medium as a suitable cultural substrate for the recovery of several *Vibrio* species, the *Vibrio* pathogenic ones included. Moreover, in this study a new way to exploit the shotgun metagenomics to investigate on the *Vibrio* biodiversity associated to the Manila clam microbiota was developed. In particular, the

performance of the culture-dependent shotgun metagenomics on the *Vibrio* community characterization was compared to one achieved by the culture-dependent *rec*A-*pyr*H metabarcoding. Results obtained showed that the cultural-dependent shotgun metagenomics applied in only a subset of plated clam samples detected in all of these samples the presence of *Vibrio* species such as *V. alginolyticus*, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. This result demonstrated the expected higher sensitivity of the shotgun metagenomics respect to the *rec*A-*pyr*H metabarcoding on the detection of these species and provided important information about the potential risk of human Vibriosis related to contaminated shellfish product. The application of shotgun metagenomics on plated clam samples, in fact, offered the advantage to detect the *Vibrio* human pathogens in alive and consequently potentially virulent state. Despite the higher sensitivity of the shotgun metagenomics respect to the new metabarcoding approach, its application on culturable-independent samples is still an open challenge. The host DNA in fact tends to overwhelm bacterial DNA on shotgun metagenomics sequencing results. On the contrary, the new metabarcoding developed in this PhD study, successfully characterized the Vibrios biodiversity on both culture-dependent and -independent clam samples. This result highlighted the robustness of *rec*A-*pyr*H metabarcoding approach and its suitability to characterize *Vibrio* microbial community studied by using culture-dependent and –independent approaches.

Then, results obtained from the methodological application of *rec*A-*pyr*H metabarcoding suggested that the combined use of cultural-independent metabarcoding with culture-dependent shotgun metagenomics could represent valid and reliable tool to monitor the occurrence of *Vibrio* human pathogens associated to several seafood products such as fish, crustaceans and clams.

To conclude, the methodological application of the *rec*A-*pyr*H metabarcoding and culture-dependent metagenomics developed in this PhD study led to the publication of a scientific paper, which original full text is attached below:

- **Zampieri, A.**, Babbucci, M.; Carraro, L.; Milan, L.; Fasolato Cardazzo, B.; Combining Culture-Dependent and Culture-Independent Methods: New Methodology Insight on the *Vibrio* Community of *Ruditapes philippinarum*. *Foods*. 2021; 10(6): 1271. doi.org/10.3390/foods10061271
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Article

Combining Culture-Dependent and Culture-Independent Methods: New Methodology Insight on the *Vibrio* Community of *Ruditapes philippinarum*

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Abstract: Vibrios represent a natural contaminant of seafood products. *V. alginolyticus*, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* are the most hazardous species to human health. Given the worldwide consumption of mollusc products, reliable detection of *Vibrio* species is recommended to prevent human vibriosis. In this study, culture-dependent and -independent methods were compared and integrated to implement knowledge of the Manila clam *Vibrio* community composition. Here, 16S and *rec*A-*pyr*H metabarcoding were applied to compare the microbial communities of homogenate clam samples (culture-independent method) and their culture-derived samples plated on three different media (culture-dependent method). In addition, a subset of plated clam samples was investigated using shotgun metagenomics. Homogenate metabarcoding characterized the most abundant taxa (16S) and *Vibrio* species (*rec*A-*pyr*H). Culture-dependent metabarcoding detected the cultivable taxa, including rare species. Moreover, marine agar medium was found to be a useful substrate for the recovery of several *Vibrio* species, including the main human pathogenic ones. The culture-dependent shotgun metagenomics detected all the main human pathogenic *Vibrio* species and a higher number of vibrios with respect to the *rec*A-*pyr*H metabarcoding. The study revealed that integration of culture-dependent and culture-independent methods might be a valid approach for the characterization of *Vibrio* biodiversity.

Keywords: culture-dependent and -independent methods; metabarcoding; shotgun metagenomics; *Ruditapes philippinarum*; microbiota; *Vibrio* spp.

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1. Introduction

Vibrios, which are Gram-negative, rod-shaped bacteria, represent a ubiquitous constituent of marine and brackish ecosystems. Within *Vibrio* biodiversity, some species are associated with aquatic animals, while others have proved to be dangerous to human health. Among the Vibrios that affected marine animals, there are species such as *V. anguillarum* and *V. salmonicida* which are pathogenic to farmed fish, while *V. tapetis* is the main etiological agent of disease for molluscs at all life stages [1]. *V. alginolyticus*, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* were found to be the species responsible for the most serious human diseases [2].

Given the significant human health hazard represented by the worldwide distribution of *Vibrio*, reliable methods are required to detect and control their biodiversity distribution, particularly in seafood products. Seafood products, in fact, could represent a vehicle for the foodborne disease vibriosis [3,4]. In addition, it is demonstrated in the literature that the emergence of vibriosis could be related to the increase in global seawater temperature [5]. This phenomenon induces changes in *Vibrio* species distribution and the spread of enteropathogenic species into marine ecosystems.

Over the years, the approaches to characterize *Vibrio* biodiversity, with specific concern regarding human pathogenic species, included culture-dependent and -independent methods. The culture-dependent methods of the Food and Drug Administration's Bacteriological Analytical Manual (FDA-BAM 2004) and those of ISO/TS 21872-1:2017 are recommended by the health organizations [6].

Different selective media are applied to improve the detection and isolation of the main human pathogenic *Vibrio* species. Thiosulfate-citrate-bile salts-sucrose agar (TCBS) medium represents one of the consolidated selective media adopted to isolate some of the potential pathogenic human *Vibrio* spp. [7]. Moreover, CHROMagar Vibrio (CV) appears to differentiate *V. cholerae*, *V.parahaemolyticus* and *V.vulnificus* by exploiting the different colony colors taken in the chromogenic substrate [8]. Unfortunately, the culture-dependent methods present some limitations. Firstly, bacteria grown on specific media represent only a small fraction of the total community [9]. Specifically, only that part of the community for which the metabolic and physiological requirements can be reproduced in vitro can grow on plates [10]. Consequently, the culturing approach fails to reproduce the entire complex bacterial community present in a natural environment or substrate. In addition, because of the different replication times among culturable bacteria, the grown fraction obtained in plates is distorted. Furthermore, it is difficult to isolate *Vibrio* species present in a viable but non-culturable (VBNC) state [11,12]. Specifically, the VBNC state is a phenotype induced by different stress factors, such as low temperature or excessive UV light exposure, which increase the survival and tolerance of bacteria to harsh environmental conditions. It is a reversible state that could potentially restore bacteria in favorable conditions [13].

Despite the well-known limits of culture-dependent methods, the developed specific media for *Vibrio* spp. allow for focused research on pathogenic targets such as the species *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, which represent a potential risk for seafood consumers, avoiding the background of resident microbiota [8,14]. In addition, culture-dependent methods provide the recovery of low-abundant taxa that could be lost to culture-independent methods [15]. Moreover, culture-dependent methods offer the possibility to store, by the alive fraction of the bacterial community, single strains of potential human pathogenic *Vibrio* species in order to perform future genomic analyses [6].

Over the years, several studies have applied PCR-based techniques as culture- independent methods for directly extracting DNA from samples instead of culture in order to obtain more realistic knowledge of the vibrios community composition or of a specific lineage, such as that belonging to human pathogenic *Vibrio* species [16]. For this purpose, in *Vibrio* research, next-generation sequencing (NGS) technology, a PCR-dependent method, has been applied to investigate *Vibrio* biodiversity in environmental and seafood samples. In particular, NGS analysis conducted on 16S rRNA combined with the sequencing of housekeeping genes such as heat shock protein 60 (*hsp60*) and *rec*A-*pyr*H allowed for the identification of *Vibrio* species that naturally contaminate water, oyster, and Manila clam samples [17–19]. Moreover, this approach was used to successfully identify potential human pathogenic species such as *V. alginolyticus*, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. PCR-dependent techniques, such as metabarcoding, are a valid tool to investigate complex microbial communities, such as the one related to the food matrix [20]. One of the disadvantages of PCR-dependent techniques is that they do not discriminate alive from dead bacteria [21]. Moreover, amplicon-based methods present an intrinsic bias in the amplification step [22]. Designing inclusive universal primers located in regions more informative than 16S for species/strains' identification is challenging, and only parts of species/strains are successfully amplified [17–19]. The PCR bias can be overcome by using a shotgun metagenomics approach. Shotgun metagenomics has the advantageous capability to sequence all the DNA extracted from a sample, but it requires a huge amount of reads to obtain a reasonable coverage of the microbial genomes as a consequence of the predominance of host DNA [23,24]. There are several commercial kits available to remove host DNA, acting prior to DNA extraction (pre-extraction methods) or after DNA

extraction (post-extraction methods), but most of them were developed for liquid samples (saliva, blood, milk) from mammalian hosts [25].

Even if culture-dependent and -independent methods present specific limits, as previously described, a combination of these two approaches could still provide a more comprehensive and accurate overview of bacterial community compositions, as suggested by several studies [26,27]. Following this idea, the objective of this study was to explore the *Vibrio* community composition of *Ruditapes philippinarum* microbiota by combining culture-dependent and -independent methods. Specifically, DNA metabarcoding, developed in a previous study [19], was applied to evaluate and compare the accuracy of isolation and discrimination of *Vibrio* species identified on homogenate clam samples and on their culture-derived samples plated on marine agar (MA), thiosulfate-citrate-bile salts-sucrose agar (TCBS) and CHROMagar Vibrio (CV) media. On a subset of these culture-derived clam samples, shotgun metagenomics was applied in order to enhance the knowledge of the *Vibrio* spp. biodiversity present in Manila clam microbiota.

2. Materials and Methods

2.1. Sample Collection and Experimental Design

Ruditapes philippinarum individuals of commercial size (weight: 12.9 ± 4.5 g; shell size: 36.2 ± 2.9 g; age: 20 months) were collected from six clam-farming sites along the northeast coast of the Adriatic Sea: (from north to south) Marano (MA), Porto Marghera (PM), Colmata (CO), two sites in Chioggia (CH), Scardovari (SC) and Goro (GO). Sample collection was performed during the summer and the winter seasons. For each site, the batch of clams was analyzed before and after the depuration treatment. The experimental study was designed in order to use a metabarcoding and a shotgun metagenomics approach to analyze and compare the *Vibrio* community composition of isolates from the plate with the first serial dilution of each medium (see Section 2.2), called plated clam samples (culture-dependent method), with the counterparts provided by the clam homogenate samples (culture-independent method). The culture-dependent method adopted three different growth media to define marine bacteria, vibrios and potential human pathogenic *Vibrio* species such as *V. alginolyticus*, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. The investigation on the *Vibrio* community was performed on both clam homogenate and plated clam samples through 16S rRNA and *rec*A-*pyr*H metabarcoding. In addition, the community composition of isolates collected on MA and TCBS plates from 16 samples was investigated using shotgun metagenomics (Figure 1, created with Biorender.com). In total, 54 homogenate clam samples were obtained, of which 26 were collected in the summer season and 28 in the winter season (Table S1, Supplementary Materials).

2.2. Microbiological Analysis

Clams were processed as described in a previous study of Zampieri et al. [19], with the addition of a microbiological analysis performed using a selective medium coupled with the temperature of incubation required for the detection of the main human pathogenic *Vibrio* species. Briefly, clams were scrubbed under running potable water and the shell cleaned with ethanol 100%. Subsequently, clams were weighted, measured and shucked to collect 25 g of flesh and intervalvular liquid clam tissues into a sterile stomacher bag. For the homogenization, 225 mL of alkaline peptone water (APW, 2% NaCl, 1% peptone, pH 8.5; Microbiol, Macchiareddu, CA, USA) was added and then tenfold serial dilution was performed. Subsequently, for samples of the summer season, 100 µL of each serial dilution was plated on marine agar (MA) (Condalab, Cagliari, CA, USA) and thiosulfate-citrate-bile salts-sucrose agar (TCBS) (Biolife, Monza, MI, USA) media. For samples collected during the winter season, in addition to TCBS and MA media, 100 µL of each serial dilution was plated on CHROMagar Vibrio media (CV) (CHROMagar Microbiology, Paris, France) for the isolation of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. The plates of TCBS and CV media were incubated at 22 and 37 °C for 24–48 h [28]. The MA plates were incubated at 22 °C for 24 h. After incubation was completed, the total bacterial

communities grown on MA, CV and TCBS media (dilution -1) were gathered by scrubbing and washing the surface of the plates with 2 mL of phosphate-buffered saline (PBS). Then, suspended cells were centrifuged at 10,000 rpm for 1 min (Eppendorf centrifuge 5424) and the pellets were stored at −80 °C until performing the DNA extraction. For determination of the homogenates' microbial community, from each homogenate, an aliquot of 2 mL was collected and then centrifuged at 10,000 rpm for 1 min to obtain a pellet (Eppendorf centrifuge 5424). Then, the pellets were stored at −80 °C until performing the DNA extraction for culture-independent analysis.

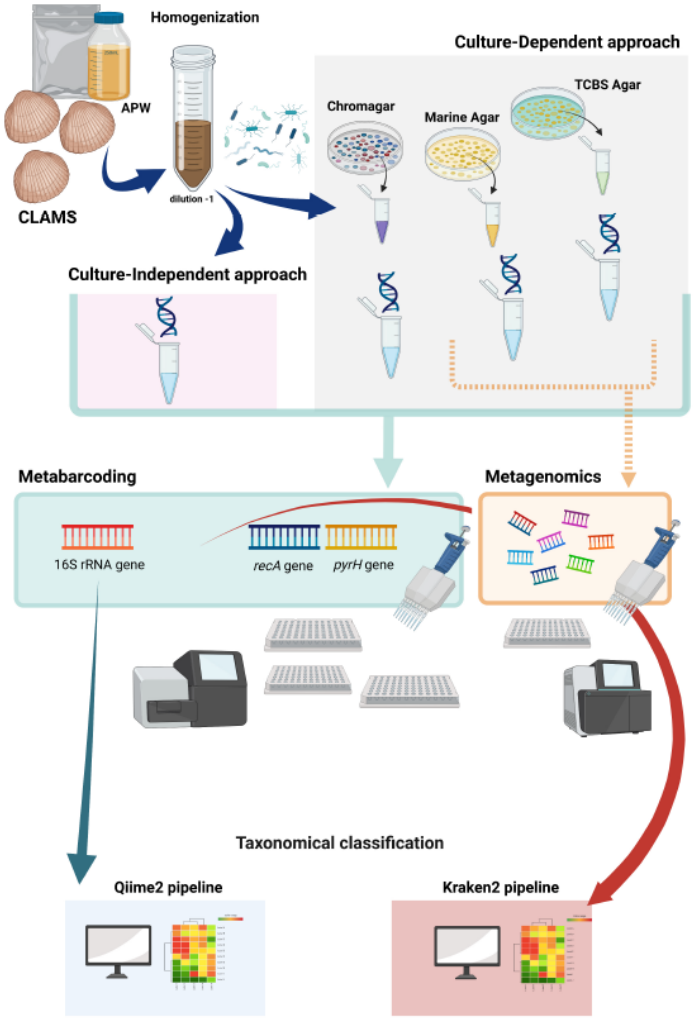


Figure 1. Flow chart of the experimental design. APW: alkaline peptone water; TCBS agar: thiosulfate-citrate-bile salts-sucrose agar.

2.3. DNA Extraction and Libraries' Preparation for Metabarcoding

DNA samples of the microbial communities collected from the MA, TCBS and CV media were extracted using the Invisorb® Spin Tissue Mini Kit (Invitek molecular, GMBH, Berlin, Germany) following the manufacturer's instructions. For the microbial community of homogenates, DNA was extracted using a DNeasy® PowerSoil® Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. As described in a previous study [19], a mock community DNA sample was included into the sequencing libraries analysis as an internal control. Subsequently, each DNA sample was diluted for Illumina library preparation, performed on 16S rRNA, *rec*A and *pyr*H genes. Specifically, the DNA of

homogenates was diluted to 1:5, while DNA from each plated clam sample was diluted to reach 2 ng/µL. Illumina libraries were prepared using a two-step approach as described in a previous study [19]. PCR composition and thermal profiling was conducted as reported in [29], using, in the first PCR step, the specific temperature of annealing for the *rec*A and *pyr*H genes as indicated by Zampieri et al. [19]. The result of each PCR step was verified on agarose gel 1.8% and purified using the SPRIselect reagent Kit (Beckman Coulter Genomics) following the manufacturer's instructions. Quantification of the purified samples was conducted using a Qubit ™ dsDNA BR Assay Kit (Invitrogen, Life Technologies, Monza, Italy)) as an end-point fluorometric detection. The equimolar pool of the final libraries was checked for the quality using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and for the quantification using a Qubit® Assay Kit BR. The 16S rRNA, *rec*A and *pyr*H libraries were sequenced at the UC Davis Genome Center (California) using the MiSeq System, Illumina (300 bp forward and reverse). The raw sequence data were deposited in the NCBI database with the following BioProject ID: PRJNA726587.

2.4. Shotgun Metagenomics Libraries Preparation

For a subset of 16 plated clam samples, DNA quantity control was performed using a Qubit dsDNA HS Assay (Invitrogen, Life Technologies, Monza, Italy). The library construction was assessed using a Nextera XT DNA Sample Preparation Kit (Illumina, Inc., San Diego, CA, USA) with IDT for Illumina Nextera DNA UD Indexes. Then, a 2100 Bioanalyzer (High Sensitivity DNA Assay, Agilent Technologies) was used to verify the quality of the libraries. Finally, the libraries were run on an Illumina Novaseq Sp500 PE250 (Illumina, Inc., San Diego, CA, USA). The raw sequence data were deposited in the NCBI database with the following BioProject ID: PRJNA726531.

2.5. Bioinformatic and Statistical Analyses for Metabarcoding

Firstly, the quality of the raw reads of homogenates and plated clam samples was checked and visualized using FastQC software (version 0.11.9). Subsequently, the bioinformatics analysis procedure followed that described in a previous study [19]. In brief, 16S rRNA raw sequence data were trimmed and merged with DADA2, and a full analysis was conducted using the QIIME2 platform (https://qiime2.org/, QIIME2 version accessed on 10 February 2021). To assign taxonomy, a Naïve Bayes classifier was employed using the SILVA138 release data. After trimming and filtering using Trim Galore v. 0.6.6 (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/, accessed on 10 April 2020), the *rec*A and *pyr*H sequences were imported to Kraken 2 software (https://ccb.jhu.edu/software/kraken2/, accessed on 10 February 2020) to perform a full data sequencing analysis against the MiniKraken2_v1_8 GB database [30]. Then, Bracken software was used to carry out a Bayesian inference of abundance of the species detected during data sequencing (https://ccb.jhu.edu/software/bracken/, accessed on 10 February 2020). This software used the taxonomy labels assigned by Kraken 2 to estimate the number of reads deriving from each species present in a sample [31]. As conducted in a previous study [19], a confidence cut-off set at 0.1 was applied in order to define species attribution inside the bacterial communities of homogenates and plated clam samples. In addition, a filter was applied to set the minimum number of reads (10) reliable for *Vibrio* species detection. The homogenate and plated clam samples produced a total of 45,000,000 raw reads and 50,000,000 raw reads for 16S Illumina Miseq and *rec*A-*pyr*H libraries sequencing, respectively. Firstly, a rarefaction curve was drawn to assess the representation of microbial communities defined by 16S rRNA gene. Subsequently, an exploratory analysis of the output files of QIIME2 and Bracken software was carried out using the CALYPSO platform [32]. The microbial alpha diversity of 16S rRNA data sequencing was studied using an ANOVA (p-value < 0.05) carried out at the feature level (ASV) according to the Richness Index. Then, the beta microbial diversity was described using a PCoA representation. The statistic test PERMANOVA was used to evaluate the different community compositions according to 16S rRNA (ADONIS +, according to the Bray–Curtis

index) and *rec*A-*pyr*H data sequencing (PERMANOVA Pairwise comparisons). As reported in a previous study, *rec*A-*pyr*H merged results were elaborated using a qualitative approach (presence/absence of species) [19]. The number of shared taxa and bacterial species, identified by 16S rRNA and *rec*A and *pyr*H genes, respectively, among homogenate and plated clam samples was visualized through Venn diagrams. Finally, the agreement among the detection of each *Vibrio* species using homogenate (*rec*A-*pyr*H culture-independent method) and clam samples plated and incubated at 22 °C on MA and TCBS media (*rec*A-*pyr*H culture-dependent method) was evaluated using kappa statistical measures [33]. Specifically, we used Fleiss' kappa, for which values were summarized as poor agreement ($k < 0$), slight agreement ($0.0 < k < 0.20$), fair agreement ($0.21 < k < 0.40$), moderate agreement ($0.41 < k < 0.60$), substantial agreement ($0.61 < k < 0.80$) and almost perfect agreement ($0.81 < k < 1$).

2.6. Bioinformatic and Explorative Analyses for Shotgun Metagenomics

The shotgun metagenomics libraries yielded a total of 120,000,000 raw reads (forward and reverse) for all samples. The shotgun metagenomics data elaboration started with the application of metaWRAP, a specific pipeline for metagenomic analysis [34], on the demultiplexed reads. Reads' trimming and human contamination removal were performed using the metaWRAP Read_qc module (including Trim Galore, Cutadapt and BMTagger tools with default parameters). Taxonomic profiling was performed on the trimmed reads with the metaWRAP Kraken module for metagenomics (cut-off set at 0.1, against MiniKraken2_v1_8 GB database). Bracken (Bayesian Reestimation of Abundance with Kraken) was used to compute the abundance of bacterial species using the taxonomy labels assigned by Kraken. The bracken report files were converted into a biom file by using the Kraken-biom tool. Subsequently, the microbial community composition was investigated with a qualitative assessment based on the presence/absence of species identified. In detail, a heat map was defined using the output Bracken files of the 16 plated clam samples investigated through shotgun metagenomics and the corresponding output Bracken files of the homogenate and plated clam samples investigated through metabarcoding. Specifically, the heat map, based on a matrix file of the presence/absence of detected species, was realized by using ComplexHeatmap package in R (version 4.0.3). As described in a previous study conducted by Zampieri et al. [19], for the creation of the matrix of presence/absence, a reliable filter was used, with a minimum of ten reads. Then, Venn diagrams were generated to visualize the number of shared species among the clam samples plated on MA 22 °C and TCBS 22 °C media according to metabarcoding and shotgun metagenomics.

3. Results

3.1. 16S Metabarcoding-Based Microbial Communities of Homogenate and Culture-Derived Clam Samples

The microbiota composition of homogenate and plated clam samples was investigated through 16S rRNA amplicon sequencing in order to evaluate the recovery capability of each culture media. The rarefaction curve of filtered reads showed a good representation of the microbial communities (Figure S1, Supplementary Materials). Furthermore, the alpha diversity analysis revealed a higher level of taxa in the homogenate samples with respect to the clam samples plated and incubated at 22 °C on MA and TCBS media. The clam samples plated on TCBS 22 °C media showed a lower level of taxa with respect to the homogenate and clam samples plated on MA 22 °C media (Figure 2a). Then, the variation in the bacterial communities (beta diversity) was explored among the homogenate and the plated clam samples. Communities from CV 22 °C and TCBS 37 °C were not included in this analysis because these media and conditions of incubation allowed the growth of a limited number of taxa. The multivariate analysis demonstrated a different community composition of homogenate samples compared to the plated clam samples (p-value < 0.001). Moreover, a different microbial composition was also observed depending on the season, where summer clam samples' results were different from those of the winter clam samples

(*p*-value < 0.001). The variation in the bacterial communities was then visualized with a PCoA representation, according to the Bray–Curtis index. The PCoA representation reported in Figure 2b shows a clear clustering of homogenate samples separated from the clam samples plated on MA 22 °C and TCBS 22 °C media. The communities from the two media instead showed a partial overlapping of samples. In addition, the effect of the season of sample collection clearly separated summer clam samples from the winter clam samples.

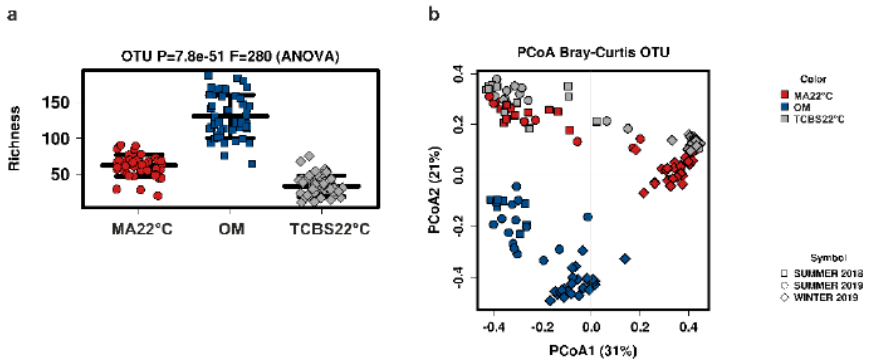


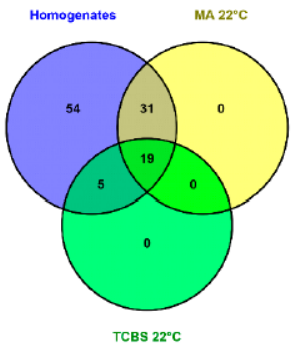
Figure 2. 16S amplicon-based microbial diversity of clam samples. (**a**) 16S amplicon-based alpha diversity tested with ANOVA according to Richness Index. (**b**) 16S amplicon-based microbial principal coordinate analysis (PCoA) representation of homogenate and plated clam samples according to the Bray–Curtis Index. In PCoA representation, the season factor corresponding to the collection time of clam samples is also reported. OM: Homogenate clam samples; MA 22 °C: clam samples plated on marine agar and incubated at 22 °C; TCBS 22 °C: clam samples plated on thiosulfate-citrate-bile salts-sucrose agar and incubated at 22 °C.

Subsequently, the number of taxa shared among the homogenate and the plated clam samples was described through a Venn diagram, according to a matrix of the presence/absence of taxa (Figure 3). As previously demonstrated in the alpha diversity analysis, the homogenate samples showed a higher taxa biodiversity, evaluated at the genus level, with respect to the clam samples plated on MA 22 °C and TCBS 22 °C media. In total, 19 taxa were shared among the homogenate clam samples and the clam samples plated on MA 22 °C and TCBS 22 °C media. Specifically, the taxa found in the homogenate samples comprised all the taxa in the clam samples plated on TCBS 22 °C and MA 22 °C media (Figure 3).

3.2. recA-pyrH Metabarcoding on Homogenate and Plated Clam Samples

The 16S metabarcoding provided a description of the complete bacterial community, while *rec*A and *pyr*H sequencing were performed to investigate the *Vibrio* and Vibrionaceae species biodiversity highlighted by the culture-dependent and -independent methods. Firstly, the diversity of microbial species composition (beta diversity) was investigated among homogenate and plated clam samples. Pairwise comparisons, according to PERMANOVA analysis, showed statistical differences in the *Vibrio* community composition between homogenate samples and plated clam samples on MA 22 °C, TCBS 22 °C, TCBS 37 °C and CV 22 °C media (*p*-value < 0.001). In addition, PERMANOVA analysis revealed a different community composition of homogenate samples (culture-independent method) in comparison with metabarcoding on culture media when also considering the season stratification (*p*-value 0.0001). Subsequently, the variation in the *Vibrio* community between samples was visualized using a PCoA representation according to the Jaccard index (Figure S2, Supplementary Materials). Specifically, the PCoA showed a higher dispersion of homogenate and clam samples plated on MA22 °C and TCBS 22 °C media. Clam samples plated on CV 22 °C and TCBS 37 °C media were more clustered with respect to

the other clam samples. Moreover, the PCoA representation showed a clear separation of samples collected in winter with respect to the ones collected in the summer season (Figure S2, Supplementary Materials). Then, Venn diagrams were generated to represent the number of *Vibrio* and Vibrionaceae species shared among the homogenate and plated clam samples. Firstly, the number of species shared among homogenate samples and clam samples plated and incubated at 22 °C was studied (Figure 4a). Subsequently, the effect of the two temperatures of incubation (22 and 37 °C) on the number of species shared among homogenate and clam samples plated on TCBS medium was investigated (Figure 4b). In total, 11 taxa including *Photobacterium* and *Vibrio* species were shared among the homogenates and the clam samples plated on MA, TCBS and CV at 22 °C (Figure 4a). Among the *Vibrio* species, the main potential human pathogenic *Vibrio* species, such as *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, were detected. All the culture media at 22 °C evidenced taxa that were not identified in the homogenate samples, and MA showed a higher number of *Vibrio* species with respect to the homogenate samples and to the other plated samples. According to the comparison of the homogenates with TCBS samples at the two temperatures of incubation, the homogenates shared 15 taxa, including 13 *Vibrio* species and 2 Vibrionaceae, with plated samples (Figure 4b). Again, the potential human pathogenic *Vibrio* species, such as *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, were included among the shared species between homogenates and clam samples plated on TCBS medium. The clam samples plated on TCBS 22 °C showed a higher number of *Vibrio* species with respect to the clam samples plated on TCBS medium and incubated at 37 °C (Figure 4b).

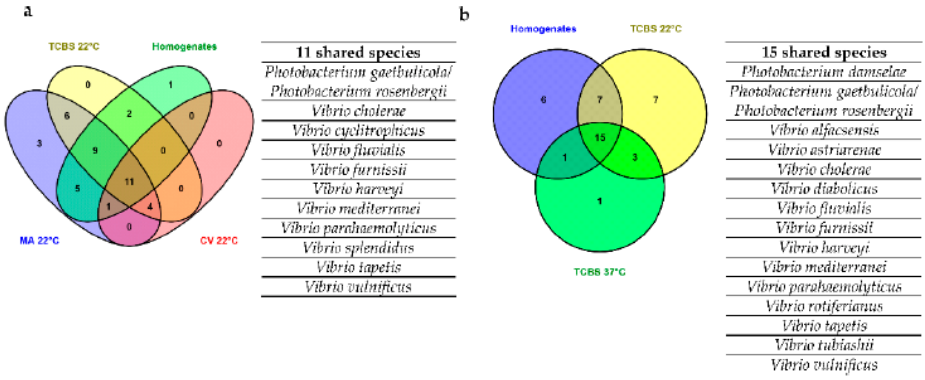


19 shared taxa
Acinetobacter
Candidatus Fritschea
Cutibacterium
Endozoicomonas
Flavobacterium
Lactobacillus
Listeria
Marinomonas
Mycoplasma
NS3a marine group
Photobacterium
Polaribacter 4
Polynucleobacter
Pseudoalteromonas
Pseudomonas
Shewanella
Spirochaeta 2
Staphylococcus
Vibrio

Figure 3. Venn diagram showing the number of taxa, identified by 16S metabarcoding at the genus level, shared among homogenate samples and clam samples plated on MA 22 °C and TCBS 22 °C media. The table report the detail of the nineteen taxa shared among homogenate and plated clam samples. MA 22 °C: clam samples plated on marine agar and incubated at 22 °C; TCBS 22 °C: clam samples plated on Thiosulfate-citrate-bile salts-sucrose agar and incubated at 22 °C.

Then, the kappa statistical measures were evaluated among homogenate and clam samples incubated at 22 °C and plated in MA and TCBS media. The kappa statistical measures revealed a significant agreement (p-value < 0.05) in the detection of thirteen *Vibrio* species and three Vibrionaceae species (Table S2, Supplementary Material). Specifically, eight *Vibrio* species reported a moderate agreement, and two *Vibrio* species reported a significant statistical agreement in terms of detection among the three approaches. Regarding human pathogenic *Vibrio* species, the detection of *V. parahaemolyticus* and *V. vulnificus* showed a significant moderate agreement among homogenate and plated clam samples. *V. cholerae* and *V. alginolyticus*, on the other hand, showed a slight and not significant agreement among the culture-independent methods and metabarcoding on MA and TCBS,

respectively. In addition, among the potential *Vibrio* species pathogenic to molluscs, species such as *V. tapetis* and *V. splendidus* showed a statistically fair agreement (Table S2, Supplementary Materials). Moreover, considering culturable and not culturable approaches, clam samples accounted for a noticeable percentage of human pathogenic *Vibrio* species. *V. alginolyticus* was detected in 16.7%, *V. cholerae* in 66.7%, *V. parahaemolyticus* in 56.3% and *V. vulnificus* in 54.2% of the samples.



11 shared species
Photobacterium gaetbulicola/ Photobacterium rosenbergii
Vibrio cholerae
Vibrio cyclitrophicus
Vibrio fluvialis
Vibrio furnissii
Vibrio harveyi
Vibrio mediterranei
Vibrio parahaemolyticus
Vibrio splendidus
Vibrio tapetis
Vibrio vulnificus

15 shared species
Photobacterium damselae
Photobacterium gaetbulicola/ Photobacterium rosenbergii
Vibrio alfacsensis
Vibrio astriarenae
Vibrio cholerae
Vibrio diabolicus
Vibrio fluvialis
Vibrio furnissii
Vibrio harveyi
Vibrio mediterranei
Vibrio parahaemolyticus
Vibrio rotiferianus
Vibrio tapetis
Vibrio tubiashii
Vibrio vulnificus

Figure 4. Venn diagram showing the number of *Vibrio* and Vibrionaceae species, identified by *rec*A-*pyr*H metabarcoding, shared among homogenate and clam samples plated on MA 22 °C, TCBS 22 °C and CV 22 °C media (**a**) and among homogenate and clam samples plated on TCBS 22 °C and TCBS 37 °C media (**b**). The two tables report the details of the number and names of shared species among homogenate and plated clam samples. MA 22 °C: clam samples plated on marine agar and incubated at 22 °C; TCBS 22 °C: clam samples plated on thiosulfate-citrate-bile salts-sucrose agar and incubated at 22 °C; CV 22 °C: clam samples plated on CHROMagar *Vibrio* media and incubated at 22 °C.

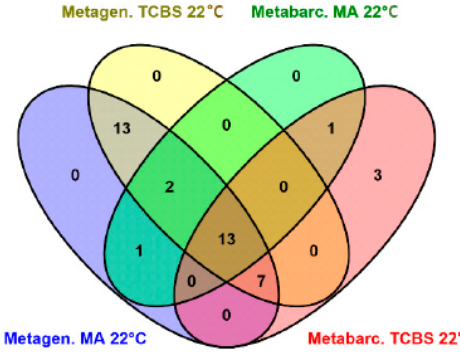
3.3. Shotgun Metagenomics of Plated Clam Samples and Comparison of Metagenomics and recA-pyrH Metabarcoding Community

Shotgun metagenomics was applied to 16 plated clam samples, including 8 from MA 22 °C and 8 from TCBS 22 °C, in order to obtain more accurate knowledge of the *Vibrio* species community composition. Three clam samples (3A, 7A, 8A) plated on MA medium produced low-quality raw reads during the shotgun metagenomics sequencing and, consequently, were not included in the analysis (Tables S1 and S3, Supplementary Materials). Then, the shotgun metagenomics data were compared to the results obtained by *rec*A-*pyr*H metabarcoding on the same plated clam samples.

The number of species commonly identified by shotgun metagenomics and *rec*A-*pyr*H metabarcoding was visualized using a Venn diagram. In total, the Venn diagram reported 12 *Vibrio* and 1 Vibrionaceae species commonly identified by metagenomics and metabarcoding in the two media, MA and TCBS (Figure 5). The potential human pathogenic *Vibrio* species, such as *V. parahaemolyticus* and *V. vulnificus*, were among the 13 shared species. In addition, metabarcoding conducted on the eight clam samples plated on TCBS medium identified two *Vibrio* (*V. ponticus*, *V. Scap*.24) and one Vibrionaceae (*P. gaetbulicola/P. rosenbergii*) species not detected by shotgun metagenomics.

From each specific medium, several species were identified by both shotgun metagenomics and metabarcoding. Specifically, in MA and TCBS media, shotgun metagenomics and metabarcoding commonly identified 13 *Vibrio* and 3 Vibrionaceae species (Figure S3, Supplementary Materials) and 19 *Vibrio* and 1 Vibrionaceae species, respectively (Figure S4, Supplementary Materials). Subsequently, these shotgun metagenomics and metabarcoding data were compared to the results obtained by *rec*A-*pyr*H metabarcoding of the homogenates of the same 16 samples. The comparison was visualized using a heat map representation, as reported in Figure 6. Specifically, shotgun metagenomics identified, in all samples, the main potential human pathogenic *Vibrio* species such as *V. alginolyticus*, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. On the contrary, metabarcoding did not

find *V. alginolyticus* in the homogenate and plated clam samples. *V. cholerae* was identified only in one homogenate sample (4). Moreover, *V. parahaemolyticus* and *V. vulnificus* were identified by metabarcoding, but not in all of the homogenate and plated clam samples (Figure 6).



13 shared species
Photobacterium damselae
Vibrio astriarenae
Vibrio campbellii
Vibrio cyclitrophicus
Vibrio diabolicus
Vibrio furnissii
Vibrio harveyi
Vibrio mediterranei
Vibrio parahaemolyticus
Vibrio rotiferianus
Vibrio splendidus
Vibrio tapetis
Vibrio vulnificus

Figure 5. Venn diagram showing the number of *Vibrio* species found in clam samples plated on MA and TCBS media according to the *rec*A-*pyr*H metabarcoding and shotgun metagenomics results. The table reports the details of the thirteen species commonly identified by metabarcoding and shotgun metagenomics. MA 22 °C: clam samples plated on marine agar and incubated at 22 °C; TCBS 22 °C: clam samples plated on thiosulfate-citrate-bile salts-sucrose agar and incubated at 22 °C; Metagen.: shotgun metagenomics; Metabarc.: metabarcoding.

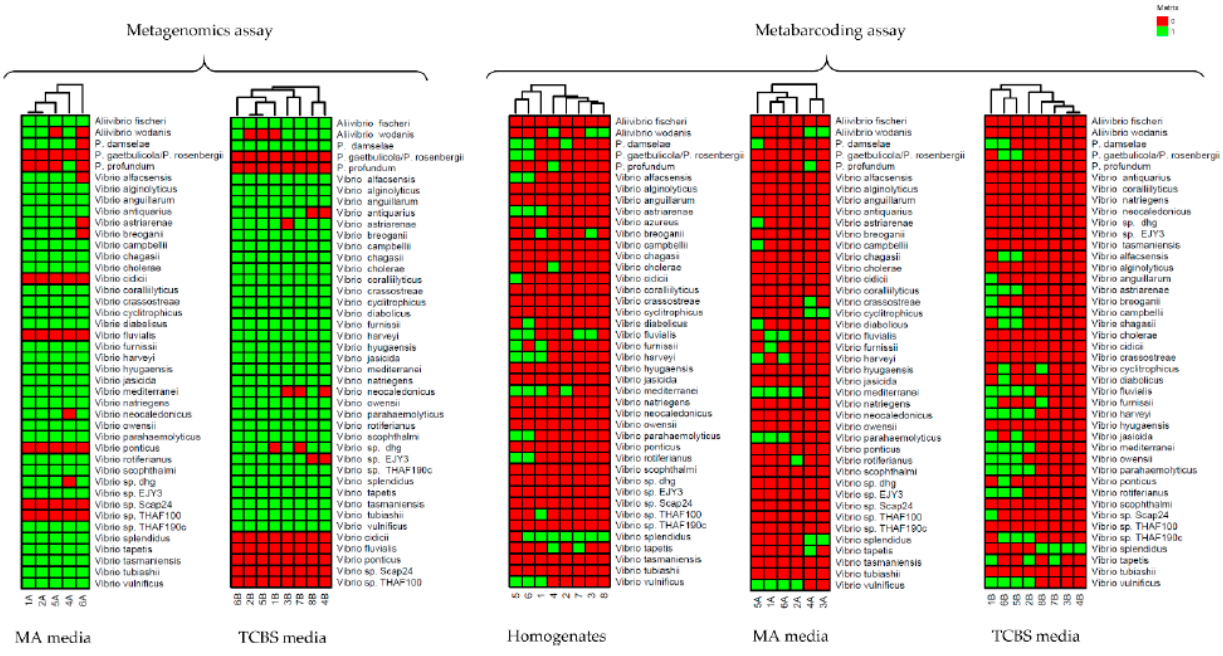


Figure 6. Heat map showing the presence (green) and the absence (red) of Vibrios found in clam samples (homogenates and clam samples plated on MA and TCBS media) according to shotgun metagenomics and *rec*A-*pyr*H metabarcoding. The letter "A" is assigned to samples plated on marine agar (MA) medium; the letter "B" is assigned to samples plated on thiosulfate-citrate-bile salts-sucrose agar (TCBS) medium. A number code (from 1 to 8) is assigned to each homogenate sample. P.: Photobacterium.

Heat map representation was also used to describe the additional species not belonging to the Vibrionaceae family that were detected by metabarcoding and shotgun metagenomics. In particular, shotgun metagenomics identified 26 and 25 marine bacteria species not belonging to the Vibrionaceae family in MA and TCBS media, respectively (Figure S5). Specifically, *rec*A-*pyr*H metabarcoding detected *Litorilituus sediminis* and *Haemophilus parainfluenzae* only in two homogenate samples and *Shewanella sp. Scap07* in one clam sample plated in TCBS medium (7B). These species were not found by shotgun metagenomics (Figure S5, Supplementary Materials).

4. Discussion

Seafood products represent reservoirs and vectors for several *Vibrio* species, including the main human pathogenic *Vibrio* species such as *V. alginolyticus*, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* [1,35–37]. Consequently, an up-to-date knowledge of the *Vibrio* species biodiversity present in seafood products requires accurate methods of detection and identification of these marine bacteria. Over the years, in order to prevent the spread of human vibriosis, the characterization of *Vibrio* species associated with seafood products has been carried out using culture-dependent and/or -independent methods [6]. A recent research field involved in the characterization of microbial communities reported the advantage of the combined use of culture-dependent methods with culture-independent ones. Specifically, these studies highlighted the complementarity of these two methods when applied for the characterization of microbial biodiversity [15,38]. Following this promising coupled approach, in this study, culture-dependent and -independent methods were used in parallel to increase the knowledge on Manila clam *Vibrio* community compositions, with specific concern regarding the *Vibrio* species that are hazardous to human health.

First, the variation in bacterial community composition was evaluated, according to 16S rRNA data sequencing, in homogenate samples (culture-independent method) and clam samples plated on MA and TCBS media (culture-dependent method). In this first evaluation, the community compositions of clam samples plated on TCBS and incubated at 37 °C (TCBS 37 °C) and clam samples plated on CV and incubated at 22 °C (CV 22 °C) were not considered. These media and temperatures of incubation showed specificity and selectivity for the detection of the main human pathogenic *Vibrio* species such as *Vibrio parahaemolyticus* [14,39] and, consequently, did not offer a general overview of the bacterial community composition.

In agreement with Manuel Anguita-Maeso et al. [26], our results suggested that microbial composition is strongly dependent on the use of culture-dependent and -independent methods. The selection performed using the culture-dependent method was corroborated by the higher biodiversity of taxa found in the homogenate samples with respect to the plated clam ones (Figures 2a and 3). This higher biodiversity could be explained by the direct extraction of DNA from the clam homogenate samples, which contributed to the detection of those marine taxa not recovered in the culture media as they were either not alive (or in a dormant VBNC state) or not able to grow in the culture media [40]. Moreover, the different microbial compositions of the homogenate and plated clam samples could derive from the effect of the different selections of cultural media, MA and TCBS, on the taxa present in clam samples (Figure 2b). The non-selective medium MA is commonly applied for the recovery of a wide range of marine bacteria [41]. TCBS medium, instead, is primarily used for the detection of human pathogenic *Vibrio* species such as *V. cholerae* and *V. parahaemolyticus*. Besides this application, TCBS medium was also useful for the recovery of several environmental vibrios [42]. All microbial communities, defined by both culture-dependent and -independent methods, could be well differentiated by season (Figure 2b). This result is in line with several studies in which the seasonal shifts of microbial communities are reported in freshwater and seawater ecosystems [43,44].

The investigation on clam-associated microbiota performed with 16S metabarcoding was completed using *Vibrio*-specific metabarcoding (based on *rec*A-*pyr*H genes) for the characterization of the *Vibrio* community, paying specific attention to the potential

human pathogenic *Vibrio* species. Clam samples plated on CV (CV 22 °C) and TCBS media (TCBS 37 °C) were also included in the analysis of *Vibrio* community composition, incubated at 22 and 37 °C, respectively. As expected, PERMANOVA analysis revealed a different *Vibrio* biodiversity community between homogenate and plated clam samples. The PCoA representation of homogenate and plated clam samples, in fact, presented a higher dispersion with the clusters of clam samples plated on TCBS 37 °C and CV 22 °C media (Figure S2). Clam samples plated on TCBS 37 °C and CV 22 °C media, indeed, showed a lower number of *Vibrio* and Vibrionaceae species with respect to the other clam samples plated on TCBS (TCBS 22 °C) and MA (MA 22 °C) media incubated at 22 °C (Figure 4a,b). This result is in accordance with the fact that culture conditions, represented by TCBS and CV media composition coupled with a specific temperature of incubation, are commonly applied to carry out investigations on restricted *Vibrio* biodiversity, represented mostly by the potential human pathogenic *Vibrio* species. The stringent selection performed using TCBS medium incubated at 37 °C represents a step in the ISO/TS 21872-1:2017 standard procedure for the detection, enumeration and isolation of enteropathogenic *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus*. Similarly, CV medium has been applied in several studies to characterize the main human pathogenic species, such as *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, by exploiting the chromogenic substrate offered by this medium [8,39]. Curiously, clam samples plated on MA 22 °C medium resulted in a higher number of *Vibrio* species identified compared to the other plated and homogenate samples (Figure 4a). This suggested that MA medium, despite not being inclusive for the general clam microbiota, is a valid substrate for the detection of several environmental *Vibrio* species, including potential human pathogenic species such as *V. alginolyticus*, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. Moreover, the MA medium allowed for characterizing rare species, such as *V. casei*, *V. hyugaensis* and *V. rumoieinsis*, which were not found in the other plated clam and homogenate samples. Consequently, despite the common application of MA medium as a non-selective medium for culturing several marine bacteria, it could also be considered adequate for application, coupled with *rec*A-*pyr*H metabarcoding, for the target isolation of *Vibrio* species. In a recent study, MA medium was applied by the authors as a positive control medium, which showed a comparable growth of environmental vibrios isolates to the one obtained from the modified TCBS medium used in the study [45]. This study demonstrated the advantage of the combined use of culture-dependent and culture-independent methods for the detection of human pathogenic *Vibrio* species (Table S1, Supplementary Materials). Specifically, despite the comparable reliability in the detection of *V. parahaemolyticus* and *V. vulnificus* (significant moderate k agreement), the combination of culture-dependent and -independent methods was necessary for the detection of *V. alginolyticus* and *V. cholerae* (slightly not significant k agreement) (Table S2, Supplementary Materials). Unlike a previous study [19], *V. alginolyticus* was not found in homogenate samples. However, this potential human pathogenic *Vibrio* species was detected by the culture approach in the clam samples plated on TCBS 22 °C, TCBS 37 °C, CV 22 °C and MA 22 °C media. This result could be explained by the low concentration of *V. alginolyticus* on homogenate samples, which, covered by the other outgrown *Vibrio* species, was not found by the *rec*A-*pyr*H metabarcoding. In addition, given the halophilic nature of *V. alginolyticus* species [46], it is possible to assume that the plating step performed on TCBS, CV and MA media, satisfying the salt requirement of this species, promoted the growth and subsequent molecular detection. This hypothesis can be corroborated by the study of Tagliavia et al. [46], in which the authors demonstrated that increasing the salt concentration of a medium used for *Vibrio* spp. detection allows for a more accurate estimation of the actual presence of *Vibrio* species in dilution plate counts.

Moreover, TCBS medium coupled with two different temperatures of incubation (22 °C and 37 °C) was also useful for the detection of several environmental *Vibrio* species not identified in the homogenate samples. Again, the *Vibrio* species not identified in the homogenate samples were probably present in these samples, but at such a low level that they were unable to compete with the other more abundant species, and consequently,

they were not detected (Figure 4b). Moreover, only in clam samples plated on TBCS 37 °C medium was *V. mimicus* found. These culturing conditions are required to isolate this species, closely related to *V. cholerae*, which represents a potential risk for marine animals and humans [47]. Moreover, as previously described, the *Vibrio* community composition among homogenate and plated clam samples showed microbial changes depending on the season of sample collection. In line with this result, several studies reported different *Vibrio* biodiversity in seafood products depending on the time of samples' collection [48,49]. In addition, the kappa statistical measures showed the complementarity of culture-dependent methods coupled with the independent ones in the detection of the juvenile/adult molluscan pathogens *V. tapetis* and *V. splendidus* [50]. Specifically, the homogenate samples and the clam samples plated on MA 22 °C and TCBS 22 °C showed a significant, fair agreement for the detection of these two *Vibrio* species. For these reasons, the application of culture-dependent methods in combination with culture-independent ones is strongly recommended to detect these species. The presence of *V. tapetis* and *V. splendidus* in *Ruditapes philippinarum* microbiota is in accordance with a previous study by Zampieri et al. [19].

In order to obtain a more complete picture of *Vibrio* species biodiversity, in this study, shotgun metagenomics was also applied on 16 MA- and TCBS-plated clam samples. The metagenomics investigation was not applied on homogenate samples because host DNA tends to overwhelm bacterial DNA on sequencing results [51].

Subsequently, the *Vibrio* communities identified by shotgun metagenomics were compared with the results of the *rec*A-*pyr*H metabarcoding analysis conducted on the same 16 plated clam samples (Figure 6). In this analysis, 16S RNA gene results were not included because they only perform taxonomic identification of the vibrios bacterial community up to the genus level [52].

The shotgun metagenomics data more accurately characterized microbial community composition. Specifically, shotgun metagenomics offered a more complete picture of the *Vibrio* and non-*Vibrio* biodiversity (Figure 6, Figure S5, Supplementary Materials) present in Manila clam microbiota. Similarly to the results achieved, other studies demonstrated that shotgun metagenomics provides a better resolution on the species taxonomic level compared to metabarcoding [53,54]. Consequently, as expected, *rec*A-*pyr*H metabarcoding identified a limited number of *Vibrio* species in clam samples plated on MA and TCBS media and on homogenate samples (Figures S3 and S4, Supplementary Materials, Figure 6). This finding suggested that the limit of metabarcoding in *Vibrio* spp. detection is not only related to the culture-dependent and -independent methods used to process clam samples; in fact, a similar number of Vibrionaceae and *Vibrio* species were identified in homogenates and on clam samples plated on MA and TCBS media (Figure 6). This result confirmed the bias of the PCR-based approach in the amplification of different species and the difficulties in designing completely inclusive genus-specific universal primers [17–19].

The main human pathogenic *Vibrio* species, such as *V. alginolyticus*, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, identified by shotgun metagenomics were not found in all of the samples with *rec*A-*pyr*H metabarcoding. This result confirms the higher level of sensitivity of shotgun metagenomics with respect to metabarcoding for the detection of these species, which are of particular concern for human health, that frequently occur in low concentrations. In addition, it is important to point out that the application of metagenomics on plated clam samples offered the advantage of detecting the main viable human pathogenic *Vibrio* species, such as *V. alginolyticus*, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, in an alive and, consequently, potentially virulent state.

Nevertheless, metabarcoding identified *P. gaetbulicola/rosenbergii*, *V. cidicii*, *V. fluvialis*, *V. ponticus*, *V.sp.Scap.24* and *V.sp.THAF100*, which were not detected by shotgun metagenomics (Figure 6). Of these, *P. gaetbulicola/rosenbergii*, *V.sp.Scap.24* and *V. ponticus* were detected only in clam samples plated on TCBS medium (Figure 5). It is likely that these six species belong to that part of rare bacterial biodiversity for which shotgun metagenomics presents a limited resolution. In accordance with these results,

Srivathsan et al. [55] achieved better detection of rare taxa by using metabarcoding with respect to shotgun metagenomics.

Shotgun metagenomics is currently applied to map microbial contamination in the food industry [24,53,56]. This study showed the usefulness of carrying out a microbial community characterization using culture-dependent shotgun metagenomics. Specifically, the application of shotgun metagenomics on plated clam samples overcomes the problem of host DNA that could overwhelm microbiota DNA. Moreover, this approach offered a less time-consuming characterization of the alive fraction of bacteria down to the species taxonomic level. For this reason, the cultural-dependent shotgun metagenomics reduced the cost of the analysis to one required by the metabarcoding approach. Given this, culture-dependent shotgun metagenomics could be applied in the seafood industry, for example, screening techniques to prevent human vibriosis that could be spread by contaminated seafood products such as fish, crustaceans or shellfish. In addition, in the aquaculture sector, both metabarcoding and shotgun metagenomics could also be useful for the characterization of the biofilm microbial composition, which could comprise *Vibrio* species pathogenic for humans and species pathogenic to farmed animals [57].

5. Conclusions

In the current study, the culture-dependent method coupled with the culture- independent method was a valid tool to achieve the characterization of several *Vibrio* species in the *Ruditapes philippinarum* microbiota. The culture-dependent and -independent methods showed a comparable reliability for the detection of *V. parahaemolyticus* and *V. vulnificus*. On the contrary, the two methods proved to be complementary for the detection of *V. alginolyticus* and *V. cholerae*. The obtained results suggested a reassessment of MA as a suitable medium for the recovery of Vibrios, including the main human *Vibrio* pathogenic species. Moreover, the study showed that metabarcoding, applied on the homogenate samples and on clam samples plated on MA media, could represent a useful screening approach for the prevention of human vibriosis related to contaminated seafood. Furthermore, the comparison of the shotgun metagenomics and metabarcoding results highlighted the higher number of species detected by shotgun metagenomics during the *Vibrio* species detection. In particular, the application of cultural-dependent shotgun metagenomics offered a reliable and accurate characterization of the alive fraction of bacteria down to the species taxonomic level.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/foods10061271/s1, Table S1: Summary of the total 54 clam samples collected during summer and winter seasons. Samples are subdivided according to the culture-independent and -dependent methods. Clam samples were plated on the following: A: MA22 °C, Marine agar incubated at 22 °C; B: TCBS 22 °C, Thiosulfate-citrate-bile salts-sucrose agar incubated at 22 °C; C: CV 22 °C, CHROMagar *Vibrio* media incubated at 22 °C: D: TCBS 37 °C, Thiosulfate-citrate-bile salts-sucrose agar incubated at 37 °C. Sampling sites: CH: Chioggia; CO: Colmata; GO: Goro; MA: Marano; PM: Porto Marghera; SC: Scardovari. 1: Pre-depuration samples; 2: Post-depuration samples. Table S2: Fleiss' kappa agreement of *Vibrio* species detection in homogenate and clam samples plated and incubated at 22 °C on MA and TCBS media according to *rec*A-*pyr*H metabarcoding. The *Vibrio* species with a significant statistical Fleiss' kappa are shown in bold. Fleiss' kappa values agreement interpretation: k < 0—poor; k = 0.01–0.20—slight; k = 0.21–0.40—fair; k = 0.41–0.60—moderate; k = 0.61–0.80—substantial; k = 0.81–1—almost perfect; Table S3. Resulting sequencing depth obtained by the shotgun metagenomics; Figure S1: Rarefaction curve of homogenate and plated clam samples reads obtained by 16S sequencing. OM: Homogenate clam samples; MA 22 °C: clam samples plated on marine agar and incubated at 22 °C; TCBS 22 °C: clam samples plated on Thiosulfate-citrate-bile salts-sucrose agar and incubated at 22 °C; CV 22 °C: clam samples plated on CHROMagar *Vibrio* media and incubated at 22 °C. Figure S2: *rec*A-*pyr*H amplicon-based microbial principal coordinate analysis (PCoA) representation of homogenate and plated clam samples according to Jaccard index. In PCoA representation, the season factor corresponding to the collection time of clam samples is also reported. OM: Homogenate clam samples; MA 22 °C: clam samples plated on marine agar

and incubated at 22 °C; TCBS 22 °C: clam samples plated on thiosulfate-citrate-bile salts-sucrose agar and incubated at 22 °C; CV 22 °C: clam samples plated on CHROMagar *Vibrio* media and incubated at 22 °C. Figure S3: Venn diagram showing the number of species identified in clam samples plated in MA medium and incubated at 22 °C, according to shotgun metagenomics and *rec*A-*pyr*H metabarcoding. The table reports the details of the 16 species shared between the two methods. MA 22 °C: clam samples plated on marine agar media and incubated at 22 °C. Figure S4: Venn diagram showing the number of species identified in clam samples plated in TCBS medium and incubated at 22° C, according to shotgun metagenomics and *rec*A-*pyr*H metabarcoding. TCBS 22 °C: clam samples plated on thiosulfate-citrate-bile salts-sucrose agar and incubated at 22 °C. Figure S5: Heat map showing the presence (green) and absence (red) of marine bacteria, *Vibrio* species excluded, found in clam samples according to metagenomics and *rec*A-*pyr*H metabarcoding.

Author Contributions: Conceptualization, B.C., L.F. and M.M.; methodology, B.C., L.F. and L.C.; formal analysis, A.Z., L.C., L.F. and M.B.; investigation, B.C., L.F. and A.Z.; resources, B.C. and L.F.; data curation, A.Z. and L.C.; writing—original draft preparation, A.Z.; writing—review and editing, B.C., L.F. and L.C.; supervision, B.C. and L.F.; project administration, B.C. and L.F.; funding acquisition, B.C. and M.M. All authors have read and agreed to the published version of the manuscript.

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Chapter 3. Broad-range application: Evaluation of the *Vibrio* community composition associated with the microbiota of *Ruditapes philippinarum* farmed along the North-east Adriatic coast

This last chapter of the PhD thesis aimed to explore and to characterise the *Vibrio* species biodiversity associated with the microbiota of the Manila clam (*R. philippinarum*), by performing a broad-range application of *rec*A-*pyr*H metabarcoding. Specifically, all the homogenate clam samples collected during the three years of the PhD study were investigated according to three fixed factors such as the clams' origin site, the season of collection and the depuration effect, which defined the experimental design.

During the three years of the PhD, the collection of clam samples was carried out in a collaboration between the University of Padua and the shellfish stakeholders within the framework of the 'PROGETTO ECCEAQUA' project. This last received a Ministero dell'istruzione, dell'università e della ricerca (MIUR) funding thanks to the recognised high-quality and innovative nature of research on the conservation of large marine vertebrates and on the health and safety of aquaculture products. Moreover, the present PhD project was carried out within the project SID 2018 "Microbial ecology, food safety and quality in seafood production" founded by the Università degli Studi di Padova. In addition, the PhD was realized within the framework of the 'PORFESR: Sostenibile, sicuro, di alta qualità: un progetto integrato di ricerca industriale per l'innovazione della filiera molluschicola del Veneto finanziato dalla regione Veneto Bando POR FESR 2014-2020' project, which supports the environmental research field.

In the experimental design were defined three fixed factors such as the clams' origin site, the season of collection and the depuration effect in order to better comprehend the *Vibrio* community composition changes in Manila clam microbiota in response to the wild and anthropic environment, respectively.

Moreover, the study was carried out on *Ruditapes philippinarum* species, which is one of the most cultured molluscs throughout the word. Within the European market, Italy produces about 55,000,000 tons of Manila clams each year (Turolla *et al.*, 2020). Specifically, in Italy the farming and harvesting activities of this shellfish are mainly distributed in the North-east Adriatic lagoons such as the Venetian Lagoon, Sacca di Goro and the Po River delta.

Given this geographical location of clam harvesting areas, the clams' origin site factor was represented by the sites of clam samples collection, which were located in seven operating shellfish farming areas along the North-east Adriatic coast (from North to South): Marano (MA), Porto Marghera (PM), Colmata (CO), Chioggia (CH) two sites, Scardovari (SC) and Goro (GO) (Figure 3).

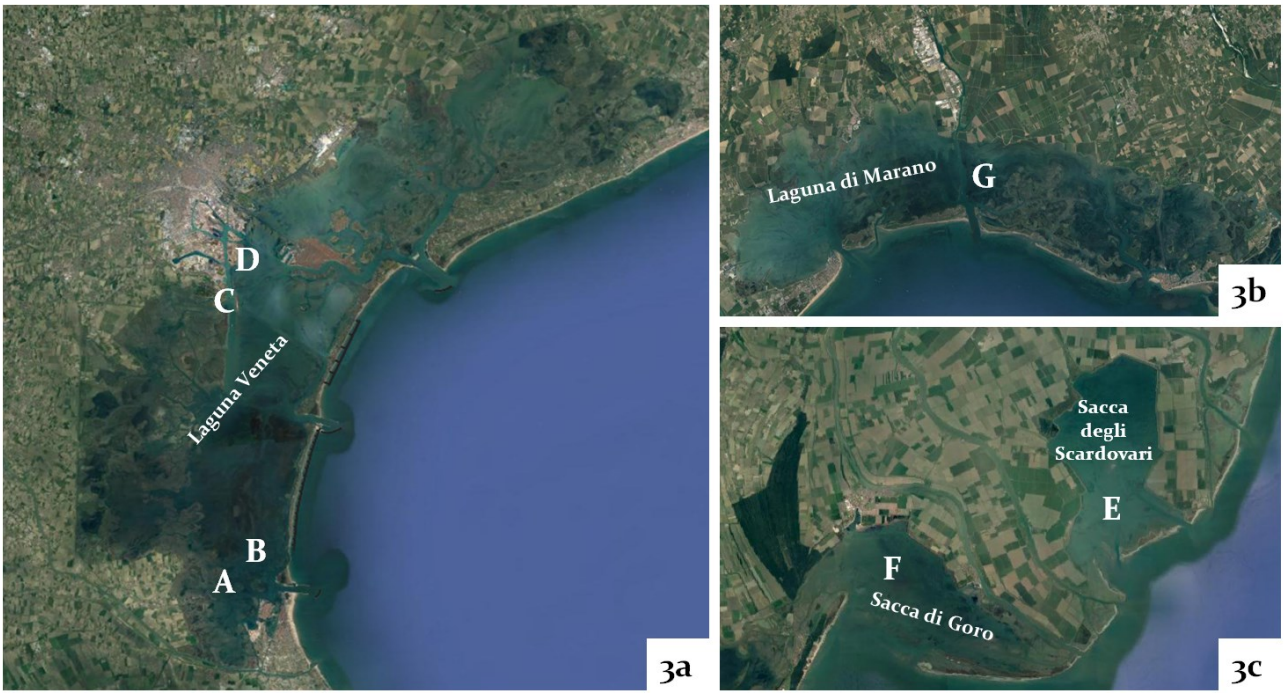


Figure 3. Map of the Venetian Lagoon (3a), the Marano Lagoon (3b) and the Po River delta (3c) indicating the seven Manila clam sampling sites. A, 2Chioggia; B, 1Chioggia; C, Colmata; D, Porto Marghera; E, Scardovari; F, Goro; G, Marano. Maps created using the Free and Open Source QGIS.

Among these seven sites, Marano, 1Chioggia, 2Chioggia, Scardovari and Goro are located in operating shellfish farming areas classified as zone B. According to European Regulation (UE) 2019/627, zone B areas are suitable for shellfish production, but the animals require a depuration treatment to guarantee a microbiologically safe product for human consumption. The Porto Marghera and Colmata sites belong to a part of the Venetian Lagoon classified as not suitable for clam farming (DGR3366/2004). In these areas of the lagoon, the high anthropogenic pressures related to the intensive industrial and harbour activities carried out in Porto Marghera have led to a severe chemical contamination of sediments, soils and inner tidal canals (Zonta *et al.*, 2007).

The location of the clam sampling sites in authorized farming area offered the possibility to carry out investigation on the potential risk represented by *Vibrio* human pathogenic species associated to shellfish seafood product, such as Manila clam, destined to the human consumption. In addition, the inclusion in the project of polluted areas in which is forbidden

the clam farming could represent an interesting point of investigation on the potential changes of *Vibrio* community in Manila clam microbiota related to the presence of chemical pollution. In literature, in fact is demonstrated that the exposure to chemical pollution affects the microbial community composition of *Ruditapes philippinarum* microbiota (Milan *et al.,* 2018) and the occurrence of the *Vibrio* human pathogens in coastal waters (Goh *et al*., 2017).
For what concern the season of clam samples collection factor, it was defined by the time of clam samples collection carried out during winter and summer season. Specifically, the sampling started in the summer of 2018 and ended in the winter of 2020. Specifically, the clam samples were collected during two summers (2018 and 2019) and two winters (2019 and 2020). Then, the depuration effect consisted in the evaluation of the *Vibrio* spp. community composition changes in response to the depuration treatment carried out in a different depuration plant for each batch of clams. The only exception was given by the clam samples collected in Porto Marghera and Colmata sites, which were both depurated in the same depuration plant. In addition, the depuration water tank parameters and the technologies applied in the facilities during the depuration process of each depuration plant were noted. These data are provided in the Appendix (Table 1a and 1b).
Given this experimental design, the broad-range application of *rec*A-*pyr*H metabarcoding was aimed to characterize the *Vibrio* community composition of the *R. philippinarum* microbiota according to the three fixed factors giving a particular concerning on the detection of *Vibrio* human pathogens namely *V. alginolyticus*, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. The results presented and discussed in this third chapter of the thesis will be integrated with additional analyses that are ongoing in the laboratory for a final publication.

Materials and methods

Batches of *R. philippinarum* individuals of commercial size were collected in the clam-farming areas located in Marano, Porto Marghera, Colmata, 1Chioggia, 2Chioggia, Scardovari and Goro. The sampling was carried out during the summer and the winter, specifically during the months of June 2018, January 2019, June 2019 and January 2020. Each batch of living clams selected for the study was split into two aliquots, one for the pre-depuration analyses and the other one to the post-depuration analyses. Specifically, each batch of clams was destined to a different depuration plant to carry out its depuration. Only Porto Marghera and Colmata batches were both depurated in the same depuration plant.

The study of the *Vibrio* community composition present in the *R. philippinarum* microbiota was carried out by using culture-dependent and culture-independent methods (Figure 4) for each homogenate clam sample. Specifically, the culture-dependent method consisted of plating each sample on two different culture media to perform the microbiological counts and to quantify the total marine bacteria and *Vibrio* concentration.

The culture-independent method comprised the direct extraction of the bacterial DNA from an aliquot of each sample. In total, during the 3-year PhD project, 120 homogenate clam samples were collected, of which 50 were collected from seven farming site during the summer and 70 during the winter (Appendix, Table 2). Moreover among these samples 60 were pre-depuration homogenate clam samples and 60 were post-depuration ones. The bacterial and *Vibrio* communities were characterised by using 16s rRNA and *rec*A–*pyr*H metabarcoding. The analysis included the effect of the three fixed factors – the depuration effect, the clams' origin site and the season of collection – on the *Vibrio* biodiversity, with particular attention to the detection of *Vibrio* human pathogens.

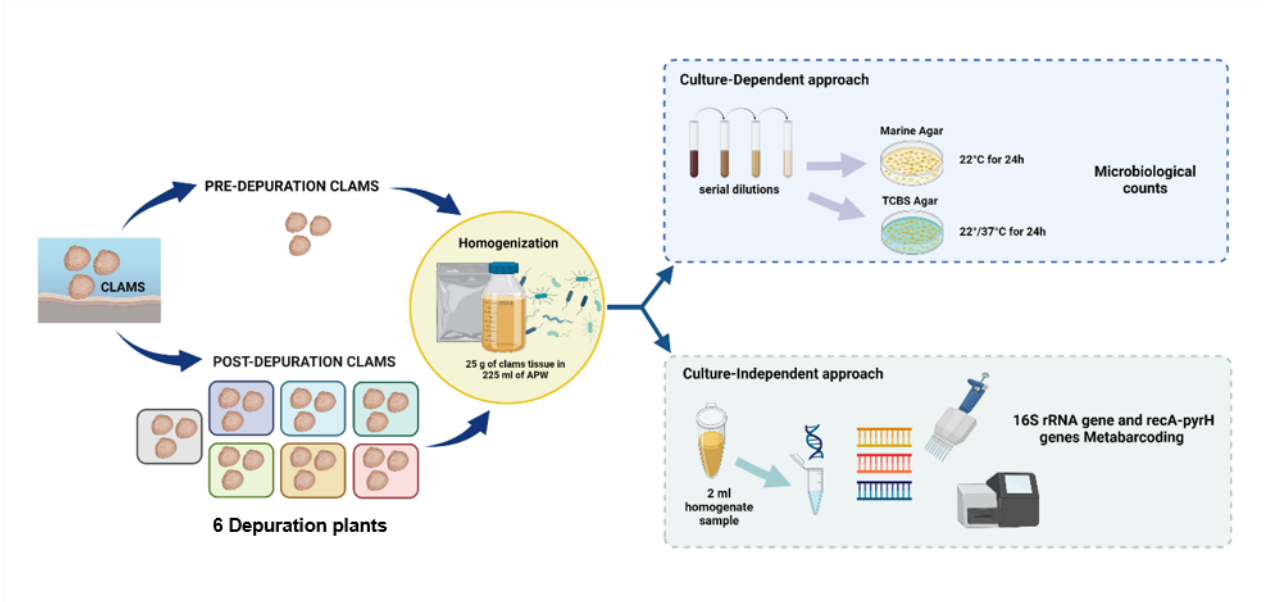


Figure 4. Flow chart of the experimental design. APW: alkaline peptone water; TCBS agar: thiosulfate-citrate-bile salts-sucrose agar.

Microbiological analyses

After collection, live depurated and non-depurated *R. philippinarum* individuals were transferred rapidly to the laboratories of the university to carry out the experimental procedures. To analyse the pre- and post-depuration clam samples at the same time, the pre-depuration clam samples were stored at 4°C for 24 h. First, after cleaning the shells, by

washing clams under running potable water and 100% ethanol, pools of clams were measured and shucked to collect 25 g of flesh and intervalvular liquid clam tissues into a sterile stomacher bag and then homogenised. The homogenisation was performed by adding 225 ml of Alcaline Peptone Water (APW, 2% NaCl, 1% peptone, pH 8.5, Microbiol, Macchiareddu, CA) inside the stomacher bags; the sample was then diluted tenfold. Subsequently, for samples collected during the two summers and two winters, 100 µl of each serial dilution was plated on MA medium (Condalab, Madrid, Spain) and TCBS medium (Biolife, Milano, Italy). MA medium was incubated at 22°C for 24 h and TCBS medium was incubated at 22 and 37°C for 24 h. The temperature of 22°C was chosen to promote the growth of several *Vibrio* species (Vezzuli *et al.*, 2013). The temperature of 37°C is a selective temperature, which promotes the growth and detection of the main *Vibrio* human pathogens (Bonnin-Jusserand *et al.*, 2017). After incubation, the microbiological counts were performed and are expressed as log colony-forming units per gram of sample (log_{10} CFU g^{-1}) for total viable counts The confirmation of the *Vibrio* colonies was performed by using the Sanger sequencing, developed on *rec*A gene, on some suspected isolates, selected according to the colour and morphologies assumed on the TCBS medium as described in Zampieri *et al*., 2020.

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). The pellets were stored at -80°C until DNA extraction.

*DNA extraction and preparation of libraries for 16r RNA and rec*A–*pyr*H *metabarcoding*

The procedure of bacterial DNA extraction from homogenate clam samples and the subsequent preparation of 16S rRNA and *rec*A–*pyr*H metabarcoding libraries have been described by Zampieri *et al.* (2020).

Bioinformatic and statistical analyses

The 120 homogenate clam samples produced a total of 19,620,571 and 24.575.865 raw reads for 16S rRNA Illumina Miseq and *rec*A-*pyr*H libraries sequencing, respectively.

The total microbial counts and the *Vibrio* counts are represented by box plots generated by using the SPSS software (https://www.ibm.com/it-it/analytics/spss-statistics-software). The non-parametric combination (NPC) test was carried out on microbiological counts to evaluate

a statistically significant effect of the singular fixed factors and of their combination on the bacterial and *Vibrio* concentration present in the homogenate clam samples. The quality check and bioinformatic analyses carried out on 16S rRNA and *rec*A–*pyr*H raw reads of homogenate clam samples followed a previous study (Zampieri *et al.*, 2021). Specifically, the CALYPSO platform was used for exploratory analysis of the output files of QIIME2 for the 16S rRNA reads and Bracken for the *rec*A–*pyr*H reads.

For 16S rRNA sequencing data, a rarefaction curve was generated to estimate the representation of the microbial community by the sequencing data. Then, the microbial alpha diversity of 16S rRNA sequencing data was investigated according to the three fixed factors: the depuration treatment, the clams' origin site and the season of collection. In detail, the alpha diversity was studied using analysis of variance (ANOVA, p-value < 0.05) carried out at the feature level (ASV) according to the richness index. Then, ANOVA was performed to evaluate the abundance of the *Vibrio* genus based on the 16S rRNA sequencing data according to the three fixed factors considered.

For the *rec*A–*pyr*H sequencing data, a three-way permutational multivariate analysis of variance using distance matrices (ADONIS +) performed with Calypso and PRIMER-e software (https://www.primer-e.com/), was carried out to evaluate whether the three fixed factors affected the *Vibrio* community composition. Then, the beta microbial biodiversity was described using a principal coordinate analysis (PCoA) according to the Jaccard index. Subsequently, PERMANOVA pairwise comparisons were carried out by using the PRIMER-e software (https://www.primer-e.com/) to test the following contrasts: pre-depuration versus post-depuration, summer versus winter and origin site. As reported by Zampieri e*t al.* (2020, 2021), the *rec*A–*pyr*H results were elaborated according to a qualitative approach (presence/absence) of species detected. For this reason, the ADONIS + analysis and the PCoA representation were carried out according to the Jaccard index. Subsequently, the percentage of homogenate clam samples positive for the main *Vibrio* human pathogens was determined for the three fixed factors considered in the study. The NPC test was carried out to determine statistically significant differences in *Vibrio* species found in homogenate clam samples according to *rec*A–*pyr*H metabarcoding. Particular attention was given to the detection of the main *Vibrio* human pathogens.

Results

Microbiological counts

In the homogenate clam samples, the marine bacterial and *Vibrio* loads were evaluated on MA and TCBS media, respectively, both incubated at 22°C. In addition, the *Vibrio* spp. concentration present in the homogenate clam samples was evaluated on TCBS medium incubated at 37°C to promote the growth of *Vibrio* human pathogens. Specifically, the microbiological counts were analysed according to the fixed factors such as the clams' origin site and season of collection. Given the refrigeration step of pre-depuration homogenate samples performed at 4°C for 24 h, only the microbiological counts results of the post-depuration homogenate clam samples were considered and analysed.

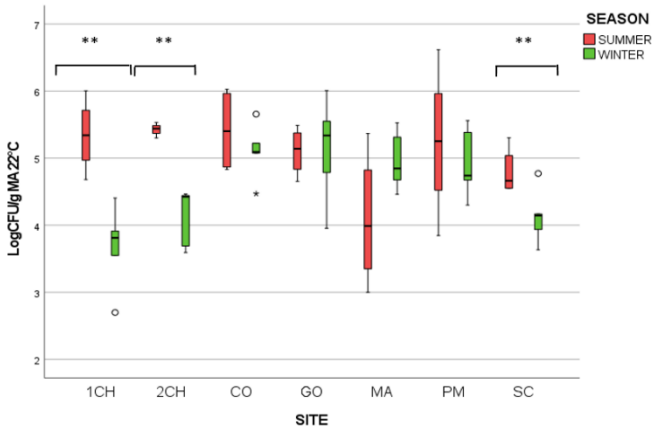
The NPC-test showed a different microbial concentration among the post-depuration clam samples according to the clam's origin site (p-value = 0.013) and the season of clam samples collection (p-value = 0.0001).

More in details regarding the depuration plant effect, the post-depuration homogenate clam samples showed a statistical different marine bacteria evaluated on MA medium incubated at 22°C (p-value = 0.05) and of *Vibrio* concentration evaluated on TCBS medium incubated at 22°C (p-value = 0.0001) among the depuration plants. In particular, the marine bacteria and *Vibrio* counts evaluated in MA and TCBS media, respectively, both incubated at 22°C, reached the higher load in clam samples depurated in PLANT A and in PLANT F, respectively (Appendix section, Table 3).

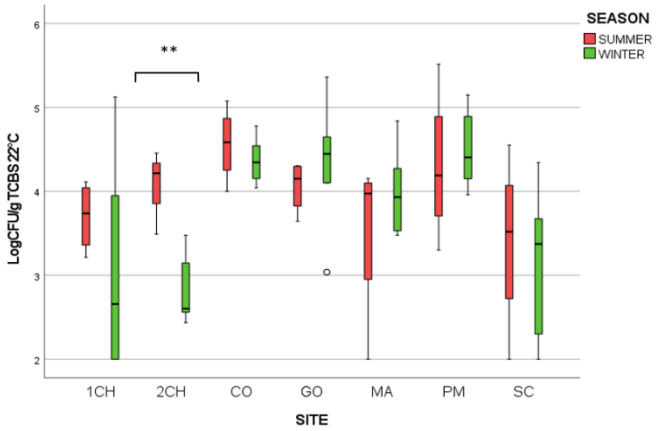
In addition, the post-depuration clam samples collected in summer season showed a statistical different marine bacteria load evaluated on MA medium incubated at 22°C (p-value = 0.02). In details, in summer post-depuration clam samples the marine bacteria load reached the higher value of 5.1 $\log_{10}$ CFU g^{-1} ±0.8 evaluated in MA media incubated at 22°C.

Regarding the clam's origin site factor the post-depuration homogenate clam samples showed a statistical significant difference in *Vibrio* load reached on the TCBS media incubated at 22° C (p-value: 0.002). In details, PM, CO and GO post-depuration clam samples presented the higher *Vibrio* concentration of 4.4 $\log_{10}$ CFU g^{-1} ±0.7, 4.5 $\log_{10}$ CFU g^{-1} ±0.4 and of 4.2 $\log_{10}$ CFU g^{-1} ±0.6 in TCBS media incubated at 22°C, respectively (Appendix section, Table 3). Moreover, the clam's origin site factor combined to the season of clam samples collection revealed a higher marine bacteria load on MA medium incubated at 22° C in post-depuration clam samples collected during the summer season in 1CH (p-value = 0.008), 2CH (p-value = 0.02)

and SC (p-value = 0.03) sites respect to the ones collected in the winter season (Figure 5a). Moreover as reported in Figure 5b, the post-depuration clam samples collected in 2CH site in summer season showed also a higher *Vibrio* concentration respect to the ones collected during the winter season (p-value = 0.04). Finally, the *Vibrio* concentration evaluated on TCBS medium incubated at 37°C showed no growth from the post-depuration homogenate clam samples collected during the winter season.



5a



5b

Figure 5. Total microbial counts and *Vibrio* spp. counts of the post-depuration homogenate clam samples collected in the seven farming areas performed on thiosulfate-citrate-bile salts-sucrose agar (TCBS) (5a) and Marine Agar (MA) (5b) media according to the season of collection. The red boxplot corresponds to statistical analysis of the homogenate clam samples collected during the summer; the green boxplot corresponds to statistical analysis of the homogenate clam samples collected during the winter. Thicker black lines in the boxes correspond to the medians. 1CH, 1Chioggia; 2CH, 2Chioggia; CO, Colmata; GO, Goro; MA, Marano; PM, Porto Marghera; SC, Scardovari.

16S-metabarcoding-based microbial communities of the homogenate clam samples

The microbial community of the homogenate clam samples was investigated using 16S rRNA amplicon sequencing data. The rarefaction curve generated from the filtered reads showed a good representation of the microbial communities (Appendix, Figure A1). Moreover, according to the 16S rRNA amplicon sequencing data, both the season of collection (winter,

summer) and the clams' origin site affected the microbial richness in the homogenate clam samples (Fig.6). The depuration treatment, instead, not affected the microbial richness in the homogenate clam samples. Moreover, the alpha diversity analysis showed more taxa in the homogenate clam samples collected in Porto Marghera and Colmata (Figure 6a) and greater microbial richness in homogenate clam samples collected during the summer (Figure 6b).

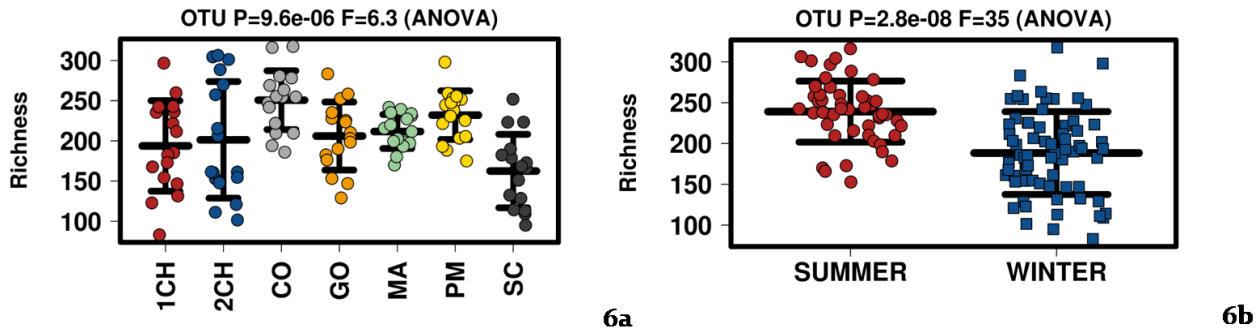


Figure 6. 16s-rRNA-amplicon-based microbial analysis of homogenate clam samples. Alpha diversity was tested with analysis of variance according to the richness index for the clams' origin site (6a) and season of collection (6b). 1CH, 1Chioggia; 2CH,2Chioggia; CO, Colmata; GO, Goro; MA, Marano; PM, Porto Marghera; SC, Scardovari.

Then, an ANOVA was performed to determine whether there were differences in *Vibrio* abundance according to the three fixed factors. Specifically, the *Vibrio* abundance in the homogenate clam samples collected during the summer was statistically higher than in the samples collected during the winter (Figure 7). Moreover, despite the clams' origin site factor did not affected the *Vibrio* abundance, the homogenate clam samples collected in Porto Marghera, Marano and Colmata showed the higher abundance of *Vibrio* spp. (Appendix, Figure A2). In addition, the depuration treatment factor did not affected the richness and the abundance of the *Vibrio* genus in the homogenate clam samples.

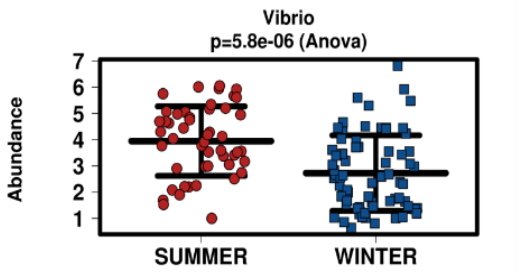


Fig.7. Analysis of variance of 16s rRNA sequencing data of the *Vibrio* genus abundance among the homogenate clam samples according to the season of collection.

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

After evaluating the bacterial community composition based on the 16S rRNA sequencing data, the *Vibrio* community composition was studied using *Vibrio*-specific metabarcoding (based on the *rec*A and *pyr*H genes) developed in the present PhD project and presented by Zampieri *et al.* (2020). The *Vibrio* community composition was investigated according to the three fixed factors. First, a three-way PERMANOVA, performed on the *rec*A–*pyr*H metabarcoding results by using the Calypso software, according to the Jaccard index, revealed that the clams' origin site and the season of collection significantly affected the *Vibrio* community composition of homogenate clam samples (Figure 8a). The three-way PERMANOVA carried out with PRIMER-e software confirmed these results (Appendix, Table 4a). The interaction among the three fixed factors was not statistically significant. Subsequently, the variation in the *Vibrio* community between the homogenate clam samples based on the depuration treatment (Figure 8b), the season of collection (Figure 8c) and the clams' origin site (Figure 8d) was visualised using a PCoA representation according to the Jaccard index.

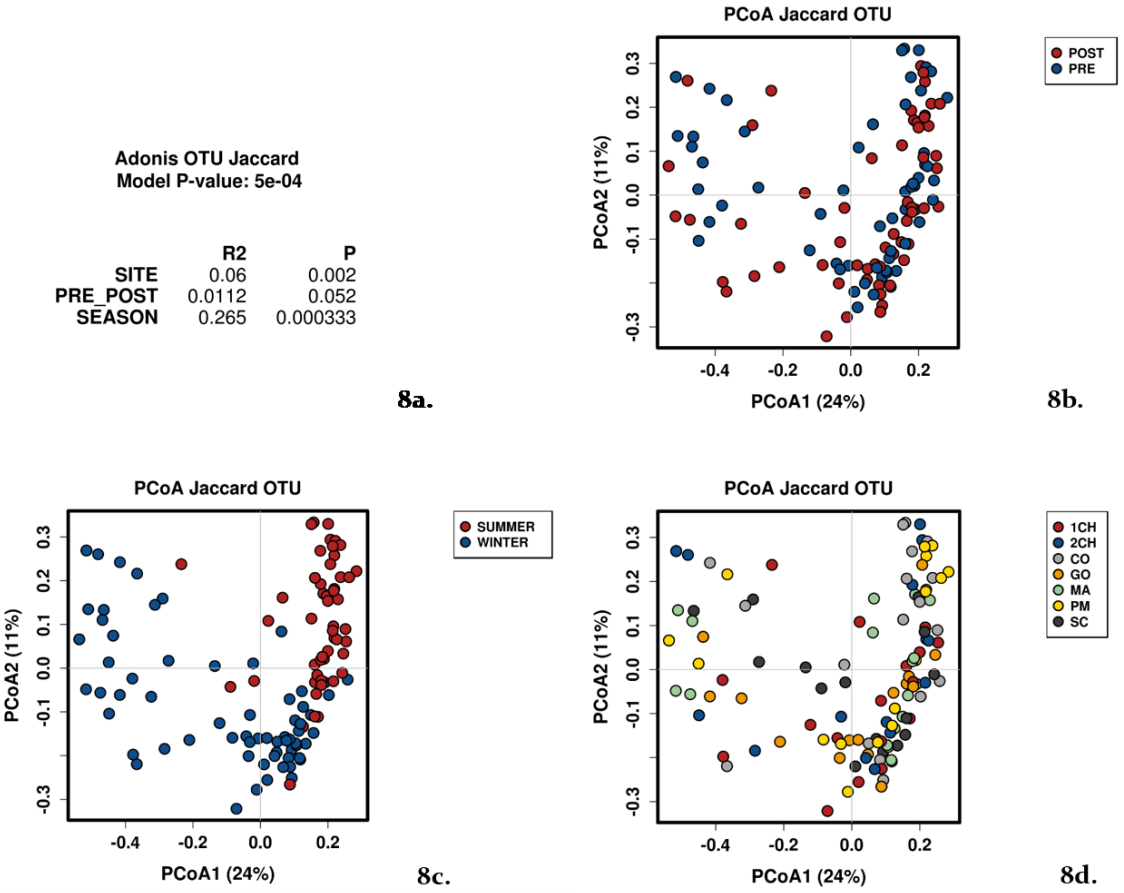


Figure 8. *rec*A–*pyr*H-amplicon-based microbial principal coordinate analysis representation of homogenate clam samples according to the depuration treatment (8b), the season of collection (8c) and the clams' origin site (8d). The PCoA representation and the permutational multivariate analysis of variance (ADONIS +) (8a) were developed according to the Jaccard index. POST, post-depuration clam samples; PRE, pre-depuration clam samples; 1CH, 1Chioggia; 2CH, 2Chioggia; CO, Colmata; GO, Goro; MA, Marano; PM, Porto Marghera; SC, Scardovari.

The PCoA representation showed a higher dispersion of homogenate clam samples according to the depuration treatment (Figure 8b) and the clams' origin site (Figure 8d). Regarding the season of collection, the PCoA showed a clear separation of samples collected during the winter and the summer (Figure 8c). Moreover, the one-way PERMANOVA pairwise comparisons showed a significant difference in the *Vibrio* community composition of the homogenate clam samples collected during the summer compared with the samples collected during the winter (p-value = 0.0001). In addition, according to the one-way PERMANOVA pairwise comparisons there were no significant differences in *Vibrio* community composition between pre- and post-depuration homogenate clam samples and among the seven shellfish farming sites (Appendix, Table 4a).

According to the three fixed factors, more *Vibrio* species were found in homogenate clam samples collected during the summer (38), the pre-depuration samples (37) and in the homogenate clam samples collected from Porto Marghera and Colmata (30) (Appendix, Table 5). The NPC test showed 14 *Vibrio* species and 3 *Vibrionaceae* that differed significantly in terms of the percentage of detection between the samples collected during the summer and winter. Among these species, only *V. splendidus* and *V. tapetis* had a higher percentage of detection in the winter samples (Appendix, Table 6).

Subsequently, the *rec*A–*pyr*H amplicon sequencing data were used to evaluate the percentage of detection (presence/absence) of the main *Vibrio* human pathogens in the homogenate clam samples. In particular, the effect of the three fixed factors was determined. The three-way PERMANOVA (ADONIS +) performed according to the Jaccard index showed that only the season of collection significantly affected the presence/absence of the main *Vibrio* human pathogens (Table 4b). Figures 9, 10 and 11 indicate the percentages of the homogenate clam samples positive for *V. alginolyticus*, *V. cholerae*, *V parahaemolyticus* and *V. vulnificus*, according to the depuration treatment, the season of collection and the clams' origin site, respectively. Figure 9 shows the persistence of the main *Vibrio* human pathogens in post-depuration homogenate clam samples. Moreover, although the depuration treatment did not significantly affect the presence/absence of the *Vibrio* human pathogens, there was a slightly higher percentage of post-depuration homogenate clam samples with *V. cholerae* (75%) and *V. vulnificus* (78%) compared with the pre-depuration samples.

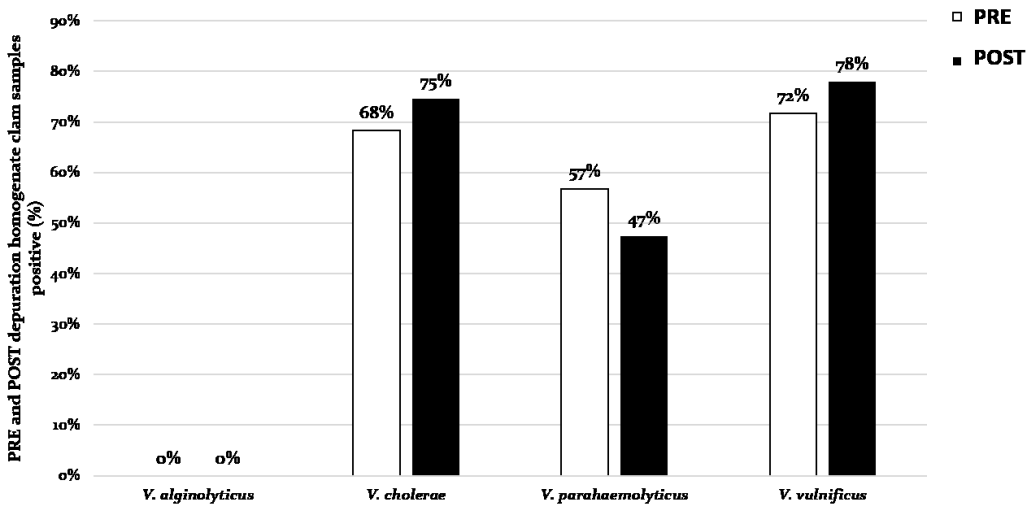


Figure 9. Bar chart representing the percentage of pre-depuration (white bar chart) and post-depuration (black bar chart) homogenate clam samples positive for the main *Vibrio* human pathogens –*Vibrio alginolyticus*, *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus*.

Figure 10 shows the percentages of homogenate clam samples positive for the main *Vibrio* human pathogens according to the season of collection. The NPC test revealed that among these human pathogens, *V. parahaemolyticus* and *V. vulnificus* were significantly higher in the homogenate clam samples collected during the summer compared with the winter. Specifically, *V. parahaemolyticus* and *V. vulnificus* were detected in the 72% and 82%, respectively, of the summer samples and 30% and 62%, respectively, of the winter samples.

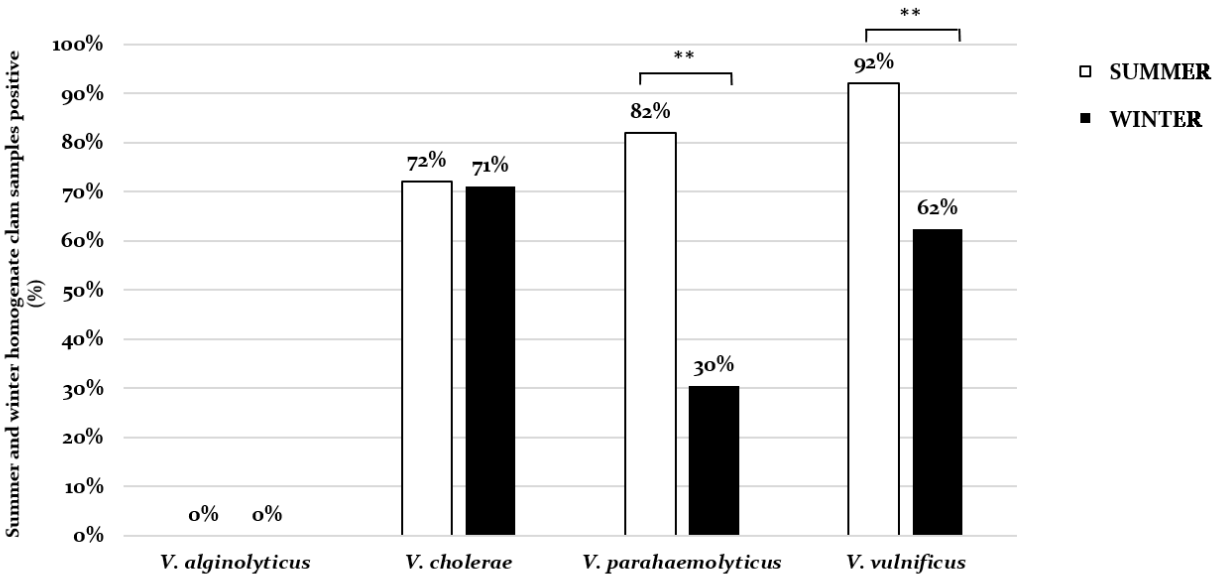


Figure 10. Bar chart representing the percentage of homogenate clam samples collected during the summer (white bar chart) and the winter (black bar chart) positive for the main *Vibrio* human pathogens – *Vibrio alginolyticus*, *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus*.

Figure 11 reports the percentages of homogenate clam samples positive for the main *Vibrio* human pathogens according to the seven shellfish farming sites selected in the study. The samples collected in Goro, Colmata and Porto Marghera (PM) had the highest percentage of samples with *V. cholerae* (81%), *V. parahaemolyticus* (67%) and *V. vulnificus* (82%), respectively. Moreover, among the main *Vibrio* human pathogens, *V. vulnificus* and *V. cholerae* were identified in more than the 50% of all the homogenate clam samples collected in the seven shellfish farming sites. Of note, *V. alginolyticus* was not found in the homogenate clam samples (Figures 9–11).

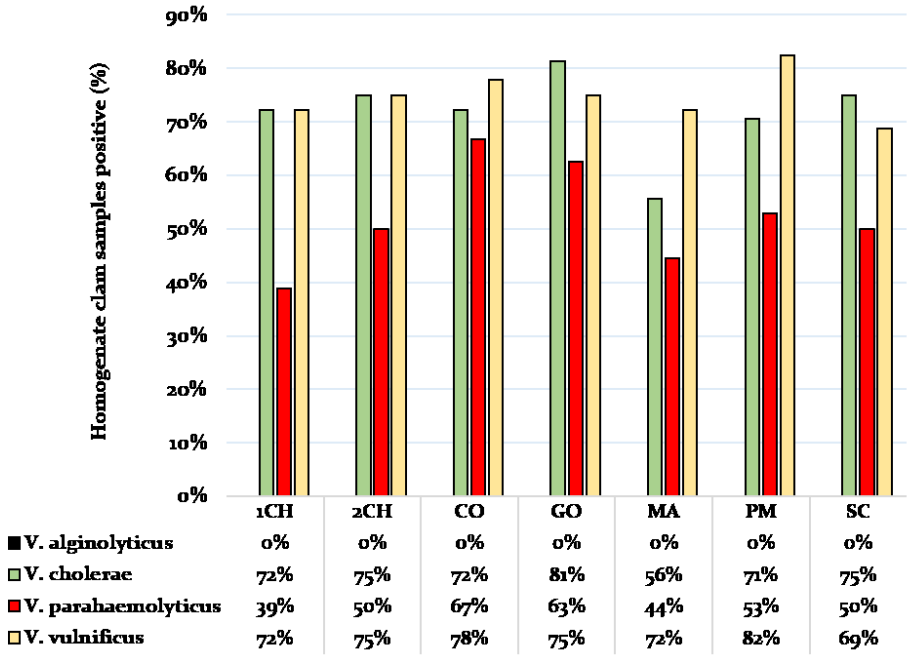


Figure 11. Bar chart representing the percentage of the homogenate clam samples positive for the *Vibrio* human pathogens – *Vibrio alginolyticus*, *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio Vulnificus* – according to the clams' origin site. 1CH, 1Chioggia; 2CH,2Chioggia; CO, Colmata; GO, Goro; MA, Marano; PM, Porto Marghera; SC, Scardovari.

Discussion

In light of the worldwide distribution of *Vibrio* spp. and of the significant human health hazards of the *Vibrio* human pathogens detected in several seafood products, homogenate clam samples collected in seven operating shellfish farming areas along the North-east Adriatic coast were analysed (Passalacqua *et al.*, 2016; Bonnin-Jusserand *et al.*, 2017). The study focused on characterising the *Vibrio* community composition associated with the *R. philippinarum* microbiota, with a specific concern for the detection of the main *Vibrio* human pathogens. For better comprehension on the variability of *Vibrio* biodiversity and the incidence of *Vibrio* human pathogens, the homogenate clam samples were analysed using culture-dependent and culture-independent methods according to three fixed factors: the depuration effect, the clams' origin site and the season of collection.

First, microbiological counts were determined to evaluate the *Vibrio* and marine bacterial concentration in homogenate clam samples according to the fixed factors.

Given the pre-depuration clam samples refrigeration step performed at 4°C for 24 h, the microbiological counts of pre-depuration clam samples were not considered in the analysis. As reported in literature (FAO/WHO, 2016), the storage of seafood products, in fact, could distort the concentration of marine bacteria reached in the cultural plates by inducing a cold stress in *Vibrio* cells. In this stressful condition, Vibrios by entering in a VBNC state are unable to grow and to be counted on a selective medium such as the TCBS.

Moreover, the analysis were focused on the microbiological counts of the post-depuration clam samples in order to implement the knowledge about the microbial load associated to the depurated shellfish product, which is destined to the human consumption according to the European Regulation (UE) 2019/627. Specifically, the marine bacteria and *Vibrio* load reached in the post-depuration clam samples was evaluated according to the fixed factors of the study such as the clams' origin site and the season of collection. Moreover, in the evaluation of the microbiological counts results the depuration plant resulted a factor related to the clams' origin site one. According to the experimental design, in fact, each batch of clams farmed in one of the seven clams' origin site was depurated in a different depuration plant. The only exception was of Porto Marghera and Colmata, which were both depurated in the same facility.

For what concern the depuration plant factor related to the clams' origin site, the statistically higher marine bacteria load was reached in summer clam samples farmed in 1Chioggia,

2Chioggia and Scardovari sites and depurated in PLANT B, PLANT C and PLANT E, respectively (Figure 5a). The statistically higher *Vibrio* concentration was achieved in clam samples farmed in 2Chioggia and depurated in PLANT B (Figure 5b). The marine bacteria and *Vibrio* loads achieved in these samples could be justified by the effect of both the factors such as the depuration plant and clams' origin site.

In particular, the clams' origin site factors could have contributed in the statistically higher marine bacteria concentration achieved in 1Chioggia, 2Chioggia homogenate clam samples. These two sites in fact are located closed to the Chioggia inlet, which is one of three major inlet of the Venice lagoon through which takes place an intense maritime traffic. The movement of ships to and from the Chioggia harbour could favour the introduction of several marine bacteria on the water column through the ballast water. The ballast water discharge represents, in fact, a potential source for the spread of several marine microorganisms (Hess-Erga et al., 2019). Moreover, the close location of 2Chioggia site to the Chioggia inlet could also explain the higher *Vibrio* load achieved in these samples. In Chioggia inlet takes place the water exchange between lagoon and northern Adriatic sea (Ghezzo et al., 2010). Consequently, this is an area with an important hydrodynamics and this could implement the resuspension of the sediments. Considering that the sediment represents a potential reservoir of several *Vibrio* species, *Vibrio* human pathogens included, its resuspension could directly affect the spread of several *Vibrio* species (Vezzulli et al., 2009; Takemura *et al*., 2014). In addition, also the ballast water could implement the occurrence of *Vibrio* species. In particular, several studies, reported the detection of the *V. cholera* in water samples collected from the ballast water (Fykse *et al*., 2012; NG *et al*., 2018; Soleimani et al., 2021).

For what concern, the high marine bacteria load achieved in summer clam samples collected in Scardovari site, these could be related to the higher temperature, which accelerated not only the bacterial metabolism and reproduction but also the rate of mussels' filtration, which like clams, are farmed in this area (Ceccherelli and Rossi, 1984). The filtration activity of mussels, as suggested by Solomonova et al., 2021, could have favoured several marine bacteria in this area, by removing their predators and competitors such as ciliates, heteroflagellates and diatoms from the water column.

Then, the different microbial and *Vibrio* concentration achieved in clam samples collected from the seven clams' origin site could also have been affected to each specific depuration plant and technologies applied during the process. In the previous study of Zampieri *et al*.,

2020, in fact, is showed that the depuration plant and the technologies applied during the depuration treatment affected both the microbial composition and concentration in the homogenate clam samples.

Moreover, the samples collected during summer and winter season in Porto Marghera (PM), Colmata (CO) and Goro (GO) reached the higher *Vibrio* loads (Figures 5b). The similar level of *Vibrio* load of Porto Marghera (4.4 $\log_{10}$ CFU g^{-1} ± 0.7) and Colmata post-depuration clams (4.5 $\log_{10}$ CFU g^{-1}± 0.4) could be justified by the geographical proximity of these two sites (see the map in Figure 3a) and to the fact that clams collected in these two sites were depurated in the same depuration plant (Table 1). In addition, it is possible to assume that the human impact present in the area of the Venetian Lagoon, in which are located Porto Marghera and Colmata sites, could have prompted the proliferation of *Vibrio* species. Indeed, researchers have demonstrated that coastal sediments and the resuspension of organic matter represent important reservoirs for *Vibrio* species, including human pathogens (Neogi *et al.*, 2018; Kopprio *et al.*, 2020). Consequently, the intensive industrial and harbour activities conduced in Porto Marghera and Colmata could have made organic matter available to promote the proliferation of several *Vibrio* species (Ciavola, 2005). The *Vibrio* load of post-depuration clam samples collected in Goro site could be related to the growth and proliferation of phytoplankton in the Sacca di Goro lagoon. The alive phytoplankton represents an environmental reservoirs for several marine bacteria, Vibrios included (Aspulnd et al, 2011). In particular in the Sacca di Goro lagoon, as described by Nizzoli *et al.*, 2005 and 2006, the proximity of mussels rope community to the clam farming areas act as a source of particulate organic matter, inorganic nitrogen and phosphate to the water column. Given these nutrients input, the authors suggested that in Sacca di Goro lagoon the phytoplankton could find the ecological framework suitable for its turnover and biomass growth and consequently could offer also a suitable substrate for the *Vibrio* colonization. Moreover, as suggested in recent study of Musella *et al.*, 2020, the mussel farming could also increase the occurrence of Vibrios in the seawater surrounding the mussel farm by releasing into water column the *Vibrionaceae* associated to their gills.

In addition, also the season of collection factor affected the microbial load of the post-depuration clam samples. Specifically, the samples collected during the summer had significantly higher marine bacterial concentration than the samples collected during the winter (p-value: 0.02). This result could find an explanation on an increased *R. philippinarum*

filtration rate, promoted by the elevated summer seawater temperature, which induced also on an increased ingestion of water and marine bacteria and Vibrios that are free living or associated with particles. Koo and Seo (2020) reported the filtration rate of *R. philippinarum* increased with a water temperature > 25°C. Moreover, the season affected the *Vibrio* spp., as denoted by the absence of bacterial growth obtained on TCBS medium incubated at 37°C for the samples collected during the winter. The incubation at 37°C was probably such a stressful condition for the potential *Vibrio* pathogens and other species able to growth at 37°C, which had adapted to the winter seawater temperature that they could not grow on the culture medium. The main *Vibrio* human pathogens are more frequently isolated during the summer (Vezzulli *et al.*, 2019).

After quantifying *Vibrio* and marine bacterial concentration, the metabarcoding approach was used to characterise the microbial biodiversity associated with the *R. philippinarum* microbiota. The microbiota of the homogenate clam samples was first investigated according to 16s rRNA metabarcoding. This approach provided information regarding bacterial biodiversity to the genus level.

Similarly, to the microbiological counts, the bacterial community composition was explored according to the fixed factors such as the clams' origin site and the season of clam samples collection. Moreover, unlike the microbiological counts, the analysis of 16s rRNA and *rec*A-*pyr*H metabarcoding sequencing data evaluate also the fixed factor such as the depuration treatment. In the 16s rRNA and *rec*A-*pyr*H metabarcoding sequencing data were, in fact included the pre-depuration homogenate clam samples. Despite the possible presence of VBNC bacteria cells in pre-depuration clam samples induced by the storage at 4°C for 24h, the direct DNA extraction from clam samples and their community characterization performed by using the NGS-approach let possible the study of complex microbial community of alive, dead or VBNC bacterial cells (Carraro et al., 2011; Boers et al., 2019). Consequently, the depuration treatment factor evaluated on 16s rRNA and *rec*A-*pyr*H metabarcoding sequencing data consisted in the comparison of microbial and *Vibrio* species community composition between pre and post-depuration clam samples.

According to the clams' origin site, the 16s rRNA metabarcoding sequencing data of homogenate clam samples collected in Porto Marghera and Colmata showed a greater microbial richness (Figure 6a). Again, this result could be related to the intensive industrial activities carried out in this area of the Venetian Lagoon. Moreover, the homogenate clam

samples collected during the winter showed lower microbial richness compared with the samples collected during the summer (Figure 6b). This result is in line with several studies that have shown a reduced bacterial diversity in the winter related to the reduced filtration rate of bivalves combined with the lower diversity of marine bacteria present in the water column (Zurel *et al.*, 2011; Pierce *et al.*, 2016; Mestre et al., 2020).

The higher *Vibrio* genus abundance obtained in homogenate samples collected during the summer (Figure 7) is consistent with several studies that have reported an increase in *Vibrio* abundance present in seawater in response to the rise in seawater temperatures (Vezzulli *et al.*, 2016, 2019).

The *Vibrio* biodiversity of the *R. philippinarum* microbiota was then examined by using *rec*A–*pyr*H metabarcoding developed in the present PhD project. The season of collection had a greater contribution to the *Vibrio* community composition of the homogenate clam samples than the other two factors (Figure 8a, 8c). Consistently, the PERMANOVA pairwise comparisons revealed a different *Vibrio* community composition between homogenate clam samples collected during the summer and the winter (Appendix section: Table 4a).

The differentiation of the *Vibrio* community composition related to the season factor is in line with several studies in which researchers have reported a different *Vibrio* biodiversity 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). Moreover, the season factor showed the major difference in terms of the number of *Vibrio* species detected between the homogenate clam samples collected during the summer and winter if compared with the number of species found in pre- and post-depuration homogenate clam samples and among the seven shellfish farming sites (Appendix, Table 5). In particular, the seasonality of 14 *Vibrio* and 3 *Vibrionaceae* species was demonstrated (Appendix, Table 6). Among these species, the high detection of *V. tapetis* in homogenate clam samples collected during the winter is consistent with the frequent cold-water prevalence of this species (Paillard *et al.*, 2004).

Subsequently, the effect of the three fixed factors on the percentage of detection of the main *Vibrio* human pathogens – *V. alginolyticus*, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* – was evaluated. Only the season of collection significantly affected the presence/absence of the *Vibrio* human pathogens in the homogenate clam samples (Table 4b). In particular, the NPC-test showed that the percentage of detection of *V. parahaemolyticus* and *V. vulnificus* in

homogenate clam samples collected during the summer was significantly higher than the samples collected during the winter (Figure 10, Table 6).

The high percentage of *V. vulnificus* (92%) in the homogenate clam samples collected during the summer is in line with other studies in which this pathogen was only found in seafood products collected during the summer (Strom and Paranjpye, 2000; Huehn *et al.*, 2014). Moreover, the detection of *V. parahaemolyticus* and *V. vulnificus* in more than half of the homogenate clam samples collected during the summer compared with 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 frequency of *V. vulnificus* and *V. parahaemolyticus* in the homogenate clam samples is in line with the results obtained by Zampieri *et al.* (2021). That study included only a part of the total samples collected during this 3-year PhD project; the results showed 56.3% and 54.2% of samples positive for *V. parahaemolyticus* and *V. vulnificus*, respectively considering the winter and summer clam samples together. Consequently, the high percentage of homogenate clam samples collected during the summer 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 the human vibriosis is becoming clearer (Vezzulli *et al.*, 2015; Kim and Chun, 2021).

As shown in Figures 9–11, *V. alginolyticus* was not found in the homogenate clam samples. As described by Zampieri *et al.* (2021), this species is halophilic and probably requires a culture step for characterisation and a more sensitivity approach such as the shotgun metagenomics to be detected.

Unlike the season factor, the depuration treatment had no effect on the percentage of samples positive for the *Vibrio* human pathogens (Figure 9). In accordance with several studies, the results obtained highlight the lack of efficiency of the depuration treatment in removing *Vibrio* spp. from the shellfish products (Sferlazzo *et al.*, 2018; Vezzulli *et al.*, 2018). Differently from the previous study in which clam samples collected in May 2020 in Chioggia were analysed (Zampieri et al., 2020), this study comprising all samples collected during this PhD project revealed that some of the pre-depuration homogenate clam samples were also positive for the *Vibrio* human pathogens (Figure 9). This difference could be related to the lower number of pre-depuration homogenate clam samples (10) surveyed in the previous study compared with this recent one (60). In addition, in the previous study Zampieri *et al.*, 2020

the clams' origin was limited to only one site of Chioggia from which was collected only one batch of clams. Nevertheless, the persistence of *Vibrio* human pathogens after the depuration treatment could be related to the formation of biofilms associated with the surface of the depuration facilities. Several *Vibrio* species form biofilm, including the human pathogens *V. cholerae* and *V. parahaemolyticus* (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 (Schreier *et al.*, 2010; Martins *et al.*, 2013). Considering that the shellfish depuration plants are equipped with a recirculation system of seawater and biological filters (Appendix, Table 1b), one could assume there are *Vibrio* species associated to these components that is similar to the contamination found in the facilities used to farm freshwater and marine fish.

Similarly to the depuration treatment, the clams' origin site did not affect the presence/absence of detection of the *Vibrio* human pathogens in homogenate clam samples. There were, however, high percentages of *V. cholerae* and *V. vulnificus* (> 50% positive samples) from homogenate clam samples collected in the seven shellfish farming sites (Figure 11). These results highlighted the wide distribution and presence of these potential human pathogenic species among the shellfish farming areas.

Given these results, metagenomics analysis are ongoing on these samples to confirm and describe in more details the *Vibrio* pathogenic strains.

Conclusion

The results presented in this PhD thesis offer new insights regarding the *Vibrio* biodiversity and the presence of *Vibrio* human pathogens associated with the *R. philippinarum* microbiota considering the effect of three fixed factors: the depuration effect, the clams' origin site and the season of collection. This last factor was the only one that affected the biodiversity of marine bacteria, the *Vibrio* community composition and the presence/absence of the main *Vibrio* human pathogens in the homogenate clam samples. The *Vibrio* human pathogens persisted after the depuration treatment. Among the post-depuration homogenate clam samples the ones collected in Porto Marghera, Colmata and Goro sites showed the highest *Vibrio* load. Moreover, there was a statistically significant increase in the detection of *V. parahaemolyticus* and *V. vulnificus* in homogenate clam samples collected during the summer. The results showed the presence of the main *Vibrio* human pathogens – *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* – in homogenate clam samples collected in the seven main shellfish farming areas operating along the North-east Adriatic coast. To conclude, given the frequency of detection of *Vibrio* human pathogens in homogenate clam samples, additional studies are necessary to achieve more information about the concentration and pathogenicity of these *Vibrio* species. However, these results suggest the importance and usefulness of detecting these species in control measures of seafood products. This addition could help to avoid the spread of human vibriosis caused by the consumption of raw or slightly cooked contaminated seafood products.

3. GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

Given the widespread distribution of *Vibrio* species associated with seafood products, this dissertation has provided new insights regarding the *Vibrio* community composition associated to the *R. philippinarum* microbiota. The development and applications of *rec*A–*pyr*H metabarcoding and culture-dependent metagenomics, presented in the two published papers, allowed a better comprehension of the modification of the *Vibrio* community composition in response to the depuration treatment and provided an evaluation of the usefulness of combining culture-dependent and culture-independent methods to characterise accurately *Vibrio* biodiversity and the main *Vibrio* human pathogens, respectively. Moreover, the broad range application of the new metabarcoding for the characterization of the *Vibrio* community composition of all the homogenate clam samples collected during the 3-year PhD project has provided new avenues for future analysis.

In the first paper, the on field use of *rec*A–*pyr*H metabarcoding demonstrated changes in the richness and biodiversity of the *Vibrio* community composition in the Manila clam microbiota according to the depuration plant. In particular, the detection of the main *Vibrio* human pathogens in depurated clams suggests that the depuration plant could represent a niche for the proliferation of these marine bacteria. Given these findings, in the future it could be interesting to apply *rec*A–*pyr*H metabarcoding to verify the presence of *Vibrio* species in the microbial biofilm community composition frequently formed over the water tank surfaces in depuration plants. This future investigation could offer suitable information for shellfish farmers and authorities involved in food safety controls to improve the management and food controls of shellfish production.

In the second paper, the methodological application of *rec*A–*pyr*H metabarcoding highlighted the complementarity of culture-dependent and culture-independent methods for the detection of the main *Vibrio* human pathogens present in the Manila clam microbiota. In particular, *rec*A–*pyr*H metabarcoding applied to homogenate clam samples and samples plated on MA medium provided the most useful screening approach for complete characterisation of the main *Vibrio* human pathogens. In the seafood industry, this combined approach could be useful as a screening tool to apply to seafood products such as fish and crustaceans to prevent the spread of human vibriosis. In addition, this study offered a new way to exploit the high resolution of shotgun metagenomics in *Vibrio* species characterisation, avoiding the problem of host DNA that tends to overwhelm the microbiota DNA. Specifically,

the culture-dependent shotgun metagenomics developed in this study allowed the characterisation of *Vibrio* biodiversity and the detection of the main *Vibrio* human pathogens present in a potentially virulent state. In conclusion, *rec*A–*pyr*H metabarcoding and culture-dependent shotgun metagenomics applied in the second paper represent reliable and accurate approaches to obtain updated knowledge about *Vibrio* biodiversity associated with shellfish products destined for human consumption.

The broad-range application of *rec*A–*pyr*H metabarcoding to all homogenate clam samples collected during the 3-year PhD project showed a high frequency of detection of the main *Vibrio* human pathogens; these findings could represent the starting point of future analysis. Specifically, the high frequency of *V. parahaemolyticus* and *V. vulnificus* in the homogenate clam samples collected during the summer and the detection of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* in the homogenate clam samples collected in the seven farming sites could be coupled with the real-time PCR to quantify these species. Moreover, shotgun metagenomics could be carried out to verify the pathogenicity and the virulence of these *Vibrio* human pathogens. In addition, to better comprehend the abundance of the *Vibrio* species related to the season and to sites such as Porto Marghera, Colmata and Goro environmental parameters should be collected and analysed. This endeavour could also provide the possibility to relate these data to the concentration and pathogenicity of *Vibrio* pathogens to identify a possible correlation between them. To conclude, these additional analyses could provide more accurate information about the food safety of the *R. philippinarum* products that are widely consumed in Italy.

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5. APPENDIX

Table 1. Depuration tank parameters (a) and technical plant information (b) of the seven depuration facilities. W, winter; S, summer, MA, Marano, PM, Porto Marghera; CO, Colmata; 1CH, Chioggia site 1; 2CH, Chioggia site 2; SC, Scardovari; GO, Goro.

Table 1a

SEASON	W	S	W	S	W	S	W	S	W	S	W	S	W	S
SITE	MA		PM		CO		1CH		2CH		SC		GO	
PLANT	PLANT D		PLANT A		PLANT A		PLANT C		PLANT B		PLANT E		PLANT F	
DEPURATION TANK PARAMETERS														
T (°C)	12.5 - 13	/	12	13.5	12	13.5	10	<12	14	14	12	12	13.8	13.4
pH	/	/	8.2	7-8	8.2	7-8	8	7-8	8.1	7-8	8.2	8.2	7.7	7.8
Salinity (‰)	35	/	36	22-30	36	22 - 30	29	22-30	28	22 - 30	31	31	33	33
OD (%)	> 100	/	100 - 101	100	100 - 105	100	93	100	100	100	95	95	98	98
NH_3(ppm)	/	/	> 0.50	<1	> 0.50	<1	0.2	<1	0.5	<1	0.6	0.6	1.5	0.7
NO_2(ppm)	/	/	> 0.50	<0.50	> 0.50	<0.50	< 0.50	< 0.50	< 0.50	< 0.50	< 0.50	< 0.50	110	110
NO_3 (ppm)	/	/	> 0.50	<50	> 0.50	<50	< 0.50	< 50	< 50	< 50	< 50	< 50	1.8	0.45
Redox potential (mV)	350	/	/	/	/	/	316	270	100	70-100	270	270	380	/

/: data not provided by the shellfish stakeholders

Table 1b

SEASON	W	S	W	S	W	S	W	S	W	S	W	S	W	S
SITE	MA		PM		CO		1CH		2CH		SC		GO	
PLANT	PLANT D		PLANT A		PLANT A		PLANT C		PLANT B		PLANT E		PLANT F	
TECHNICAL PLANT INFORMATION														
Depuration time (h)	18	23	12	20	12	20	18	20	14	20	12-15	12-15	17	24
Mechanical filter	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Biological filter	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
UV water treatment	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Ozone (O_3)	Yes	Yes	No	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Chlorine (Cl)	No	No	No	No	No	No	No	No	Yes	Yes	No	No	No	No
Refrigeration	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Recirculation-system	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No

Table 2. Summary of the 120 homogenate clam samples collected during the PORFESR project. The samples are subdivided according to the depuration, site and season.
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.

		SUMMER	WINTER
1CH	PRE	4	5
	POST	4	5
2CH	PRE	3	5
	POST	3	5
PM	PRE	4	5
	POST	4	5
CO	PRE	4	5
	POST	4	5
MA	PRE	4	5
	POST	4	5
SC	PRE	3	5
	POST	3	5
GO	PRE	3	5
	POST	3	5
Total SUMMER samples		50	
Total WINTER samples			70
Total PRE - depuration samples		60	
Total POST - depuration samples		60	

Table 3. *Vibrio* and marine bacteria counts, expressed in log_{10} CFU g-1 of homogenate clam sample, performed on TCBS and MA media both incubated at 22°C, according to the three fixed factor: depuration plant, clams' origin site and season of collection. (TCBS: Thiosulfate-citrate-bile salts sucrose agar; MA: Marine Agar)

		***Vibrio* concentration reached in TCBS media incubated at 22°C (log_{10} CFU g-1)**	**Marine bacteria concentration reached in MA media incubated at 22°C (log_{10} CFU g-1)**
Depuration plant	A	4.4 ± 0.5	5.2 ± 0.7
	B	3.3 ± 0.8	4.6 ± 0.8
	C	3.4 ± 1.0	4.4 ± 1.0
	D	3.8 ± 0.8	4.6 ± 0.8
	E	3.3 ± 1.0	4.4 ± 0.5
	F	4.2 ± 0.6	5.1 ± 0.6
Clams' origin site	1CH	3.4 ± 1.0	4.4 ± 1.0
	2CH	3.3 ± 0.8	4.6 ± 0.8
	CO	4.5 ± 0.4	5.2 ± 0.5
	GO	4.2 ± 0.6	5.1 ± 0.6
	MA	3.8 ± 0.8	4.6 ± 0.8
	PM	4.4 ± 0.7	5.1 ± 0.8
	SC	3.3 ± 1.0	4.4 ± 0.5
Season of collection	SUMMER	3.9 ± 0.8	5.1 ± 0.8
	WINTER	3.8 ± 1.0	4.6 ± 0.7

Table 4. PERMANOVA analysis tests performed with PRIMER-e and Calypso software on the *rec*A-*pyr*H metabarcoding results about the *Vibrio* community composition **(4a)** and presence/absence of the *Vibrio* human pathogens **(4b)**, according to the three fixed factors: depuration treatment, season of collection and clams' origin site.

Table 4a.

	PRIMER-e software				**Calypso software**	
	One-way pairwise PERMANOVA		**Three-way PERMANOVA**		**Three-way PERMANOVA**	
Fixed factor	**p-*value***		**p-*value***		**p-*value***	
Depuration treatment	0.149	*ns*	0.077	*ns*	0.052	*ns*
Season of collection	0.0001	***	0.00005	***	0.000333	***
Clams' origin site	0.146	*ns*	0.035	*	0.02	*

ns: non-significant

Table 4b.

	Calypso software	
	Three-way PERMANOVA	
Fixed factor	**p-*value***	
Depuration treatment	0.265	*ns*
Season of collection	0.000333	***
Clams' origin site	0.361	*ns*

ns: non-significant

Table 5. Number of *Vibrio* species detected by *rec*A–*pyr*H metabarcoding according to the three fixed factors of the study: depuration, season and site. PRE, pre-depuration homogenate clam samples; POST, post-depuration homogenate clam samples; 1CH, Chioggia site 1; 2CH, Chioggia site 2; PM, Porto Marghera; CO, Colmata; MA, Marano, 2; SC, Scardovari; GO, Goro.

	Depuration		Season		Site						
	PRE	POST	S	W	1CH	2CH	CO	GO	MA	PM	SC
No. of *Vibrio* species detected	37	35	38	26	29	28	30	26	26	30	27

Table 6. *Vibrio* species with a significantly different percentage of detection in homogenate clam samples collected during the summer and winter according to *rec*A–*pyr*H metabarcoding.

Species	**Summer homogenate clam samples positive (%)**	**Winter homogenate clam samples positive (%)**	***p*-value**
Vibrio nigripulchritudo	8	0	0.0310
Vibrio vulnificus	92	61	0.0004
Vibrio rotiferianus	70	20	0.0001
Vibrio spp. THAF100	8	0	0.0274
Vibrio splendidus	82	99	0.0019
Photobacterium damselae	54	10	0.0001
Vibrio azureus	12	1	0.0206
Vibrio harveyi	96	49	0.0001
Vibrio mediterranei	94	54	0.0001
Aliivibrio fischeri	46	7	0.0001
Vibrio diabolicus	16	1	0.0040
Vibrio parahaemolyticus	82	30	0.0002
Vibrio alfacsensis	40	1	0.0002
Vibrio astriarenae	18	0	0.0006
Vibrio tapetis	20	41	0.0172
Vibrio panuliri	18	3	0.0099

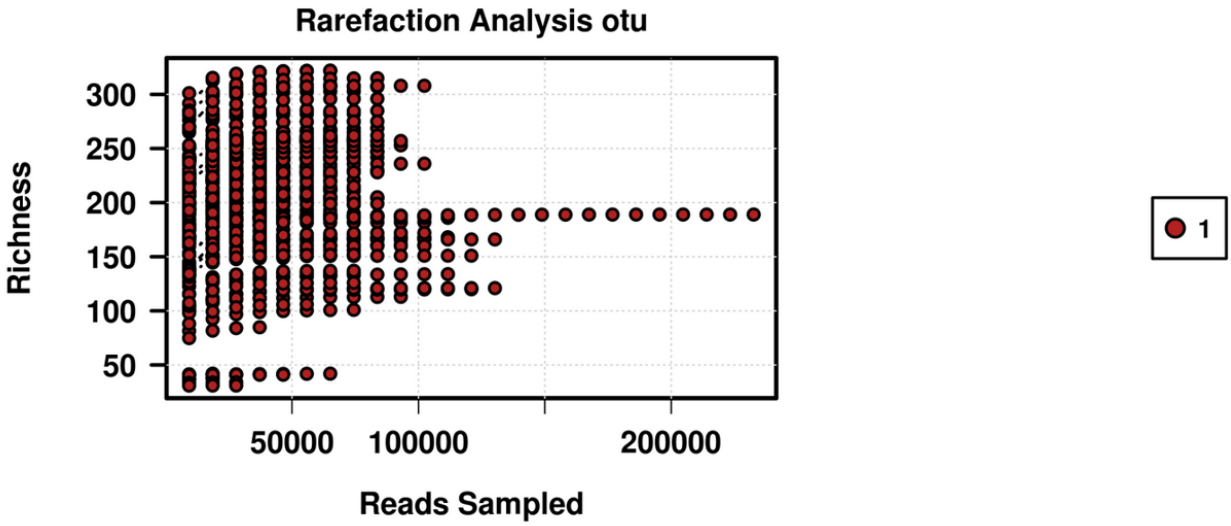

Figure A1. Rarefaction curve of the homogenate clam sample reads obtained by using 16S rRNA sequencing data.

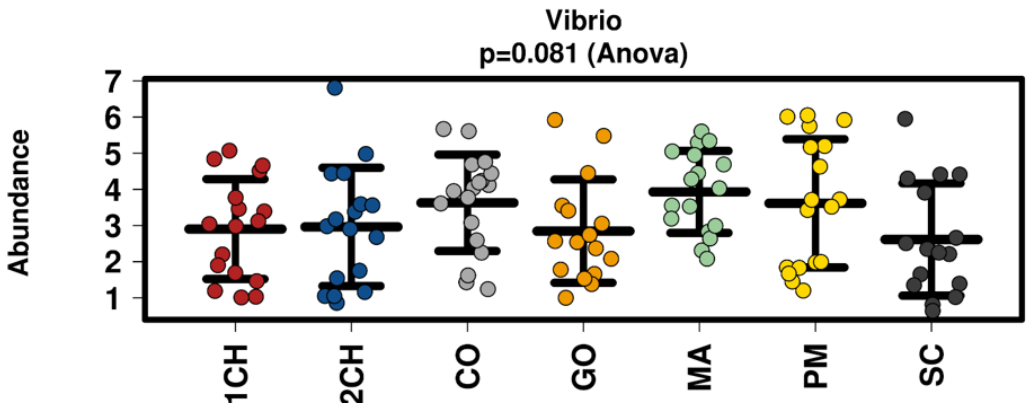

Figure A2. Analysis of variance of the 16s rRNA sequencing data of the *Vibrio* genus abundance among homogenate clam samples according to the clams' origin site. 1CH, 1Chioggia; 2CH, 2Chioggia; CO, Colmata; GO, Goro; MA, Marano; PM, Porto Marghera; SC, Scardovari

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