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DYNAMICS OF VIBRIO DURING EGESTION IN CLAMS (*RUDITAPES PHILIPPINARUM*) OBSERVED IN LABORATORY CONDITIONS

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ABSTRACT

Bivalve molluscs are aquatic invertebrates that have anatomical and structural characteristics that are typical of the phylum *Mollusca*.

Since clams are molluscs, they have filtered feeders' habits, a mechanism of feeding that allows the accumulation of contaminants and microorganisms to a much higher level than that of the surrounding seawater. Between microorganisms, a large number of species of the genus *Vibrio* are associated with marine organisms such as fish, molluscs and crustaceans, with commensal or pathogenic relationships.

Depuration is a procedure used with the purpose of removing dangerous contaminating microorganisms from shellfish. To better understand this process, microcosms were simulated in the laboratory in this study with the aim of quantifying the *Vibrio* released during a predetermined observation period.

The objectives of the project were firstly to study the release of *Vibrio* species during the deportation process in an experimental and controlled environment and then, use and compare different methods to understand if and how, the composition of these bacteria change during the time inside different samples of bivalve molluscs considered. This study just represents one part of the project, and its aim is to study the concentration of vibrios within the bivalve mollusc *Ruditapes philippinarum*. Specifically, the study aimed to assess the timing of the *Vibrio* release within the ingested and released material produced during the purification process.

The combination of both cultural and non-culturable methods (e.g. real time) allowed a definition of different pictures of the bacteria release into the microcosm.

The cultural techniques are not able to detect the VBNC, on the other part the non-cultural approaches are based on the DNA analyses with an overestimation of the living cells.

NPC test showed that variables considered do not differ if only the variable time is considered, but, considering also the matrix of the experiment (6 matrices were considered in this study), it is possible to observe that each matrix influences the trend of the target and they are different.

The observed discrepancy present between some samples is due to the diversity of the methods applied, which have different sensitivities and are therefore not perfectly superimposable.

Comparing the contamination level of ingestion content, then, it is possible to conclude that the temperature of lagoon water is a parameter that could influence the level of contamination because the concentration of *Vibrio* inside molluscs is higher in summer months than in spring.

The results obtained by this study highlighted the necessity of another set of the same tests to have comparable results. To achieve this goal, the experiments and all analyses must be done in the same period of ones made in this study.

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1. INTRODUCTION

1.1. Bivalve molluscs

Bivalve molluscs are aquatic invertebrates that have anatomical and structural characteristics that are typical of the phylum *Mollusca*.

Often dominant members of the macrobenthos in coastal ecosystems providing numerous pivotal ecosystem services such as nutrient cycling, benthic-pelagic coupling, and exerting top-down control on phytoplankton communities. They are laterally compressed organisms with a sturdy ovoid shell made up of two equal valves hinged together on the dorsal side. The valves are connected by elastic ligaments and teeth to form a hinge, the closing and opening of which is controlled by the adductor muscles' contraction or relaxation. The body and mantle cavity are enclosed within the two valves (Bocchi B., 2018/2019).

The shell is made up of several calcareous layers that are covered with an organic protein material known as periostracum. The shell begins to form on each valva's dorsal protuberance, known as the umbo, which is the oldest part and the point from which the concentric lines of growth radiate. The colour of the shell is given by the presence of different types of pigments (e.g., pyrroles and porphyrins) while melanin contribute to the colour of the internal organs. (FAO, 2014).

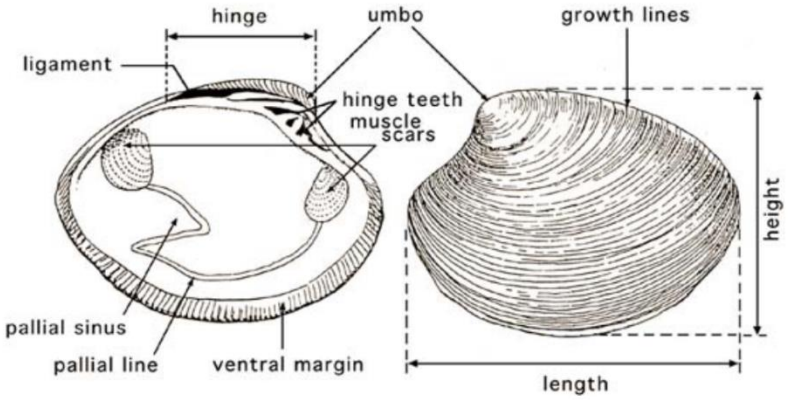

Figure 1: External and internal features of the shell valves of the hard shell clam, *Mercenaria mercenaria*. Modified from Cesari and Pellizzato, 1990.

Like all molluscs, bivalves are bilaterally symmetrical coelomate protostomes with a reduced coelom around the main organs.

Their body is covered by a mantle, a thin epidermal-cuticular membrane that envelops the inner surface of the valves and isolates the visceral mass from the shell. The two halves of the mantle are attached to the shell from the hinge ventral to the pallial line but are free at their edges. The mantle edge often has tentacles; in clams the tentacles are at the tips of the siphon (FAO, 2014).

The main function of the mantle is to secrete the shell, but it also has a sensory function and can initiate closure of the valves if environmental conditions are not suitable. It can control the water intake and takes part in the respiration process.

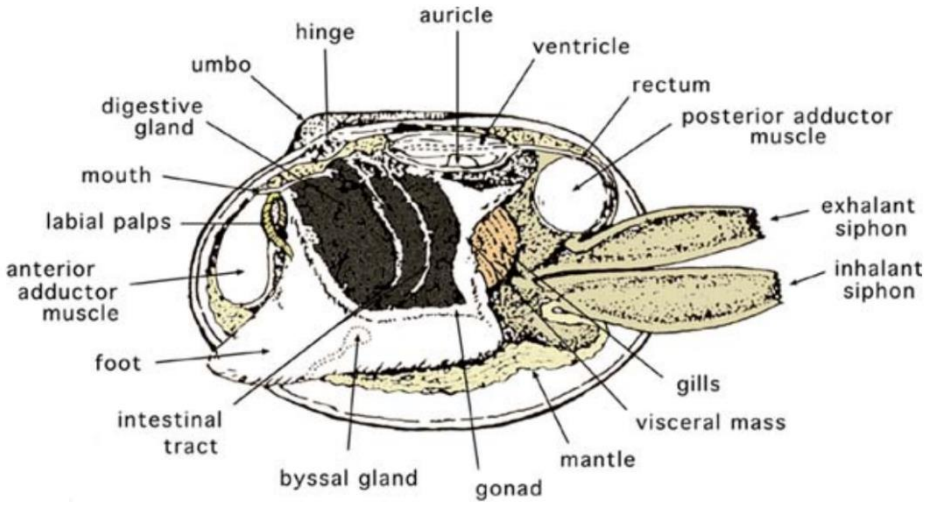

Figure 2: The internal, soft tissue anatomy of a clam of the genus Tapes. In this view, the uppermost gill lamellae have been removed to reveal the foot and other adjacent tissues.

The mantle and the body share a region known as mantle cavity which hosts also gills (known as ctenidia) and where water, nutrients, faeces, and excretory and reproductive system products converge.

The mantle is jointed at the posterior border to produce the inhaling and exhaling siphons, which ensure water flow in and outside the bivalve. The nutrient-rich water, in fact enters by the inhaling siphon and runs across the ctenidia, which are responsible for retaining food particles. The cilia thrust it through reproductive systems and the anus, collecting egg waste particles, before exiting through the exhaling siphon.

The large gills filter food from the water and direct it to the labial palps, which surround the mouth. Food is sorted and passed into the mouth. Bivalves have the ability to select food filtered from the water. Boluses of food, bound with mucous, that are passed to the mouth are sometimes rejected by the palps and discarded from the animal as what is termed "pseudofaeces". A short oesophagus leads from the mouth to the stomach, which is a hollow, chambered sac with several openings. The stomach is completely surrounded by the digestive diverticulum (gland), a dark mass of tissue that is frequently called the "liver".

The stomach contains ducts that lead to glandular digestive tracts, which produce enzymes for nutrition processing. The hepatopancreas is a gland linked to the digestive system that performs functions unique to the liver and pancreas in vertebrates, such as the release of digesting enzymes. The anus is positioned at the exhaling siphon in the mantle cavity at the end of the gut.

An opening from the stomach leads to a closed, sac-like tube containing the crystalline style. The style is a clear, gelatinous rod that can be up to 8 cm in length in some species. It is round at one end and pointed at the other. The round end impinges on the gastric shield in the stomach. It is believed it assists in mixing food in the stomach and releases enzymes that assist in digestion (FAO, 2014).

The circulatory system is open and it is made up of many sinuses and veins in the gills. The blood is called haemolymph and it is composed by haemocyanins, respiratory pigments that are able to bind oxygen thanks to their content of copper.

The heart, which pumps haemolymph into the veins, is found dorsally, inside the pericardial chamber, and is made up of two atria and one ventricle.

The afferent channel carries oxygen-depleted haemolymph to the gills, where oxygen-rich water travels in the reverse way. Because of the diffusion gradient, oxygen then goes into the haemolymph, which gets oxygenated again and may be delivered from the gills to the atria of the heart through the efferent channel.

The excretory system consists of two nephridia under the pericardial cavity, whereas the neurological system consists mostly of three pairs of ganglia (pedal, cerebra-pleural, visceral) and two pairs of nerve cords.

The renal system is difficult to observe in some bivalves, but is evident in such species as scallops where the two kidneys are two small, brown, sac-like bodies that lie flattened against the anterior part of the adductor muscle. The kidneys empty through large slits into the mantle chamber (FAO, 2014).

The presence of a laterally compressed blade-shaped foot allows the bivalves to move. The combined action of muscles and hydraulic pressure directs the foot forward and then retracts it, allowing the bivalve to sink into the sediment for up to fifteen centimetres and anchor itself to the surface.

The sense organs are positioned on the mantle's border. There may be ocelli and receptors, including both tactile and chemoreceptor cells. Statocysts, on the other hand, are responsible for the geo-reception of fossorial bivalves in the foot.

Bivalve mollusc reproduction is gonochoric (with separate sexes), with occasional incidences of hermaphroditism. When mature, the gonads are made up of tissue that forms a whitish, homogenous, soft mass around the digesting tract. Fecundation is external, and development is indirect, as in the early stages of development, a trochophore larva is formed from the fertilised egg, and at the end of the metamorphosis process, it settles on the bottom. The larval stage is transported by the water current, allowing the bivalves to colonize greater and bigger areas suitable for the species' survival.

1.1.1. Ruditapes philippinarum

Ruditapes philippinarum is a species belonging to the bivalve class, family *Veneridae*, also known as “Vongola verace”.

Table 1: Decreto Ministeriale n°19105 del 22 settembre 2017 - Denominazioni in lingua italiana delle specie ittiche di interesse commerciale. Allegato I.

Ordine	Famiglia	Denominazione Scientifica	Denominazione Commerciale	ALFA3
Siluriformes	Bagridae	*Rita spp.*	**Siluro asiatico**	
Sepiolida	Sepiolidae	*Rossia macrosoma*	**Seppiola**	ROA
Veneroida	Veneridae	*Ruditapes decussatus*	**Vongola verace**	CTG
Veneroida	Veneridae	*Ruditapes philippinarum*	**Vongola verace**	CLJ
Veneroida	Veneridae	*Ruditapes variegatus*	**Vongola verace indopacifica**	RTV

The species is classified as native to the Sea of Japan and was found across the Indo-Pacific. In the Mediterranean Sea it was commercialized for the first time in 1980 and, in the following years, a large amount of juveniles obtained in a British laboratory under controlled reproductive conditions was released in the Chioggia basin, sited in the Venice lagoon. The main aim of these consecutive releases was to extend and enhance the production sector of shellfish culture (Lazzari G., Rinaldi E., 1994).

From the morphological point of view, *R. philippinarum* is characterized by a large, strong shell with obvious sculpture, produced by numerous radial and concentric stripes that become nodular at the places of intersection, more pronounced in the back. Coloration varies greatly: yellowish or light hazelnut backdrop with more or less fused brown maculations forming varied ornamentations or real radial bands. The inside of the valves is frequently reddish or brownish, occasionally covering the entire surface, but more commonly extending beyond the palleal line. The length ranges from 25 to 57 mm. The sculpture is more apparent than in the indigenous *Ruditapes decussatus*, but the fundamental distinctions are anatomical: the inhaling and exhaling siphons appear to be welded together, whereas in *R. decussatus* they are disconnected (Cesari P., Pellizzato M., 1985).

R. philippinarum is a species defined rustic, able to adapt to changes in environmental parameters and to sediments of different types. Usually this mollusc prefers sandy or silty-sandy substrates in calm seas, often between the mean tide level and a few metres deep. It is a filter-feeding creature that feeds primarily on phytoplankton; as a result, it has an advantage in eutrophication settings, frequently reaching significant sizes and exceeding the maximum size specified for its locations of origin.

Another feature that contributes to define this species is that it grows faster than the native congener and it also appears to be more tolerant to the fluctuations in physical and chemical parameters, as well as environmental anoxic crises, making it competitive against less tolerant native species.

In European waters, the harvesting is carried out when the shell length is in a range from 30 to 40mm, this length is usually reached after 16 to 30 months. The

harvesting procedure in Europe is different from China, where clams are usually harvested when their shell length reaches 30mm or more, so after 10 to 16 months. The reason is the exchange value, higher in Europe due to the later time of harvesting (FAO, 2022).

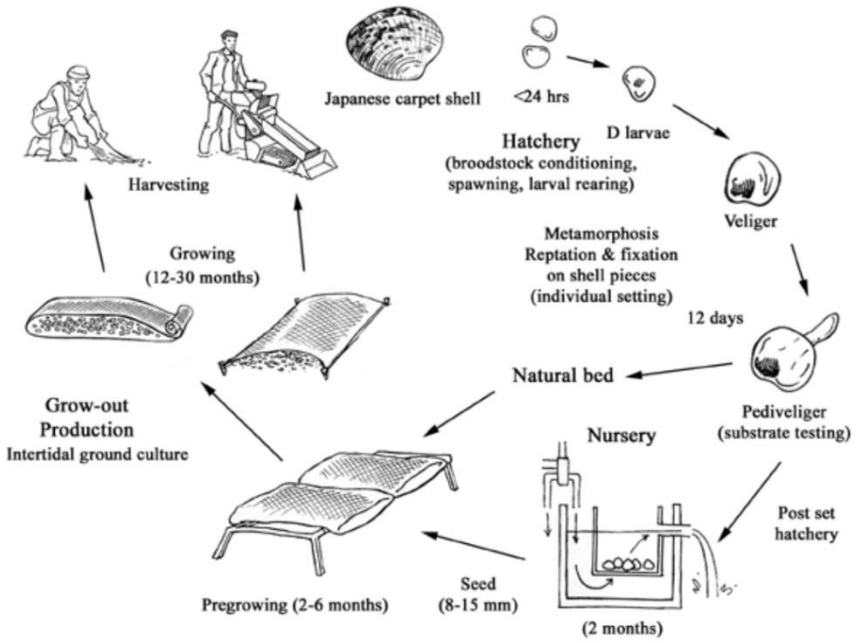

Figure 3: Production cycle of *Ruditapes philippinarum*

At the beginning of the production cycle there is the reproductive stage, followed by the hatchery and metamorphosis to a veliger larvae that is reached after 2 weeks from the beginning of the reproduction. The next stage, the pediveliger larvae is the one that is shipped away after a counting process and distribution into different types of material allowed for the shipping. An alternative way is to keep them into setting tanks or micro-nursery trays.

The pediveliger larvae is then kept into a nursery system that is different according to the shell length. They can be kept during the pre-growing phase in a nursery system that uses an up-welling approach if their shell length is in a range from 10 to 15mm. Alternatively, if the shell is bigger, they can be distributed in the seabed enclosed in mesh bags. Otherwise, other cultural practices for pre-growing exist.

During the pre-growing phase is important to measure all the clams to prevent the competition between bigger and smaller individuals, another thing concerns the

removal of predators such as crabs and all the structures and facilities used during the nursery stage must be kept clean.

When the shell length is in a range from 10 to 15mm, clams can be seed into the substrate (usually made of gravel, sand, mud and shell to facilitate the equipment used). Also, in this phase the area selected for the seeding must be treated, cleaned and predators removed.

In European waters clams are harvested after 16 to 30 months, when the shell length has reached a range from 30 to 40mm (larger size if compared to Chine, where clams are harvested after 10 to 16 months and their shell length is smaller). This because at a larger size, the commercial and exchange value is higher if compared to a smaller size (FAO, 2022).

Ruditapes philippinarum aquaculture productivity is expected to increase in the foreseeable future, either through increased acreage or new introductions into suitable places and countries. However, the influence of sickness has been the primary source of output shifts in some countries (FAO, 2022).

Perkinsus-like parasites are thought to be to blame for the fall in Japanese carpet shell output in the Republic of Korea, with huge mortalities happening every summer since the early 1990s, as well as in Japan and China (Yellow Sea). Furthermore, the bacterial disease known as "brown ring" has hampered production in some traditional producing countries (European Atlantic waters). Aside from disease issues, the growth of wild populations following the introduction of this species has caused numerous changes in production trends, either by facilitating the seed supply (Italy) or by competing economically with culture (France), favouring public fisheries. Non-indigenous predators (green crabs) represent a possible concern to commercial production throughout the western North American coast (FAO, 2022).

In 2008 FAO Fisheries and Aquaculture Department has published a technical paper titled “Bivalve depuration: fundamental and practical aspects” to furnish a sort of guide to the public health problems associated with shellfish consumption.

Regarding the consumption, in the paper is highlighted the fact that many species of bivalves are consumed alive or raw or, in some cases, lightly cooked. These ways lead bivalves to be part of the category high risk food products, which requires appropriate control measures in order to avoid or reduce to a level defined "acceptable" the biological, chemical and physical hazard (Lee R., Lovatelli A., Ababouch L.; 2008).

Depuration is a procedure used to remove dangerous contaminating microorganisms from shellfish. The mollusc is placed in a tank filled with clean saltwater, where it can restart filter-feeding and cleanse itself of toxins. Temperature, flow rates, oxygenation, salinity, and the water-to-shellfish ratio all have an impact on this process (Lee and Younger, 2002).

According to the paper, the depuration procedure enables the removal of microbial contaminants from light or moderately contaminated bivalves but if on one hand it is an effective procedure for removing faecal bacterial contaminants from shellfish, on the other hand it is less effective or ineffective if the aim is to remove other types of contaminants (such as marine biotoxins, heavy metals or organic chemicals or other viral contaminants (Lee R., Lovatelli A., Ababouch L., 2008).

The major concern about the effectiveness of depuration procedure regards the removal of some *Vibrio* species that are classified as pathogenic for humans and that can cause damage to health and illness; it is highlighted in the paper that the problem is when the salinity is in the right range suitable for shellfish and the temperature is high enough, this condition can lead to an increase in the concentration of vibrios during a depuration cycle (see paragraph 1.2.3. *Vibrio* in microbiota of bivalve molluscs and in *Ruditapes philippinarum*) (Lee R., Lovatelli A., Ababouch L., 2008).

Since clams are molluscs, they have filter feeders' habits. This mechanism of feeding analysed in the next paragraphs allows the accumulation of contaminants to a much higher level than that of the surrounding seawater. Contamination with bacteria and viruses in the growing area therefore determines the processing that

the shellfish need to undergo in order to remove or reduce the risks from these sources before consumption.

Table 2: Hazards associated with bivalve mollusc consumption (Lee, R.; Lovatelli, A.; Ababouch, L. Bivalve depuration: fundamental and practical aspects. FAO Fisheries Technical Paper. No. 511. Rome, FAO. 2008. 139p.).

Class of hazard		Contaminant
Infections	Bacteria	*Salmonella* spp., *Shigella* spp., *Vibrio parahaemolyticus, Vibrio vulnificus, Vibrio cholerae, Campylobacter* spp., *Listeria monocytogenes*
	Viruses	Norovirus, Hepatitis A virus
Intoxications	Chemical	Heavy metals: including Mercury (Hg), Cadmium (Cd), Lead (Pb). Organics: Dioxins, Polychlorinated Biphenyls (PCBs), Polycyclic Aromatic Hydrocarbons (PAHs), pesticides
	Biotoxin	Paralytic shellfish poisoning (PSP), Diarrhetic shellfish poisoning (DSP), Amnesic shellfish poisoning (ASP), Neurotoxic shellfish poisoning (NSP)

Other risks are linked to naturally occurring species in the maritime environment. Infections caused by pathogenic marine *Vibrio* bacteria and biotoxins produced by some single-celled algae can cause a variety of poisonings, including paralytic shellfish poisoning ecc (Lee R., Lovatelli A., Ababouch L., 2008).

Table 3: Microbial causes of bivalve shellfish-associated illness.

Microorganism	Incubation period	Duration	Principal signs and symptoms	Principal source of contamination of shellfish
Bacteria				
Salmonella typhi and *S. paratyphi*	*Typhi*: 1–3 weeks *Paratyphi*: 1-10 days Other source: 7 to 28 days, mean14 days	*Typhi*: up to 4 weeks *Paratyphi*: 2-3 weeks	Malaise, headache, fever, cough, nausea, vomiting, constipation, abdominal pain, chills, rose spots, bloody stools	Human faeces/ sewage
Other *Salmonella*	6 to 72 hours, mean18 to 36 hours	4–7 days	Abdominal pain, diarrhoea, chills, fever, nausea, vomiting, malaise	Human faeces/ sewage or animal/ bird faeces/slurry
Campylobacter	2 to 7 days	3–6 days	Diarrhoea (often bloody), severe abdominal pain, fever anorexia, malaise, headache, vomiting	Animal/bird faeces/slurry
Shigella	24 to 72 hours	5–7 days	Abdominal pain, diarrhoea, bloody & mucoid stools, fever	Human faeces/ sewage
Vibrio parahaemolyticus	2 to 48 hours, mean 12 hours	2–14 days (average 2.5)	Abdominal pain, diarrhoea, nausea, vomiting, fever, chills, headache	Marine environment
Vibrio vulnificus	16 hours mean < 24 hours	2–3 days	Malaise, chills, fever, prostration, cutaneous lesions, fatalities occur	Marine environment
Vibrio cholerae O1 and O139 serotypes	1–5 days, usually 2–3 days	2–5 days	Profuse, watery diarrhoea (rice-water stools), vomiting, abdominal pain, dehydration	Human faeces/ sewage
Vibrio cholerae non-O1/nonO139	2 to 3 days	Up to 1 week	Watery diarrhoea (varies from loose stools to cholera-like diarrhoea)	Marine environment
Viruses				
Norovirus	1–3 days mean 36 hours	20 to 72 hours	Diarrhoea, nausea, vomiting, abdominal pain, abdominal cramps	Human faeces/ sewage
Hepatitis A virus	10 to 50 days, mean 25 days	10 to 30 days 10% of infected persons will have prolonged or relapsing symptoms over a 6–9-month period	Fever, malaise, lassitude, anorexia, nausea, abdominal pain, jaundice	Human faeces/ sewage
Astrovirus[1]	1 to 2 days	48 to 72 hours	Diarrhoea, some times accompanied by one or more enteric signs or symptoms	Human faeces/ sewage

In Table 3 are listed microbes linked to bivalve mollusc-associated illness. The majority of these are related to the faecal contamination and, among them, Norovirus (responsible of viral gastroenteritis) is the most common illness associated with the consumption of bivalve molluscs that occur in many developed temperate countries; also, if in the United States of America the highest amount of infection is caused by pathogenic vibrios such as *V. parahaemolyticus* and *V. vulnificus* (Lee R., Lovatelli A., Ababouch L., 2008).

1.1.2. Egestion process in *Ruditapes philippinarum*

Given the biological hazards that the filter-feeding activity of clams, and bivalve molluscs in general, can entail, it is useful to understand how the feeding, digestion and the expulsion of particulate matter from and through the molluscs' bodies work.

The digestive tract of bivalves is anatomically organized with digestive diverticula, gut tubules ending in blind sacs that serve as sites for phagocytosis and intracellular digestion. This intricate structural aspect of the gut is a perfect environment for resident microbes (Kueh CS, Chan KY, 1985).

The digestive system of the clam is responsible for breaking down food to obtain the nutrition that will later be used as an energy source. It accomplishes this task by using enzymes and sifting mechanisms to filter what the body of the clam needs, and what can be excreted from the body as waste.

Bivalves are filter feeders and pass large volumes of water across their gills to obtain oxygen and food. Particulate matter from the water, including microorganisms, is trapped in mucus in the gills and transported toward the mouth by ciliary action. As the mucus passes the labial palps, particles are sorted, and non-food items are rejected as pseudo faeces. The remaining items entrapped in the mucus enter the mouth. The food passes through a short oesophagus to the stomach where it is mixed with digestive enzymes by the rotating action of the crystalline style. Small food particles are transported into the blind tubules of the digestive diverticula where they are absorbed by the cells lining the tubules, or are ingested by phagocytic cells that migrate into the tubules. Particles that do not enter the digestive diverticula are passed out of the stomach into the midgut and are

eventually discharged through the anus. This process requires <2 hours in actively feeding adult oysters (Galtsoff, 1964).

Whatever is left over at this point travels to the rectum to awaiting being excreted from the body.

In a study conducted by J.-M. Defossez and A. J. S. Hawkins (1997), they were able to understand how bivalve molluscs select ingested particles according to different characteristics of the particles. Despite the particle selection in filter-feeding bivalve molluscs is well established (e.g. Newell and Jordan 1983; Hawkins et al. 1997, 1998), the emerging techniques as, in particular the development of low cytometry, allowed the confirmation of preferential feeding in bivalves.

In the classic literature, three different levels of selection are known: (1) selective retention on the gill, (2) preferential ingestion following selective rejection of filtered material as pseudo faeces, and (3) preferential digestion of ingested matter.

Differential selection can obviously influence the effect of filter-feeding bivalves on both natural suspensions and on nutrient flux and, in addition, selective feeding means that the nutritional value of available seston depends at least as much on the relative abundance of different particles within that seston as on the total concentration of particles (Defossez & Hawkins, 1997).

During their experiment Defossez & Hawkins fed the mussel *Mytilus edulis* and the clams *Tapes decussatus* and *Ruditapes philippinarum* were fed a mixture of SiO_2 particles that were of the same shape, density and chemical composition, but with diameters that ranged from 5 to 37 μm. What they found is that particle size is a significant criterion by which particles are preferentially rejected as pseudofaeces prior to ingestion.

1.2. Microbiota of bivalve molluscs

Before focusing on bivalve molluscs, it is important to clarify that the terms microbiome and microbiota that will be found in this study have been used after some researches on the difference between the two terms due to many definitions that were published in the past by other authors.

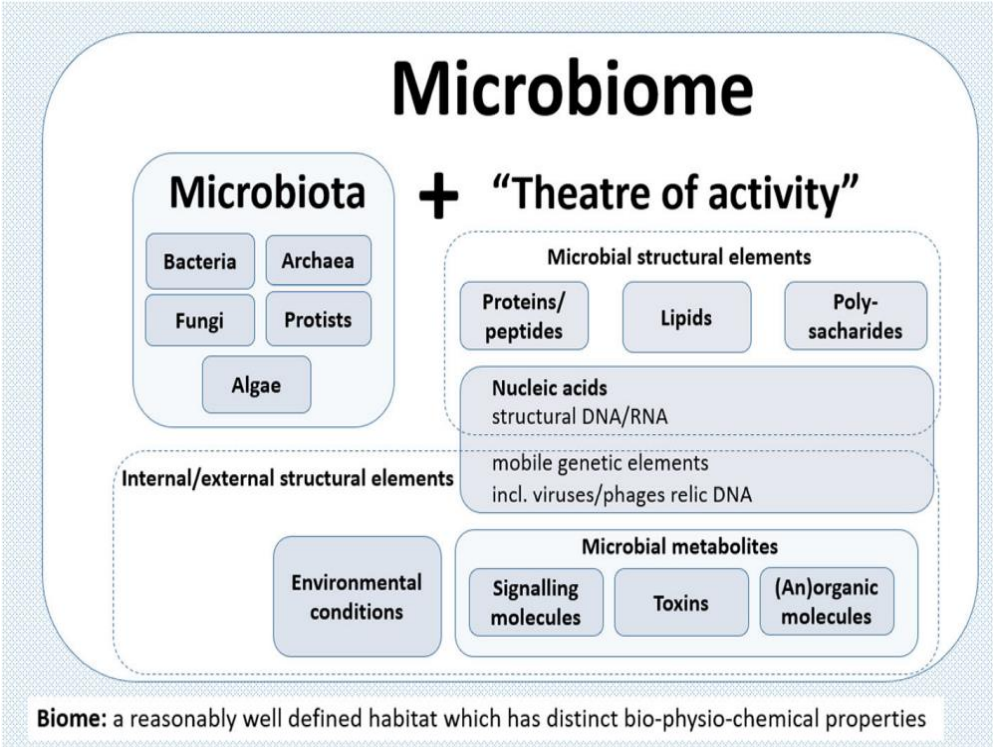

Figure 4: A schematic highlighting the composition of the term microbiome containing both the microbiota (community of microorganisms) and their "theatre of activity" (structural elements, metabolites/signal molecules, and the surrounding environmental conditions). Berg et al., 2020.

One of the first advancements in the clarification of the difference between the term microbiome and microbiota was given in 1988 by Whipps and colleagues who were studying the microorganisms present in the rhizosphere of plants. In this contest, microbiome was defined as a term composed by the words "micro" and "biome" which together identify a characteristic microbial community with its own physio-chemical properties, so as a consequence it also has different interactions with the environment in which it is found.

Although there are many other definitions of microbiome, nowadays the most cited one was given by Lederberg in 2001 and defines microbiomes as a collection of different microorganisms (so that can be pathogenic, with commensal attitude or that live in symbiosis) that form a community characterized by a body space.

So definitely microbiome is the collection of microorganisms that live in a specific environment and which has its properties and functions. Differently from microbiome, the microbiota is defined usually as an assemblage of organisms, that

specifically are living microorganisms (Berg et al., 2020) and so all the other features and elements such as proteins, nucleic acids, phages, viruses or prions are not part of microbiota because classified usually as non-living microorganisms.

According to the definition of microbiome and microbiota, living microorganisms present in the environment, whether marine or terrestrial, are known as the microbiota while these living microorganisms, if studied together with their products (and so non-living components), are called microbiome.

In recent decades, the symbiotic relationships between bacteria and eukaryotes have been extensively studied, showing how the microbiota (i.e. the set of microbial communities associated with a host) plays a key role in the development and health of the host, as well as in its responses to environmental changes (Milan et al., 2018; Meisterhans et al., 2016). Bivalve molluscs host a rich bacterial microbiota on their surface and in their internal organs, which is currently poorly documented due to the few studies on the subject (Leite et al., 2017; Meisterhans et al., 2016). The colonisation of microorganisms forming part of the microbiota occurs in marine habitats, including offshore aquaculture (Paillard et al., 2022). In this case, the gut microbial community is determined by the aquatic environment and the conditions present especially in aquaculture systems, such as water chemical parameters (pH, COD - chemical oxygen demand -, temperature, salinity total phosphorus, total nitrogen and inorganic nitrogen), which influence microbial community composition and disease occurrence (Sun et al., 2019).

Microbial communities within the gut of aquatic organisms are also closely linked to host development, nutritional status, immune response and disease resistance (Sun et al., 2019).

In general, in marine invertebrates, the microbiota can be acquired vertically or laterally (in this work the attention is for laterally acquired microbiota). In the former case, transmission occurs from parents to offspring, whereas in lateral transmission it occurs from the environment to the host and is subject to environmental fluctuations (Meisterhans et al., 2016).

It has been proposed that the composition of the microbiota is useful to the host not only for survival, but also for homeostasis and development. Studies on

microorganisms residing in the haemolymph of healthy bivalves, for example, show that the host microbiome can limit the establishment of pathogenic strains and play a role in preventing dysbiosis and promoting health recovery.

Furthermore, comparative research has been conducted on oysters and mussels, and to a lesser extent on clams. Species from similar environments showed a significant degree of similarity and a high number of major OTUs (Operational Taxonomic Unit, the definition used when the aim is to classify individuals that are strictly correlated between each other) demonstrating that uptake of environmental strains through suspension feeding is an important predictor of microbiome composition. However, bivalve species obviously differ in microbiome composition and relative abundance of taxa, which may explain why a certain species is better able to protect itself from pathogens or survive in various environmental conditions.

The influence of environmental factors on bivalve microbiota can be explained by the benthic lifestyle of bivalves, its heterogeneity at small spatial scales (ecological niches, sessile versus free-living life stages), and by the spatial and temporal variation of abiotic and biotic factors (season, transplantation, pollution, pathogens, water quality, and so on). Microbiota-environment interactions have repeatedly been studied by monitoring bivalves in the field, either over short or long periods (Paillard et al., 2022).

Specific diversity patterns suggest that bivalves select the microorganisms from the water column or sediment, depending on their microbiota compartment. This microbiota selection process is modified with the time of exposure for intertidal bivalves, but also with the seasons. The influence of stochastic processes may also be a factor for microbiota diversity. The transfer of bivalves from hatcheries to rearing sites plays an essential role in shaping the microbiome from juveniles to adults (Paillard et al., 2022).

The initial microbiome (bacteria, protist and virus) is deeply modified by transplantation (strongly for transient microbiota, and less for resident microbiota such as those from digestive gland), according to the environmental conditions of the new rearing sites.

Water temperature seems to be a major driver and strongly influences the composition of oyster microbiota both in the haemolymph and other tissues, and only a small fraction of the microbial species persists as a 'core' community throughout the different seasons.

Accordingly, climate change was observed to alter the microbiome of the Sydney rock oyster *S. glomerata*. Warmer temperatures are also favouring the proliferation of potential pathogenic bacteria (e.g. *Vibrio*) within bivalve microbiota, thus increasing susceptibility to microbial diseases (Paillard et al., 2022).

1.2.1. Transient and "core" microbiota

In some studies, the distinction between transient and resident bacteria was done by considering the "residency" of the microbes inside their hosts and the "function" that they could have. In the study conducted by Hammer et al., 2019 they use the term "residency" to refer to the degree to which a microbial population remains stably associated with a host. Resident microbes, also known as symbionts (Douglas., 1994), differ from transient microbes in that the replicate inside a host at a rate exceeding loss due to death or excretion (Hammer et al., 2019).

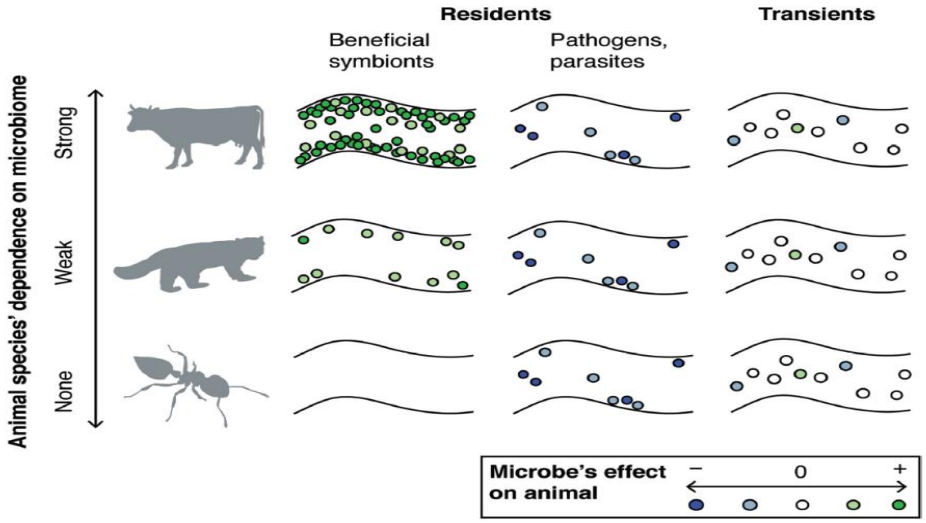

Figure 5: A schematic of microbial associations for three animal species across the microbial dependency spectrum, exemplified by a cow, red panda, and *Crematogaster* ant. Shown is a section of the gut, but the same principles could apply to non-gut symbioses as well. Inset: classification of individual microbes by their effect on host fitness, from negative (−) to neutral (0) to positive (+). Note that microbial residency and function categories can be fluid and context-dependent (not shown). For clarity, we do not depict the case of an animal highly dependent on transient microbes as food.

By “function” indeed, they refer to the types and degree of microbial effects on host fitness. Transient microbes could have positive, negative, or negligible effects on hosts. Due to their growth and metabolic activity, resident microbes are likely to have some effect on their host, but it could be negative, in which case they would traditionally be described as parasites or pathogens. Alternatively, resident microbes could increase host fitness; in these cases, the benefits to hosts can take many forms, and can vary in their importance depending on the microbial and host taxa involved and on environmental conditions (Hammer et al., 2019).

By looking at temporal and spatial dynamics of microbiomes, researchers decided to try to define a “core” microbiota; this because although the distinction between residency and function referred to microbial populations seems to be clear; often native microbiomes can be made up of many different kingdoms and this can make the distinction between resident and transient microbes not so easy. To support this necessity, useful also in the present study, Berg et al. provide a definition of transient microbiota in their publication in 2020 sustaining that transient microbiota can change over time and this change is due to different factors such as, for example, environmental conditions.

If on one hand transient microbiota changes over time, on the other hand “core” microbiota seems to remain stable and constant, characterized by a microbial community that live in association with a host genotype or in a specific environment with changes in the composition when passing from an environment to another (Berg et al., 2020).

A study conducted on oyster samples can help to better understand the difference between transient and resident microbes with a particular focus on bivalve molluscs. In order to understand how animal microbiomes are structured it can be helpful to determine which members of the microbiome are strictly governed by the host, and which members are governed by external environmental conditions or by stochastic processes that are unrelated to the host. Microbial members that are explicitly selected by the host can be considered resident associates of their host, whereas microbial members driven by external factors (e.g., the local environment or stochastic processes) can be considered transient associates of their host. For example, some oyster haemolymph bacteria such as *Vibrio spp.* can persist, despite

the high filtration activity of oysters, in the absence of an environmental source population (e.g., when held in sterile seawater) and over a range of environmental conditions, suggesting that host factors maintain this resident relationship (Vasconcelos and Lee, 1972; Lokmer et al., 2016).

Although there have not been many studies on the microbiota of clams, studies on other bivalve molluscs, such as oysters, can be considered. In the haemolymph of some oysters it has been observed that, for example, vibrios are able to persist in the absence of an environmental source population (e.g., when maintained in sterile seawater) and under a variety of environmental conditions, implying that host factors maintain this resident connection (Unzueta-Martínez, 2022).

1.2.2. *Vibrio*

Vibrio is a well-known and extensive genus of Gram-negative marine bacteria belonging to the family *Vibrionaceae*, part of the *Gammaproteobacteria* group (Zampieri et al., 2020). The genus *Vibrio* consists of more than 100 species grouped into 14 clades that are widely distributed in aquatic environments such as estuaries, coastal waters, and sediments (Romalde et al., 2014).

Figure 6: *Vibrio cholerae* bacteria, Locatus

Their morphology resembles that of a comma equipped with 1 to 3 flagella, and their size varies from 0.5 to 0.8 μm in width and 2 to 3 μm in length.

The presence of flagella enables these marine bacteria to exhibit active motility in aquatic environments and also confers the ability to perform chemotaxis along the nutrient gradient (Stocker et al., 2012).

Among marine bacteria, *Vibrio* exhibit great genomic plasticity, allowing them to adapt to new ecological niches as defined by constantly changing marine environmental conditions.

In marine ecosystems, the encounter of *Vibrio spp.* with other bacteria may require coexistence in the same ecological niche and, consequently, the coordination of biological activities. This coexistence is possible due to the mechanism of quorum sensing, by which vibrios respond to changing aquatic conditions and maintain associations with animal hosts (Milton, 2006).

Quorum sensing thus allows *Vibrio* to have a range of adaptive responses to the marine environment to survive restrictions in nutrition, UV exposure, temperature fluctuations, protozoan predation, viral infections and salinity variations. These quorum-sensing systems function as coordinate regulators of a wide range of phenotypes of different cell densities and mediate the response of the *Vibrionaceae* to environmental stress, as well as regulating such phenotypes as biofilm formation and virulence.

Biofilm formation and phase variation have a crucial role in niche persistence of the *Vibrio* species in otherwise deleterious environments.

Reversible phase variation between the rugose and smooth colony variants is important for the survival of *Vibrio* species in natural aquatic habitats. Epidemic strains of *V. cholerae* switch from the smooth to the rugose phase more frequently than environmental isolates, and this phase transition increases the resistance of the organism to osmotic, acid and oxidative stress, and enhances its capacity to form a biofilm (Reen et al., 2006).

As bacteria, *Vibrio* exhibit the typical bacterial growth curve (when grown in a closed system) characterized by a first adaptational or lag phase in which the bacteria adapt to the new medium; then a new exponential phase begins, in which the cells start dividing by binary fission at a regular interval of time. The bacterial cells stop their growth when they reach the stationary phase, in which the growth becomes limited until there is an eventual loss of viability (also called death phase). The limitation of the growth is also known as VBNC (viable, but non-culturable) that is a particular phase in which the bacteria is trying to face environmental

changes that can happen in the marine environment and so it is a survival strategy. In this phase bacteria are metabolically active, but they are not detectable when cultured because their ability of reproduce is absent (Oliver, 2005).

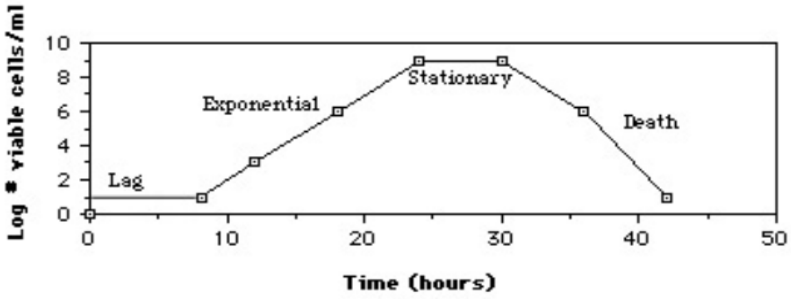

Figure 7: The typical bacterial growth curve. When bacteria are grown in a closed system (also called a batch culture), like a test tube, the population of cells almost always exhibits these growth dynamics: cells initially adjust to the new medium (lag phase) until they can start dividing regularly by the process of binary fission (exponential phase). When their growth becomes limited, the cells stop dividing (stationary phase), until eventually they show loss of viability (death phase). Note the parameters of the x and y axes. Growth is expressed as change in the number viable cells vs time. Generation times are calculated during the exponential phase of growth. Time measurements are in hours for bacteria with short generation times.

Growth is expressed as a change in the number viable cells vs. time. Generation times are calculated during the exponential phase of growth. Time measurements are in hours for bacteria with short generation times (Todar, 2020).

A large number of species of the genus *Vibrio* are associated with marine organisms such as fish, molluscs and crustaceans, in commensal or pathogenic relationships. This ability to be found in relationships with different marine organisms is due to the fact that bacteria belonging to this genus are capable of both free-living in water and of remaining attached to biotic or abiotic surfaces. In this sense, they can specifically attach themselves to the body surface of both animals and plants in order to advance through the various stages of development and simultaneously escape predators. On the other hand, vibrios are also associated with a wide variety of poikilotherm and homoiotherm animals, including humans, for some of which are pathogens. Paillard et al. (2004, Romalde et al., 2014)) recognized that the emergence of vibrios as etiological agents of diseases is likely to increase over the coming years due to ocean warming.

The hosts of *Vibrio* include bivalve molluscs, the latter of which, due to their filter-feeding activity, are able to accumulate a rich and diverse microbiota composed of different species belonging to genera such as *Vibrio, Pseudomonas, Acinetobacter, Photobacterium, Moraxella, Aeromonas, Micrococcus,* and *Bacillus* (Murchelano and Brown, 1970; Kueh and Chan, 1985; Prieur et al., 1990).

The filter-feeding activity of molluscs thus increases the association with *Vibrio spp.* Because in addition to food particles, taken from the water column, the tissues of bivalve molluscs also incorporate these marine bacteria (Zampieri, 2020).

Some researchers suggest that the biodiversity of *Vibrio* found within bivalve molluscs differs depending on which tissue of the bivalves is colonised (Lokmer et al., 2016; Destoumieux-Garzón et al., 2020), suggesting that this biodiversity is unpredictable and depends on the adaptations of these marine bacteria to both the surrounding environment and the conditions of the host body.

Since the 1990s, a large amount of studies have been conducted in order to understand the diversity of *Vibrio* species that are associated with bivalves (Montilla et al., 1994; Hariharan et al., 1995;Arias et al., 1999; Pujalte et al., 1999; Maugeri et al., 2000; Caballo and Stabili, 2002; Castro et al., 2002; Beaz-Hidalgo et al., 2008, 2010a; Lafisca et al., 2008). The most important point that was highlighted by these studies was that the variations in environmental parameters (such as temperature and/or salinity) can change the diversity of *Vibrio* spp. Present in the environment. On the other hand, these environmental changes that can happen randomly have influences also on the physiological state of the bivalve and on its tolerance, especially to bacterial infections (Arias et al., 1999; Pujalte et al., 1999; Maugeri et al., 2000; Paillard et al., 2004; Garnier et al., 2007).

In later developmental stages, tissue type becomes a strong determinant of bacterial community composition. In particular, microbiota vary between internal organs such as the gut and those that are in closer contact with the environment (e.g. haemolymph or gills). This pattern, consistent across individuals of different species, might suggest a role of microbiota in different physiological processes specific to the investigated organ (Paillard et al., 2022).

Thus, the *Vibrio* community is linked to the haemolymph and digestive glands of various bivalve molluscs (Milan et al., 2018; Vezzulli et al., 2018; Milan et al., 2019) and the species belonging to this genus of bacteria are different, and include both non-pathogenic and pathogenic species capable of causing disease to both humans and molluscs.

About the type of relationship with the host, some studies have shown the existence of neutral *Vibrio*-bivalve associations, as in the case of oysters. Indeed, in the study conducted by Wegner et al., 2019, it is shown that the natural contact of bivalves with these marine bacteria has led to *Vibrio* being an integral part of the oyster's natural microbiota.

On the other hand, however, many *Vibrio* species pose a health risk to the growth of bivalves during harvest management. Regarding this point, it became necessary to classify *Vibrio* species into primary and opportunistic pathogens. In the former case, disease outbreaks occur that kill healthy bivalves, while in the latter case, disease outbreaks occur when the host's (the bivalve's) defence barriers have already been lowered and compromised by other infections or environmental stressors (such as that caused by rising temperatures, which make bivalves more vulnerable to pathogenic opportunistic *Vibrio*). The distribution of biodiversity is in fact directly controlled by environmental elements such as the water temperature, which is important for the spread of pathogenic *Vibrio* species (Romalde et al., 2014); for proper development, water temperature must be between 10° and 30°C. Salinity, on the other hand, varies between 5 and 30%.

Pathogenic *Vibrio* species that can cause disease during both larval and adult stages of bivalves include (Zampieri et al., 2020):

- Species such as *Vibrio alginolyticus, Vibrio 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).
- *V. splendidus* strains are the main cause of disease and mortality of adult *Mytilus edulis* (Ben Cheikh et al., 2016).

- In 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). In this case, the host fails to develop properly and consequently its reproduction is halted. In addition, proliferation of *V. tapetis* in the clam tissues could led to mortality (Rodrigues et al., 2015). In 'Summer mortality', which affects the Pacific oyster *Crassostrea gigas*, 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 far as humans are concerned, vibriosis is divided into choleric and non-choleric infections (Baker-Austin et al., 2018). In the former case, infection occurs following the ingestion of contaminated food and water; the main aetiological agent responsible for these infections is *V. cholerae*. It causes watery diarrhoea that, if untreated, could lead to death (Morris, 2003).

Non-cholera infections are, unlike the former, transmitted by species such as *V. parahaemolyticus* and *V. vulnificus* and cause mild gastroenteritis and primary septicemia, respectively, as clinical manifestations (Baker-Austin et al., 2018).

A lot of studies described the influence of environmental parameters (i.e., salinity and water temperature) on the diversity and alternance of *Vibrio* species (Kaspar and Tamplin, 1993; Motes et al., 1998; Arias et al., 1999; Pujalte et al., 1999). For instance, in bivalves from the Mediterranean Sea, *V. splendidus* has been found to be dominant during winter and spring and *V. harveyi* during the warmer months (Arias et al., 1999; Pujalte et al., 1999). Another example, in shellfish growing areas of the US Northern Gulf Coast, the densities of *V. vulnificus* were high and almost constant at temperatures above 26◦C and/or at salinity below 25 ppt, but decreased drastically below this temperature and/or above this salinity (Motes et al., 1998).

Vibrio contamination has been a major source of worry in Italy. The 2016 guidelines include information on the identification of *V. cholerae* O1, *V. cholerae* 0139, *V. cholerae* non-O1, *V. cholerae* non-O139 (possibly enteropathogenic), and *V.*

parahaemolyticus in edible shellfish tissues. Despite the fact that (EC) Nos. 882/2004 and 854/2004 were repealed by (EC) No 13 2017/625, the rules remain in effect in Italy, according to the Ministero della Salute DI.GI.SAN 0069887 of 18/12/2019 (Zampieri et al., 2020).

1.2.3. *Vibrio* in microbiota of bivalve molluscs and in *Ruditapes philippinarum*

Bivalves can accumulate significant amounts of *Vibrio* species in their tissues due to their filter-feeding behaviour (Pruzzo et al., 2005). Microbiological risk control is applied to the agricultural regions of bivalve molluscs.

In Europe, European Regulation (EU) 2019/627 classifies bivalve mollusc farming areas as zones A, B or C, based on the concentration of *Escherichia coli* in mollusc flesh and intravalvular fluids. Bivalve molluscs collected from zone A, in particular, could only be harvested for direct human consumption if they contained 230 *E. coli* per 100 g of flesh and intravalvular fluids. In 90% of the samples, *E. coli* levels in bivalve molluscs collected in Zone B must not exceed 4,600 E. coli/100 g flesh and intravalvular fluids. In addition, these animals must undergo a depuration procedure before being sold as human food. In 90% of the samples, *E. coli* levels in bivalve molluscs collected in zone C must not exceed 46,000 per 100 g of flesh and intravalvular fluids. In this situation, the animals must be kept in a zone A area for at least two months to meet the microbiological standards; this action guarantees their suitability for human consumption. Furthermore, these shellfish can only be consumed as cooked seafood (Zampieri et al., 2020).

*V.splendidus, V.alginolyticus and V.*harveyi are the *Vibrio* species most commonly associated with bivalve molluscs. The combination of these species (or some of them) is the most common cause of diseases affecting bivalves at all life stages. Furthermore, *V.tapetis* has been identified as a pathogenic bacterium for clams (Rahman et al., 2014).

Analysis of the relationship between molluscs and the bacterial communities of their living environment has established that, although the concentration of microorganisms in bivalves is higher than that of the surrounding environment in

which they grow (Huss, 1994), the composition of their microbiota reflects that of the harvesting waters (Richards, 2001). The families *Vibrionaceae*, *Moranxellaceae* and *Legionellaceae* are decreased or undetected after the depuration process.

Many differences were observed between bacterial populations that live in association with tissues of bivalves (such as digestive gland or haemolymph) and those populations that are usually found in marine environments. In addition, it has been observed that such populations are dominated by a smaller number of species if compared to the ones present in salt water, these include *Vibrio* and *Pseudoalteromonas*.

In general, *Vibrio* represented a greater fraction of the microbiota in *C. gigas* (on average 1.7 times in the haemolymph) than in *M. galloprovincialis* so it is evident that *C. gigas* may provide better survival conditions for these bacteria, including also potentially harmful species such as *V. aestuarianus*. (Vezzulli et al., 2018).

In the study conducted by Milan et al., 2018, the microbial community of the hepatopancreas of the clam *R.philippinarum* was characterised by sequencing the 16s rRNA gene amplicon. The samples analysed came from the Venice lagoon. The microbiota in the digestive gland can be partially modified through the acquisition of microbial species present in the environment.

However, several studies have shown that the host-microbiota association in the digestive tract and gills of some invertebrate species, including crustaceans and oysters, is stable. The bacterial communities associated with *R.philippinarum* were found to consist mainly of the phylum *Proteobacteria, Tenericutes, Fusobacteria, Bacteroidetes, Spirochaetes, Firmicutes* and *Chlamydiae*. The most abundant classes in both seasons when sampling was performed (winter and summer) were *Alphaproteobacteria* and *Gammaproteobacteria* (comprising the genera *Vibrio* and *Pseudomonas*) (Milan et al., 2018).

Rahman et al. (2014) showed that *Vibrio* isolated from mollusc samples (including *R.philippinarum*) from the Venice lagoon mostly comprised species belonging to the group of *Vibrio* considered ‘low risk’, including *V.alginolyticus, V.fluvialis, V.harveyi* and *V.mimicus*. However, *Vibrio* belonging to the ‘high-risk’ group have

also been found, including *V.parahaemoliticus* and *V.vulnificus*; these are capable of interacting with the mollusc's haemolymph, causing it to remain in the mollusc for longer and be difficult to eliminate (Cattaneo and Bernardi, 2010).

Refrigeration of molluscs as soon as possible after harvest and maintaining low temperatures (less than or equal to 10 °C) have been shown to be important in preventing pathogenic vibrios from multiplying at high levels. In areas of the world prone to these problems, controls on harvesting, post-harvest transport conditions or post-harvest treatment (pasteurisation, high-pressure treatment, freezing or irradiation) may be carried out during the summer months, when the risk is highest.

"Under these conditions it may not be possible to achieve consistent removal of marine vibrios and there is concern that increased temperature may increase the possibility of proliferation of marine vibrios within a purification system." (Lee R., Lovatelli A., Ababouch L., 2008).

Depuration has been shown to be ineffective in reducing a number of *Vibrio* species that are pathogenic to humans, and there is concern that if salinity is in the right range (e.g. 10 to 30 ppt) and temperature is high enough (e.g. above 20 °C), an increase in *Vibrio* concentration may occur during a depuration cycle. This point is particularly important because it represents the basis of observations of the present study, from which the aims were then developed.

1.2.4. Study of *Vibrio* present in microbiota of *Ruditapes philippinarum*

The communities of *Vibrio* and the different species that characterize the microbiota of *Ruditapes philippinarum* can be identified by using culture dependent methods and independent culture methods.

1.2.4.1. Culture dependent methods

Culture dependent methods consist of isolating and cultivating microorganisms and then identifying them through morphological, biochemical or genetic characteristics (Jany and Barbier, 2008).

They start with an aliquot of the sample to be analysed, from which subsequent dilutions are prepared (in varying numbers according to need); then the plates of medium in which the sample and dilutions have been sown will be incubated for a set period of time, after which the presence, type and number of micro-organisms can be determined (J.M. Jay et al., 2009).

Analyses for finding microorganisms in samples can be divided into:

- Qualitative analyses, which involve determining the presence or absence of specific bacteria;
- Quantitative analyses, whereby the number of microbial cells in the reference matrix is determined and will later be tested in the laboratory.

Several methods exist to determine the number of microorganisms or colony-forming units (CFU), each with limitations related to their use.

However, these methods are known to have numerous disadvantages, such as the fact that some microorganisms can only be cultivated in media that meet their exact metabolic and physiological requirements. Furthermore, an artificial culture medium generally only allows the growth of certain species and the composition of the cultivable fraction is distorted during cultivation because replication times vary in different species (Carraro et al., 2011).

Conventional culture methods are unable to detect non-culturable microbial cells, so independent culture techniques have been developed to overcome this limitation (Justè et al., 2008).

1.2.4.2. Culture independent methods

The term ‘culture-independent’ refers to all cultivation-based methods used to study microorganisms in a specific ecosystem. These offer many advantages over culture-dependent methods.

Micro-organisms are detected by their DNA, RNA and proteins, not because they are able to grow on a specific microbiological medium. Furthermore, the physiological state of the bacterial cell does not influence the outcome of the investigation (Cocolin et al., 2013), as the DNA used for analysis can come from both live and dead bacteria. These molecular methods allow the distinction between

species with similar phenotypic characteristics and also between strains belonging to the same species (Carraro et al., 2011).

However, technical problems may arise: DNA may not be collected from all genotypes, some genotypes may not be detected due to the low abundance of the species in the substrate or due to inadequate cell lysis. Furthermore, 'PCR amplification could be inaccurate or inhibited (Jany and Barbier, 2018).

The molecular techniques developed involve cloning and subsequent sequencing of DNA fragments, or amplification of target sequences using PCR, prior to their sequencing. PCR products can be cloned and sequenced or used to obtain genetic profiles (Carraro et al., 2011).

2. OBJECTIVES

2.1. The project

This study is part of a larger project, divided into several phases and aimed at studying the changes in the natural microbial community of the microbiota of *Ruditapes philippinarum* with a focus on human pathogenic species.

The objectives of the project were firstly to study the release of *Vibrio* species during the depuration process in an experimental and controlled environment and then, use and compare different methods to understand if and how, the composition of these bacteria change during time inside different samples of bivalve molluscs considered.

The project is divided into two main activities: the first phase focuses on the retrospective study of *Vibrio* and *Arcobacter* populations from different production sites, while the second phase consists of a study of the colonization by *Vibrio* inside a real depuration centre.

To achieve the objectives of the two different phases, TASK1 is organised to study the community dynamics of *Vibrio*/*Arcobacter* within samples of different origin (before and after depuration). On the other hand, TASK2 aimed to explore the colonization of depuration centres by *Vibrio* species.

In TASK1, a shotgun metagenomics approach will be applied to samples of different origin to define the complete community of *Vibrio* at the strain level and of Arcobacter at the species level. Batches of clams collected from the same survey areas will be analysed before and after different depuration treatments. The Shotgun metagenomic approach not only provides a more precise taxonomic definition of the community at the strain level, but also a functional characterisation of the community and microbiota. In particular, data on the presence of virulence and antimicrobial resistance (AMR) genes in the entire microbial population are essential to improve quantitative risk assessments for food safety purposes.

For Arcobacters, the TASK project involves a more in-depth genetic characterisation of strains isolated from clams. The whole genome sequencing study will clarify the differences between strains collected from contaminated and

non-contaminated areas of the Venice lagoon. A genomic approach will also provide new insights into the contribution of the spread of antibiotic resistance and virulence factors within these microbial populations closely linked to clams.

TASK2 involves assessing the microbial community within biofilms on the surfaces of the depuration center and their contribute to the maintenance of *Vibrio* in this environment.

One of the main objectives of this activity is also the definition of different factors (co-depuration and biofilms) in the spread and maintenance of *Vibrio* communities within depuration centre. The application of egested animals will clarify the presence of *Vibrio* as transient or resident bacteria within shellfish (before and after purification processes). New metabarcoding for housekeeping genes (pyrH and recA) will be applied to specifically assess the dynamics of *Vibrio* within molluscs during purification processes.

The results of the project will provide new scientific knowledge and tools to improve the management and safety of the Italian shellfish industry.

2.2. Experimental thesis

The main purpose of this study is to study the concentration of vibrios within the bivalve mollusc *Ruditapes philippinarum*. Specifically, the study aimed to assess the timing of *Vibrio* release within the egested and released material produced during the purification process.

In order to carry out this evaluation, the clams were placed within laboratory-prepared sterile microcosms for a previously defined period of time (experimental set-up). The egested content was collected from the bottom of the microcosms at various times during the period of observation of the clams, in order to assess the change (increase and decrease) in *Vibrio* concentration along the time.

Thus, both dependent and independent culture methods were used in order to characterise the *Vibrio* load present in the organ tissues taken before and after the experimental period of observation during the permanence in the sterile microcosms. The egestion content was sampled at different times during the process.

Culture dependent approaches were applied in order to define the colony forming unit (CFU) in different selective and non-selective media; A representative number of colonies were selected for the identification of the Genus *Vibrio*.

Culture independent methods were used to evaluate the quantitative loads of *Vibrio* and total microbial count in each sample. The Real Time PCR was applied to define the copy number of the gene targets in order to evaluate the microbial contamination of each matrix.

3. MATERIALS AND METHODS

3.1. Experimental set-up

A first series of set-up experiments were conducted to define the environmental conditions of the experiments, the behaviours of animals and to establish the time-thresholds for the final experimental trial. The pre-experimental set-up was replicated several times to observe the release of *Vibrio* spp. Along the time and to reduce the number of sampling points.

3.2. Standardization of microcosms

The method used in the present study was set-up according to the study by (Griffin et al., 2021)in which a marine microcosm was reproduced under laboratory conditions. The microcosms conditions (temperature, salinity, pH and oxygen) were defined according to the standard parameters applied for the depuration in a commercial depuration plant.

Figure 8: Microcosms during final experiment with clams.

In this experiment, microcosms were used to isolate groups of clams during the egestion period. The microcosms consisted of glass vessels filled with 1.7L of laboratory-prepared salt water and fitted with glass lids. Each microcosm was also equipped with an aeration system explained in detail in the following paragraphs. Finally, an additional structure consisting of nets closed with cable ties was used to keep the clams in suspension, preventing them from coming into contact with the bio deposits accumulated at the bottom of the jar.

3.2.1. Salted water and salinity

To reproduce the purification conditions as closely as possible, sea water was prepared in the laboratory using an artificial salt (Aquaforest), which was dissolved in 1 litre of distilled water in a 1-litre glass bottle with the help of a magnetic stirrer. The amount of salt to be used varies depending on the desired saltiness level, the moisture content of the salt at the time of use and its purity.

Ten minutes on the magnetic stirrer (scientific Velp – ARE) is sufficient to dissolve all the salt; it is possible to observe the presence of particulate matter, due to possible impurities in the salt used, which however do not affect the salinity level.

The optimal salinity range for clams is between 38 and 40%; salt is added gradually and measured frequently with a refractometer (AQL MR100ATC) to obtain the appropriate salinity level. Generally, if a dry salt is used, a larger quantity is required, while with a salt that has been exposed to air, a smaller quantity will be needed to achieve the desired salinity level. Usually, 45 g of dry sea salt (Aquaforest) is needed to prepare a 1-litre bottle.

3.2.2. pH

The optimal pH range of water for clams is between 7.8 and 8. To obtain this value, it is necessary to measure the pH of the freshly prepared seawater after dissolving all the salt, and if necessary correct it by adding dilute solutions of HCl or NaOH.

The pH was measured with a pH meter (Metter Toledo five easy) previously calibrated with acid, basic and neutral buffers.

In the set-up stages, it was necessary to make some corrections to the pH of the prepared saltwater bottles, using a 1:1000 diluted solution of HCl 6M, when the pH was too basic compared to the desired range. In this case, HCl was added with a 100µl pipette, 100µl at a time and measuring the pH after each addition.

It was rarely necessary to use NaOH to correct the pH. In these cases, a tablet weighing 21g was dissolved in 35ml of distilled water to obtain a basic solution with a pH between 13 and 14. The solution was added to the bottles that needed correction in proportion to the acidity of the pH already present.

The prepared seawater, with an acceptable salinity and pH level, was immediately autoclaved (at 121°C for 15 minutes) to prevent the proliferation of any bacteria.

3.2.3. Oxygenation

In this experiment, the ideal oxygen concentration to allow clam survival varies between 70 and 100%; an 33elease33 (OxyGuard – Handy Gamma) was used to measure the oxygenation level of the lab-prepared seawater.

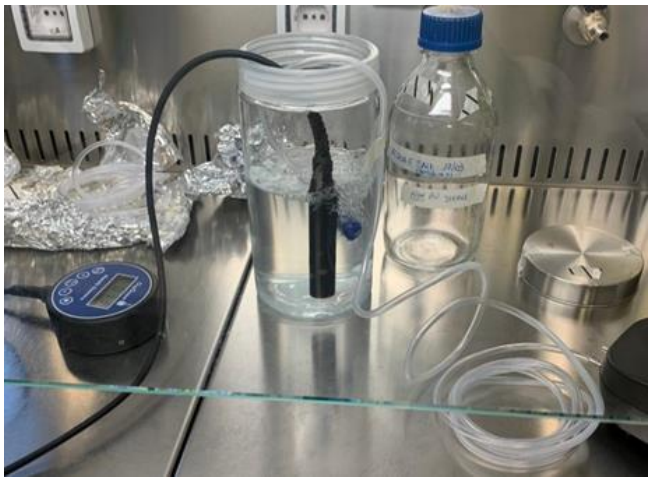

Figure 9: Use of the oxymeter to measure the oxygenation level of the water prepared in laboratory.

The calibration of the instrument must be carried out in air and keeping the probe at the same temperature as the air.

To measure in mg/l (ppm) in salt water it is necessary to make a salinity correction by selecting the reading in mg/l on the dial. While holding down the button on 'Sal Set', press the up or down arrow buttons until the exact salinity value is read on the display. Next switch to the % saturation reading and calibrate the instrument to 101.

3.2.4. Set up of experimental tools

Each microcosm was composed by a jar filled with salted water; a porous stone connected to a circuit of pipes that provide filtered air by being connected to an insufflator as shown in Figures 6 and 8.

3.2.4.1. Preparation of the nets

In this experiment two type of nets were used. The first type was used during the set up and was made of a metallic net closed with a clip. Then during the experiment, a nylon net was used, closed by a plastic cable tie and fixed to the wall of the jars with a clip. All the materials of both types of net were sterilized in autoclave at 121°C for 15 minutes.

3.2.4.2. Preparation of the circuit of pipes

To supply air to each jar forming microcosm, two set of pipes of different diameters (in order to be connected to the filter).

Smaller pipes were connected to the insufflator (Sera precision – Air 275R plus) and to the porous stone, then by using a metallic connector they were bound to the larger tubes that were both connected to an air filter (Whataman Rezist 50mm/2µm PTFE).

All the materials were sterilized in autoclave or under UV rays to avoid contaminations that can influence *Vibrio* growth.

Figure 10: Air filter connected to the small pipes. This picture was taken during the set up, when the use of plastic and metallic connectors were both considered.

Figure 11: The metallic connector used to connect pipes during the final experiment.

3.3. Experimental design

A lot of non-depurated clams was collected at the depuration centre. Per each lot of products 4 clams were directly used to obtain the homogenate and 1 was opened and the organs were extracted. Three different aliquots (4 clams x 3 sampling) were analysed before the egestion (Figure 12).

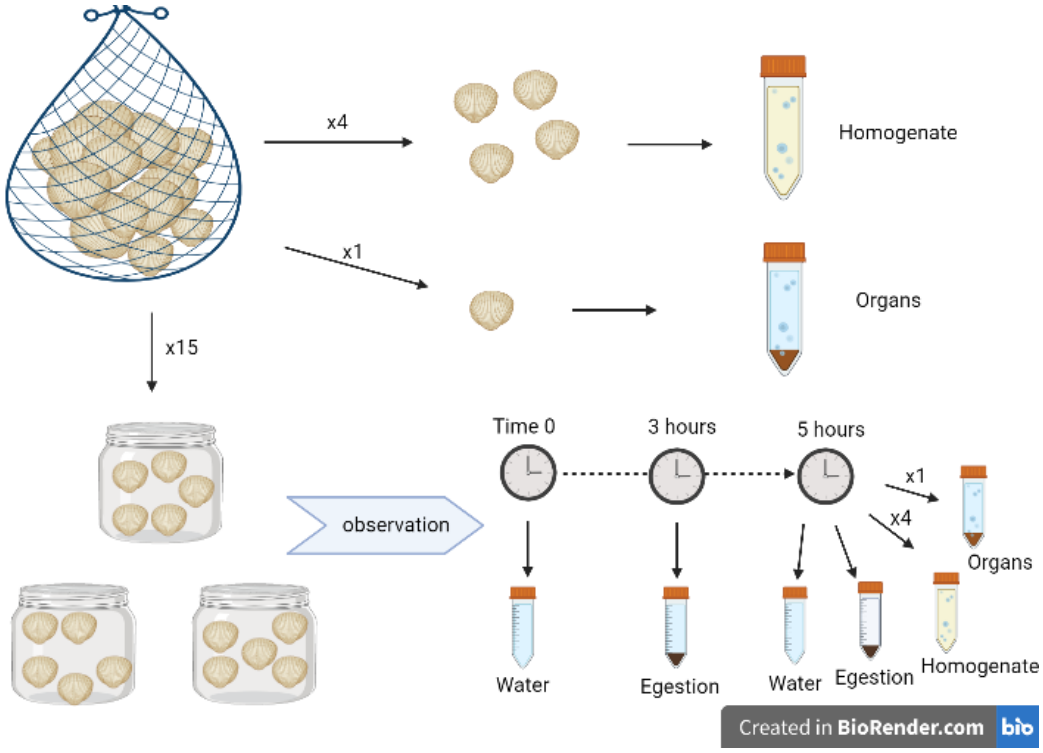

Figure 12: The procedure followed in the lab is showed.

Fifteen clams were then randomly selected, properly treated (weighted, measured in width and length and cleaned on the surface of valves with 96% alcohol) and placed into three different sterile jars named differently for each day of experiment. As showed in Figure 10, after 3 and 5 hours different samples were taken and used in the molecular biology procedure, described in Figure 13.

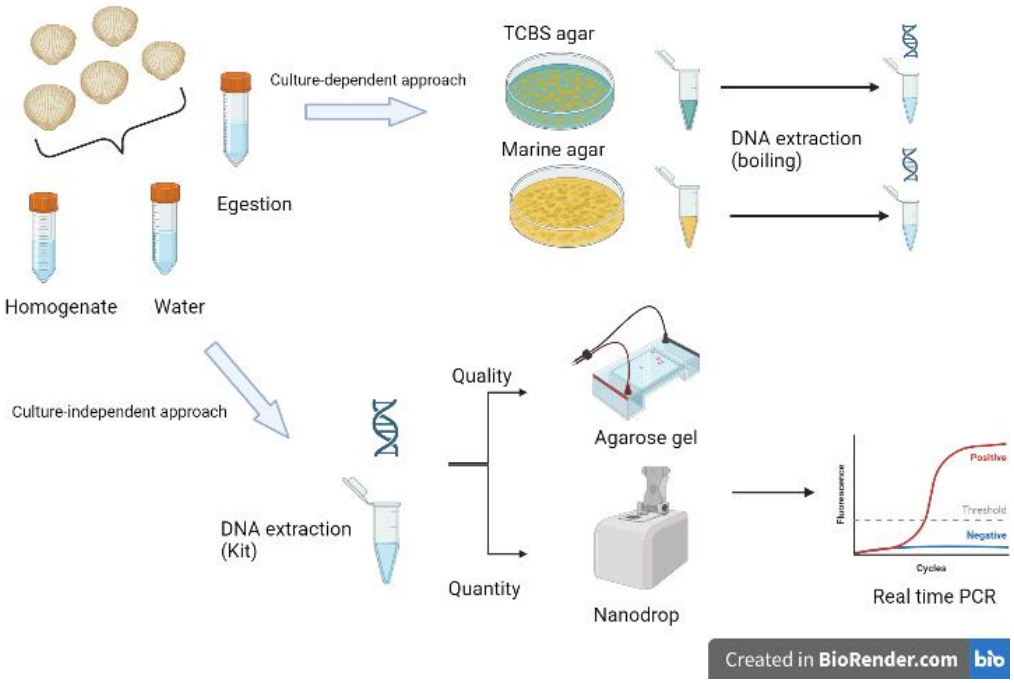

Figure 13: Molecular biology procedure applied to analyse the samples.

The first step after the samples were taken is showed in this image and will be treated in detail in the next paragraphs. Here the molecular biology steps are showed. Starting from the samples obtained from each jar, culturable and non-culturable methods were applied in order to extract and analyse the DNA present in each sample from a qualitative and quantitative point of view.

3.3.1. Sampling units

Three microcosms were prepared during each experiment, and each independent experiment was replicated two times (04/05/2022 and 18/05/2022) during the set-up. For these trials the lot of clams were collected in the Chioggia basin and identified with the code 14L004.

During the final experiment (07/06/2022, 08/06/2022 and 09/06/2022) the three lot of clams were collected at the Blupesca depuration centre that took the clams from the same areal during the three days, identified with the code 14L010.

A net with five clams each was placed inside each microcosm.

A representative group of clams were randomly selected in three pools from each lot in order to define the group of non-treated clams. Each pool was constituted by 4 animals for the preparation of the homogenates plus 1 that was dissected to collect organs. The preparation of the homogenates and the removal of the organs will be described in next session.

Before being placed in the net, each clam was measured in height and length with a calibre (METRICA) and weighed with a balance (Kern – EMB 1000 – 2). All measurements were tabulated in the results.

After the egestion period, clams from each microcosm were divided into:

- 4 clams were used to prepare the homogenate,

- 1 clam was opened and organs such as gills, intervalvar liquid and digestive gland were extracted.

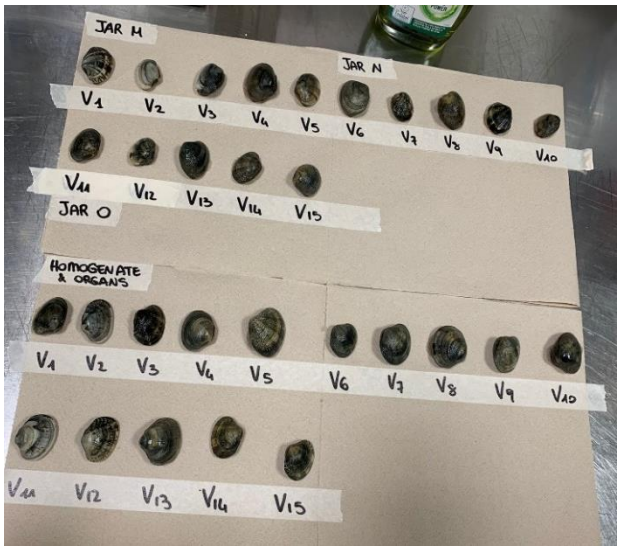

Figure 14: Fifteen clams that were later placed in each microcosm, each microcosm was identified by using a letter of the alphabet; below, fifteen clams used for the preparation of the homogenate and organ extraction.

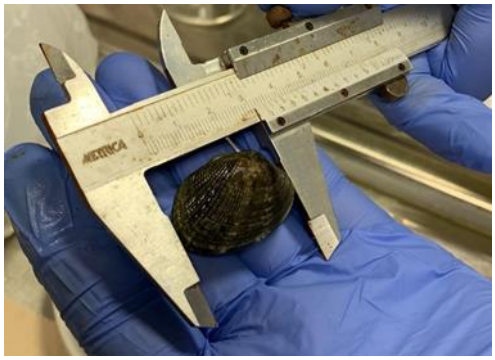

Figure 15: Measurement of a clam before it was placed inside the microcosm.

3.4. Survey of environmental parameters

As mentioned in the previous paragraphs, the concentration of *Vibrio* within bivalve molluscs varies with various environmental parameters such as temperature, salinity, oxygen, etc.

To be able to observe this variation, environmental data referring to the clams' area of origin were also analysed during the setting of the experiment in the laboratory

The periods taken as reference in the tables showed in the results are related to days when the clams were taken from the areal to carry out the experiment setting. The clams were taken on three different days, 04/05/2022 and 18/05/2022.

In addition, environmental parameters were also investigated in the period before and during the final experiment carried out in June.

3.5. Microbiological analysis

The microbiological analysis enabled a quantitative assessment of the micro-organisms present in different matrixes (in the organ and homogenate samples taken before and after the observation period) and in the egestion content produced by the clams during the observation period in the laboratory.

In this study two different media were used during the application of the culture dependent method, in order to define the concentration of *Vibrio* inside all the samples:

1) Marine agar (Marine broth, 1.5% Agar) Kobayashi Agar is a generic media used for the cultivation and isolation of heterotrophic marine bacteria. It contains mineral salts, peptones and yeast extract, that ensure the optimal growth of these bacteria (*Microbiol*, n.d.). The colonies appear white coloured or transparent.

2) TCBS (Thiosulfate-Citrate-Bile-Salts-Sucrose Agar) is a differential selective media used for the cultivation and isolation of bacteria belonging to genus *Vibrio*. This media inhibits the growth of non-halophilic microorganisms and, in addition, it is possible to distinguish between *Vibrio cholerae* which forms yellow colonies and *Vibrio parahaemolyticus* which forms blue to green colonies.

3.5.1. Sampling

All the samples in this experiment were divided according to the scheme showed in the experimental design.

Samples of 25 ml of water from each microcosm were collected in Falcon tubes before placing the clams (T0) and after 5h (T5). Also, in Falcon tubes, samples of egestion contents were collected after 3h and after 5h. The volume of these samples varied according to the amount of egestion content released from the clams during the observation period and, on average, the volume taken as sample of egestion was 35ml.

According to the scheme presented in the experimental design, two types of samples were collected from the clams.

Homogenate samples of 4 clams prior to the observation period were prepared by using a stomacher bag, into which non depurated and weighed clams (without valves) were placed, together with a 1:10ml dilution of MRD (maximum recovery diluent) prepared by mixing 1g of pepton and 8,5g of NaCl (both provided by Condalab). The same procedure was performed with 4 clams from each microcosm at the end of the observation period (after 5 hours). From the stomacher bag, 1 ml of homogenate was taken to prepare the dilutions and then plated on Marine agar and TCBS.

The clams were also used to take organ samples such as digestive gland, gills and intravalvular fluid. For organ extraction, it was sufficient to take 3 non depurated

clams from the bag provided by the depuration centre, and 3 clams one from each microcosm, after 5 hours of observation.

All extracted organs were stored at -20°C for further analysis in the future.

3.5.2. Use of the samples

Samples of water, egestion content and homogenate were used to prepare serial dilutions in MRD broth by taking 1ml of the sample and adding it to 9ml of MRD.

- For samples of water: 10^{-1} serial dilution;
- For samples of egestion content: serial dilutions 10^{-1} and 10^{-2};
- For samples of homogenate: serial dilutions from 10^{-1} to 10^{-4}.

Afterwards, 100µl of each dilution was plated in Petri plates with Marine agar (MA) media and TCBS media. All plates were then incubated at room temperature for 48 hours.

After the incubation time the count of the colonies grown in MA and TCBS plates was performed. For each count, and for each sample, only the successive dilutions that had a number of colonies between 10 and 300 was considered, and the applied formula was:

$$N = \frac{\sum C}{V(n1 + 0{,}1n2)d}$$

Where:

N: is the number of microorganisms, expressed in colonies forming units (CFU);

∑C: is the sum of the counted colonies in two following plates;

V: is the volume used for the inoculation;

n1 and n2: are the number of the plates used for each considered dilution;

d: is the first considered dilution during the count.

Then from MA and TCBS plates, all the colonies morphologically relevant and more frequent were selected and numbered with a progressive number. These were plated in MA plates to isolate them and obtain pure colonies.

3.6. Strain identification

3.6.1. Dependent culture method

In order to perform culture-dependent analysis, the colonies grown in MA and TCBS plates were used for the DNA extraction as previously said in paragraph related to microbiological analysis.

The DNA extraction was not performed in the same days in which the colonies were counted.

Before starting with the procedure of DNA extraction, among all the plates, only the first dilutions were considered. These were melted in 2ml of PBS and centrifuged for 5 minutes at 12000 rpm to obtain the pellet that will be used later to perform the DNA extraction.

The tubes with the obtained pellet were stored at -80°C until DNA extraction was performed.

3.6.1.1. DNA extraction by boiling

Pellets obtained from isolated colonies, plated on MA plates were resuspended in 100µl of sterilized water (DNAse and RNAse free) in 1,5ml microtubes. Afterwards the DNA was extracted by boiling using a thermal block (Orbital mixing, chilling/heating plate – TORREY PINES SCIENTIFIC INC).

The microtubes where boiled at 95°C for 10 minutes and centrifuged at 12000 rpm for 5 minutes.

The surnatant with the DNA was placed in new microtubes that were then stored at -20°C.

3.7. Molecular biology

3.7.1. Independent culture method

With the independent culture method, the DNA could be extracted directly from homogenate samples by using the Dneasy power soil pro kit (QIAGEN).

3.7.1.1. Sampling and preparation of the samples

Samples taken for the microbiological analysis 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 5 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.

3.7.1.2. Clams

From each homogenate, an aliquot of 2 mL was collected and then centrifuged at 12,000 rpm for 5 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.

3.7.1.3. DNA extraction with kit

With the independent culture method, the DNA could be extracted directly from homogenate samples by using the Dneasy power soil pro kit (QIAGEN) and by following the given protocol with some adaptations:

The beads were placed inside the cryotube containing the pellet of the sample and 800µl of Solution CD1 were then added. The cryotubes were vortex briefly to mix. All the cryotubes were then placed in the Tissue Lyser II (QIAGEN) and a set of 3 cycles of the duration of 30 seconds with a frequency of 30 s^{-1} were performed. After, the cryotubes were placed in the Centrifuge (Eppendorf centrifuge 5425) at 15,000 x g for 1 min. 550µl of the supernatant were transferred to a clean 2ml microcentrifuge tube and 200µl of solution CD2 were added. The cryotubes were put in the vortex for 5s and then centrifuged at 15,000 x g for 1 min at room

temperature. Avoiding the pellet, 600µl of supernatant were transferred to a clean 2ml microcentrifuge tube and subsequently 600µl of solution CD3 were added. The cryotubes were then vortex for 5s. After, 650µl of the lysate were loaded onto a MB Spin Column and centrifuged at 15,000 x g for 1 min. The flow-through was discarded and the previous step was repeated to ensure that all the lysate has passed through the MB Spin Column. The MB Spin Column was then placed into a clean 2ml collection tube with 500µl of solution EA and centrifuged at 15,000 x g for 1 min. The flow-through was discarded and the MB Spin Column was replaced into the same 2ml collection tube together with 500µl of solution C5. A cycle in the Centrifuge at 15,000 x g for 1 min was then performed. After discarded the flow-through, the MB Spin Column was placed into a new 2 ml collection tube and centrifuged at up to 16,000 x g for 2 min. The MB Spin Column was then placed into a new 1.5ml elution tube and 80µl of solution C6 were added to the centre of the white filter membrane. The MB Spin Column was then centrifuged at 15,000 x g for 1 min for 2 times to concentrate the DNA. From this moment, all the tubes were kept on ice. An aliquot of 8µl was taken and transferred to a new tube in order to preserve the mother and use the aliquot for further analysis.

3.7.1.4. Qualitative analysis

In order to perform a qualitative evaluation of the extracted DNA fragments through the use of a DNA extraction kit (Dneasy power soil pro kit (QIAGEN)) the subsequent Nanodrop analysis (Nanodrop ND-1000) was applied.

3.7.1.4.1. Nanodrop

After the extraction, the DNA was subjected to spectrophotometric analysis using Nanodrop ND-1000 spectrophotometer to perform both qualitative and quantitative analysis.

This instrument consists of an instrumental part and a software installed on a computer. After having pipetted 1µl of sample taken from the aliquot prepared during the DNA extraction on the optical surface and closed the lever arm, a command is given to the computer and the software measures the absorbance at the

different wavelengths and in this way calculates the ratio between the concentration (ng/µl) and the purity of the DNA extracted.

The wavelengths appear on the screen in ratios:

- 260/280 nm indicates the presence of proteins in the sample;

- 260/230 nm indicates the presence of non-protein components in the sample.

This evaluation gives information on the presence or absence of the contaminants (e.g. proteins) but does not give information about DNA integrity.

The qualitative analysis performed by using the Nanodrop is made by looking at the ratios 260/280 and 260/230 nm while the quantitative analysis can be performed by looking at the concentration expressed by the ratio ng/µl.

3.7.1.4.2. Agarose gel

The quality of the extracted DNA was also evaluated by placing each sample in 1% agarose gel. This allows to evaluate the degradation level of the DNA molecules.

It is essential to have good quality DNA to proceed with subsequent molecular analyses.

Agarose gel was prepared by mixing 100ml of water and 1g of TAE (boiled in microwave) with 10µl of Cyber safe (1µl of Cyber safe for each 10ml of gel). TAE is a salt buffer with 1g of pure agarose used for gel.

The samples were centrifuge for a short period and then 5µl of Loading Dye 2X (Green Go Taq Flexi Buffer Promega) were added to 5µl of the sample.

The samples were centrifuge again to mix the Loading Dye with the sample and then they were placed inside the wells of the agarose gel.

3.7.1.5. Real time PCR

Quantification of total 16S rDNA and 16S rRNA genes specific to the *Vibrio* genus were performed using quantitative PCR (qPCR). All amplification reactions were analysed using a Roche LightCycler 480 Real-Time thermocycler.

The total qPCR reaction volume was 10 µl and consisted of 2.5 µl DNA (10–50 ng/µl) and 7.5 µl LightCycler 480 SYBR Green I Master mix (Roche) containing 10 µM PCR primer (Eurofins). Total bacteria specific primer pairs were:

- 16S_Fw—TCCTACGGGAGGCAGCAGT
- 16S_Rev—GGACTACCAGGGTATCTAATCCTGTT,

targeting the variable V3V4 loops for bacterial communities (Nadkarni, Martin, Jacques, & Hunter, 2002).

Total *Vibrio* specific primer pairs were:

- Vib1-Fw GGCGTAAAGCGCATGCAGGT
- Vib2- Rev GAAATTCTACCCCCCTCTACAG

(Siboni, Balaraju, Carney, Labbate, & Seymour, 2016), targeting the 16S rRNA of *Vibrio* genus.

The reaction volume was 10 µl containing 5 µl Platinum SYBR Green qPCR SuperMixUDG (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.

SYBR Green is a dye capable of intercalating with dsDNA (double stranded DNA). The fluorescence signal measured in this way at the end of each amplification cycle is proportional to the amount of dsDNA produced, but is not specific for the amplicons of interest; for this reason, at the end of the PCR, the Melting Curve analysis was performed, which allows to estimate the melting temperature I of the PCR products and thus to identify the presence of primers or non-specific dimers.

The thermal cycle uIcluded a first step of 2 'at 50 °C (necessary for the UDG system), fol'owed by 2' at 95 °C (initial denaturation) and then by 45 cycles, each comp‘’sing 10 " at 95 °C (denaturati’’) and 40" at 60 °C (annealing and extension), to end with a cycle, necessary for the construction of the Melting Curve, in which the temperature was slowly brought to 95 °C.

3.8. Absolute quantification

Absolute quantification was made starting by cp values, so from the crossing points obtained from the qPCR. The aim of absolute quantification is to extrapolate a data that in this case is the copy number that indicates how many DNA copies that represent cells in the sample are present.

Another way can be made by relative quantification but this method is only an observation of which sample contains more copies of DNA and so it is less precise if compared to absolute quantification.

In order to be able to perform an absolute quantification, in the present study two standard curves were used and are reported in Figures 16 and 17.

Vibrio parahaemolyticus pure genomic DNA was serially diluted in molecular-grade water and dilutions were used to construct standard curves for 16S assay and *Vibrio spp*. assay.

Standard curves were generated by plotting the Cp vs the number of bacterial copies of dilutions. The bacterial copy number was estimated by dividing DNA quantity (ng) in the dilutions with the molecular weight of the *Vibrio parahaemolyticus* genome (5.6 fg), as described by Pathak, Awuh, Leversen, Flo 2012.

For each sample, bacterial copy number was determined by interpolation of the resulting Cp values to the standard curve.

y = -3,5167x + 35,999 for *Vibrio* spp. assay

y = -3,736x + 35,943 for 16S total bacteria assay

for interpolation:

y = Cp of each sample

x = Log copies bacterial cells in sample.

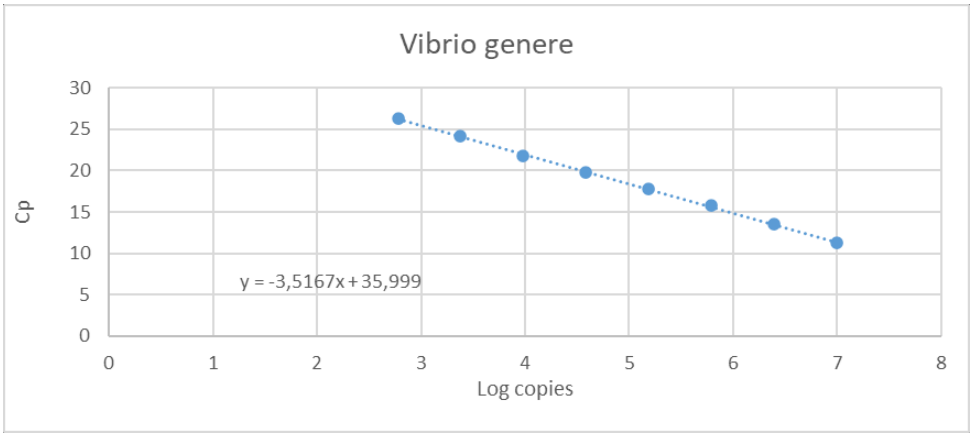

Figure 16: Standard curve used for the *Vibrio* genere.

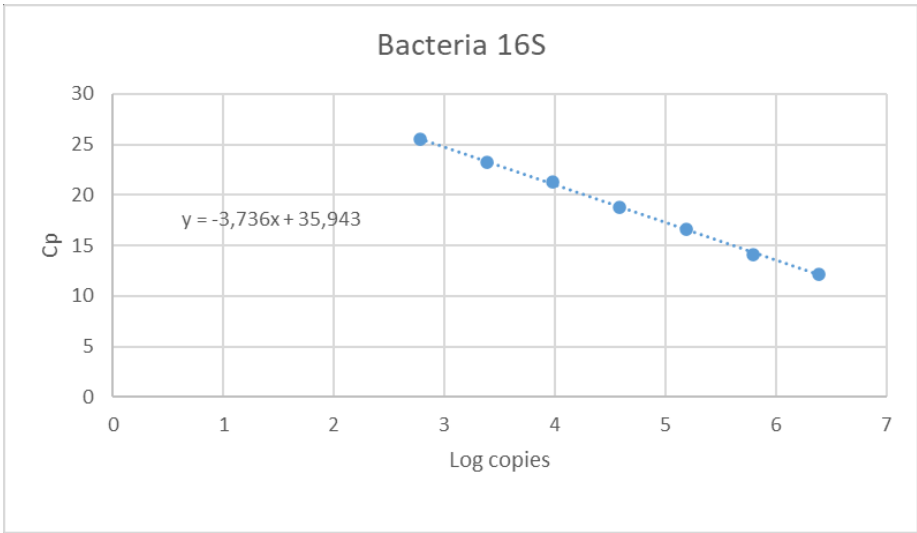

Figure 17: Standard curve used for Bacteria.

In both curves presented, on the y axis cp cycles are reported, while on x axis values of logarithms of copy numbers are showed.

Each graph line is obtained by taking the logarithms of copy numbers and cp values and both were used to find the copy number of samples taken during egestion final experiment made in June.

Entering the values of cp into the formula of each graph line instead of y gives x, the logarithm of the copies (bp) to be calculated and after calculation is possible to obtain the final values, expressed in logarithm of the corresponding amount of bacteria present in the sample.

3.9. Statistical analysis

The non-parametric combination (NPC) test was conducted with the free software NPC Test R10 (http://www.wiley.com/legacy/wileychi/pesarin/material.html). The partial and global p-values were determined for the microbial count profiles (MA and TCBS $\log_{10}$ CFU/g) and for the log copies features obtained by real time PCR, considering the three experiments (days 1, 2 and 3) and the matrixes (water, egestion, homogenate) before and after the stabilization in microcosms. Matrixes and experimental days were also applied as a stratification block according to th' NPC Test's C-sample procedure for dependent variables. Partial p values were corrected for multiplicity and the global p-values were obtained using the Tippet combining function.

4. RESULTS

In the next paragraphs all results obtained in this study are reported. For ease of reading and understanding, the results have been divided into paragraphs referring to trials made in May (set-up of experiments) and the final trial in June.

4.1. Set-up of experimental tools

The first phase of the present study was identified as set up phase, because of the need to have replicable and standardised experiments.

In this phase all the parameters that can influence the stability of microcosms were measured several times in order to understand their variations and which factors or other parameters can have influence on their values.

The standardization of the procedure for the preparation of microcosms is particularly useful if the experiment will be replicated, because thanks to the standard conditions, all the parameters can be set up without the necessity of many trials before.

4.1.1. Variability of parameters during the set-up

In Table 3 the average and the standard deviation of parameters that were measured during the set-up of experimental tools are shown.

During this set-up phase was particularly important that each parameter that can influence the microcosm is measured in order to obtain standardized experiments when working with molluscs.

The higher standard deviation is related to the Oxygen percentage, this can be explained by the fact that the oximeter that was used for measurements, despite the calibration procedure that was carried out before starting, didn't give stable values or measured values too different between each other, also if related to the same sample of salted water.

The level of salinity was the more stable value, together with temperature (because of the laboratory rules). The salinity level was easily measurable and, if not in the appropriate range (38-40‰ for clams) could be adjusted by simply add salt to water time by time in small rate to be sure to don't exceed the range value. The standard deviation is not so low and this value can be justified by the fact that these results refer to the high number of trials that were done, with different rates of salt, again with the aim to obtain a standardized and replicable procedure.

Another concern regards the standardization of the procedure to obtain a stable value of pH suitable for clams (that have a narrow range of pH included in the interval 7,8-8) was during the calibration of pH meter.

Three different pH meters were used before taking the decision to use only one of them that measured the right values.

If during the set-up and during all the trials with salted water, the pH was not in the range suitable for clams, some adjustments with 6M HCl diluted 1:1000 were done. The major problem with this parameter was that it is highly influenced by the temperature and by time. In fact at room temperature, as reported in Table 4, it was 0,2 points higher respect to the range for clams while when the microcosms were kept at 15°C during the trials and during the final experiments, the measures of pH were a bit lower if compared with the optimal range of clams (provided by the depuration centre where all clams were taken for trials and final experiment)but, during trials and final experiment, all molluscs after one hour of observation (in both trials and final experiment) presented open valves and exposed siphons.

Table 4: Average and standard deviation of parameters that were measured during the set-up of experimental tools.

T°C	pH	Oxygen (%	Salinity (‰)
20.8±0.50	8.2±0.55	81.1±6.71	37.4±2.23

4.1.2. Results of set-up assessment of microbiological features

In this section all the results related to trials with clams made on 04/05/2022 and 18/05/2022 are showed and described.

4.1.2.1. Environmental parameters

In the tables below are reported all the data related to the environmental parameters that were monitored during the period of the setting of the experiment, when the clams were taken to be used in trials in the laboratory.

The two trials were done on 04/05 and on 18/05 but the second day the clams were not available because of the bad weather conditions and for this reason they were taken from the depuration centre the successive day.

Table 5: Environmental parameters measured in the areal present in Chioggia during the first trial with clams done on 04/05.

Date	Temperature	Oxysat (%)	Salinity (‰)	Ph
30/04/2022	15.9	109	36	8.08
01/05/2022	16.3	110	36	8.08
02/05/2022	16.8	111	36	8.08
03/05/2022	17.4	108	36	8.09
04/05/2022	17.7	110	36	8.08
Average	16.8±0.738	109.6±1.331	36±0.284	8.1±0.005

Table 6: Environmental parameters measured in the areal present in Chioggia during the second trial with clams done on 18/05.

Date	Temperature	Oxysat (%)	Salinity (‰)	Ph
14/05/2022	19.7	110	35	8.06
15/05/2022	19.9	108	33	8.05
16/05/2022	19.7	104	35	8.04
17/05/2022	20.5	104	37	8.06
18/05/2022	20.9	110	38	8.09
Average	20.10±0.542	107.2±2.772	35.7±1.679	8.1±0.018

The oxygen level expressed in mg/L was not represented because in this study was not considered. The average and standard deviation for the considered periods are: 8.6±0.153 for the week of 04/05 and 7.9±0.211 for the week of 04/05.

Focusing on the 17/05 and 18/05, when clams were not taken because of the weather, it is possible to observe that the variation was very limited and so, weather conditions did not influence the environmental parameters.

Another thing that emerges from these parameters is that the pH remains fairly constant during the time and always in the range suitable for clams while when

looking at values of salinity, these are often under the optimal range of clams (FAO, 2022; information provided by Dr. Luciano Boffo).

4.1.2.2. Results of microbiology and statistical analysis

During the experimental set-up were conducted several trials to define the dynamics of microorganisms inside the egested materials. Samples were analysed each hour for a total of 6 observations.

The microbiological counts were performed and are expressed as log colony forming units per gram of sample (log10 CFU g^{-1}) for total viable counts.

In the plots below are reported the results of the microbiological analysis performed on MA and TCBS, to define log10 CFU g^{-1} for Bacteria (on marine agar) and for *Vibrio* spp. (on TCBS).

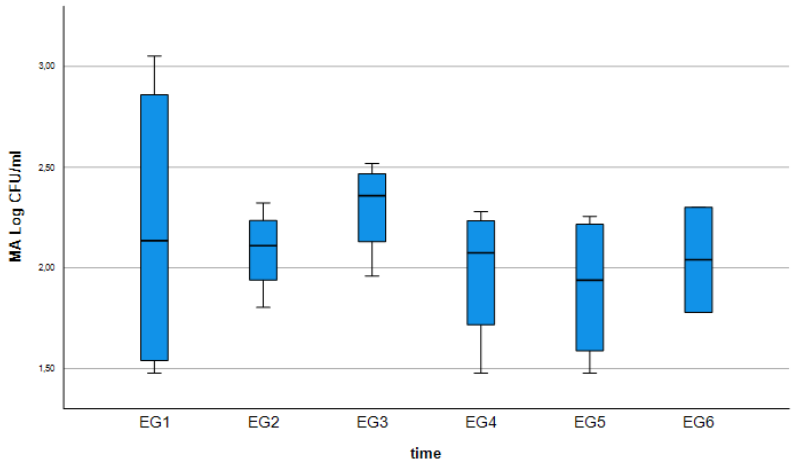

Figure 18: Logarithm of MA of CFU per g or ml.

In Figure 18 are represented with box plots 6 hours of observation made during 04/05/2022 and 18/05/2022. This first image is referred to log of CFU on marine agar and so all bacteria present in the samples were considered.

The choice of using this type of diagram is related to the fact that a box plot uses segments and a rectangle to visualize the median, quartiles, extreme values and the range in which data vary. In addition, it is useful to visualize both the centre and the distribution of the data present. The central black line represented in each

rectangle refers to the median of data while in the two extremes of the rectangle (upper and lower) the 25th and 75th quantile are reported. The lines that extend from the rectangle are also named whiskers and they represent the expected variation of data.

It is observable that between the matrices (Egestion materials) the medians are very similar (at around 2-2.5 Log CFU/ml) even if in the first matrix there is a higher excursion respect to all the others. Also, by looking at y axis, in which log of CFU on marine agar are reported, the median is only slightly different between the matrices, indicating that Bacteria release is not changing during the time. The NPC test did not show any statistical differences between times.

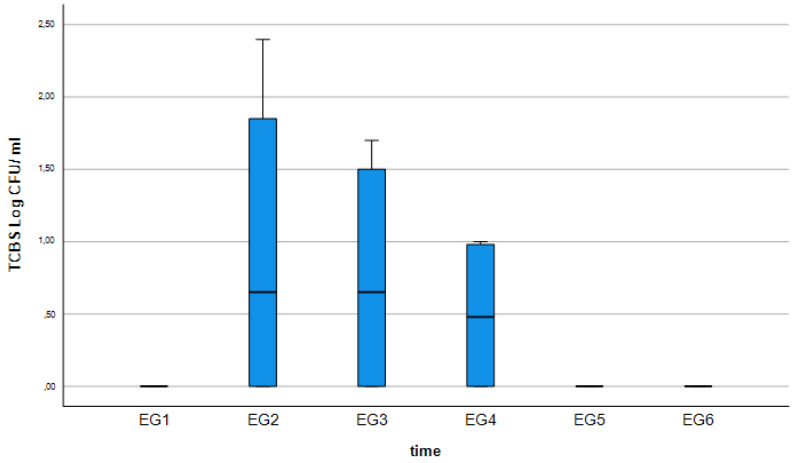

Figure 19: Logarithm of TCBS of CFU per g or ml.

In Figure 19, again with box plots, *Vibrio* release is represented. What is obvious at first sight is that at the beginning and at the end of the observation period made during trials of 04/05 and 18/05 the release of *Vibrio* is absent. The maximum excursion is present after two hours of observation, indicating that the higher amount of *Vibrio* is found in samples that were taken after this period of observation.

The median of matrices 2, 3 and 4 is quite same, especially in 2 and 3, indicating that the release of *Vibrio* is maximum after this period of observation, this sentence is justified also by the fact that the median of 3 is lower if compared with the previous two and so is the release of *Vibrio*.

Comparing Figure 18 and 19 what is evident is that the release of Bacteria starts before the release of *Vibrio* and remain during all the observation period. *Vibrio* release instead reaches its maximum during the central period of an observation conducted for 6 hours. This suggested that 6 hours of experimental trial in sterile microcosms are enough to study the release of *Vibrio* present inside the molluscs.

4.2. Results of final experiment

4.2.1. Environmental parameters

In following plots are reported the trend of different environmental parameters considered in this study. The final experiment was performed in three different days (07/06/2022, 08/06/2022 and 09/06/2022). During this week some changes in the environmental condition of the sampling site are observable.

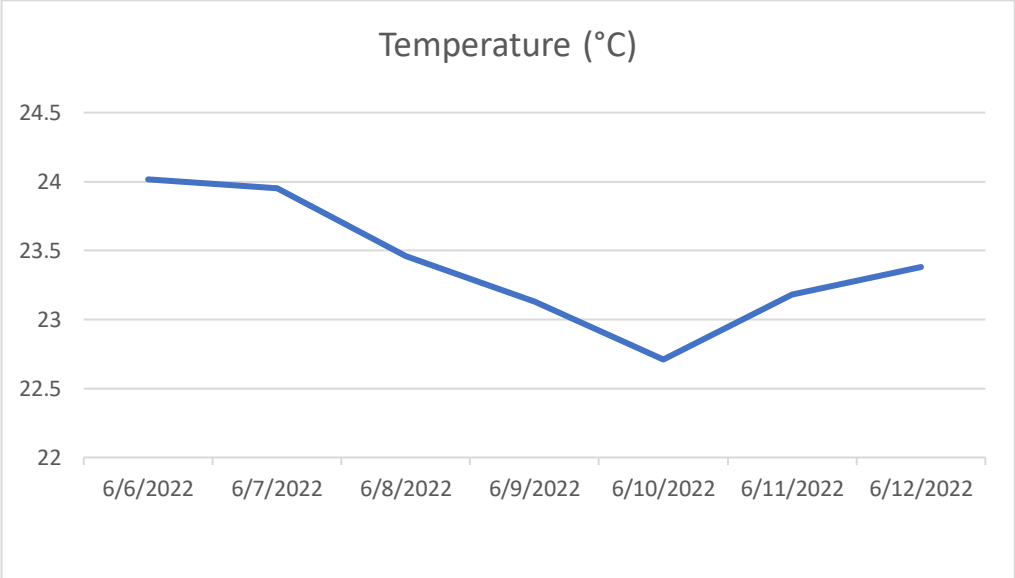

Figure 20: Temperature values registered in site of Chioggia during the week of the final experiment.

In Figure 20 is presented the trend of temperature during the week of the final experiment, as shown in the graph it is possible to observe that there is a general decline in values especially during the three days of the experiment. The lowest value was registered on 10/06/2022, right after the last day of experiment, so during

this period clams were taken when temperature values were lower day by day (this means that the temperature was lower for each subsequent day of experiment).

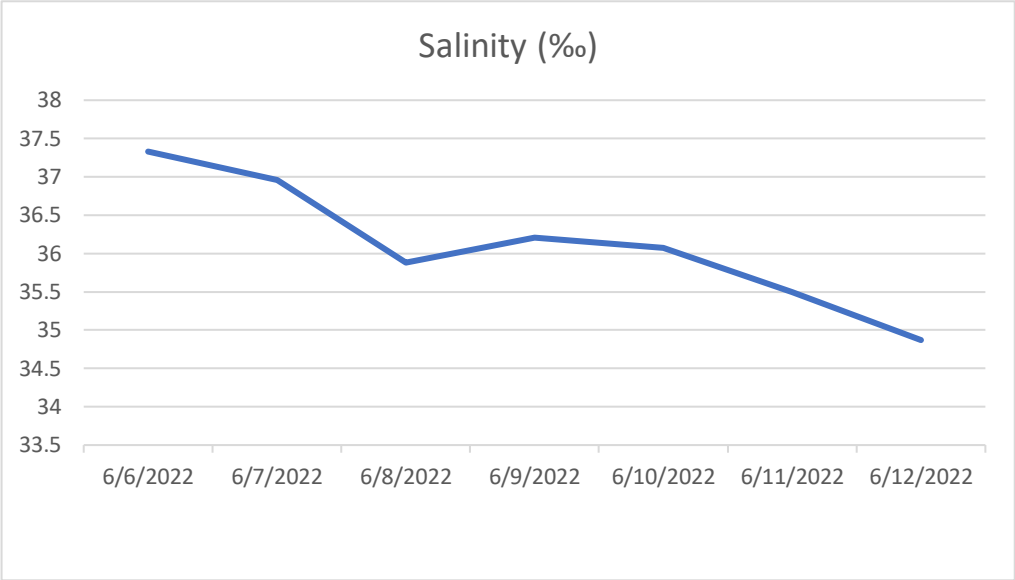

Figure 21: Salinity values registered for the marine water during the week of the final experiment in the areal of Chioggia were the lot of clams were taken.

In Figure 21 the trend of Salinity during the week of final experiment is presented. As showed, the general trend follows the declining trend of temperature but without the final increase and, focusing on the three days of final experiment t is possible to observe that on second day (so 08/06/2022) there is a first peak of decrease in the salinity level, that for this reason was different from day 1, but also from day 2 in which an increase was registered.

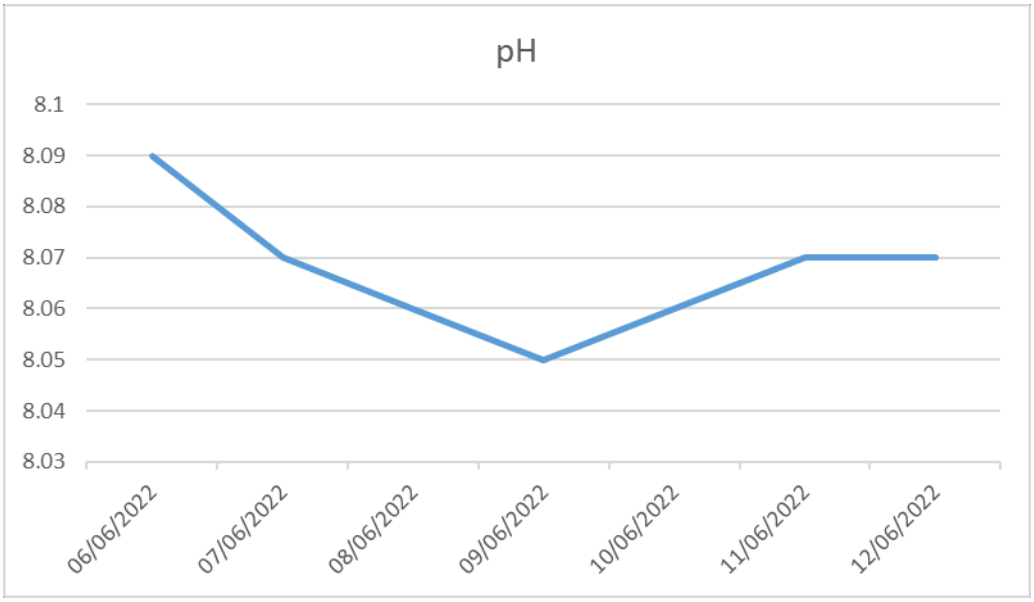

Figure 22: pH values register in the areal of Chioggia were lots of clams were taken for the final experiment.

The pH values showed in Figure 22 describe an initial decrease in pH values, with the lowest peak registered in correspondence of the final day of the experiment.

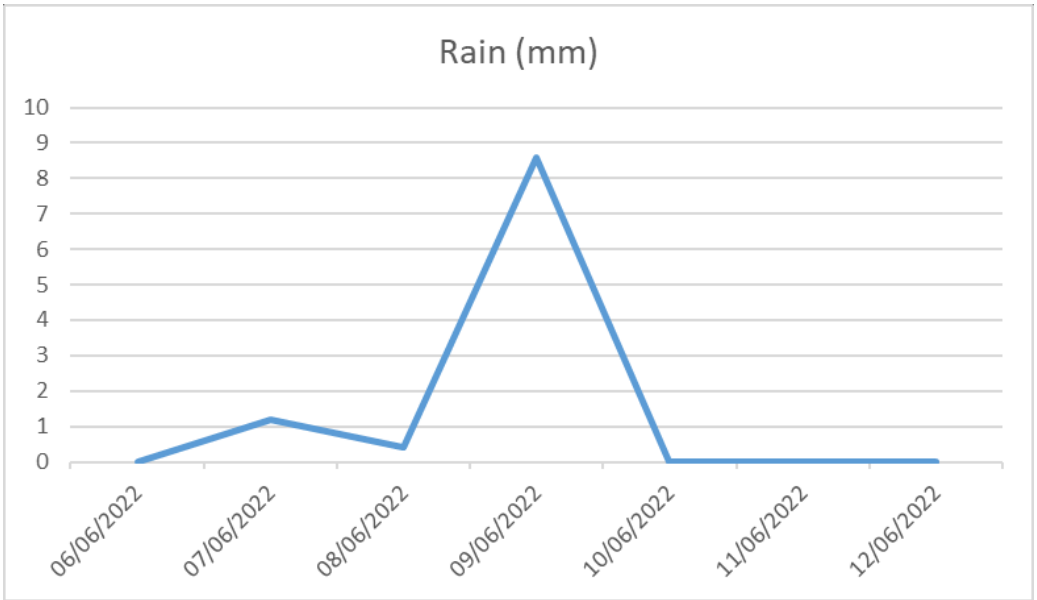

Figure 23: Rain data referred to the week of the final experiment specific of the areal where lots of clams were taken. Data provided by ARPA Veneto.

In Figure 23 are reported data relative to precipitations that were registered in the same period of all other environmental parameters previously showed and

described. The incidence of meteorological conditions on the parameters of the water is observable by looking at day 09/06/2022. During this day (that was also the last day of the experiment) the highest peak of precipitations was registered, and it corresponds also to the lower peak of pH registered (Figure 22). This comparison is particularly useful to understand how environmental parameters are influenced by each other.

4.2.2. Results of microbiology

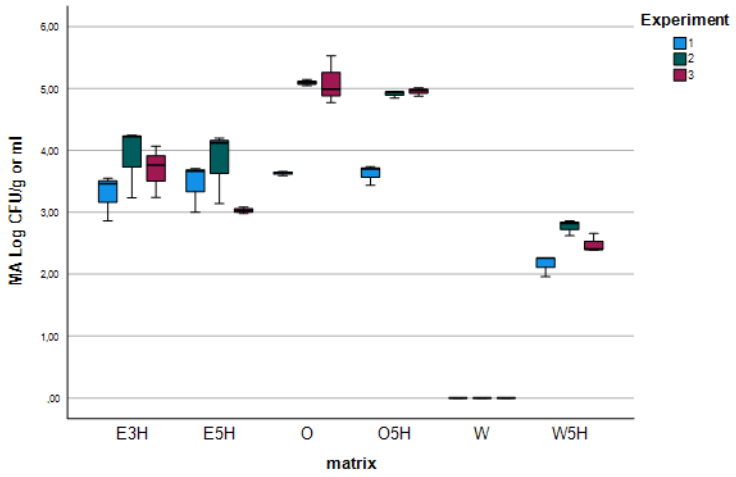

Figure 24: MA Log CFU for the final experiment (for further information see Supplementary materials).

The results of microbiological features are reported in boxplots (Figure 24-25). The data are stratified according to the days of Experiments (day 1-2-3) and defined the microbial contamination in each matrix examined in the study. With statistical analysis it was possible to verify which factors had influenced the most what it is possible to observe in the following plots and so, understand if during the experiment there was a trend or all the observations were due by chance. To see if there is any effect that has an influence on the observed trend, it was necessary to apply some statistical tests, in particular, all the analysis in the present study were based on non-parametric tests, useful tests that compare the medians of data and do not require a normal distribution of them.

Another thing that made the use of non-parametric tests necessary is the low numerosity in samples. In the present study, there is no sorting by ranking. Therefore, the approach using a test such as Kruskal-Wallis could distort the results

since the samples would be sorted by ranking starting with the lowest and ending with the highest.

The method used for this analysis was a permutational test, able to simulate comparisons of permutations made n number of times between all the groups to assess whether they are different from each other and obtain at the end a p-value.

If the p-value is less than 0.05, the studied effect is not due to chance, but it is a result of the influence of the observed factor. If the p-value is higher than 0.05, the two sample populations are similar and there is no statistical difference.

In this study, an NPC test (non-parametric permutation test) was used, which is a non-parametric software developed by the University of Padua. Two factors that are inherent in this study (experiment day and matrix) can influence the variables that are studied. The response variable is identified in MA, TCBS, values of log copies (DNA copies) for both bacteria and *Vibrio*. First two variables are related to the microbiology while the DNA copies of Bacteria and *Vibrio* are related to molecular biology steps that also include the qPCR.

These considered variables can vary in function of the experiment (referred to the three days), and in this case the aim was to understand if they differ during time or if they are different when the matrix changes (so egestion at the beginning is different from egestion content taken after 5 hours of observation).

What was highlighted by using the NPC test is that these 4 variables do not differ if only the variable time is considered, but, considering also the matrix of the experiment (6 matrices were considered in this study), it is possible to observe that each matrix influences the trend of the target and they are different. The applied test does not tell which matrix is different. To understand such difference a Pairwise comparison was applied, and the results are shown in Table in Supplementary materials.

In general, there are no differences according to the main factor day of experiment (Table 7), however, the stratification according to the matrix highlighted differences in different pairwise comparison (Table 8-9).

In Figure 24 it is possible to observe that, in MA, day 1 of the experiment was the one with lower contamination in each matrix considered, and in comparison with day 2 and 3. In this case also, homogenate samples taken before the starting of experiment and after 5 hours of observation (O and O5H) seem to be different, but, by looking at Table 8 in Supplementary materials, they are not different as the p-value in the Pairwise comparison is higher than 0.05.

In this plot is also possible to observe that the second day of the experiment has values of log CFU in some matrices that are higher respect to other days of the experiment.

Looking at water samples, in the sample taken before clams were put inside the microcosms, the log CFU was under the limit of detection indicating that non-culturable microorganisms were present inside microcosms. This is in accordance with the assumption of maintaining sterile conditions in microcosms until clams were put inside.

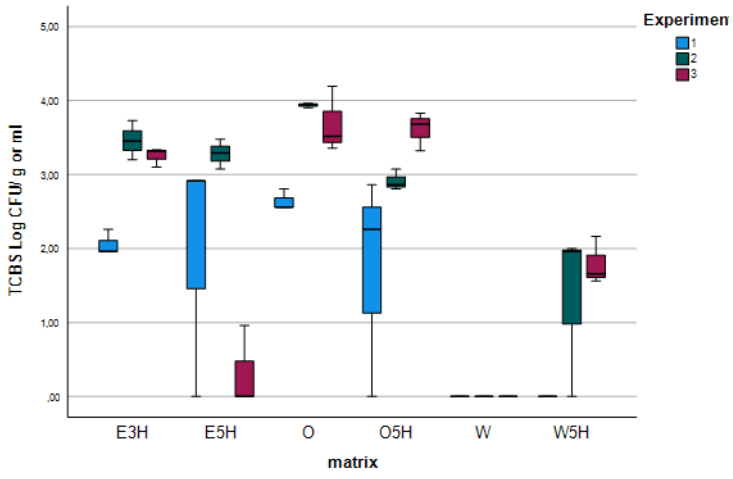

Figure 25: TCBS Log CFU for the final experiment (for further information see Supplementary materials).

In Figure 25 it is possible to notice that, in TCBS, *Vibrio spp.* changed both in egestion and homogenate samples (E3H, E5H, O and O5H) because the p-value reported in Table 9 is lower than 0.05, and it is possible to conclude that between the three days of experiment in these matrices there are statistical differences that can be observed, and they are due to experimental days.

It is possible to observe that in the first day of experiment the excursion is higher respect to other days (variability intra-replicates of different microcosms) and, looking at *Vibrio* release between the three days, it is possible to notice a progressive release that increase in the first 2 days but decrease in day 3 except for homogenate samples (O5H), in which the visualized trend reflects an increm60eleasethe release of *Vibrio* spp.

These graphs are not giving significance indications, but they furnish visive indications on the trend of the experiment.

4.2.3. Results of qPCR

What emerges from Tables 8 and 9 is that for e.g. the water sample that was taken before putting the clams in the microcosms and after 5 hours of observation is similar for MA and for TCBS but is statistically different for BacLog (p-value < 0,05).

What emerges from Table 8 is that egestion content sample taken after 3 hours of observation (E3H) and egestion content sample taken after 5 hours of observation (E5H) are similar for all the variables considered (so the p-value is > 0.05 every time).

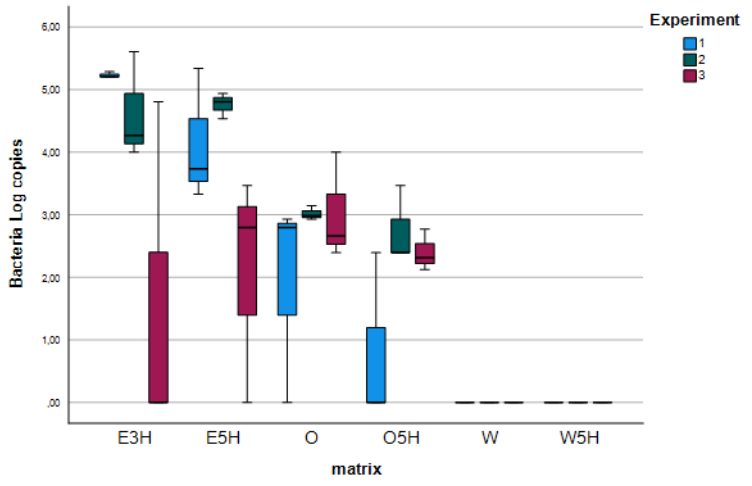

Figure 26: Bacteria Log copies related to different matrices considered in the final experiment (for further information see Supplementary materials).

It is possible to observe that the samples related to water (W and W5H) are always under the limit of detection (Figure 26), although in microbiology results, in the graph (Figure 25) a contamination was present and detectable in the water sample W5H.

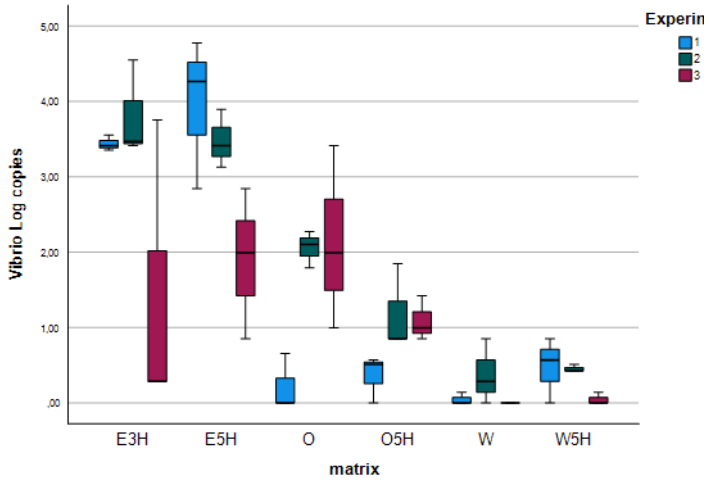

Figure 27: *Vibrio* Log copies related to different matrices considered in the final experiment (for further information see Supplementary materials).

It is evident in this case the presence of DNA belonging to *Vibrio spp.* in water samples, taken before clams were put inside the microcosms while in TCBS log CFU (Figure 25) this data was not present because with culture independent methods it is possible to also detect the presence of *Vibrio* that are in VBNC phase (see paragraph 1.2.2. *Vibrio*). This means that in W only DNA was present, but, in W5H the results were given by DNA already present (and so of dead bacteria) and by Bacteria (*Vibrio* in case of TCBS) that are present in VBCN phase.

4.3. Comparison of methods

To compare the two different methodologies applied in this study, the graphs in Figures 28 and 29 can be compared.

In both graphs shown are all the samples analysed in the study. In Figure 28, in y-axis are the CFU of the microbial counts while in Figure 29 are presented the logarithms of the copy numbers, i.e. the bacterial copies present in the sample calculated using Real Time PCR (see section 3.8. Absolute quantification).

In Figure 28, the results obtained in TCBS are presented in orange, while Figure 29 shows the *Vibrio* copy numbers.

In light blue, the MA with the total marine bacteria count is presented in Figure 28, while Figure 29 shows the bacteria count in general.

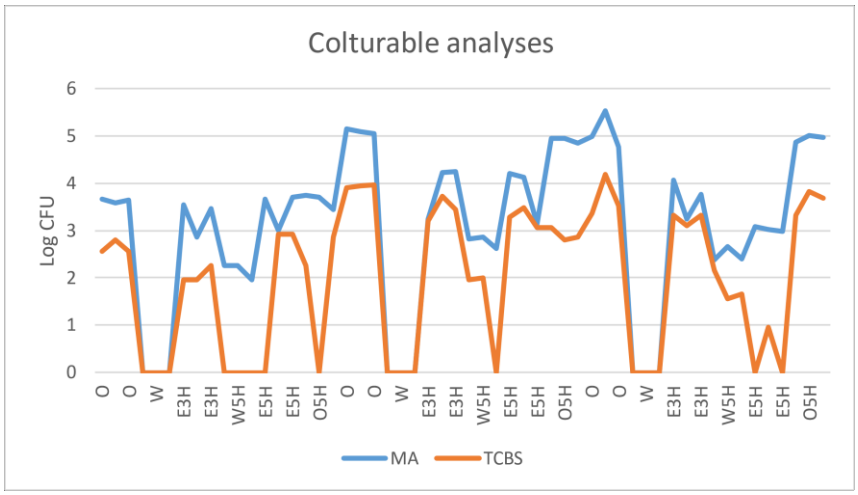

Figure 28: Results obtained with application of culturable analyses.

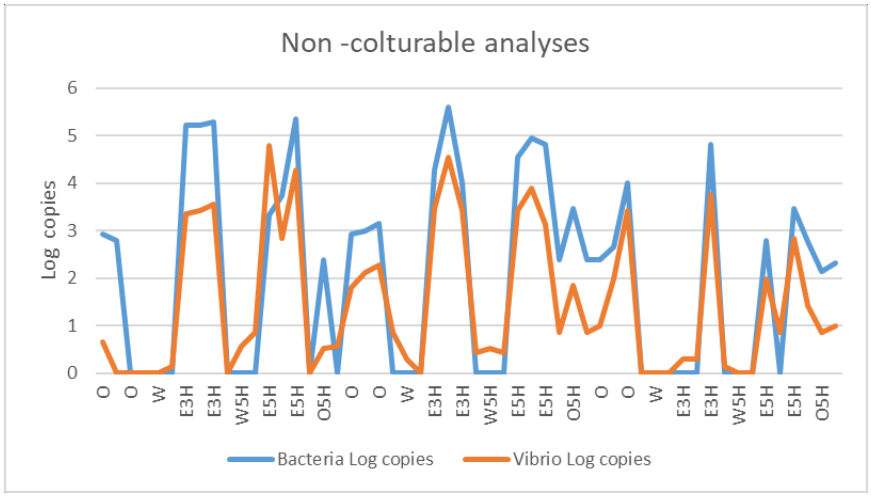

Figure 29: Results obtained with application of non-culturable methods.

5. DISCUSSION

Environmental parameters registered during the week of the final experiment were useful to understand if a variation in one or more of them can influence the way in which bivalve molluscs release their contents during the depuration period.

Rain and pH were two factors that showed a fast variation that was registered on the same day (09/06/2022) that coincided with the last day of the experiment. Comparing Figures 22 and 23 it is possible to observe that the higher peak of precipitations corresponds to the lower peak of pH, but in any case, the value of pH was never below the optimal range for clams furnished by Dr. Luciano Boffo (personal communication) or by FAO, 2022.

Looking at temperature and salinity (Figures 20 and 21) they were always out of the optimal range of clams that are maintained in a depuration plant. Values taken into account and considered to be the optimal range for clams were furnished by Dr. Luciano Boffo and refer to parameters registered in Bluepesca depuration plant sited in Chioggia. Temperature range varies between 13 and 15°C, salinity varies between 38-40 ‰, oxygenation between 70-100% and pH is in the range of 7,8-8.

Although parameters were out of the optimal range, this seems not to be an influent condition for the release of the egestion content that differ only in the composition between day 1 and 2 of the experiment. This difference is not related to environmental parameters, although the general trend demonstrates a decline in temperature, salinity and pH during the three days, but despite this change that was kind same in all three parameters, the egestion content was quite same between day 1 and 3.

Focusing on the experiment and on observations made day by day during it; it can be difficult to explain some of the variations that have been reported during the observation period. Every day of experiment in fact, all clams after a certain period (approximatively after 2 hours from when they were placed inside microcosms) were inside microcosms with open valves and exposed siphons. The egestion content released during the period of observation was of different consistence, sometimes the solid component was prevalent, but sometimes it was composed only

of mucoid components. The difference in the content of egestion was noticed in day 2, when it was in prevalence constituted of mucoid components.

NPC test showed that everything that was observed is not related only to a single experiment and that each matrix influences the target considered. Otherwise, this observation is not complete because the only information given is that the matrices are different from each other.

E3H and E5H as shown in the results do not present a statistical difference ($p>0.05$, see Table 8 in Supplementary materials), this means that there is no difference in *Vibrio* release after 3 or after 5 hours of observation. The similarity between samples taken after different time periods suggests that the microbiota within the mollusc did not change over time, even though they were not fed from the beginning to the end of the experiment. What may have changed, however, is the microbiome, which may differ in composition between the two samples given the absence of food.

The presence of *Vibrio* in both periods (set-up experiments May and final experiment June), indicate that it is present in the environment and in the microbiota of bivalve molluscs in both May and June, what differ between trials and final experiment is the concentration of this bacteria founded in TCBS Log CFU (Figure 19 and Figure 25). In trials after 2 hours, there is the maximum release of *Vibrio* that decrease in the following hours. In final experiment, the situation is different because *Vibrio* was found also after 5 hours and with a large excursion. The periods in which samples were taken were different and this suggests that there is something that allows *Vibrio* presence in higher concentration during summer months. Looking at environmental parameters to justify this increment in the concentration of *Vibrio*, salinity and pH were similar in May and June while the temperature is higher in June. This means that in summer months, with higher temperatures, the concentration of *Vibrio* is higher. Temperature had been already studied in other researches as a factor that can influence and that actually has an influence on the concentration of bacteria and on the filtration rate of bivalve molluscs (Dame, 2011).

A possible explanation for the difference in the level of contamination is that the samples were taken at one month and, with higher temperatures (see tables with environmental parameters in previous paragraphs) the contamination by *Vibrio* is higher.

To conclude, although variability between trials and final experiment is described and observable in plots, from a statistical point of view, the differences are mainly due to the variability observed between days and in certain cases probably due to a certain degree of variability between replicates.

In Figure 27 where *Vibrio* Log copies of final experiment are plotted the presence of *Vibrio* in samples of water at the beginning of the experiment (reference matrix: W) is due to the contamination of this bacteria of the marine salt used for the preparation of water. This means that its DNA molecule was already present in the sample and underline the resistance and stability of this molecule to persist in the external environment.

The similarity presence in the samples taken in the final experiment at different times highlighted the fact that also after a certain period of time, *Vibrio* had been already present (Figure 25). Focusing on a depuration plant then, this result highlights the fact that it is possible that in this contest, molluscs can be responsible for their own contamination in the first hours of depuration process and so, if the depuration period is no longer enough, the problem of the high contamination can persist, making this process less effective (Lee R., Lovatelli A., Ababouch L., 2008).

Another result that may be discussed in this study regards the presence of the DNA molecule of *Vibrio* also before the starting of the experiment. This condition is relevant since the molecular methods are able to detect the presence of DNA in a better way if compared with microbiological tests (see graphs in the paragraph dedicated to results obtained in final experiment). The persistence of the DNA molecule can also influence the results of the study because, especially in plots where it is possible to have a comparative visual of the trends, in one of the two methods applied, *Vibrio* spp. was present, but not in the other. This can represent a

problem since it is difficult to understand if the concentration of log copies was under the limit of detection, and so sterility conditions were maintained, or not.

In Figures 28 and 29, both graphs present the results obtained in the study, then the counts obtained with and analysis. The observed discrepancy present between some samples is due to the diversity of the methods applied, which have different sensitivities and are therefore not perfectly superimposable.

Furthermore, in the microbiology part (Figure 28), it must be taken into account that the media do not allow all bacteria to be recovered (there are in fact some cells that may have difficulty growing or are in the VBNC phase because of the different or unstable environmental conditions in which they are (see paragraph 1.2.2 *Vibrio*).

TCBS medium, although specific for *Vibrio*, also allows the growth of other bacteria while in MA, marine bacteria grow due to the presence of salt, but, in general, for both microbiology and Real Time, it is not necessarily the case that all the bacteria in the sample are recovered.

The real time primers were taken from the literature and should amplify all the *Vibrio* present, however, as in the example of the homogenate samples (O and O5H) there is a low charge because the presence of the Host DNA (clams) can reduce the efficiencies of the real time and so it is a little underestimated.

At the same time, results for the same sample can also be underestimated in microbiology due to the presence of VBNCs.

6. CONCLUSION

Comparing the contamination level of egestion content (E3H and E5H) it is possible to conclude that the temperature is a parameter that could influence the level of contamination because, as shown in the previous paragraphs, the concentration of *Vibrio* inside molluscs is higher in summer months than in spring.

The difference observed in the samples of W5H in MA and in Bacteria log copies highlighted the limitation of the methods applied because it couldn't detect all the *Vibrio* present in the sample, or *Vibrio* was present in a smaller number of copies that the molecular method wasn't able to detect it, while the microbiological one was capable and showed results in plots. Both microbiological analyses and molecular methods showed some limitations in detecting the DNA molecules/live cells present in samples that may have led to an underestimation of the effective presence of *Vibrio*.

This conclusion highlights the necessity of another set of tests, that will be done next year in the same months, to have a direct comparison with the results obtained in this study and to see if the temperature has a significant influence on the release of *Vibrio* spp. from bivalves.

However, this study was the bases for additional analyses as microbial communities or metagenomics analyses. Moreover, this study highlighted the importance to study the transient microbiota (especially vibrios) that occurs also in the early stages of Egestion process.

7. SUPPLEMENTARY MATERIALS

Table 7: NPC test of the two main factors (Experiments and Matrix) considered in the final experiment related to both microbiology results and qPCR results.

	MA	TCBS	BacLog	VibrioLog
Exp	NS	NS	NS	NS
Matrix	0.001	0.001	0.001	0.001

Table 8: Pairwise comparison of the different matrices considered in the final experiment related to both microbiology results and qPCR results.

Matrix	Pairwise comparison			
	MA	TCBS	BacLog	VibrioLog
W-W5H	0.221	0.322	0.001	0.475
W-E5H	0.001	0.018	0.001	0.028
W-E3H	0.001	0.001	0.001	0.008
W-O5H	0.001	0.001	0.001	0.001
W-O	0.001	0.001	0.001	0.004
W5H-E5H	0.001	0.268	0.001	0.001
W5H-E3H	0.001	0.001	0.001	0.001
W5H-O5H	0.001	0.005	0.002	0.01
W5H-O	0.001	0.001	0.009	0.001
E5H-E3H	0.68	0.28	0.338	0.663
E5H-O5H	0.003	0.175	0.023	0.001
E5H-O	0.002	0.014	0.143	0.01
E3H-O5H	0.007	0.784	0.046	0.003
E3H-O	0.006	0.218	0.252	0.043
O5H-O	0.787	0.338	0.937	0.724

Table 9: Pairwise comparison of the different matrices considered in the final experiment related to both microbiology results and qPCR results. Stratification Variables Matrix per experiment.

	MA	TCBS	BacLog	VibrioLog
O	0.035	0.0145	0.518	0.046
O5H	0.0176	0.0082	0.1248	0.103
W	0.9	0.9	0.9	0.9
W5H	0.9	0.9	0.9	0.9
E3H	0.339	0.0143	0.085	0.197
E5H	0.1317	0.042	0.051	0.048

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