OCCURRENCE OF VIBRIOS IN THE SOUTHERN ADRIATIC SEA ITALIAN COASTS

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VIBRIO SPP
MICROBIAL POLLUTION INDICATORS
ABIOTIC VARIABLES
SOUTHERN ADRIATIC SEA

ABSTRACT. – *Vibrio* spp from Southern Adriatic Sea (Italy) are investigated from February 1997 to January 1998. 659 strains were isolated. The data obtained show that *Vibrio alginolyticus* was the most prevalent species (40 % of isolates), followed by *Vibrio vulnificus* (31.1 %). Recovery of vibrios was correlated with temperature but not with microbial pollution indicators. Aeromonads were not correlated with abiotic parameters and microbial pollution indicators. In all the sites examined the bacterial distribution was not related to stations or depth. Present study contributed to further characterize Southern Adriatic Sea showing the predominant vibrio species of sanitary interest present in this temperate marine environment.

VIBRIO SPP
INDICATEURS MICROBIOLOGIQUES DE POLLUTION
FACTEURS ABIOTIQUES
MER ADRIATIQUE DU SUD

RÉSUMÉ. – La présence de *Vibrio* spp dans les eaux côtières de la Mer Adriatique Sud (Italie) a été recherchée de février 1997 à janvier 1998. 659 souches ont été isolées. Les résultats obtenus démontrent que *Vibrio alginolyticus* est l'espèce la plus fréquemment identifiée (40 % des isolats); suivie de *Vibrio vulnificus* (31,1 %). La présence de *Vibrio* spp est liée à la température mais non aux indicateurs microbiologiques de pollution. Dans tous les sites examinés la distribution bactérienne n'est liée ni aux stations ni à la profondeur. La présente étude contribuera à caractériser ultérieurement le Sud de la Mer Adriatique en indiquant les espèces prédominantes de *Vibrio* spp d'intérêt sanitaire dans cet environnement marin tempéré.

INTRODUCTION

Several studies on the role of *Vibrio* spp in the marine environment have shown their importance in biodegradation, nutrient regeneration and biogeochemical cycling (Ducklow 1983, Jørgensen 1983, Colwell 1994). Vibrios are readily cultivable from seawater, marine animals and seaweeds, leading to the hypothesis that they comprise a dominant component of the microbial community structure. Some vibrios are pathogenic for fish and shellfish, as well as for humans (e.g. *V. cholerae*, *V. vulnificus*, *V. parahaemolyticus*, etc.).

Previous researches indicated that species found in the sea are numerous and their variability depends on environmental conditions; in fact, different ecological parameters such as nutrient availability, temperature and salinity influence the presence of different *Vibrio* species. Nutrient insufficiency is the most common environmental stress which microorganisms routinely encounter in natural ecosystems. Many marine bacteria, especially *Vibrio* spp, can survive for a long time during starvation by sequential changes in cell physiology and gradual changes in morphology. However, some pathogenic vibrios, such as *V. cholerae* and *V. vulnificus*, have been found to enter into a viable but nonculturable stage rather than die when exposed to a low nutrient environment (Jiang & Chai 1996). Vibrios are recovered less frequently when temperature declines and salinity increases. However, factors such as temperature, dissolved oxygen, salinity etc. are interactive thus effects of a single environmental parameter are difficult to assess.

The fraction of pathogenic vibrios able to grow at 35 °C is even more linked to the coastal environment and it shows a direct relation with the water temperature.

In the present study the presence of *Vibrio* spp in water samples from four different coastal sites in the Southern Adriatic Sea was investigated. Attention was focused especially on those species of *Vibrio* which have sanitary interest. Understanding the ecology of *Vibrio* spp of clinical interest and tracing virulence determinants in environmental isolates are important issues, and several studies addressing these subjects have been performed in various geographic areas and with samples from various environmental sources (Colwell *et al.* 1977, Chowdhury *et al.* 1990, Hoge *et al.* 1990, Ramamurthy *et al.* 1994, Dalsgaard *et al.* 1995). In order to evaluate the degree of microbial pollution, total coliforms, fecal coliforms and fecal streptococci were determined too.

MATERIAL AND METHODS

Sampling procedure: This study was carried out from February 1997 to January 1998 in four sites of the Southern Adriatic Sea: Brindisi, S. Cataldo, Otranto and S. M. di Leuca (Fig. 1). Water samples were collected monthly at 0.5 m below the water surface and at 0.5 m above the bottom (between 5 and 50 m depth depending on the bathymetry of each site), using a 5 l Niskin sterile bottle. For each examined site four sampling points were fixed. They were referred as stations 1, 2, 3, and 4 located on transects perpendicular to the shore, starting from a distance of 0.3 to 5.4 miles.

Abiotic and biotic parameters: Temperature, pH, salinity and dissolved oxygen concentration were measured *in situ* using a «Ocean Seven Idronaut» multiparametric Sounding-line. Total coliforms, fecal coliforms, fecal streptococci were determined by the most-probable-number method (APHA 1992).

Isolation and identification of vibrios:Vibrio spp. research was carried out by filtering 500 ml of seawater on 0.45 μm pore size filter and placing the membrane in 100 ml of alkaline peptone water incubated at 24°C for 24 hours. Subcultures were plated on thiosulfate-citrate-bile-salts-sucrose (TCBS) agar plus 2 % NaCl. Plates were incubated at 24 °C for 24 h. Emerging colonies were isolated «at random» and then submitted to the following tests: mobility, Gram staining, oxidase and catalase assay, carbohydrates fermentation on triple sugar iron (TSI) agar plus 2 % NaCl, growth at 4, 35, 40 °C, growth in 0, 6, 8 % NaCl, aminoacids decarboxylase reaction, sensitivity to 0/129 vibriostatic agent (10 and 150 μg). After that a representative sample of vibrios was submitted to further biochemical assay by the API 20E system (Bio Merieux Sa, France), using 2 % NaCl as the diluent, in order to identify the strains to the species level.

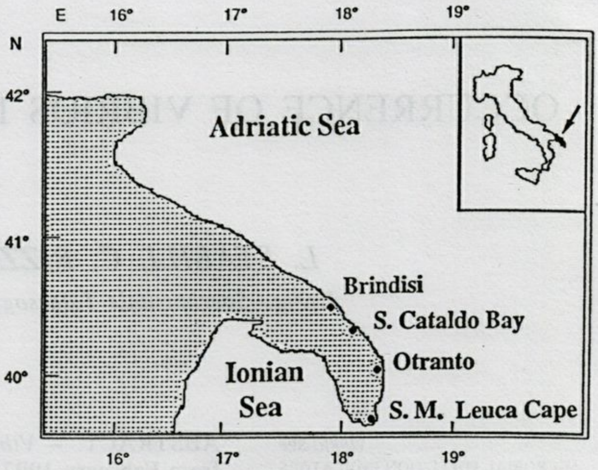

Fig. 1. – Map of the Southern Adriatic Sea, Italian coast showing the locations of the sampling sites.

Statistical analysis: Data were analyzed to identify linear regression and relationships between variables. Correlations coefficients between hydrological or microbiological pollution data and vibrios species were computed and significance was tested by the Student's t-test.

RESULTS

During a year of sampling, 659 *Vibrio* spp. strains were isolated on TCBS plus 2 % NaCl from the Southern Adriatic Sea: 173 in Brindisi, 152 in S. Cataldo, 187 in Otranto and 147 in S. M. di Leuca. The percentages of isolation were: 40 % *V. alginolyticus,* 31.1 % *V. vulnificus*, 14.5 % *V. parahaemolyticus,* 10.2 % *V. cholerae* non-O1, 2.4 % *V. mimicus*, 1 % *V. splendidus*, 0.7 % *V. harvey* (Fig. 2). The main physico-chemical characteristics of the sampling points (surface and bottom) such as, temperature, salinity, dissolved oxygen, are reported in Fig. 3. pH ranged between 7.9 and 8.37 at the surface and between 7.9 and 8.41 at the bottom. Statistical analysis revealed

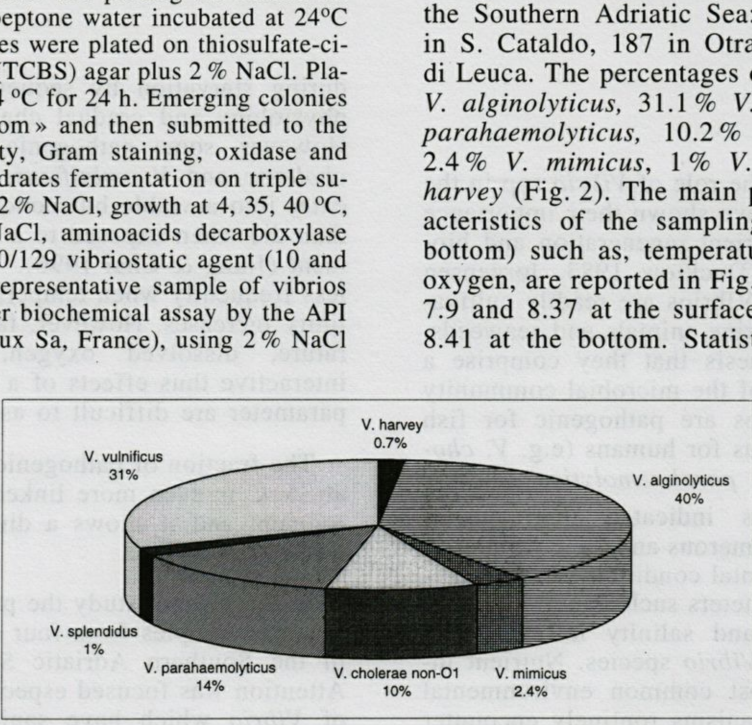

Fig. 2. – Percentages of isolated *Vibrio* spp strains.

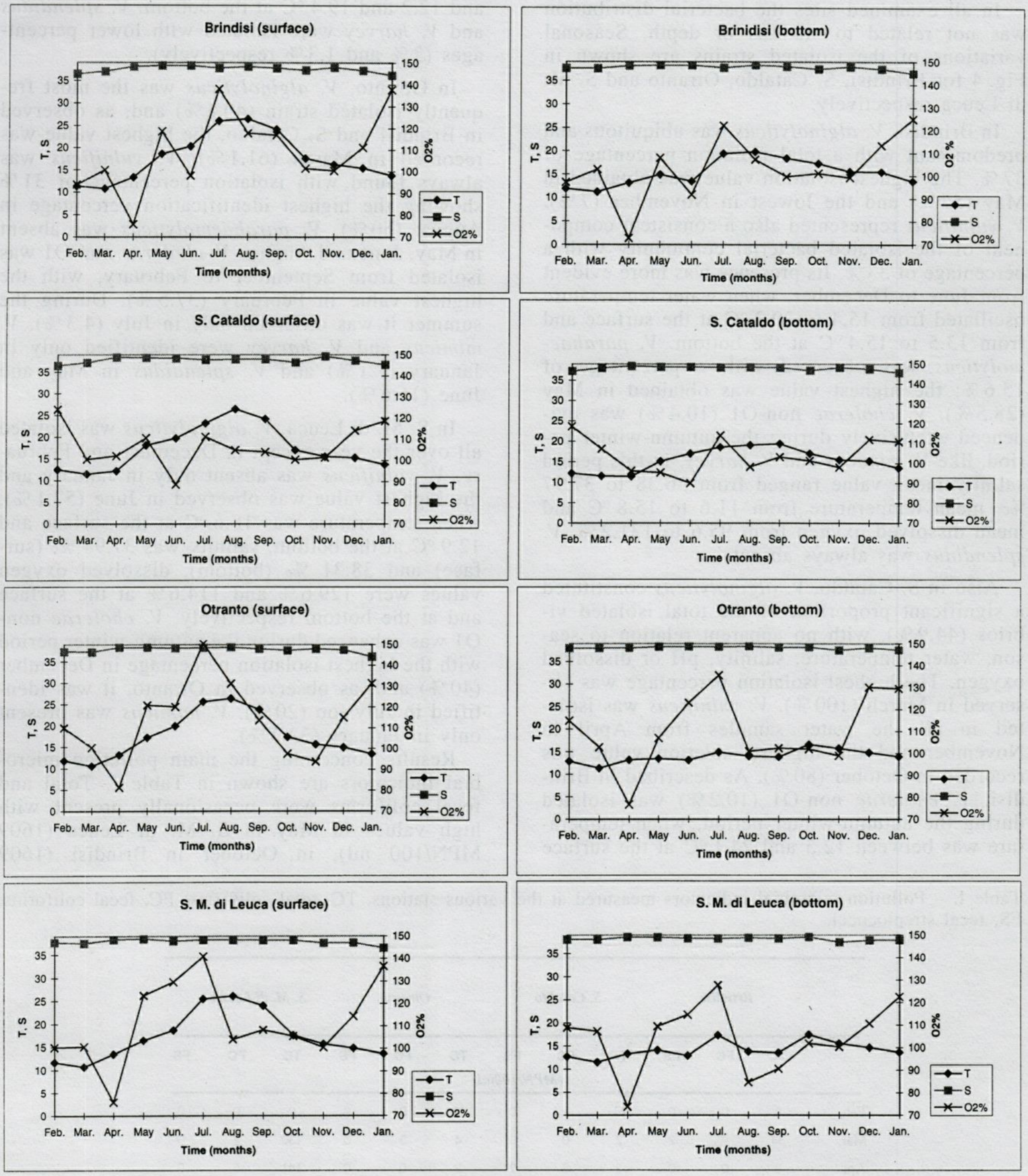

Fig. 3. – Abiotic parameters : T = Temperature (°C), O_2 % = dissolved oxygen (Sat %), S = salinity (‰), measured at the various sampling stations.

that *Vibrio* spp isolation was directly related to water temperature ($r^2 = 0.693$, $P < 0.001$) but not to pH ($r^2 = 0.070$), salinity ($r^2 = 0.202$) and dissolved oxygen ($r^2 = 0.036$). By the analysis of individual species, only *V. vulnificus* was directly related to water temperature ($r^2 = 0.4931$, $P < 0.05$). This species was also directly related to salinity ($r^2 = 0.43$, $P < 0.001$). On the contrary, *V. parahaemolyticus* resulted directly related to dissolved oxygen ($r^2 = 0.48$, $P < 0.001$).

In all examined sites the bacterial distribution was not related to stations or depth. Seasonal variations of the isolated strains are shown in Fig. 4 for Brindisi, S. Cataldo, Otranto and S. M. di Leuca respectively.

In Brindisi, *V. alginolyticus* was ubiquitous and predominant with a total isolation percentage of 37 %. The highest isolation value was obtained in May (57 %) and the lowest in November (7 %). *V. vulnificus* represented also a consistent component of the isolated bacterial community with a percentage of 33 %. Its presence was more evident from June to December, when water temperature oscillated from 15.4 to 20.3 °C at the surface and from 13.5 to 15.4 °C at the bottom. *V. parahaemolyticus* was observed with a percentage of 15.6 %; the highest value was obtained in May (28.5 %). *V. cholerae* non-O1 (10.4 %) was evidenced exclusively during the autumn-winter period, like *V. mimicus* and *V. harvey*. In this period salinity mean value ranged from 36.38 to 37.97 ‰, mean temperature from 11.6 to 15.8 °C and mean dissolved oxygen from 95.6 to 131.2 %. *V. splendidus* was always absent.

Also in S. Cataldo, *V. alginolyticus* constituted a significant proportion of the total isolated vibrios (44.9 %), with no apparent relation to season, water temperature, salinity, pH or dissolved oxygen. The highest isolation percentage was observed in March (100 %). *V. vulnificus* was isolated in all the water samples from April to November and the highest isolation value was recorded in October (80 %). As described in Brindisi, *V. cholerae* non-O1 (10.2 %) was isolated during the autumn-winter period, when temperature was between 12.5 and 24.1 °C at the surface and 12.2 and 19.4 °C at the bottom. *V. splendidus* and *V. harvey* were isolated with lower percentages (2 % and 1.3 % respectively).

In Otranto, *V. alginolyticus* was the most frequently isolated strain (40.6 %) and, as observed in Brindisi and S. Cataldo, the highest value was recorded in March (61.1 %). *V. vulnificus* was always found with isolation percentage of 31 % showing the highest identification percentage in August (50 %). *V. parahaemolyticus* was absent in May, June and August. *V. cholerae* non-O1 was isolated from September to February, with the highest value in February (37.5 %). During the summer it was observed only in July (4.3 %). *V. mimicus* and *V. harvey* were identified only in January (7.1 %) and *V. splendidus* in May and June (16.6 %).

In S. M. di Leuca, *V. alginolyticus* was isolated all over the year except in December and February. *V. vulnificus* was absent only in January and the highest value was observed in June (57.1 %), when temperature was 18.6 °C at the surface and 12.9 °C at the bottom, salinity was 37.94 ‰ (surface) and 38.31 ‰ (bottom), dissolved oxygen values were 129.6 % and 114.6 % at the surface and at the bottom respectively. *V. cholerae* non-O1 was enhanced during the autumn-winter period with the highest isolation percentage in December (40 %) and, as observed in Otranto, it was identified in July too (20 %). *V. mimicus* was present only in January (33.3 %).

Results concerning the main pollution microbial indicators are shown in Table I. Total and fecal coliforms were occasionally present with high values in May in S. M. di Leuca (1609 MPN/100 ml), in October in Brindisi (1609

Table I. – Pollution microbial indicators measured at the various stations. TC, total coliforms; FC, fecal coliforms; FS, fecal streptococci.

	Brindisi			*S. Cataldo*			*Otranto*			*S. M. di Leuca*		
	TC	FC	FS	TC	FC	FS	TC	FC	FS	TC	FC	FS
	(MPN/100ml)											
Feb.	2	0	0	11	2	0	17	0	9	0	0	0
Mar.	34	9	2	2	0	0	4	2	0	130	2	0
Apr.	2	0	0	2	2	2	2	0	0	141	5	0
May	5	5	0	5	5	0	5	0	0	>1609	>1609	46
Jun.	2	2	0	2	2	0	0	0	4	5	5	17
Jul.	0	0	2	0	0	2	109	0	9	0	0	0
Aug.	5	2	0	2	2	0	0	0	6	2	2	17
Sep.	0	0	0	0	0	0	4	0	4	9	9	7
Oct.	1609	1609	0	34	11	0	11	5	0	0	0	0
Nov.	2	0	0	49	2	4	9	0	0	0	0	0
Dec.	0	0	0	0	0	0	0	0	17	0	0	0
Jan.	0	0	2	0	0	4	5	0	0	0	0	0

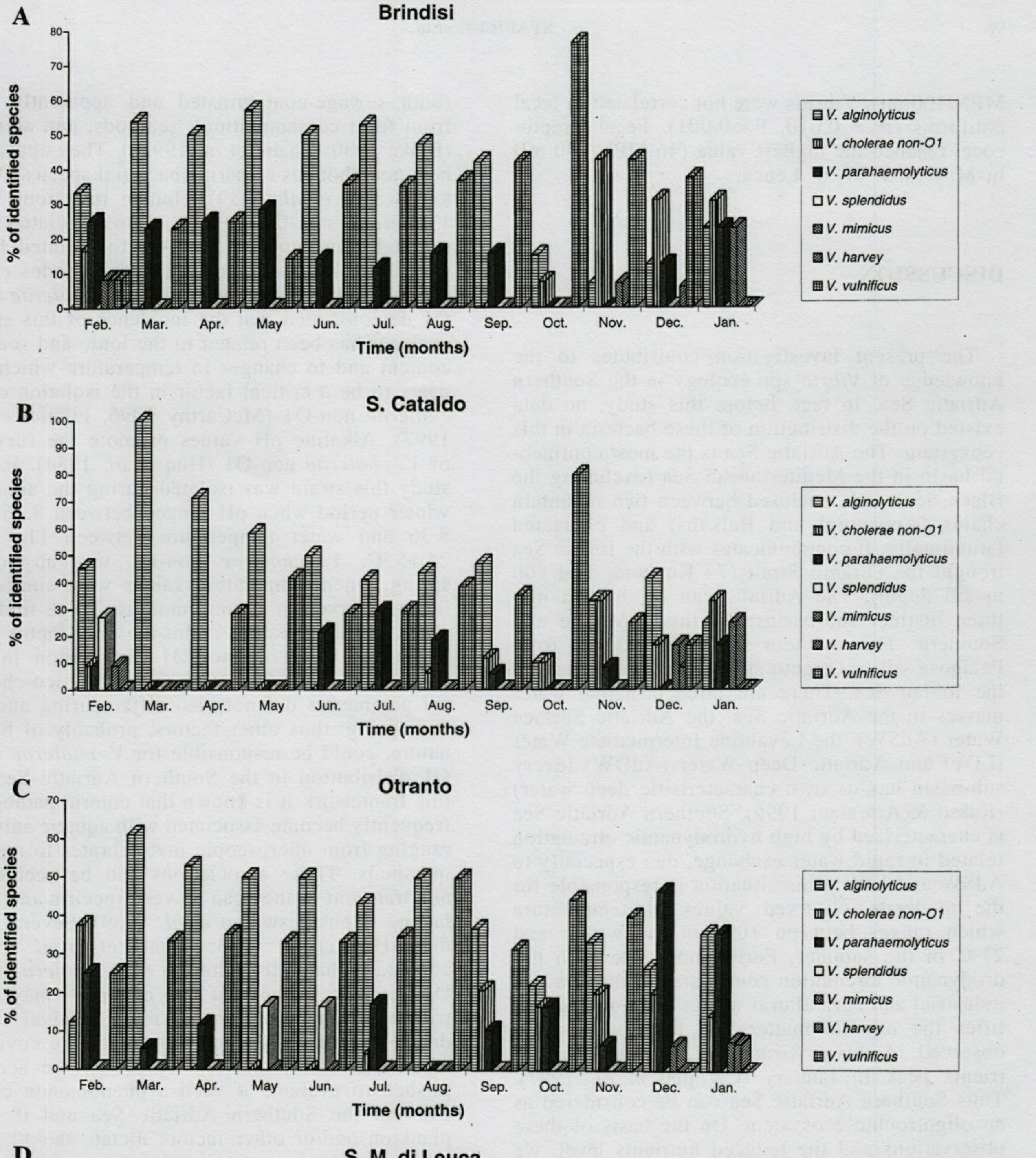

Fig. 4. – Mounthly percentages of *Vibrio* identified species during a 12-month period. Water samples have been taken both at the surface and bottom. A, in Brindisi; B, in S. Cataldo; C, in Otranto; D, in S.M. di Leuca.

MPN/100 ml). Vibrios were not correlated to fecal coliforms (r^2 = 0.010, $P < 0.001$). Fecal streptococci reached the highest value (46 MPN/100 ml) in May in S. M. di Leuca.

DISCUSSION

The present investigation contributes to the knowledge of *Vibrio* spp ecology in the Southern Adriatic Sea. In fact, before this study, no data existed on the distribution of these bacteria in this ecosystem. The Adriatic Sea is the most continental basin in the Mediterranean Sea (excluding the Black Sea), it is enclosed between two mountain chains (Appennini and Balkans) and elongated latitudinally. It communicates with the Ionian Sea trought the Otranto Strait (74 Km wide and 800 m sill depth). The Adriatic can be divided into three distinct sub-basins: Northern, Middle and Southern. The southern sub-basin extends from Pelagosa sill to Otranto sill which divides it from the Ionian Sea. There are three principal water masses in the Adriatic Sea: the Adriatic Surface Water (AdSW), the Levantine Intermediate Water (LIW) and Adriatic Deep Water (AdDW) (every sub-basin has its own characterisitic deep water) (Russo & Artegiani 1996). Southern Adriatic Sea is characterized by high hydrodynamic circulation related to rapid water exchange, due especially to AdSW and LIW. This situation is responsible for the moderate observed values of temperature which ranges between 10°C in the winter and 27°C in the summer. Furthermore, the high hydrodynamic circulation contributes to the sewage, industrial and agricultural wastes dilution and justifies the organic matter low level which was observed in this environment, with only a nutrients peak in January (Caroppo *et al.* 1997). Thus Southern Adriatic Sea can be considered as an oligotrophic ecosystem. On the basis of these observations and the reduced nutrients level, we especially considered the effect of pH, temperature, salinity and dissolved oxygen on vibrios ecology.

There has been a remarkable interest in the occurrence of potentially pathogenic vibrios species in coastal environments, since a close relationship between the consumption of raw seafood and the occurrence of gastroenteritis or alimentary toxic infection episodes attributed to some species of *Vibrio* (*V. cholerae* non-O1, *V. alginolyticus*, *V. parahaemolyticus*, *V. vulnificus*) has been observed (Wong *et al.* 1992, Matte *et al.* 1994)

V. cholerae non-O1 strains are widely distributed in the environment in Europe, Asia and United States. They have been found in sewage, sewage-contaminated surface water, estuarine waters (both sewage-contaminated and apparently free from fecal contamination), seafoods, and animals (Blake 1980; Chang *et al.* 1995a). They appear to be autochthonous estuarine bacterial species (Venkateswaran *et al.* 1989). Human infections with *V. cholerae* non-O1 are most often associated with seafood consumption, exposure to polluted fresh water, brackish water or seawater (Rhodes *et al.* 1986). Studies on the ecology of *V. cholerae* non-O1 demonstrated that the incidence of this strain in water has been related to the ionic and sodium content and to changes in temperature which appears to be a critical factor in the isolation of *V. cholerae* non-O1 (McCarthy 1996, Filetici *et al.* 1997). Alkaline pH values promote the survival of *V. cholerae* non-O1 (Huq *et al.* 1984). In our study this strain was isolated during the autumn-winter period when pH ranged between 8.16 and 8.36 and water temperature between 11.3 and 24.15 °C. *V. cholerae* non-O1 was absent in spring, when temperature values were similar to those observed in autumn months. These findings show that temperature is not the only factor controlling *V. cholerae* non-O1 distribution in the Southern Adriatic Sea. The other physico-chemical parameters did not also differ during autumn and spring; thus other factors, probably of biotic nature, could be responsible for *V. cholerae* non-O1 distribution in the Southern Adriatic Sea. In this framework it is known that enteric pathogens frequently become associated with aquatic animals ranging from microscopic invertebrates to marine mammals. These associations can be accidental and transient or they can be very specific and long lasting (Venkateswaran *et al.* 1989). Several studies (Huq *et al.* 1983; Dumontet *et al.* 1996) concluded that cells adhesion of *V. cholerae* non-O1 onto the surfaces of live copepods may contribute significantly to the survival and distribution of this specie in the aquatic environment. Therefore, further studies will be accomplished to evaluate if such a phenomenon could exist in the Southern Adriatic Sea and if zooplankton and/or other factors dictate the *V. cholerae* non-O1 seasonal distribution.

V. parahaemolyticus is part of the normal flora of estuarine and other coastal waters throughout most of the world. It has been isolated from seawater, sea mud or seafoods (De Paola *et al.* 1990). Associated to *V. parahaemolyticus gastroenteritis* appear to be transmitted exclusively by food, usually raw or cooked seafood, although sometimes other foods presumably cross-contaminated by raw sea food have been thought to transmit the infection. *V. parahaemolyticus* is typical of environments with organic matter high content. Furthermore, the occurrence of *V. parahaemolyticus* is not governed by any single biotic or abiotic environmental factor (Venkateswaran *et al.* 1990). Chowdhury *et al.* (1990) observed that *V. para-*

haemolyticus in a temperate region aquatic environment was detected quite frequently during the winter months, at a temperature ranging from 10 to 14 °C. Consistent with these observations, in our investigation *V. parahaemolyticus* was frequently isolated with a maximum during the winter period, when the highest content of organic substance was recorded and mean temperature was comprised between 10.8 and 14.7 °C in Brindisi, Otranto and S. M. di Leuca.

V. vulnificus is a common bacterium in estuarine waters in temperate and tropical climates and in different coastal areas. It is found in water column as well in sediment and associated with various marine life forms (Kelly 1982, Oliver *et al.* 1982, Tamplin *et al.* 1982, 1983, Kaysner *et al.* 1987). Occasionally *V. vulnificus* causes invasive disease and life-threatening infections in humans who suffer from preexisting illnesses and consume raw oysters (Klontz *et al.* 1988). Environmental parameters that correlate with the recovery of *V. vulnificus* from seawater, generally correspond to estuarine conditions having relatively lower salinity (7 to 16 ‰) and high temperature (above 20 °C) (Wright *et al.* 1996). O'Neill *et al.* (1992) were able to detect *V. vulnificus* in oysters from water samples at temperatures of ca. 11°C, suggesting that this strain can adapt to the cold too. We were able to identify *V. vulnificus* all over the year, in all the examined stations, with a decline in isolation percentage when mean temperature value was 12.8 °C and salinity 37 ‰. In this study *V. vulnificus* was found to be one of the dominant species composing the vibrios population in the Southern Adriatic Sea.*V. vulnificus* requires low contents of organic substance, compared to *V. parahaemolyticus*. These different requirements could explain why *V. vulnificus* was isolated more frequently in comparison to *V. parahaemolyticus* (24.4 and 11.3 % respectively).

V. alginolyticus is an environmental species, frequently observed in temperate coastal areas, and it has been isolated from seawater and seafoods in many parts of the world (Ortigosa *et al.* 1989, Buck 1990). In our study its recovery did not appear influenced by temperature, salinity and pH as revealed by the r^2 and P values (not shown). The lack of statistical correlation between temperature and occurrence of *V. alginolyticus* may be explained by the fact that the minimum growth temperature for this species is 8 °C (Blake 1980), and this temperature value was never reached in the Southern Adriatic Sea, where the minimum temperature value was 10.57 °C.

V. mimicus is a bacterium detected frequently in freshwater, seawater and brackish water. It is a causative agent of human gastroenteritis and has recently been described as new food poisoning. *V. mimicus* as *V. cholerae* non-O1 show a reduced survival when temperature increases. Researches accomplished by Chang *et al.* (1995b) evidenced the presence of *V. mimicus* in January. Our results show that *V. mimicus* was mainly found in January, when mean temperature value was 13.6 °C.

V. splendidus and *V. harvey* were occasionally recovered thus it is difficult to evidence a correlation with their presence and abiotic parameters.

No relationship was observed between *Vibrio* spp. and fecal pollution. It is well known that vibrios are ubiquitous in the aquatic environment and these organisms are not introduced significantly into the marine realm by sewage or the adjacent freshwaters. Grimes *et al.* (1986) pointed out that many investigators erroneously interpret the wastewater discharge as the source of the pathogens rather than as a source of nutrients, which may stimulate the growth of the autochthonous pathogens. For this reason, the traditional indices of fecal contamination alone are not reliable indicators of the quality of water. Thus authorities responsible for controlling bathing water quality should pay particular attention to potential pathogenic *Vibrio* spp and even when microbial pollution indicators show low detectable levels.

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