Research Article

Microbiological controls in polyculture farming: A pilot case study in the Castellammare Gulf (Sicily)

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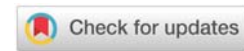
Abstract

Background: Integrated multi-trophic aquaculture (IMTA) experiences, based on the co-cultivation of two or more organisms, have proliferated in recent years; nevertheless, studies regarding the microbiological implications of these farming systems are not available yet.

The context and purpose of the study: The microbiological conditions of shellfish and surrounding rearing environment were investigated during a pilot polyculture system (fish and shellfish farming) performed in the Castellammare Gulf (Sicily). The quantitative distribution of faecal coliforms and enterococci as faecal pollution indicators, together with that of environmental and potentially pathogenic halophilic vibrios, was determined in two different seasons (spring and autumn). Samplings of surface waters and bivalves (oysters, *Cassostrea gigas* and *Ostrea edulis*; mussel, *Mytilus galloprovincialis*, and clam, *Tapes philippinarum*) were performed at stations where integrated polyculture was performed (Impact sites) compared to others (Control sites) where farming activities regarded shellfish only.

Results: Higher numbers of faecal pollution indicators were detected in waters at the Impact than at Control ones, generally in spring, although polyculture seemed to have only a low impact over the area. Shellfish products were characterized by vibrios concentrations higher than in waters, due to their filter-feeding activity. They ranged in the order of- 10^2-10^3 CFU/g, with higher abundances in spring in the specimens reared at the Impact sites; conversely, in shellfish farmed at the Control sites, no significant quantitative variations were found between the two samplings. The qualitative study of vibrios isolated from both shellfish and environment showed the ubiquitous distribution of *V. alginolyticus*, and the predominance of *V. vulnificus* at the Impact sites in both shellfish and waters, representing the 40% and 27% of the total isolates, respectively.

Main findings: Overall, the low levels of microbial contamination detected in the area showed that polyculture did not result in a heavy impact on the surrounding environment.

Conclusions: The results obtained in the polyculture experiment performed in Castellammare Gulf suggest the use of this system as a sustainable farming strategy for productive purposes.

Brief summary: During a pilot experiment of integrated aquaculture, the microbiological quality of the shellfish products was assessed, proving that this productive practice did not give negative results on the concentrations of total heterotrophic bacteria, faecal pollution indicators and potentially pathogenic vibrios.

Any potential implications: Polyculture is suggested as an ecofriendly farming system, that allows the production of shellfish safe for consumers.

Introduction

Aquaculture (including fish, shellfish and macroalgae farming) represents a very important sector for the world economic growth, that can lead to social benefits in terms of new occupational perspectives [1]. Productive activities, however, have well-known impacts on the environment, mainly due to the release of feed wastes, chemical and pharmaceuticals (i.e.

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antibiotics), as well as to potential transmission of diseases, dispersal of non-native species, and destruction of habitats [2,3].

The severity of aquaculture impacts varies in relation to the reared species, local environmental conditions, farming and management techniques. Future developments in the aquaculture field depend on the selection of rearing practices able to meet the principles of environmental sustainability [3,4]. In this context, particular interest has recently been addressed to to Integrated Multi-Trophic Aquaculture (IMTA) systems, where organisms belonging to different trophic levels and inhabiting different ecological niches are co-cultured. IMTA includes several aquaculture practices based on the complementarity of different productive compartments. By IMTA, inorganic and organic wastes from finfish are assimilated respectively by autotrophic (i.e. phytoplankton, macroalgae, plants) and heterotrophic species (e.g. oysters, mussels, sea cucumbers) that are co-cultured with the target reared species. It can represent a key alternative in the evolution of aquaculture systems, allowing to face a double challenge: sustaining the growing demand of aquatic products and preserving the environment by reducing wastes [5,6]. IMTA has also been suggested as a possible ecofriendly strategy to mitigate the effects of multiple stressors [7].

In Asian Countries, the integrated cultivation of fish with organisms of different trophic levels, as well as the rearing of both shellfish and seaweed in lagoons or bays close to fish farming structures is an old practice [8,9]. In China, the polyculture systems with the macroalga *Eucheuma gelatinae* and the bivalve *Gafrarium tumidum* provided a suitable tool for the purification of eutrophic seawater and control of algal bloom [10]. In other Countries, such as Canada, Reid, et al. [11], reviewed shellfish production in the context of open-water IMTA; later, Chopin [12] underlined the advantages of IMTA systems, able to increase economic profitability per cultivation unit through co-cultivation of many species (fish, seaweeds, invertebrates), characterized by environmental sustainability and societal acceptability. More recently, Buck, et al. [13], have provided an in-depth review of the main variables affecting IMTA and the potential benefits/limitations of offshore mariculture plants. The feasibility of integrated fish, shellfish and/or seaweed aquaculture within offshore wind farming areas has been reported to be affected by factors such as biological feasibility, technological implementation, environmental sustainability and economic feasibility of the farming systems.

In Europe, Alexander, et al. [14], studied the main incentives and barriers to the development of IMTA, highlighting that moving from IMTA at a pilot scale to commercial scale developments requires changes in policy and legislation to promote innovation and technologies for aquaculture and simplification of the regulations regarding licensing or spatial planning for aquaculture. Although attention to IMTA systems is more recent compared to Asia, in Europe integrated polyculture production still faces inherent difficulties which limit the adoption of this practice across this Continent [15]. In Italy integrated polyculture of fish and low-trophic-level organisms such as shellfish is a still scarcely exploited

practice [16]. Among shellfish, mussels, oysters and clams are organisms highly appreciated by consumers; oysters are commonly cultured in shallow waters or in intertidal zones near estuaries, while mussels and clams are cultured also in brackish inland ponds. Although the impacts on the environment of bivalve cultivation are well known in terms of local benthic (i.e. physical disturbance, changes in sediment topography and sedimentation, accumulation of debris, biodeposition) and water column effects (alteration in water quality and nutrient cycling) as well as of wider ecological effects (on fish, seabirds, transmission of diseases) [17], the effects of bivalve cultivation in polyculture have been underestimated [18]. The limited knowledge of growth performance of the reared organisms explains the delayed success of IMTA experiences and of the economic performance of this practice. Polyculture experiences have proliferated in recent years [15], nevertheless studies regarding the microbiological implications of polyculture systems are not available yet. Microbiological controls in aquaculture systems play a crucial role to prevent possible transmission to man of pathogenic bacteria or toxins *via* food consumption [19-23]. Compliance to high quality microbiological criteria is particularly important in shellfish farming areas, in relation to the filtering-feeding behavior of these organisms that results in the concentration of bacteria and polluting substances inside the animal body. Consequently, hygienico-sanitary monitoring of shellfish microbiological quality must be extended also to the waters of the farming environments [24-27].

The Gulf of Castellammare (Trapani, Sicily) is a 70 Km wide coastal area that extends along the Tyrrhenian coast between Cape S. Vito and Terrasini. Thanks to its particular morphological and hydrological characteristics - which ensures an efficient water exchange and low urban and industrial loading - this area has traditionally been designed for mariculture purposes. Some reports on the microbiological conditions of this ecosystem are those available from Caruso, et al. [28-30]; nevertheless, knowledge of the health risks related to aquaculture farming is still scarce. In the framework of a pilot IMTA experiment of mussels and fish co-cultivation, an investigation of the bacteriological quality of shellfish products was undertaken. With the aim of assessing the microbiological quality of both shellfish and environment, the bacterial indicators of faecal pollution (faecal coliforms, *Escherichia coli* and *Salmonella* spp.) as well as heterotrophic microflora (marine and not marine bacteria), autochthonous bacteria (*Vibrio* spp. and their potentially pathogenic members) were estimated. In addition, the expression of hydrolytic enzymes (caseinolytic, lipolytic, haemolytic and lecithinolytic activities) as presumptive virulence factors involved in the pathogenicity mechanisms of bacteria, was also studied.

Materials and methods

Study site

The Castellammare Gulf was chosen as a polyculture site due to the intense aquaculture practiced in this area; the wastes released by productive plants such as faecal pellets and food residuals cause the organic enrichment of the waters [29].

In this context, shellfish farming could represent a suitable tool to convert the trophic inputs into biomass production. The study area was located 3 miles offshore Balestrate, where the polyculture pilot plant was set up (Figure 1). The geographical coordinates of the sampling sites are reported in Table 1.

The experimental design of integrated cultivation consisted of five submersible cages (Farmocean, Sweden; volume = 4,500 m³) and 6 smaller cages (volume = 1,000 m³) filled with seabass (*Dicentrarchus labrax*) and seabream (*Sparis aurata*) for a total annual production of about 600 tons of biomass. All the species selected for the study were bivalves highly appreciated

due to their high nutritional value: Japanese oyster (*Cassostrea gigas*), European oyster (*Ostrea edulis*), common mussel (*Mytilus galloprovincialis*), Philippine clam (*Tapes philippinarum*), which belong to Ostreidae (*Ostrea* genus), Mytilidae (*Mytilus* genus) and Veneridae (*Tapes* genus) families, respectively. Mussel seed was cultivated in tight nylon net bags for 12 months in sites close to fish cages (hereafter indicated as Impact sites) and 1 Km far from fish cages (hereafter indicated as Controls).

Collection of shellfish and water samples

Shellfish and surface waters were collected at both Impact and Control sites, during two different samplings, performed in spring and autumn. Surface seawater samples were drawn using sterile 10 litres Niskin bottles. Both seawater and shellfish samples were stored at +5°C in fridge containers until their analysis at the CNR laboratory. They were examined within 4 hours of sampling.

Physico-chemical parameters

The main physico-chemical parameters, temperature and salinity, were recorded using a multiparametric probe.

Shellfish samples

The bacteriological analysis of shellfish was carried out on a sample constituted by 6-8 individuals for oysters, 15 for mussels and 20-30 for clams. The shellfish samples were carefully cleaned from mud, removing the epiphytes; the individuals showing open valves were excluded from analysis. To open shellfish, a sterile scalpel was used, then the tissues and the intravalvular liquid were collected in a sterile container and homogenised using a Stomacher. The homogenate was diluted in sterile saline solution in a 1:10 ratio (weight/volume), and treated differently depending on the target bacteria to be searched.

Each shellfish sample was examined for the quantitative determination of Faecal Coliforms (FC), *Escherichia coli*, faecal streptococci (ENT), culturable not marine and marine heterotrophic bacteria, and *Vibrio* spp. at 24°C and 35°C. The search for *Salmonella* spp. was carried out. The qualitative composition of *Vibrio* spp. was determined too.

For the determination of FC abundance, the Most Probable Number (MPN) method of fermentation in multiple tubes was applied; briefly, for each shellfish homogenate three dilutions (1:10, 1:100, 1:1000 in sterile saline solution, each dilution in five replicates) were prepared and inoculated into tubes of lactose broth – each dilution in five replicates – containing a Durham tube. The tubes showing turbidity and lactose fermentation with gas production after incubation at 35°C for 24 hours were counted.

The abundance of *E. coli* was estimated through indole production test; small volumes (0.1-0.2 ml) of the broth cultures positive for FC were inoculated in tryptone water, incubated at 44°C for 24 hours. The tubes showing a typical pink colouration after addition of the Kovacs' reagent were considered as positive.
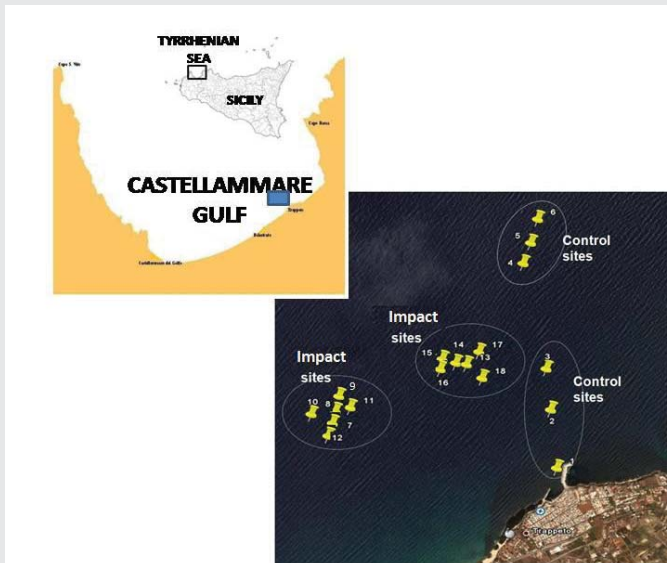

Figure 1: Sampling sites in Castellammare Gulf. Reported are as Impact sites the sites where polyculture (fish- and shellfish farming) activities were performed, while Control sites were those with shellfish farming activities only.

Table 1: Coordinates of the sampling sites in the Castellammare Gulf.

Stations		Latitude	Longitude
1	Control	38.0725 N	13.0380 E
2	Control	38.0784 N	13.0374 E
3	Control	38.0826N	13.0369 E
4	Control	38.0932 N	13.0345 E
5	Control	38.0953 N	13.0353 E
6	Control	38.0977 N	13.0360 E
7	Impact	38.0782 N	13.0146 E
8	Impact	38.0788 N	13.0148 E
9	Impact	38.0792 N	13.0149 E
10	Impact	38.0779 N	13.0120 E
11	Impact	38.0787 N	13.0162 E
12	Impact	38.0770 N	13.0143 E
13	Impact	38.0830 N	13.0285 E
14	Impact	38.0832 N	13.0273 E
15	Impact	38.0835 N	13.0259 E
16	Impact	38.0823 N	13.0258 E
17	Impact	38.0843 N	13.0298 E
18	Impact	38.0816 N	13.0301 E

For the qualitative search of *Salmonella* spp., 25 g of shellfish were inoculated for 20 hours at 35°C into 225 ml of buffered peptone water as a pre-enrichment step. After further enrichment in Rappaport-Vassiliadis and Tetrathionate broths (Oxoid), incubated at 42°C for 20-24 hours, isolation was performed by sub-culturing on plates of Hektoen Enteric and SS (Salmonella-Shigella) agar (Oxoid), incubated at 35°C for 20-24 hours as selective media for *Salmonella* spp. Suspected colonies were identified according to their biochemical profiles as obtained by API 20E strips and confirmed by serological tests (Omni-O anti-*Salmonella* polyvalent serum, Sanofi Pasteur).

Culturable not marine bacteria were counted by spreading 0.1 ml of sample homogenate on the surface of Plate Count Agar plates, further incubated at 22°C for 24-48 hours. The quantification of heterotrophic marine bacteria was carried out by spreading of Marine Agar 2216 plates (Difco), incubated at 22°C for 7 days.

The abundance of halophilic *Vibrio* spp. was estimated by spreading 0.1 ml of sample homogenate on the surface of Thiosulfate Citrate Bile Salts (TCBS, Oxoid) agar plates added with 2% sodium chloride and incubated at 24°C for 48 hours and at 35°C for 24 hours, for the determination of total presumptive vibrios (VP) - microorganisms autochthonous in the marine environment - and of the fraction of potentially pathogenic species (VPP) respectively.

For the qualitative study of *Vibrio* spp, bacterial strains were isolated "at random" from TCBS agar plates, by streaking on fresh plates of the same medium until axenic cultures were obtained. The phenotypic characteristics examined were: Gram staining, glucose fermentation on Triple Sugar Iron agar (Difco), cytocrome-oxidase activity, acid from sucrose on TCBS agar, sensitivity to the vibriostatic agent O129 (150 μg), biochemical profiling on API 20 E (Biomerieux) strips.

Water samples

Quantitative analyses for the bacteriological characterization of seawater samples were performed by filtration through a sterile 0.45 μm pore size membrane (Millipore); the filter was further placed in culture media different depending on the target microorganism to be evaluated. Particularly, for FC counts, incubation was carried out on m-FC agar (Oxoid) plates at 44.5°C for 24 hours; for *Escherichia coli*, incubation was performed on TBX agar (Oxoid) plates at 35°C for 24 hours and colonies grown were confirmed by indole test. The abundance of ENT was determined on Slanetz-Bartley agar plates incubated at 35°C for 48 hours.

For the search of *Salmonella* spp., 300 ml of seawater were filtered and the membrane was pre-enriched in buffered peptone water incubated at 35°C for 20 hours. The further steps of the analytical procedure were similar to those described for shellfish samples.

For the determination of culturable non-marine bacteria and heterotrophic marine bacteria, 0.1 ml seawater samples were streaked on the same culture media as reported for shellfish samples; for VP and VPP counts, 100 ml of seawater were filtered through 0.45 μm pore size sterile membranes and the filters incubated on TCBS agar plates as for shellfish samples.

Assays for enzymatic activities as proxies of virulence factors

All the *Vibrio* spp. isolates were screened for the presence of enzymes involved in virulence. Before such assays, bacterial strains were grown overnight in Tryptic Soy agar with 2% NaCl at 35° C, and further inoculated into different solid culture media according to García Moreno and Landgraf [31]. In detail, protease (caseinolytic) activity was determined by incubation at 35°C in skim milk agar plates (2% final concentration). Bacterial isolates able to produce clearing zones into the medium containing the casein substrate were recorded as positive.

Lecithinolytic activity was determined by streaking bacteria onto a nutrient basal agar containing 10% (v/v) egg yolk emulsion, further incubated at 35°C for up to 7 days. Zones of precipitation surrounding the colonies indicated the production of lecithinase.

Lipase production was tested by inclusion into a nutrient basal agar of Tween 80 (polyoxyethylene sorbitan mono-oleate, 1% final concentration) as a substrate for bacterial growth.

The production of haemolysins (i.e. Kanagawa phenomenon) was assayed on plates of Columbia agar base added with a 5% suspension of sheep red blood cells. The colonies able to produce haemolysis after incubation at 35°C for 24 hours were considered as positive.

Expression of the results and statistical data elaboration

For the shellfish samples, the number of positive tubes in the MPN procedure was recorded and the results were reported as MPN index per 100 grams of homogenate after comparison with a Mc Grady probability table. Not marine and marine bacteria as well as *Vibrio* spp. were reported as Colony Forming Units (CFU) per gram of sample.

Bacteriological counts of the seawater samples were expressed as the mean value of duplicate plates, and reported as CFU per 100 ml of water, except for not marine and marine bacteria, which were reported in CFU per ml of water.

Analysis of Variance (ANOVA) test was performed by Excel software to evaluate the occurrence of significant spatial variations in the bacterial abundances among the Control and Impact sites. When data failed the assumption of normal distribution, logarithmic transformation was performed before ANOVA was applied.

Pearson's correlation was used to assess whether the abundance of bacteria was related to the environmental parameters.

Results

Temperature values ranged from a minimum of 14.10 to a maximum of 27.84°C; salinity from 35.88 to 38.12 and oxygen from 50.55 to 91.25 %.

Shellfish samples

The results obtained in the examined shellfish are shown in Table 2a,b.

In spring (Table 2a) the values of FC were generally ≤20 MPN/100 g in all the samples. Only one sample of *O. edulis* harvested from the Impact site showed higher concentrations (130 MPN/100 g) that, however, were still lower than 300 MPN/100 g (i.e. the value fixed for shellfish coming from areas classified as A zone). Only in this species a significant difference between the Control and the Impact sites was found, with FC values higher at these latter. The distribution of *E. coli* reflected that of FC, showing peak values in the *O. edulis* sample collected from the Impact site (40 MPN/100 g). No positive results for *Salmonella* spp. were obtained in the examined samples. The biochemical profiling by API 20E strips of the bacterial isolates allowed the identification of a strain of *Citrobacter freundii*, an Enterobacterium with biochemical characteristics similar to *Salmonella* spp., that was isolated from one sample of *C. gigas* harvested from the Impact site.

Marine and not marine bacteria showed values in the range of 10^2–10^3CFU/g, with higher concentrations for the marine bacteria and a peak of 1.1 x 10^4 CFU/g in *M. galloprovincialis* collected at Impact sites.

VP concentrations varied between 2.0 x 10^1 and 1.0 x 10^4 CFU/g

recorded in *C. gigas* from Control site and in *M. galloprovincialis* from Impact site, respectively. VPP values (data not shown) were in the order of 10^1–10^2 CFU/g (2.7 x 10^1– 1.2 x 10^2 CFU/g), peaking in the mussel sample collected from Impact sites.

Regarding the qualitative composition of vibrios (Table 3), most of the isolates were assigned to *Vibrio* spp., while the species *V. alginolyticus* and *V. vulnificus* were predominant within the VPP fraction of shellfish collected from the Control and Impact sites, respectively.

FC, ENT, VP and VPP abundances correlated positively with temperature (Pearson correlation coefficients r= +0.55, +0.54, +0.58 and +0.64, P<0.05, respectively).

In autumn (Table 2b), FC concentrations in shellfish collected from the Control sites were ≤ 30 MPN/100g; only *C. gigas* and *M. galloprovincialis* harvested from the Impact sites showed high concentrations (130 MPN/100g and 110 MPN/100g respectively). *E. coli* distribution showed values not exceeding 20 MPN/100 g in all the examined samples. Shellfish samples were also negative for *Salmonella* spp. The biochemical profiling by API 20E strips of the bacterial isolates identified six strains classified as *Proteus*, isolated from *O. edulis* and *M. galloprovincialis* collected from the Impact sites (data not shown).

A decrease in the abundance of not-marine and marine bacteria was observed, compared to the spring numbers. The highest concentrations of not marine bacteria were found in *O. edulis* collected from the Impact site (1.1×10^3 CFU/g), while the lowest ones were recorded in one *C. gigas* sample collected from the Impact site (3.75×10^2 CFU/g).

Table 2a: Microbiological characteristics of the shellfish in spring.

	Samples	Faecal coliforms	*Escherichia coli*	*Salmonella* spp.	Not marine bacteria	Marine bacteria	*Vibrio* spp. (24°C)
		MPN/100g	MPN/100g	in 25 g	CFU/g	CFU/g	CFU/g
Impact	1 – Cassostrea gigas	<20	<20	Absent	1860	1035	720
	2 – Cassostrea gigas	20	<20	Absent*	2190	3760	591
	3 - Ostrea edulis	130	40	Absent	2320	5440	255
	4 - Mytilus galloprovincialis	<20	<20	Absent	1010	11300	10500
	5 - Tapes philippinarum	<20	<20	Absent	855	8000	2633
Control	1 – Cassostrea gigas	<20	<20	Absent	1000	4940	973
	2 – Cassostrea gigas	20	<20	Absent	1090	3440	20
	3 - Ostrea edulis	40	<20	Absent	515	3020	310
	4 - Mytilus galloprovincialis	<20	<20	Absent	1130	3920	600

*Isolation of a strain of *Citrobacter freundii*

Table 2b: Microbiological characteristics of the shellfish in autumn.

	Samples	Faecal coliforms	*Escherichia coli*	*Salmonella* spp.	Not marine bacteria	Marine bacteria	*Vibrio* spp (24°C)
		MPN/100g	MPN/100g	in 25g	CFU/g	CFU/g	CFU/g
Impact	1 – Cassostrea gigas	130	<20	Absent	585	1950	980
	2 – Cassostrea gigas	40	20	Absent	375	1680	290
	3 - Ostrea edulis	70	<20	Absent	1112	2700	635
	4 - Mytilus galloprovincialis	110	20	Absent	759	2044	1161
Control	1 – Cassostrea gigas	40	<20	Absent	470	1620	800
	2 – Cassostrea gigas	20	<20	Absent	420	1200	100
	3 - Ostrea edulis	40	<20	Absent	700	1800	350
	4 - Mytilus galloprovincialis	20	<20	Absent	550	1500	600

Table 3: Qualitative study of the *Vibrio* spp. isolated in spring from shellfish and water.

Shellfish	Species	Origin	No. of isolates	% of the total isolates	*Vibrio* spp.	*V. alginolyticus*	*V. vulnificus*	*V. metschnikovii*
Control	1 – *Cassostrea gigas*	AP	2	20,0	1	1		
	2 – *Cassostrea gigas*	AP	2	20,0	1	1		
	3 - *Ostrea edulis*	TCBS/AP	3	30,0	1			2
	4 - *Mytilus galloprovincialis*	TCBS/AP	3	30,0	1	2		
	Total		10	100	4	4	0	2
Impact	1 – *Cassostrea gigas*	TCBS	2	13,3	2			
	2 – *Cassostrea gigas*	TCBS	1	6,7			1	
	3 - *Ostrea edulis*	TCBS/AP	5	33,3	4		1	
	4 - *Mytilus galloprovincialis*	TCBS	4	26,7	1	1	2	
	5 - *Tapes philippinarum*	TCBS/AP	3	20,0	1		2	
	Total		15	100	8	1	6	

Water	Station	Origin	No. of isolates	% of the total isolates	*Vibrio* spp.	*V. alginolyticus*	*V. vulnificus*	*V. metschnikovii*
Control	1	AP	1	14,3	1			
	2	AP	2	28,6	1	1		
	3	AP	1	14,3	1			
	5	AP	1	14,3				1
	6	AP	2	28,6	2			
	Total		7	100	5	1	0	1
Impact	9	AP	1	9,1	1			
	10	AP	3	27,3	1		1	1
	13	AP	1	9,1			1	
	14	AP	1	9,1	1			
	15	AP	2	18,2	1		1	
	17	AP	2	18,2	1	1		
	18	AP	1	9,1		1		
	Total		11	100	5	2	3	1

Regarding marine bacteria, a peak value was measured in the *O. edulis* sample collected from the Impact site (2.7×10^3 CFU/g), while the minimum concentration was reached in *C. gigas* collected from the Control site (1.2×10^3 CFU/g).

VP counts showed the constant presence of these microorganisms with densities in the order of 10^2–10^3 CFU/g; the highest microbial concentrations were recorded in the mussel sample collected from the Impact sites, while the lowest ones occurred in one sample of the oyster *C. gigas* (10^2 CFU/g from both the Control and Impact sites). VPP were mostly absent in the shellfish collected during autumn (data not shown).

The qualitative analysis of vibrios (Table 4) highlighted a more diversified composition compared to the spring, with the predominance in the shellfish collected at the Impact sites of *Vibrio* spp., *V. vulnificus* and small numbers of *V. alginolyticus*, *V. mimicus* and *V. parahaemolyticus*. Conversely, at the Control sites, strains of *V. alginolyticus*, followed by *Vibrio* spp., *V. mimicus* and *V. vulnificus* were isolated and *V. parahaemolyticus* was substituted by *V. fluvialis*.

Water samples

The results obtained from the seawater samples are reported in Table 5a,b and shown in Figures 2 and 3.

In spring (Table 5a and Figure 2) the examined area did not show significant faecal contamination inputs; the abundance of FC was comprised between 2×10^0 and 1.47×10^3 CFU/100 ml; the maximum value was recorded at the station 1.

E. coli was recorded at concentrations generally low, <7

CFU/100 ml, except for the station 1, where its abundance reached values two orders of magnitude higher than those of the other stations, similarly to FC distribution. No strains were identified as *Salmonella* spp. in the examined samples. A strain of *Citrobacter freundii* was isolated from the enrichment in Tetrathionate broth of the water sample drawn from station 1. Values of ENT were <10 CFU/100 ml, except for station 1.

The abundance of not marine bacteria ranged from 1.5×10^1 to 1.24×10^3 CFU/100 ml, recorded at stations 18 and 1, respectively.

Marine bacteria showed concentrations comprised between a minimum of 3.0×10^2 (station 9) and a maximum of 1.27×10^3 CFU/100 ml recorded at station 1.

VP showed the lowest concentrations at stations 6 and 9 (1.95×10^2 CFU/100 ml), while the peak value (9.0×10^2 CFU/100 ml) was found at station 1. The lowest value of VPP was measured at station 8 (2.7×10^1 CFU/100 ml), the highest one (1.22×10^2 CFU/100 ml) at station 1 (Figure 2).

The qualitative study of the vibrios community highlighted that *Vibrio* spp., *V. vulnificus* and *V. alginolyticus* were predominant at the Impact sites, while *V. vulnificus* was not detected at the Control sites (Table 3).

In autumn (Table 5b and Figure 3) the Castellammare Gulf exhibited very low faecal pollution levels. The highest densities of FC (1.2×10^1 CFU/100 ml) were detected at the station 1, while at the other stations FC were mostly absent or equal to 1 CFU/100 ml (stations 6 and 7).

E. coli was generally absent, except for the station 1, where

Table 4: Qualitative study of the *Vibrio* spp. isolated in autumn from shellfish and water.

Shellfish	Species	Origin	No. of isolates	% of the total isolates	*Vibrio* spp.	*V. alginolyticus*	*V. vulnificus*	*V. parahaemolyticus*	*V. mimicus*	*V. fluvialis*
Control	1 – *Cassostrea gigas*	TCBS	13	40,6	4	6	3			
	2 – *Cassostrea gigas*	TCBS	7	21,9	2	2	1		2	
	3 - *Ostrea edulis*	TCBS	2	6,3	1		1			
	4 - *Mytilus galloprovincialis*	TCBS	10	31,3		1	1		5	3
	Total		32	100	7	9	6	0	7	3
Impact	1 – *Cassostrea gigas*	TCBS	11	26,2	7		1	3		
	2 – *Cassostrea gigas*	TCBS	7	16,7	6		1			
	3 - *Ostrea edulis*	TCBS	15	35,7	4	3	6		2	
	4 - *Mytilus galloprovincialis*	TCBS	9	21,4	2	2	2		3	
	Total		42	100	19	5	10	3	5	0

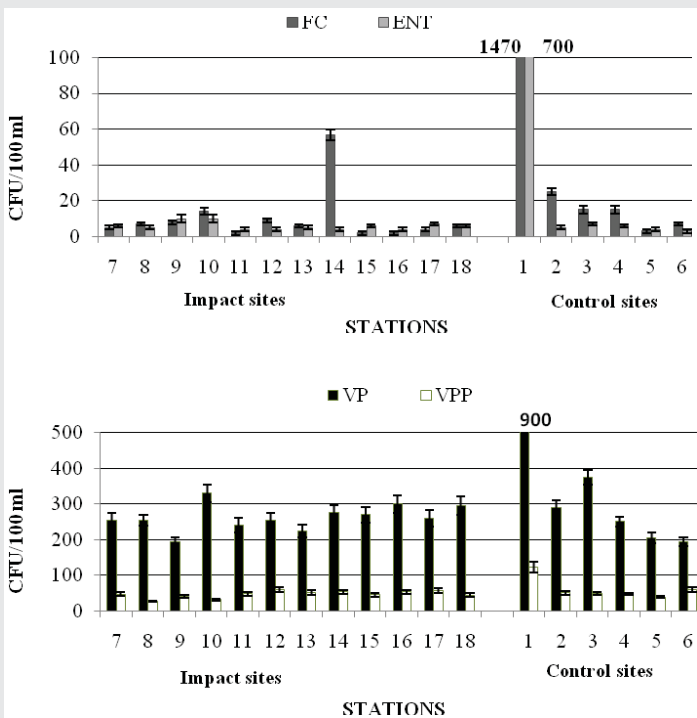| Water	Station	Origin	No. of isolates	% of the total isolates	*Vibrio* spp.	*V. alginolyticus*	*V. vulnificus*	*V. parahaemolyticus*	*V. mimicus*	
Control	2	TCBS	5	38,5		2			3	
	3	TCBS	4	30,8	1	2	1			
	4	TCBS	1	7,7				1		
	6	TCBS	3	23,1			1	2		
	Total		13	100	1	4	2	3	3	
Impact	7	TCBS	2	5,7		2				
	8	TCBS	4	11,4	2	1	1			
	9	TCBS	4	11,4	1	1	2			
	10	TCBS	3	8,6		2		1		
	11	TCBS	4	11,4	1	2	1			
	12	TCBS	2	5,7				1	1	
	13	TCBS	2	5,7			2			
	14	TCBS	3	8,6	3					
	15	TCBS	4	11,4	2	2				
	16	TCBS	3	8,6		1	2			
	17	TCBS	4	11,4	3		1			
	Total		35	100	12	11	9	2	1	

Figure 2: Spring sampling. Abundance of faecal pollution indicators (FC, Faecal Coliforms; ENT, Faecal Streptococci) and *Vibrio* spp. (VP, *Vibrio* spp. able to grow at 24°C; VPP, potentially pathogenic *Vibrio* spp.) found in the Castellammare Gulf waters
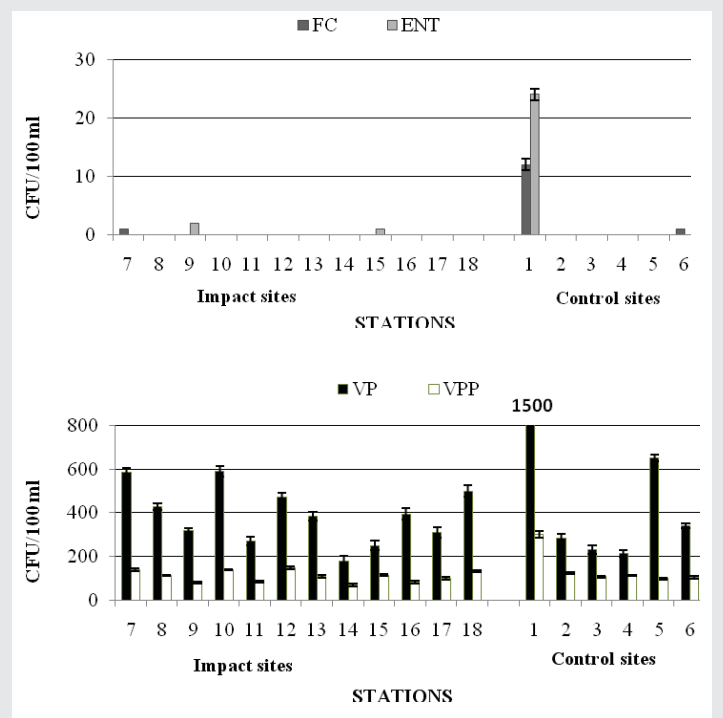

Figure 3: Autumn sampling. Abundance of faecal pollution indicators (FC, Faecal Coliforms; ENT, Faecal Streptococci) and *Vibrio* spp. (VP, *Vibrio* spp. able to grow at 24°C; VPP, potentially pathogenic *Vibrio* spp.) found in the Castellammare Gulf waters.

Table 5(A,B): Microbiological characteristics of waters in spring (A) and autumn (B).

Sample (A)	Faecal coliforms	*Escherichia coli*	*Salmonella* spp.	Faecal streptococci	Not marine bacteria	Marine bacteria	*Vibrio* spp. (24°C)	*Vibrio* spp. (35°C)
	CFU/100ml	CFU/100ml	CFU/100ml	CFU/100ml	CFU/ml	CFU/ml	CFU/100ml	CFU/100ml
1	1470	800	0	700	1245	1275	900	122
2	25	5	0	5	95	615	290	51
3	15	7	0	7	30	830	375	49
4	15	1	0	6	30	665	250	48
5	3	1	0	4	20	620	205	39
6	7	4	0	3	75	575	195	60
7	5	3	0	6	30	485	255	47
8	7	1	0	5	65	870	255	27
9	8	3	0	10	35	300	195	41
10	14	5	0	10	75	895	330	32
11	2	2	0	4	90	780	240	48
12	9	1	0	4	80	845	255	59
13	6	4	0	5	45	455	225	52
14	57	5	0	4	45	470	275	54
15	2	2	0	6	70	370	270	46
16	2	1	0	4	35	1030	300	54
17	4	2	0	7	35	520	260	57
18	6	1	0	6	15	615	295	45

Sample (B)	Faecal coliforms	*Escherichia coli*	*Salmonella* spp.	Faecal streptococci	Not marine bacteria	Marine bacteria	*Vibrio* spp. (24°C)	*Vibrio* spp. (35°C)
	CFU/100ml	CFU/100ml	CFU/100ml	CFU/100ml	CFU/ml	CFU/ml	CFU/100ml	CFU/100ml
1	12	10	0	24	1440	7850	1500	300
2		0	0	0	10	285	285	125
3	0	0	0	0		230	230	109
4	0	0	0	0	0	215	215	115
5	0	0	0	0		650	650	99
6	1	0	0	0	1400	940	340	105
7	1	1	0	0	10	1085	585	140
8	0	0	0	0	990	3040	430	112
9	0	0	0	2		320	320	81
10	0	0	0	0	10	590	590	138
11	0	0	0	0		270	270	84
12	0	0	0	0		470	470	147
13	0	0	0	0	70	385	385	111
14	0	0	0	0		180	180	69
15	0	0	0	1	120	250	250	115
16	0	0	0	0	1140	395	395	83
17	0	0	0	0	0	310	310	99
18	0	0	0	0	220	500	500	134

this bacterium reached a maximum value of 10 CFU/100 ml, like FC distribution. All the examined water samples were negative for *Salmonella* spp. Values of ENT were generally absent except for station 1, where a maximum value of 2.4×10^1 CFU/100 ml was recorded.

Regarding not marine bacteria, the highest concentrations (1.44×10^3 CFU/100 ml) were found at the station 1; negative values were recorded at two stations (4 and 17). Station 1 was also characterized by the highest concentration of marine bacteria (7.85×10^3 CFU/100 ml), while the lowest numbers were recorded at the station 14 (1.8×10^2 CFU/100 ml).

VP were everywhere detected at high densities, ranging around 10^2–10^3 CFU/100 ml, with the highest concentrations at the station 1 and lower values at the station 14. The quantitative

distribution of VPP, with values one order of magnitude lower than VP, showed a similar course (Figure 3).

The qualitative study of vibrios isolates (Table 4) showed that *V. alginolyticus* and *V. vulnificus* represented the predominant species within the *Vibrio* community at the Impact sites; at the Control ones, *V. alginolyticus*, *V. parahaemolyticus* and *V. vulnificus* were the most frequent isolates. No strains of *V. fluvialis* were isolated from the waters.

Statistical data elaboration

ANOVA calculated per each microbiological variable for shellfish and water samples showed that there were no statistically significant differences between Impact and Control sites, except for FC in shellfish sampled during the autumn (F= 7.52, P=0.033).

Virulence factors of *Vibrio* spp

The results of the screening for enzymatic activities as proxies of potential virulence of *Vibrio* spp. isolates are shown in Figures 4 and 5 for spring and autumn samplings, respectively.

In spring (Figure 4), most of the strains exhibited lipolytic and lecithinolytic activities, reaching at the control sites percentages of 70% of the total for both shellfish and water samples. Conversely, for proteolytic and haemolytic activities bacterial isolates were almost negative (water origin) or slightly positive (shellfish origin, with positive strains accounting for < 13% of the total).

In autumn (Figure 5) there was an increase in the percentage of strains showing enzymatic abilities. The most evident increases in shellfish bacterial isolates were observed in the percentage of caseinolytic bacteria, which reached the 68.9% of total at the control sites, and in water bacterial isolates in the percentage of lipolytic bacteria, which reached values close to 100% at both impact and control sites. Both in shellfish and water samples the percentages of lecithinolytic bacteria increased too.

Globally, higher expression of enzymatic activities as virulence proxies were observed in bacterial strains isolated from water compared to shellfish samples. The bacterial strains isolated from shellfish samples showed higher ability to produce potential virulence factors at the impact sites compared to the control ones in summer, while an opposite trend was observed in autumn.

Discussion

The sustainable use and a correct management of resources, also through the application of eco-friendly production methods [32], could represent the winning strategies for future innovation in aquaculture sector, that is now considered a pillar for Blue Growth [33]. Accurate marine planning of the space dedicated to sea farming, together with the application of good practices for animal welfare, are priority measures to ensure sustainable aquaculture growth.

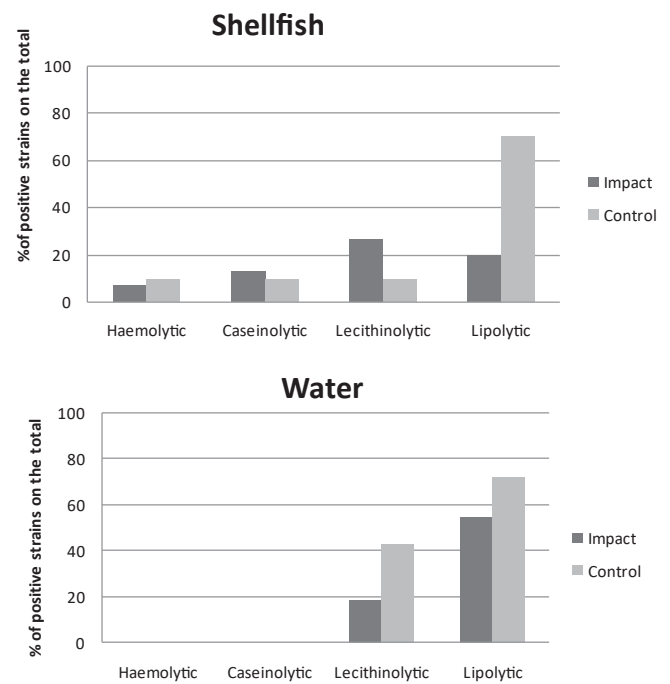To date, European aquaculture relies on the farming of a

Figure 4: Presumptive virulence factors in *Vibrio* spp. isolated from shellfish and water in spring. The percentage(on the total) of the strains showing enzymatic activities is shown.
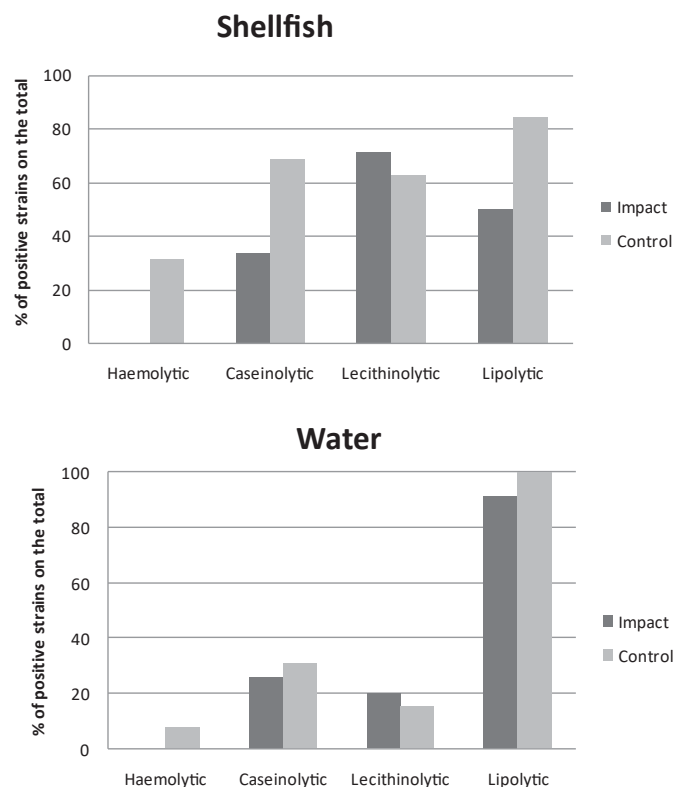

Figure 5: Presumptive virulence factors in *Vibrio* spp. isolated from shellfish and water in autumn. The percentage(on the total) of the strains showing enzymatic activities is shown.

few major species, such as sea bass, sea bream, oyster, mussel, salmon, and others minor productions, such as carp [34]. In Italy

shellfish farming represents an important sector of aquaculture, with clams and mussels representing the 94.2% and 70.8% of the European aquaculture productions, respectively. In Sicily, however, the production of mussels (about 700 tons, equivalent to 0.5% of national production) is still low. Polyculture has now been regarded as an environmentally sustainable practice to increase local mussel production [16]. For shellfish farmers, however, microbiological quality of the products is often a cause of concern, causing severe economic losses. In this context, this study has aimed at determining the potential implications of a polyculture pilot experiment regarding shellfish bacteriological safety. The bacteriological quality of shellfish and of the waters surrounding the site, was assessed during two seasonal samplings by covering a wide range of parameters that included not only faecal pollution indicators, released through faecal pellets of farmed specimens, but also autochthonous microorganisms such as halophilic vibrios. This has allowed to draw a more complete picture of the effects of IMTA on the quality of shellfish products and environment.

Faecal pollution indicators: spatial and temporal distribution

Faecal pollution indicators (FC, *E.coli*, ENT) do not represent by themselves pathogenic bacteria, but they provide information on the potential risk to consumers associated with the possible presence of enteric pathogens [35]. Furthermore, compared to FC - that may have different origins - *E. coli* is regarded as a more meaningful indicator of faecal contamination, being the main component of the intestinal flora of humans and warm-blooded animals [36]. *E. coli* concentrations recorded in the shellfish reared at the Impact stations were higher than those at the Control ones; nevertheless, their values fall within the threshold values (≤230 MPN/100 g of molluscs) prescribed by the Shellfish Hygiene Regulations for areas designed to aquaculture purposes (CE 853/2004 and 854/2004 [37,38]). Such low concentrations, together with the absence of *Salmonella* spp. in 25 g of shellfish, suggested the optimal hygienico-sanitary quality level of the production area, that could be classified as A zone. Therefore, IMTA did not have negative effects on the health quality of shellfish production. Also in the waters of the entire farming area, except for the station 1, faecal pollution indicators exhibited very low abundances, suggesting that terrigenous inputs of faecal origin were limited in the study area.

In the Castellammare Gulf waters, FC and ENT were more abundant during the spring period, however, compared to previous investigations in the same area [21,28,30], an improvement in the overall microbiological conditions was found, perhaps in relation to the start of efficient depuration systems. Moreover the absence of strains referable to *Salmonella* spp. confirmed the suitability of the area for shellfish farming, a productive activity which involves higher risks to human health compared to fish farming, due to the high filtering capacity of molluscs and their consequent concentration of pathogenic microorganisms.

The abundance of FC and ENT recorded in Castellammare fall in a magnitude order similar to the Mar Piccolo (Ionian Sea) by Cavallo and Stabili [27]; range: 2 - 94 MPN/ 100 ml), but significantly lower than those measured by Zaccone. et al. (log FC: 2.8 CFU/ml) [22]. Low concentrations of ENT (range: 0.55-10.70 CFU/g) were also found in the sediments of a mussel-farming area of the Gulf of Gaeta [26].

Distribution patterns of heterotrophic marine and not-marine bacteria

Bacteria have always been considered as the normal inhabitants of molluscan microflora; in oysters the presence of heterotrophic bacteria has implications not only for hatchery production but also for environmental and human health [39].

In the polyculture experiment, heterotrophic bacteria in shellfish collected at the Impact sites exhibited in both samplings almost doubled abundances than those at the Control sites, although these quantitative variations were not statistically significant. This finding could be explained by a stimulation of heterotrophic bacterial growth by feed wastes and excretions released by fish farming practices, that enriched waters with nutrients and organic substrates. At polyculture sites, mussel and clam were the species concentrating inside their body the highest bacterial abundance in spring, while in autumn *O. edulis* was more rich in heterotrophic bacteria.

At impact sites in spring allochthonous not-marine bacteria correlated with *E. coli* only weakly (r= 0.56, P<0.05), suggesting that microorganisms other than *E. coli* contributed to the bacterial flora present in these sites. In this season, inverse significant relationships between marine and not-marine bacteria were found, as expected (r= -0.72, P<0.01), while in autumn marine and allochtonous not-marine bacteria were directly correlated (r= 0.78, P<0.01), indicating that the reciprocal relationships between these microorganisms was modulated not only by fish farming wastes but also by other organic inputs, such as continental terrigenous matter.

Like in shellfish, in water heterotrophic bacterial abundances reached their highest values during the spring than the autumn period; bacterial growth was supported by the favorable temperature conditions (r= 0.54, P<0.05, between T and marine bacteria). In spring, marine and not-marine bacterial concentrations in water were unaffected by polyculture activities, as shown by the similar values recorded at both Impact and control sites. In autumn, higher bacterial abundances (marine and not-marine) measured at control than at impact sites were probably related to their different geographical location, being the control sites located in proximity of the coast, and therefore affected by organic inputs introduced into the marine environment.

Distribution patterns of *Vibrio* spp.

Members belonging to *Vibrio* genus are an ubiquitous component of the heterotrophic microflora autochthonous of marine and brackish environments. In shellfish collected from Castellammare, these microorganisms in spring accounted for an average percentage of the 43.2% and 11.5% of the heterotrophic bacteria at Impact and Control sites, respectively, while in autumn for the 37.0% and 29.3% of the heterotrophic

bacteria at Impact and Control sites, respectively. Percentages close to 70% and 90% of heterotrophic bacteria were reached in *C. gigas* and *M. galloprovincialis* in spring, while in autumn in the same species they approached the 50% of the heterotrophic bacteria. These findings confirmed the widespread occurrence of pathogenic *vibrio* species that has been documented in shellfish and water samples from several European Countries [24,27-28,40,41]. Bivalves have been regarded as an important ecological niche for vibrios, since these bacteria can persist inside molluscan tissues even after their depuration process [42]. Indeed, molluscs normally host a diversified microbial community that varies in relation with environmental conditions, in agreement with the filtering feeding capacity of these organisms [43]. In the shellfish samples examined in our study, significantly higher concentrations than those recorded in water were observed for all microbial parameters (for VP as well as for FC, *E. coli*, not-marine and marine bacteria); in summer, this concentration ability was more relevant for *E. coli*, not-marine and marine bacteria (from 5.55 to 31.8 times), while in autumn for FC and marine bacteria (up to 5 orders of magnitude and 3 times, respectively).

Vibrio species are very frequent in aquatic ecosystems [21,44-49]**,** and in coastal environments their concentrations can reach values higher than 10^3CFU/100 ml, supported by the availability of organic substrates. The abundance of vibrios found in Castellammare waters was in the same range of magnitude as reported in a previous study in the same area [30], namely of 10^3 CFU/100 ml and 10^2 CFU/100 ml for VP and VPP respectively. *Vibrio* spp. concentrations of 10^4 CFU/100 ml were detected in seawater samples collected at a hatchery plant of *Ostrea edulis* located off the Mediterranean Spanish coast [50]. Higher concentrations of VP (up to 1.64x10^4 CFU/g) were reported in the sediments of a mussel farm in the Gulf of Gaeta (Tyrrhenian Sea) [22].

Spatial and temporal variations characterized the distribution of halophilic vibrios in shellfish produced in the Castellammare Gulf; in spring vibrios were about 6 times more abundant at impact than at control stations, while in autumn their spatial variations became more narrow (about 2 times between impact and control stations). In both samplings, however, ANOVA suggested that their quantitative variations in shellfish samples were not related to polyculture activities. Seasonal fluctuations in the abundance of halophilic vibrios, with higher concentrations in summer compared to autumn, reflected the trends observed in the distribution of total heterotrophic bacteria and were consistent with the typical ecology of these bacteria, whose growth is supported by warm temperature and high nutrient availability [51]. The concentrations measured at the most coastal stations reflected the preferential distribution of vibrios in coastal habitats rich in organic matter [28]. Moreover, in autumn at Impact sites *Vibrio* spp. abundances correlated significantly with FC (r= 0.92, P<0.01), indicating that both autochtonous and allochtonous bacteria were stimulated by organic fish wastes. On the other hand, the relatively low concentration of halophilic vibrios found in waters confirmed the good health conditions of Castellammare Gulf area, already reported by Caruso, et al.

[28,30]. The good bacteriological quality of farming waters is a basic requisite for productive activities, that is required also in order to limit the massive use of antibiotics and prevent possible related antibiotic resistance phenomena in aquaculture [52].

Qualitative study of *Vibrio* community and virulence factors

Several studies have addressed the study of *Vibrio* biodiversity in shellfish, given their relevance as aetiological agents of foodborne diseases [53]. In fact, other than the well-known pathogenic species *V. cholerae*, some vibrios species such as *V. parahaemolyticus*, *V. vulnificus*, are primary or opportunistic pathogens responsible for severe gastroenteritis usually associated with the consumption of raw or under-cooked seafood that, through their filtering activity, concentrate these microorganisms inside their body [21,23-25,27,54]. Also, vibrios species such as *V. vulnificus* are aetiological agents of wound infections and lethal septicemia [53,55] and other species (i.e. *V. alginolyticus*, *V. anguillarum*) are opportunistic pathogens responsible for animal diseases [56].

Vibrio species composition in Castellammare shellfish samples revealed that *V. alginolyticus*, *V. vulnificus* and *V. parahaemolyticus* were the most frequently found species. Even if a clear association was not found between these species and the site of isolation, *V. vulnificus* predominated at the Impact sites in spring season; conversely, in autumn the *Vibrio* community showed a more diversified composition, with the detection of other species such as *V. mimicus* and *V. fluvialis*.

V. alginolyticus is a common member of microflora of temperate and tropical marine environments and has been isolated from guts of different marine organisms and seafood [57]; however, it has been found to act as an opportunistic pathogen for aquatic organisms being the aetiological agent of infectious diseases in marine fish cultured in the Mediterranean Sea and of high mortality episodes in bivalve larvae [56]. *V. alginolyticus* was the dominant *Vibrio* species in several Mediterranean waters [44,45]; it is favored by haline conditions, but it can tolerate up to salinity levels of 11 [58,59].

In the Castellammare waters *V. alginolyticus* and *V. vulnificus* predominated within the *Vibrio* community, especially in autumn. *V. vulnificus*, probably favored by the low temperature compared to spring, includes three biotypes, all able to cause human infection; biotype 1 is of greatest importance to oyster producers and consumers, biotype 2 infects eels, while biotype 3 has been isolated in Israel in association with tilapia fish [60]. Infection by *V. vulnificus* produces pathological changes of bacterial hemorrhagic septicaemia similar to *V. anguillarum* in Japanese eels in Japan, cobia, seabass, rainbow trout and European eels in England, Spain, Denmark and the Netherlands [61].

V. vulnificus is often recovered from Mediterranean seawaters [62]; temperatures outside the range of 13 to 22°C and salinities greater than 25 ppt reduce the survival of this bacterium in seawater [63,64]. In Mexico, it was isolated from seawater and sediments of the Gulf of Mexico estuary [65] as

well as in 31% of oysters and water samples collected from Pueblo Viejo Lagoon, Veracruz [66]; the detection of vvhA gene and some putative virulence factors suggested that this microorganism posed a health risk to both oyster consumers and fishermen.

V. parahaemolyticus is a natural inhabitant of coastal marine and estuarine environments; there is no correlation between its distribution and faecal pollution [67-69]. This species is involved in the outbreak of acute gastroenteritis associated with the consumption of raw contaminated seafood [53]. *V. parahaemolyticus* is also responsible for serious infections in fish species [61]. High incidence of *V. parahaemolyticus* was documented in oyster specimens from various Countries, including Brazil [70], Mexico [71] and USA [72]. It was also commonly isolated from shellfish (i.e. oyster and clam) collected from several Asian [73-77] and European Countries [41]. In shellfish collected from coastal areas of Southern Italy strains of *V. parahaemolyticus* accounted for percentages of 5.2-6.2% of the total *Vibrio* community [27, 78].

Another emerging human foodborne pathogen, responsible for sporadic extraintestinal diseases [79], found in the shellfish samples from Castellammare was *V. fluvialis.* This microorganism was first detected in oysters from Pacific Northwest during warmer seasons [80]; Chan, et al. [73] also reported *V. fluvialis* among the most important pathogenic vibrios in seafood sold in the Hong Hong markets.

V. fluvialis was isolated from mussels from Brazil [58] and from bivalves from Costa Rica [81]; in Italy, Ripabelli, et al. [45] found that 11%–27% of the shellfish and shrimps contained *V. fluvialis* without any association between this pathogen and conventional fecal pollution indicators. Commonly found in coastal marine, estuarine and brackish environments [82], *V. fluvialis* was isolated in high percentages (29% of the total flora) in the Toulon harbor (France) [83], as well as in suburban effluents of South Africa (41.4% of the total [84]), where a positive relationship with seawater temperature, salinity and dissolved oxygen was observed.

The *Vibrio* community biodiversity found in shellfish samples collected in Castellammare polyculture area reflected that of the waters. Indeed, in oysters, *V. parahaemolyticus* has been found to concentrate by up to 10⁴ times compared to the surrounding water [60], even if the mechanism of association (i.e. specific gut microbionts, resulting from selective enrichment, or aspecific uptake of transient microbiota from the aquatic environment) remains unclear [56]. Also in water the distribution of this species, together with *V. alginolyticus*, is closely related to temperature and salinity [71,85]. *V. parahaemolyticus* prefers water at temperature higher than 20°C [86], while below 16°C *V. parahaemolyticus* disappears from waters, surviving in the sediments [87]. *V. parahaemolyticus* and *V. alginolyticus* growth is also favoured by organic matter, therefore these species usually prefer coastal and brackish eutrophic temperate environments [51].

Through a meta-analysis of environmental variables affecting vibrios distribution, temperature and salinity were found to be the main variables explaining the variance of total *Vibrio* abundance in the water, while other variables were only marginally predictive of vibrios proliferation in the environment [56].

Seasonal variability in the vibrios composition was found in Cape Peloro lakes by Zaccone, et al. [49], who reported high percentages (approaching 70% of the total) of *V. vulnificus* in winter, at temperatures of 16.11 °C, while *V. parahaemolyticus* was detected in autumn, winter and early spring seasons with percentages of 10-14% of the total.

The predominance of the species *V. alginolyticus* in Castellammare waters confirmed the ubiquitous distribution of this facultative pathogen in coastal marine waters. Similarly to this result, *V. alginolyticus* was the predominant component of the total culturable vibrios in the Mar Piccolo of Taranto (Ionian Sea, Italy) [27].

The study of the expression of virulence factors as determinants of virulence has been suggested as a support tool for a better surveillance of the environmental spread of pathogens such as vibrios. Indeed, previous studies showed the existence of a relationship between the expression of different enzymatic activities and the virulence mechanisms of bacterial organisms [31]. Particularly, virulence factors of *V. parahaemolyticus* are a thermostable direct hemolysin (TDH) and aTDH related hemolysin (TRH) [88], while proteases and hemolysins are considered to be relevant for the pathogenesis of *V. vulnificus* [53] and *V. fluvialis* [79]. In the vibrios isolated from Castellammare Gulf shellfish and water, proteases, hemolysins, lipases and lecithinases were widespread. In the bacteria isolated from shellfish, higher percentages of positive responses were found in spring at impact sites, while in autumn the polyculture practice was associated to a reduction in the expression of enzymes related to proteolysis and haemolysis. The expression of enzymatic activities –as a proxy of virulence– in spring was higher in the bacterial strains isolated from water than from shellfish. In Mexico, strains of *V. vulnificus* isolated from oysters were found to be 100% proteolytic, 97.8% were lecithinase-positive and 79.8% lipase positive [66].

Conclusions

To our knowledge, only a few papers have dealt with the microbiological quality of shellfish produced in polyculture experiments and knowledge of bacterial communities growing in these conditions is still scarce [89]. Therefore, our study aimed at contributing to this topic in marine environments.

The analysis of whole quantitative data obtained in Castellammare Gulf confirmed that, due to their filterfeeding behaviour, shellfish were able to concentrate bacteria inside their body, compared to the surrounding waters. The simultaneous increase of faecal pollution indicators and marine heterotrophic bacteria close to polyculture activities suggested that the inputs of organic matter (such as from food residuals and faecal pellets) stimulated the bacterial growth. In spite of this, no detrimental effects due to the polyculture experiment were observed in the bacteriological quality of both

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shellfish and waters that fell within the standards required by current Directives for shellfish farming sites. Within the *Vibrio* community, *V. alginolyticus* and *V. vulnificus* displayed a mostly ubiquitous distribution in both the examined biological matrices (water and shellfish), similarly to other marine ecosystems. Future specific environmental research could be proposed to improve current understanding of shellfish–*Vibrio* ecological interactions.

In the light of these considerations, IMTA systems could represent an attractive, ecofriendly, practice to diversify local production, promoting the competitiveness of aquaculture and reducing the weight of importation from other Countries.

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