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Temporal Evolution of the Microbiological Conditions of a Sicilian Area Designed for Aquaculture (Castellammare Gulf, Southern Tyrrhenian Sea)

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Abstract

Microbiological controls of coastal seawaters are a common practice to verify their safety for human recreational and productive uses. The aim of the present study is to describe the microbiological conditions of the Castellammare Gulf (Trapani, Sicily), an area traditionally used for aquaculture purposes. Particularly, the results obtained from three surveys (1993-1995, 2000-2001 and 2007) are compared in order to depict how the hygienic-sanitary conditions of this marine ecosystem have evolved over time.

Attention has been given to the abundance and distribution of the bacterial indicators of faecal pollution (faecal coliforms, enterococci and *Salmonella* spp.) as well as halophilic vibrios, using the culture methods conventionally indicated by current legislation in force for the sanitary control of waters designed for aquaculture productions. The obtained results shows that the sanitary conditions of seawater samples met the criteria recommended by current legislations for shellfish farming approved areas. The low concentrations of faecal pollution indicators and of halophilic vibrios confirm the general suitability of the Gulf of Castellammare for seafood production. Nevertheless, the detection of low percentages of potentially pathogenic species of halophilic vibrios stresses the importance of extending microbiological controls also to these emerging pathogens.

Keywords: Microbiological controls; Aquaculture; Faecal coliforms; Enterococci; Halophilic vibrios; Mediterranean area

Abbreviations: FC: Faecal Coliforms; ENT: Enterococci; VP: Presumptive Vibrios (growing at 24°C); VPP: Potentially Pathogenic Vibrios (growing at 35°C)

Introduction

Assessment of the microbiological characteristics of coastal marine waters allows evaluating their safety, especially in areas where aquaculture is practiced. Aquaculture and any other productive activity based on the exploitation of sea resources represent a challenge for future generations. Generally, the good quality of final aquaculture products strongly depends on the conformity of the rearing environment to qualitative microbiological standards [1-3]. In the light of this consideration, the importance to carry out sanitary controls throughout the different steps of the productive cycle, starting from the environment to final products [4], is underlined. The main features that stress the need to intensify microbiological controls where seafood are produced can be summarised as follows: - both waterborne and food borne diseases represent issues of global concern; - risks for human health must be prevented; - there is a strict relationship linking the anthropic pressure over the coasts and the occurrence of high concentrations of microbiological pathogens; -the setup of proper remediation measures to reduce or eliminate any pollution sources over the marine ecosystem can be reached after the start and/or implementation of environmental monitoring programmers, specifically designed to achieve the required sanitary targets.

In Europe, Italy is the third seafood producing Country, considering that a number of more than 600 farming areas are classified as production sites (according to the data available from SINVSA. the Veterinary Information System for food security, provided by Italian Ministry for Health), therefore Italian fish and shellfish farming activities hold a great social and economical importance/significance in the National and European frame/context.

In the field of veterinary inspection, monitoring of microbial pathogens in shellfish waters is under the Shellfish Hygiene Regulations (Regulation 853/2004/EC and 854/2004/EC, Regulations 2073/2005/EC [5-9] which establish several microbiological controls to be performed from rearing to harvesting of seafood products, in order to verify the absence of risks for consumers' health. According to the Regulation 854/04 EC, the Member States have to establish a surveillance system of the farming areas and the production facilities, in order to allow continuous monitoring of the health quality of water and safety of seafood products. Bivalve molluscs, as filter feeders, can concentrate pathogens which are released in the marine environment from the anthropic activities, and become a frequent cause of foodborne diseases. Therefore, the bacteriological monitoring of fish products has also suggested as a critical feature to be considered within the recent Marine Strategy Framework Directive (2008/56/CE) [10] under the Descriptor 9- Contaminants in shellfish, as a tool to warrant that these products are safe for human consumption.

According to the Laws currently in force in Italy, the monitoring of the microbiological quality of marine ecosystems is generally made through the evaluation of bacterial indicators, such as faecal

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contamination indicators (faecal coliforms, enterococci and *Salmonella* spp.). Particularly, the Shellfish Hygiene Regulations look at the concentrations of *Escherichia coli* and *Salmonella* spp. in shellfish products. The enumeration of faecal coliforms is the first criterion to be satisfied to meet both environmental protection and human health. In addition, bacterial pathogens which are recognized as responsible for the outbreaks of intestinal diseases such as Vibrios, some of which are natural inhabitants of marine environments (*Vibrio cholerae, V. parahaemoluticus*) [11,12] require further attention, since these bacteria may constitute a potential health hazard, even if not under the above cited legislation [13-17].

In Sicily, the Gulf of Castellammare, located along the north-western Tyrrhenian coast, has been traditionally considered as a fishing area, which is intensively practiced [18-21]. It is a wide bay about 70 Km long, comprised between Capo S. Vito and Terrasini, characterized by the presence of extensive mussel beds and by the outflows of alcohol distilleries which enrich waters with organic substrates; also stream inflows cause a persistent turbidity of waters, which have sporadically undergone to eutrophication. In spite of the great potentiality of the Gulf for aquaculture and fish farming, information on the microbiological conditions of the Gulf is still fragmentary; to our knowledge, only a few scientific reports are available [4-22].

The aim of the present study was to describe the temporal evolution of the microbiological conditions in the Castellammare Gulf through a comparison of the results obtained from a total of nine surveys spanning from 1993 to 2007, in order to assess the evolutionary trends of the hygienic-sanitary conditions of this marine ecosystem.

Materials and Methods

Since 1993 a series of microbiological surveys were undertaken in the Gulf of Castellammare to explore the possible utilization of this area as an aquaculture site. Surface water samples were collected from 9 stations at a bathymetry of 20 meters (Figure 1); their geographical coordinates are reported in Table 1.

The first survey (1993-1995) covered four seasonal cruises (namely November 1993, June 1994, March 1995 and August 1995, corresponding to autumn, spring, winter and summer, respectively, for a total of 168 surface water samples. These underwent to analyses to determine the quantitative distribution of fecal coliforms, enterococci, *Salmonella* spp., and halophilic vibrios. A qualitative study of Vibrio species, to search for potentially pathogenic species (such as Vibrio *parahaemolyticus* and *V. cholerae*) was also carried out [22].

The second survey in the Castellammare area was performed in 2000-2001. Samplings were performed during March 2000, May and July 2001, for a total number of 48 samples [4].

The third survey (2007) was carried out in Castellammare during April and October 2007, corresponding to spring and autumn periods.

Culture methods

For the determination of faecal coliforms (FC) and enterococci (ENT), suitable volumes of water samples (100 ml) were filtered on 0.45 micron- pore sized Millipore filters ((Millipore Corp., Bedford, MA, USA), and incubated on m-FC (Bekton Dickinson, New Yersey, USA) and Enterococcus agar (Bekton Dickinson) plates, respectively at 35°C for 24 and 48 hours, respectively [23].

The abundance of halophilic vibrios was estimated by membrane filtration of 10 and 100 ml of seawater, followed by incubation of the filters on plates of Thiosulfate Citrate Bile Sucrose -TCBS agar (Oxoid, Milan, Italy) added with 2% sodium chloride. Counts were performed after 48 hours at 24°C for presumptive vibrios (VP), and after 24 hours at 35°C for potentially pathogenic species (*Vibrio parahaemolyticus* and *V. cholerae*).

Water samples taken during the second and the third surveys were also examined for the possible presence of *Salmonella* spp. The qualitative search of *Salmonella* spp. was carried out by pre-enrichment of a filtered water sample (200 ml) in buffered peptone water at 35°C for 18-24 h, followed by enrichment in Rappaport Vassiliadis Broth (Oxoid) and Tetrationate Broth (Oxoid) added with an iodine solution (ICN Pharmaceuticals, New York, USA) incubated for 24-48 hours at 42°C and 35°C respectively. Isolation was performed on plates of Salmonella-Shigella (SS, Oxoid) and Hektoen enteric agar (Oxoid) selective culture media. All the colonies presumed to be *Salmonella* spp. (lactose not fermenting and hydrogen sulphide producing) were biochemically characterized through inoculation in Triple Sugar Iron agar (Difco) tubes. Typical strains were further identified through API 20E strips (Biomerieux, Marcy l'Etoile, France) incubated at 35°C for 24 hours.

During 1993-1995 and 2007 survey, halophilic vibrios were also examined for their species composition. Colonies growing on TCBS

Figure 1: Map showing the location of the sampling stations.

Stations	Latitude N	Longitude E
1	38° 02' 31"	12° 55' 34"
2	38° 02' 42"	12° 56' 52"
3	38° 03' 03"	12° 57' 59"
4	38° 03' 33"	12° 59' 09"
5	38° 04' 06"	13° 00' 15"
6	38° 04' 36"	13° 01' 24"
7	38° 05' 09"	13° 02' 34"
8	38° 05' 57"	13° 03' 30"
9	38° 07' 03"	13° 03' 42"

Table 1: Geographical coordinates of the sampled stations.

agar were isolated at random and identified according to phenotypical tests: morphology, Gram staining, glucose fermentation on Triple Sugar Iron Agar (Difco), oxidase activity, sensitivity to O129 (150 micrograms) vibriostatic agent, amino acids assimilation and sugars fermentation on API 20E strips (Biomerieux). Strains biochemically similar to *V. cholerae* were tested for agglutination with O1 polyvalent antiserum (Difco) and for the production of the choleric toxin by a latex VET-RLA (Oxoid) reagent. The strains identified as *V. parahaemolyticus* were tested for their hemolytic capacity on Wagatsuma agar (Kanagawa phenomenon).

Statistical analysis

The bacterial counts obtained during the different surveys are reported as the range of variation (minimum-maximum) and the geometric mean value calculated during each period. To get more indication of the variability among samples collected within each period, a coefficient of variation (CV) was calculated using the formula CV= (Standard deviation/Mean)*100.

To assess the statistical significance of differences among quantitative data, the one-way analysis of variance (ANOVA) was carried out using the "sampling time" as the main factor. Prior to analysis, all the values were normalized by logarithmic transformation.

Pearson correlations coefficients (r) were calculated for each bacterial parameter *versus* the main environmental factors such as temperature, salinity, Total Suspended Matter, Particulate Organic Carbon and Chlorophyll-concentrations.

Results

The concentrations of FC, ENT, VP and VPP recorded in the Gulf of Castellammare are reported in Table 2A, where the range of variation, the geometric mean and the coefficient of variation obtained at each sampling are indicated. In Table 2B the average, the minimum and the maximum value of the environmental factors measured in each sampling are reported. The spatial distribution of microbial indicators of faecal contamination is shown in (Figures 2A and 2B), while that of halophilic vibrios in (Figures 3A and 3B).

1993-1995 surveys

During the first survey (1993-1995), FC concentrations were always in a range of 10E+00-10E+02 CFU/100 ml (Table 2A), with sporadic peaks and the highest variability in bacterial load in autumn and winter, as suggested by the high CV values (exceeding 200). More in detail, faecal coliforms were detected in high concentrations in autumn at Station 4 (8.0E+02 CFU/100 ml); high numbers of FC were also recorded in winter at station 9, while negative values were observed in spring and summer (Figure 2A).

Throughout the survey, enterococci were mostly below 3.3E+01 CFU/100 ml (Table 2A and Figure 2B); in winter their abundance was highly variable (CV=235, Table 2A).

Mean temperature values ranged from 14.31 (winter) to 20.07°C (autumn) while salinity from 37.68 (winter) to 37.80 (spring) (Table 2B). During autumn, FC were significantly correlated to temperature (r=+0.513, P<0.05) and with nutrients (r=+0.336, P<0.05, with NH4+).

	No. of samples	Range (min-max)	Geometric mean	CV	Range (min-max)	Geometric mean	CV
		CFU/100 ml			CFU/100 ml		
		FC			ENT		
Autumn 1993	9	0.78-2.90	1.43	241	0.00-1.20	0.58	89
Spring 1994	9	0.00-0.30	0	0	0.00-0.30	0.03	28
Summer 1995	9	0.00-0.30	0	0	0.00-0.30	0	0
Winter 1995	9	0.00-2.30	0.7	202	0.00-1.52	0.22	235
Spring 2000	6	0.00-0.90	0.15	132	0.00-0.90	0.57	59
Spring 2001	6	0.00-1.98	1.08	112	0.00-2.28	1.49	78
Summer 2001	6	0.00-0.30	0	0	0.00-0.70	0.17	87
Spring 2007	9	0.48-3.18	1.18	281	0.48-2.84	0.97	370
Autumn 2007	9	0.00-1.08	0.13	165	0.00-1.38	0.19	232
Overall	72	0.00-3.18	0.5	460	0.00-2.84	0.44	401
	No. of samples	Range (min-max)	Geometric mean	CV	Range (min-max)	Geometric mean	CV
		CFU/100 ml			CFU/100 ml		
		VP			VPP		
Autumn 1993	9	1.71-3.20	2.36	124	1.50-2.81	2.07	106
Spring 1994	9	0.00-2.20	1.71	61	0.00-1.60	0.86	96
Summer 1995	9	3.20-3.51	3.44	25	2.50-2.51	2.5	0
Winter 1995	9	1.81-2.60	2.17	65	0.00-1.81	1.37	61
Spring 2000	6	2.45-3.46	3.62	70	2.11-3.15	2.5	107
Spring 2001	6	2.88-3.45	3.23	39	0.30-2.56	1.68	100
Summer 2001	6	2.66-5.48	4.11	109	2.04-3.70	2.5	197
Spring 2007	9	2.28-2.95	2.45	69	1.43-2.08	1.69	51
Autumn 2007	9	2.32-3.18	2.62	79	1.91-2.48	2.09	49
Overall	72	0.00-5.48	4.71	451	0.00-3.70	1.88	265

Table 2A: Range of variation (minimum-maximum), geometric mean and coefficient of variation (CV, calculated by the formula 100*standard deviation/mean) obtained during each sampling for each microbial parameter: Faecal Coliforms (FC), *Enterococcus* spp. (ENT), Vibrios growing at 24 and 35°C (VP and VPP respectively). All data are reported as Logarithmic values.

Temperature (°C)				**Salinity**			
	Min	Max	Mean		Min	Max	Mean
Nov-93	14.19	22.09	20.07	Nov-93	37.37	37.96	37.77
Jun-94	13.93	21.49	17.19	Jun-94	37.56	38.46	37.8
Mar-95	14.25	14.92	14.31	Mar-95	37.58	37.98	37.68
Aug-95	13.84	28.36	18.7	Aug-95	37.49	38.47	37.76
Mar-00	17.8	17.9	17.8	Mar-00	37.3	37.4	37.72
May-01	23.3	24.6	24.35	May-01	37.3	37.38	37.35
Jul-01	25.18	25.48	26.16	Jul-01	36.2	36.53	36.24
Apr-07	12.8	18.38	14	Apr-07	35.88	37.77	37.1
Oct-07	26.5	27.84	27.7	Oct-07	37.81	38.12	38
Particulate Organic Carbon (µgC/l)				Total Suspended Matter (mg/l)			
	Min	Max	Mean		Min	Max	Mean
Nov-93	36.4	96.2	65.94	Nov-93	9.45	11.61	10.11
Jun-94	96.4	171	111.6	Jun-94	6.38	11.82	9.98
Mar-95	42.3	67.3	52.56	Mar-95	5.95	9.55	8.3
Aug-95	40.2	80.6	63.12	Aug-95	7.38	9.65	8.24
Mar-00	181.5	293.2	244.8	Mar-00	1.67	3.81	2.85
May-01	62.6	147.1	104.5	May-01	2.24	5.37	4.11
Jul-01	74.2	104.9	87.6	Jul-01	1.87	36.05	18.24
Apr-07	101.16	853.1	323.86	Apr-07	0.48	83.37	16.28
Oct-07	31.05	95.32	67.41	Oct-07	9.44	10.72	11.61
Chlorophyll-a (µgC/l)				Ammonium- NH3 (µM)			
	Min	Max	Mean		Min	Max	Mean
Nov-93	0.7	3.1	1.67	Nov-93	0.7	1.44	1.02
Jun-94	0.6	2.1	1.21	Jun-94	2.78	3.45	3.02
Mar-95	0.9	1.3	1.18	Mar-95	0.55	1.67	0.79
Aug-95	1.5	5.5	3.17	Aug-95	0.35	1.43	0.69
Mar-00	0.05	1.2	0.67	Mar-00	0.24	1.01	0.78
May-01	0.08	3.95	2.25	May-01	3.41	3.6	3.52
Jul-01	0.02	0.17	0.09	Jul-01	0.39	1.48	0.78
Apr-07	0.02	0.09	0.05	Apr-07	0.41	1.72	0.84
Oct-07	0.08	1.3	0.66	Oct-07	0.6	1.8	0.95

Table 2B: Minimum, maximum and mean values of the main environmental parameters recorded in the Gulf of Castellammare during each sampling.

Halophilic vibrios (VP) were always found in high densities, mostly above 10E+02 CFU/100 ml; they were more abundant during the warm months (summer-autumn), whereas in winter-spring their concentrations were lower. The quantitative distribution of VPP reflected this trend, but these organisms were found at concentrations one order of magnitude lower than VP. The spatial distribution of Vibrios, reported in (Figures 3A and 3B), showed the widespread occurrence of high bacterial concentrations (3.22E+03 CFU/100 ml) during summer 1995 at stations 4 to 8, and a minimum value of 1.00E+00 CFU/100 ml found at station 4 during spring 1994.

During summer, VP abundance was positively correlated with Particulate Organic Carbon (POC) and Total Suspended Matter (r=+0.65 and +0.59, P<0.05, respectively), and negatively with temperature (r= -0.576, P<0.05), suggesting that the organic matter content rather than temperature affected the presence of these microorganisms. Particularly, POC distribution showed a seasonal trend of organic inputs, with a peak in spring, and lower values in summer.

Within the *Vibrio* population, *V. fluvialis* and *V. anguillarum* accounted for the highest percentages of the total of the strains isolated at 24°C (37 and 13%, respectively), while at 35°C *V. alginolyticus* prevailed (55% of the total), followed by *V. cholerae* non O1 and *V. vulnificus* (16 and 10% of the total, respectively) (Figure 4). Strains identified as *V. parahaemolyticus* were not positive to the Kanagawa test.

2000-2001 surveys

During the 2000-2001 survey, FC were found at very low densities (Table 2A and Figure 2A). CV values close to 100 were calculated, suggesting a lower variability in FC distribution during this survey (Table 2A). ENT showed concentrations always lower than 1.9E+02 CFU/100 ml, and bacterial dispersion was lower compared to the previous year's CV=59-87, (Table 2A). No strains of *Salmonella* spp. were isolated during this period.

The concentrations of VP were comprised between a minimum of 2.8E+02 CFU/100 ml found at station 6 during spring 2000 and a maximum of 3.0E+05 CFU/100 ml recorded during summer 2001 at stations 1, 4 and 5 (Table 2A and Figure 3A). VPP concentrations ranged from 2.0E+00 during spring 2001 at station 6 to 5.0E+03 CFU/100 ml during summer 2001 at station 1 (Table 2A and Figure 3B). As shown by CV values (Table 2A), high variability was observed in VP and VPP counts in summer 2001.

During 2000-2001, temperature values ranged from 17.8 (spring 2000) to 26.16°C (summer 2001) while salinity from 36.24 (summer 2001) to 37.72 (spring 2000) (Table 2B). FC and ENT were positively related with salinity (r= +0.46 and 0.62, P<0.05, respectively) and

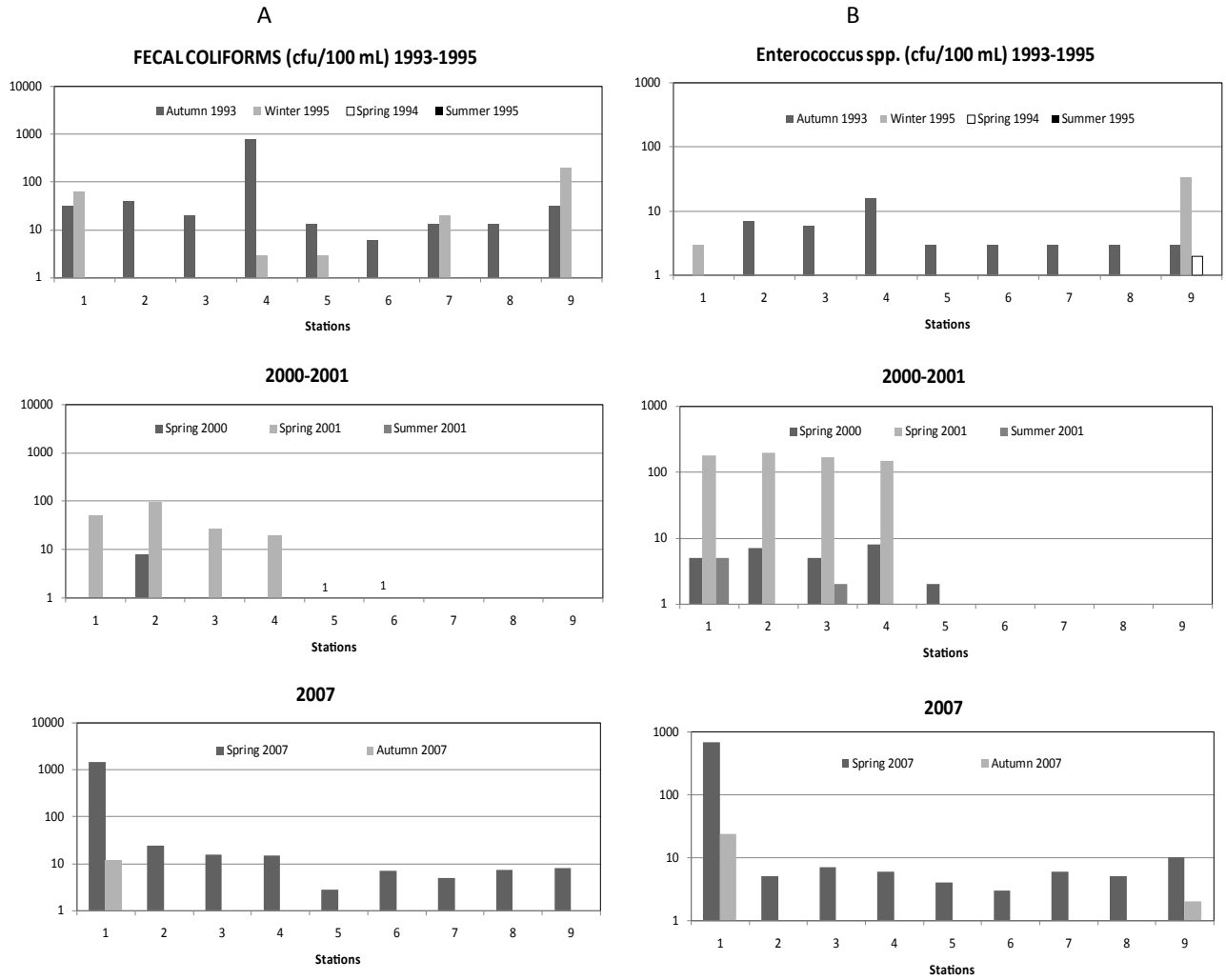

Figure 2: Temporal variations of the abundances of faecal coliforms (A, on the left) and *Enterococcus* spp. (B, on the right) in the Gulf of Castellammare from 1993 to 2007.

Chlorophyll-a (+0.70 and +0.68, respectively), but negatively with temperature (r= -0.55 and -0.77, P<0.05, respectively).

2007 surveys

During 2007, the abundances of faecal coliforms were consistent with those recorded in the previous surveys (Figure 2A). Low numbers were recorded, except for station 1 during spring when bacterial concentrations were about two orders of magnitude higher than at the other stations (1.5E+03 CFU/100 ml, Table 2A).

Very low values of ENT, less than 1.0E+01 CFU/100 ml, were recorded, except for station 1 where a peak value of 7.0E+02 CFU/100 ml was reached (Figure 2B). During this period, no strains ascribable to *Salmonella* spp. were isolated from the Castellammare waters. The concentrations of VP ranged from 1.9E+02 CFU/100 ml (station 9, spring) and 1.5E+03 (station 1, autumn) (Table 2A and Figure 3A). VPP values were in the order of 10E+01- 10E+02 CFU/100 ml, with higher counts at station 1, and in autumn than in spring (Figure 3B). Low CV suggested that data dispersion was low.

During 2007, temperature values varied between 14.0 and 27.7°C, recorded in spring and autumn, respectively (Table 2B); temperature was correlated positively with FC, ENT, VP and VPP (r= +0.55, +0.54, +0.58 and +0.64, P<0.05, respectively). Salinity values ranged from 37.1 to 38, measured in spring and autumn, respectively (Table 2B).

Concerning the species composition of the *Vibrio* community, most of the strains isolated at 24°C were identified as *Vibrio* spp. (88% of the total), while at 35°C *V. alginolyticus* and *V. vulnificus* accounted for 66 and 24% of the total, respectively (Figure 4).

Statistical analysis

Table 3 shows the results of statistical analysis performed by ANOVA test. No significant differences were observed when FC, ENT and VPP values were compared over time, while highly significant differences (F=7.18, P<10E-06) occurred in VP abundances, which were higher during the 2000-2001 surveys.

Discussion

This paper is a contribution to the characterization of the hygienic-sanitary conditions of the Gulf of Castellammare, a body of water about which information is still very limited. The microbiological controls performed in the Castellammare Gulf allowed to get a picture of this

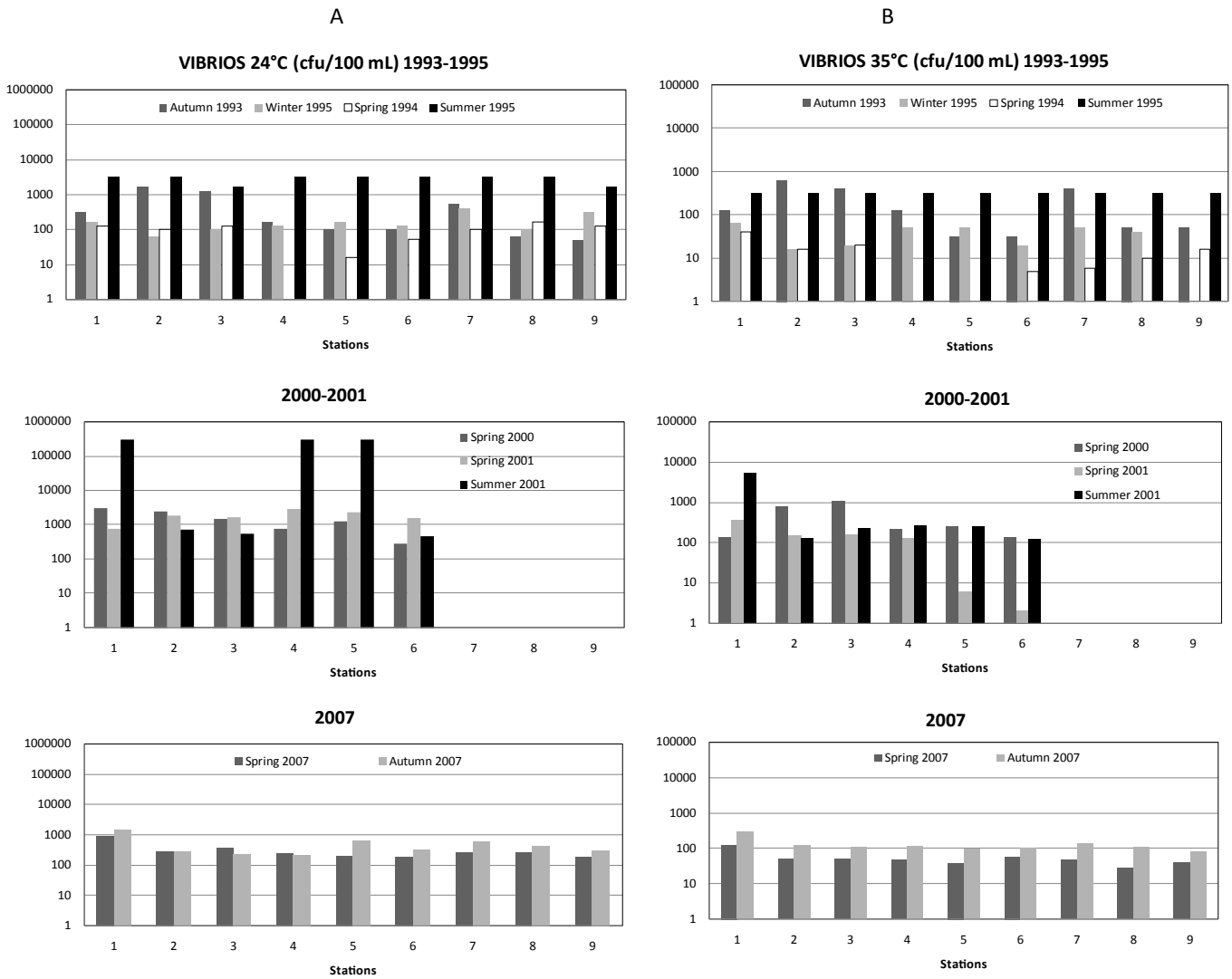

Figure 3: Temporal variations of the abundances of halophilic vibrios growing at 24°C-VP (A, on the left) and at 35°C-VPP (B, on the right) in the Gulf of Castellammare from 1993 to 2007.

area in terms to its possible utilization in aquaculture and, at the same time, helped to prevent health risks from microbial pollution. Within the Marine Strategy Framework Directive [10], the present data can also be used to estimate Good Environmental Status for microbiological contaminants in an area of the Western Mediterranean sub-region.

Bacterial indicators of fecal contamination (fecal coliforms, *Escherichia coli*) do not themselves represent pathogenic bacteria, but provide information on the potential risk to consumers associated with the possible presence of enteric pathogens. Christensen and Larsen [24] first pointed out the need for microbiological controls in aquaculture. In 1991, the Commission of the European Community established the water quality criteria for natural and farming environments from where shellfish are harvested (limit value FC: <300 CFU/100 ml of waters; Directive 91/492/CEE [25]). Low FC and ENT counts obtained from 1993 to 2007 have shown that the Gulf of Castellammare was mostly unaffected by faecal wastes, with the exception of the station 1 where both bacterial parameters were over two orders of magnitude per 100 ml. Bacterial concentrations recorded in the Gulf of Castellammare may be used for background levels for intra-specific comparison with other Italian aquaculture sites; the abundance of FC and ENT recorded in Castellammare fall in a quantitative range similar to that reported in the Mar Piccolo (Ionian Sea), ranging from 2 to 94 MPN/100 ml (Cavallo and Stabili [15]). In this same last site, Zaccone et al. [26] reported the occurrence of FC values exceeding the legal limits for approved mussel farming areas (log FC: 2.8 CFU/ml). Enterococci ranged from 0.55 to 10.70 CFU/g of sediment in a mussel-farm area in the Gulf of Gaeta [23].

Moreover, the distribution of these bacterial indicators of pollution followed generally a seasonal trend, increasing during the autumn season (except for 2007) in relation to the presence of riverine and continental runoff which may introduce into seawater some bacterial pathogens.

The maps shown in (Figures 5 and 6) summarize the health conditions of the Castellammare Gulf in terms of bacterial indicators of faecal pollution. In the study area, most of the samples were in compliance with the mandatory limit value of FC set up by current Shellfish Hygiene Regulations for areas designed to aquaculture

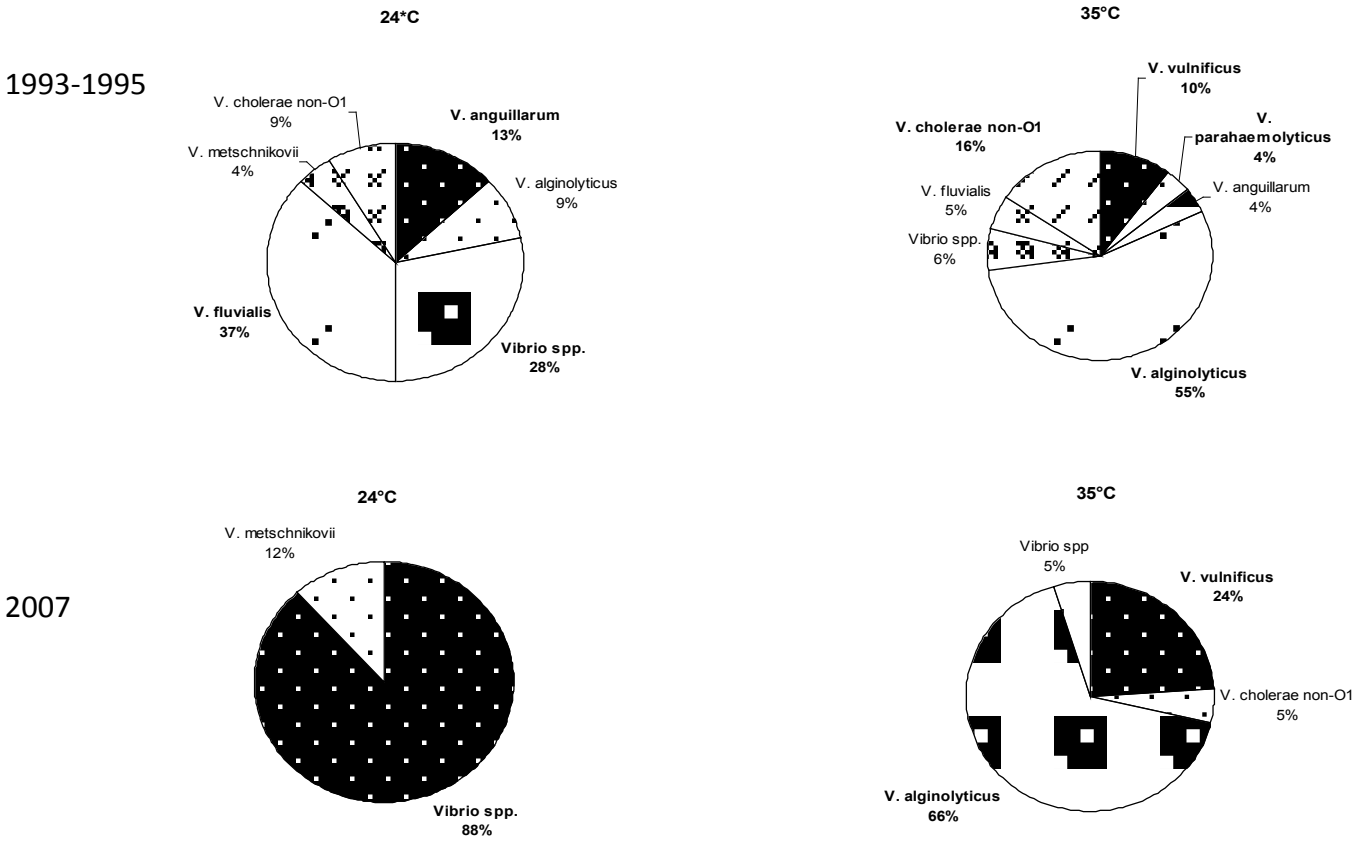

Figure 4: Species composition of halophilic vibrios isolated from the waters of the Gulf of Castellammare during 1993-1995 and 2007.

FC							
Variance	Sum of squares	degrees of freedom	Mean of squares	F	Probability	F critical	
Among times	2.58E+05	8	3.23E+04	0.821	0.589	2.089	not significant
Within times	2.48E+05	63	3.93E+04				
Total	2.73E+05	71					
ENT							
Variance	Sum of squares	degrees of freedom	Mean of squares	F	Probability	F critical	
Among times	1.05E+05	8	1.32E+04	1.765	0.101	2.089	not significant
Within times	4.70E+05	63	7.46E+03				
Total	5.75E+05	71					
VP							
Variance	Sum of squares	degrees of freedom	Mean of squares	F	Probability	F critical	
Among times	1.23E+11	8	1.53E+10	7.188	1.01E-06	2.089	highly significant***
Within times	1.35E+11	63	2.14E+09				
Total	2.57E+11	71					
VPP							
Variance	Sum of squares	degrees of freedom	Mean of squares	F	Probability	F critical	
Among times	5.06E+06	8	6.33E+05	1.931	0.07	2.089	not significant
Within times	2.06E+07	63	3.28E+05				
Total	2.57E+07	71					

Table 3: Results of analysis of variance (ANOVA, F value) performed on each microbial parameter: Faecal Coliforms (FC), *Enterococcus* spp. (ENT), Vibrios growing at 24 and 35°C (VP and VPP respectively).

purposes (<300 CFU/100 ml), therefore the Castellammare area could be classified as zone A. Only a small number of samples did not meet the recommended criterion and were quantified in a percentage of 2.7 of the total in 1993-1995 and of 5.6% in 2007. The samples which were not compliant with mandatory levels were found only sporadically during autumn 1993 for FC, in relation to rainfalls that increase the supply of organic matter from rivers or untreated urban waste waters, as also observed in a Sicilian brackish environment [27].

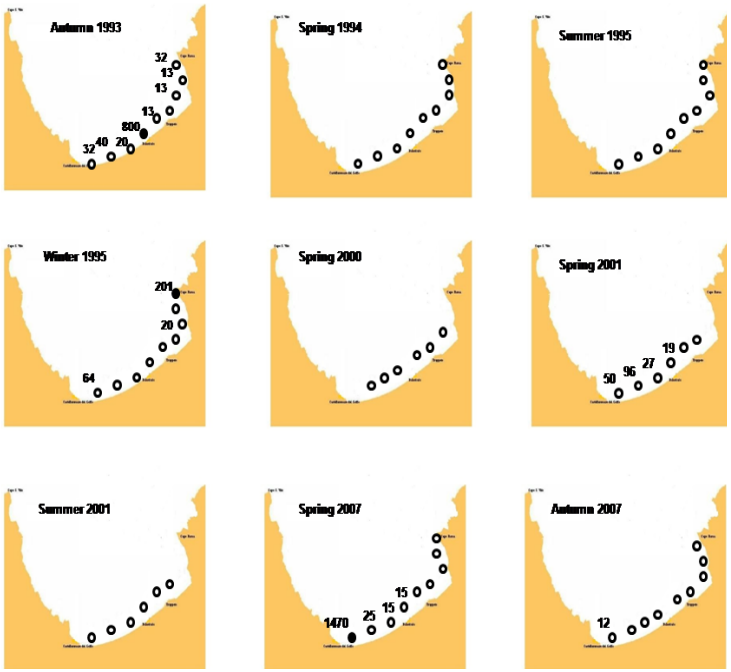

Figure 5: Spatial distribution of the faecal coliforms values higher than 10 CFU/100 ml detected in the waters of the Gulf of Castellammare from 1993 to 2007. Empty dots indicate the sampling stations, filled dots show the peak value of bacterial concentrations.

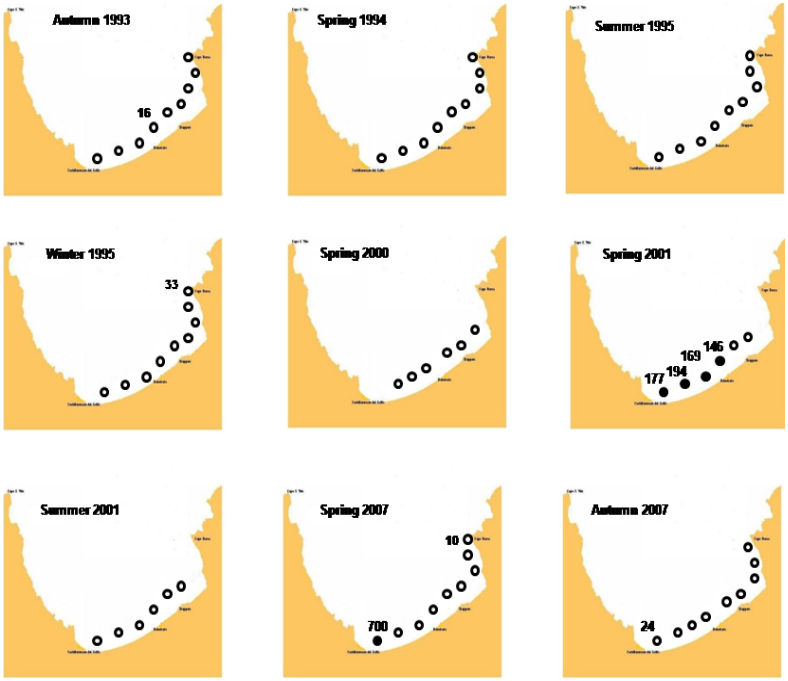

Figure 6: Spatial distribution of the *Enterococcus* spp. values higher than 10 CFU/100 ml detected in the waters of the Gulf of Castellammare from 1993 to 2007. Empty dots indicate the sampling stations, filled dots show the peak value of bacterial concentrations.

Also the absence in the analyzed water samples of *Salmonella* spp. or *Vibrio parahaemolyticus* strains producing a Kanagawa haemolysin or thermostable direct haemolysis, considered as an important virulence factor for this species, confirmed the general suitability of the area for productive activities such as shellfish and fish farming activities.

The search for faecal pollution bacterial indicators, however, does not give indication on the risk of certain species of indigenous microorganisms considered potentially pathogenic - like vibrios and toxic microalgae - whose evaluation it is not yet covered by the current regulations, while they may be concentrated inside the shellfish body.

Several studies [14-28] highlighted that the abundance of vibrios is often unrelated to that of faecal pollution indicators; therefore a complete assessment of the hygienic-sanitary quality of coastal waters should include these microorganisms to get a complete picture of the health risks associated to diseases from contaminated seafood. Halophilic vibrios are microorganisms autochthonous of marine environments; their abundance follows a seasonal trend, increasing during summer months [27]; moreover, coastal and estuarine habitats rich in organic polymers are typical habitats for vibrios whose growth depends on the availability of organic matter [11]. Some marine vibrio species have recently been recognized as possible indicators of organic pollution and as human pathogens, responsible for gastroenteritis or death following the consumption of raw seafood or exposure to contaminated waters [29], as well as for mass mortalities in fish and shellfish farming areas [30]. Several studies confirmed the widespread occurrence of pathogenic *Vibrio* spp. in coastal and estuarine areas [12-32]. In the Gulf of Castellammare waters, the abundance of VP was in the order of 10E+03 CFU/100 ml, while VPP values varied within 10E+02 CFU/100 ml. In the sediments of a mussel farm in the Gulf of Gaeta (Tyrrhenian Sea), La Rosa et al. [23] reported that VP ranged from 0.08 to 1.64x10E+04 CFU/g of sediment. Pujalte et al. [33] recorded *Vibrio* spp. concentrations of 10E+04 CFU/100 ml in seawater samples taken from a hatchery of *Ostrea edulis* located off the Mediterranean Spanish coast.

With respect to the qualitative study of halophilic vibrios, the predominance of the species *V. alginolyticus* within the VPP fraction able to grow at 35°C suggests the ubiquitous distribution of such a facultative pathogen in coastal marine waters. Similar findings are available in literature: Croci et al. [14] found that in seawater samples from two coastal areas of the Adriatic Sea (Cesenatico and Goro), *V. alginolyticus* represented 31.8% of the total, while *V. parahaemolyticus* and *V. vulnificus* accounted both for a percentage of 4.7%. Also Cavallo and Stabili [15] reported that *V. alginolyticus* was the predominant component of the total culturable vibrios in the Mar Piccolo of Taranto (Ionian Sea, Italy). The isolation of *V. cholerae* serotype non-O1 confirms the wide distribution of this microorganism in marine and brackish environments, which prefers moderate temperatures (21-28°C) and low salinities (<5) [22].

Further bacteriological controls of the Gulf of Castellammare area, repeated at shorter time intervals, and aimed also at detecting the viability status of pathogens, could allow to formulate a more precise quality judgment. More attention should be paid to the natural factors, such as temperature, season, nutrient load, which may affect the concentration of bacterial pathogens and their species composition. Nevertheless, the results till now available in terms of microbial indicators of fecal pollution and organic enrichment indicate that the Gulf of Castellammare area could be designated for aquaculture purposes with beneficial effects from a biological and naturalistic point of view.

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