First multi-year retrospective study on *Vibrio parahaemolyticus* and *Vibrio vulnificus* prevalence in *Ruditapes philippinarum* harvested in Sacca di Goro, Italy

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Abstract

The present work describes a retrospective study aiming to verify a possible correlation between the environmental conditions (temperature, salinity and dissolved oxygen), the abundance of *Vibrio* spp., and the prevalence of *V. parahaemolyticus* and *V. vulnificus* in the Manila clam *R. philippinarum* harvested in Sacca di Goro, Emilia-Romagna Region, Northern Italy. On the whole, 104 samples, collected in the period 2007-2015 and submitted to microbiological analyses (isolation and genotyping), have been reconsidered for *Vibrio* spp. load, *V. parahaemolyticus* prevalence (total, gene marker *tox*RP; potentially pathogenic, gene markers *tdh* and/or *trh*) and *V. vulnificus* prevalence (total, gene markers *vvh*A and *hsp*) together with environmental data obtained from the monitoring activity of the Emilia-Romagna Regional Agency for the Prevention, the Environment and the Energy. Environmental data have been processed to calculate the median of each, assessing the seasonal range of seawater temperature (warmer months: April-October, T°C >16.45°C; cooler months November-March, T°C <16.45°C), salinity (<or>27 psu), and dissolved oxygen (< or >8.2 mg/L). Total *V. vulnificus*, total and potentially pathogenic *V. parahaemolyticus* were present respectively in the 11.5, 29.8 and 6.7% of the samples. The *Vibrio* spp. load (mean value of 4.69±0.65 $\log_{10}$ colony forming unit g^{-1}) and the prevalence of potentially pathogenic *V. parahaemolyticus*, were not significantly correlated to the environmental conditions (P>0.05), whereas the prevalence of both total *V. vulnificus* and total *V. parahaemolyticus* was significantly higher in the warmer period (P<0.05), without correlation with salinity and dissolved oxygen values (P>0.05).

Introduction

Vibrio parahaemolyticus and *Vibrio vulnificus* are ubiquitous Gram-negative bacterial pathogens found naturally in marine and estuarine waters, representing a leading cause of seafood-associated bacterial illness. The most important vehicle for these microorganisms are raw or lightly cooked bivalve shellfish (Drake *et al*., 2007), hereafter indicated simply shellfish. Although both *V. vulnificus* and *V. parahaemolyticus* cases occur sporadically, the former are almost always sporadic while the latter can also occur in outbreak settings (Drake *et al*., 2007).

V. parahaemolyticus infection commonly include abdominal cramps, diarrhea, nausea, headaches, fever and chills (Baker-Austin *et al*., 2010). Most environmental *V. parahaemolyticus* strains are considered to be non-pathogenic due to low detection frequencies of *tdh* and *trh* genes encoding respectively for the thermostable direct haemolysin (TDH) and the TDH-related haemolysin (TRH), therefore these gene markers continues to be the simplest and most frequently used diagnostic indicator of pathogenicity (Gutierrez West *et al*., 2013; Raghunath, 2015).

V. vulnificus infection may be acquired via wound infections or consumption of raw seafood, particularly shellfish, and may result highly invasive, causing respectively secondary or primary septicemia, particularly in high-risk populations, including patients with chronic liver disease, immunodeficiency, iron storage disorders, end-stage renal disease, and diabetes mellitus (Horseman and Surani, 2011). The presence/absence of many gene target have been used to differentiate clinical from environmental *V. vulnificus* strains, and among them *vcg*C and *vcg*E (Han *et al*., 2011); 16S rRNA type A, B or AB and CPS operon group 1 allele 1 (CPS1) and allele 2 (CPS2), (Chatzidaki-Livanis *et al*., 2006). In any case, unique virulence gene markers that are present exclusively in virulent *V. vulnificus* strains have not yet been identified (Han *et al*., 2009), therefore according to FAO/WHO (2005), all *V. vulnificus* strains may be considered virulent.

The abundance and distribution of *Vibrio parahaemolyticus* and *Vibrio vulnificus* have been linked to environmental factors, most notably temperature, salinity and dissolved oxygen (Parveen *et al*., 2008; Ramirez *et al*., 2009), even if predictive relationships may vary across Regions due to differences in ecology. Currently there is scant information on both the spatial distribution and seasonal detection of *Vibrio* spp. in shellfish harvested in the Emilia-Romagna Region, and other Italian production areas as well, giving that only a few data are available, mostly from limited research activities. Clearly, human exposure to these pathogens cannot be completely eliminated, but the incidence of illness can be reduced if environmental conditions that significantly elevate risk can be identified and monitored (Johnson *et al*., 2012). To our knowledge, this study represent the first attempt to determine the relationships between environmental conditions (seawater temperature, salinity and dissolved oxygen) and *Vibrio* population in the Manila clam *Ruditapes philippinarum*, hereafter indicated simply clam, utilizing a multi years (2007-2015) retrospective study, where *Vibrio* spp. abundance, the total *V. parahaemolyticus* (*tox*RP+), the potentially pathogenic *V. parahaemolyticus* (*tdh*+ and or *trh*+), and the total *V. vulnificus* (*vvh*A+ and *hsp*+) have been considered.

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Key words: *Vibrio parahaemolyticus*; *Vibrio vulnificus*; *Ruditapes philippinarum*; Environmental conditions; Italy.

Contributions: the multi-year survey (2007-2015) on the prevalence of *V. parahaemolyticus* and *V. vulnificus* in *R. philippinarum*, was realized by PS, EZ, GB, PLP. Environmental data processing and statistical analyses were performed by FO, FG and AS.

Conflict of interest: the authors declare no potential conflict of interest.

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Materials and Methods

Study area

All clam samples were collected at the Sacca di Goro, Emilia-Romagna Region, Northern Italy (Figure 1), a wide sandy-bottomed lagoon of the Po river delta characterized by particular features suitable for the shellfish productions, particularly the Manila clam *Ruditapes philippinarum* and the mussel *Mytilus galloprovincialis*. The lagoon extends over 2000 hectares of shallow water, 60-70 cm on average, with a maximum depth of 200 cm.

Microbiological analyses

On the whole, 104 samples of *R. philippinarum*, were collected in the study area, approximately on monthly basis, excluding August, in the period 2007-2015. The analytical protocols utilized at that time to ascertain the *Vibrio* spp. load, the prevalence of total (*tox*RP+) and potentially pathogenic *V. parahaemolyticus* (*tdh*+ and or *trh*+), and the prevalence of total *V. vulnificus* (*vvh*A+ and *hsp*+) have been reported elsewhere (Passalacqua *et al*., 2016), including a more detailed genotyping approach for *V. vulnificus* isolates, characterized beyond the species level by means of other gene markers: *vcg*C, *vcg*E, 16S rRNA type A/B/AB, CPS operon group 1 allele 1 (CPS1) and allele 2 (CPS2).

Environmental parameters

Data of interest on seawater temperature, salinity and dissolved oxygen, were made available by the oceanographic vessel Daphne II (annual reports) of the Regional Agency for the Prevention, the Environment and the Energy - Emilia-Romagna (ARPAE).

Statistical analyses

Preliminarily, environmental parameters (seawater temperature, salinity and dissolved oxygen), collected monthly, were arbitrarily divided into two categories based on the median (warmer months: April-October, T°C>16.45°C; cooler months November-March, T°C<16.45°C); salinity (< or>27 psu), and dissolved oxygen (< or>8.2 mg/L). The *Vibrio* spp. load was log-transformed prior to the analysis. The Kolmogorov–Smirnov test for goodness of adaptation was used to verify distribution normality. On the basis of the results of this test, Student's t-test was used to compare the $\log_{10}$ *Vibrio* spp. load. Qualitative data [prevalence of *V. parahaemolyticus* (total, gene marker *tox*RP; potentially pathogenic, gene markers *tdh* and/or *trh*) and *V. vulnificus* (total, gene markers *vvh*A and *hsp*)] were analyzed using chi-square test. All statistical analyses were performed using the software SPSS 23 (SPSS Inc., Chicago, IL, USA).

Results

In order to be concise, detail on the microbiological results of each of the 104 samples of clams are omitted. The *Vibrio* spp. load of each sample, expressed as Colony Forming Units (CFU g^{-1}) have been log-transformed prior to calculate the mean value, resulting 4.69±0.65 $\log_{10}$ CFU g^{-1}. With respect to the specific bacterial targets, on the whole 11.5% samples were positive for total *V. vulnificus* (*vvh*A+ and *hsp*+), 29.8% were positive for total *V. parahaemolyticus* (*tox*RP+), and 6.7% were positive for potentially pathogenic *V. parahaemolyticus* (*tdh*+ or *trh*+), none of the strains showing the double positivity). A total of 8 out of 43 positive samples showed the concurrent presence of *V. parahaemolyticus* and *V. vulnificus*, and, overall, 50 strains, respectively 16 *V. vulnificus* and 34 *V. parahaemolyticus*, were isolated. The isolation of strains with different virulence genes profiles in three samples for *V. parahaemolyticus* and in three samples for *V. vulnificus* should be noted; details on the number of isolates of both *V. parahaemolyticus* and *V. vulnificus* with different virulence genes profiles in positive samples are reported in Table 1.

The environmental parameters considered in the present study are those collected from 2007 to 2013, being data from 2014 to 2015 unavailable, through 68 sampling campaigns, by the oceanographic vessel Daphne II (ARPAE). Most of the samples were collected from January to December, and none of them was collected in August; one year (2007) showed a noticeable lower number of samples. Summary data of seawater temperature, salinity and dissolved oxygen are reported in Table 2. Considering the different years and the different months of clam sampling from 2007 to 2015, the statistical analyses showed a mean prevalence of 29.81% for *V. parahaemolyticus* (total) and 11.5% of samples positive *V. vulnificus* (total), with a range respectively of 0.0-50 and 0.0-27.3% in different years, and with a range respectively of 0.0-100 and 0.0-66.6% in different months. More details are reported in Table 3. The majority of *V. vulnificus* strains (13 out of 16) were isolated during summer (June and July) whereas the majority of *V. parahaemolyticus* strains (28 out of 34) were isolated in a longer period, from June to October. The statistical analysis of the whole dataset (n=104) showed a significant differences (P=0.00 and P<0.01) of the prevalence of samples positive for *V. parahaemolyticus* (total) and *V. vulnificus* (total) between the warmer months period and the cooler months period (respectively 49.1 *vs* 6.3% and 21.4 *vs* 0%). The same association was not shown (P>0.05) for potentially pathogenic *V. parahaemolyticus* (8.8 *vs* 4.2%) and for *Vibrio* spp. load (4.71±0.10 *vs* 4.70±0.29 $\log_{10}$ g^{-1}).

No significant relationships (P>0.05) were found between the prevalence of *V. parahaemolyticus* (total and potentially pathogen-

Table 1. Virulence genes profiles of *Vibrio parahaemolyticus* and *Vibrio vulnificus* isolates detected in 43 out of 104 samples of clams.

Isolates (n)	*Vibrio vulnificus*							
	vvhA	*hsp*	*vcgE*	*vcgC*	CPS1	CPS2	16SA	16SB
8	+	+	+	n.d.	n.d.	+	+	n.d.
2	+	+	nd	+	+	n.d.	n.d.	+
2	+	+	+	n.d.	+	n.d.	+	n.d.
2	+	+	+	n.d.	+	n.d.	+	n.d.
1	+	+	+	n.d.	n.d.	n.d.	+	n.d.
1	+	+	n.d.	+	+	+	+	n.d.
	Vibrio parahaemolyticus							
	toxRP			*tdh*			*trh*	
5	+			n.d.			+	
4	+			+			n.d.	
25	+			n.d.			n.d.	

n.d., not detected.

ic), *V. vulnificus* (total) and *Vibrio* spp. load and the others environmental parameters, namely seawater salinity and dissolved oxygen.

Discussion

In the present study, a sizeable number of samples (104) of *R. philippinarum* harvested in the same area has been considered over a very long period (from 2007 to 2015), therefore our results may be considered robust and suitable for a comparison with other extensive studies carried out in the Mediterranean area. Among the few reports, this is the case of the multiyear study performed on different shellfish, including *R. philippinarum* (120 samples) harvested in the Ebro Delta, Spain, from 2006 to 2010 (Lopez-Joven *et al*., 2014, 2015), but our results are not in agreement, because we found an higher prevalence of total *V. parahaemolyticus* (29.8 *vs* 14.2%) and potentially pathogenic *V. parahaemolyticus* (6.7 *vs* 3.3%), but a lower prevalence of total *V. vulnificus* (11.5 *vs* 22.5%).

It is largely recognized that in coastal environments, as well as estuaries and coastal rivers, elevated water temperatures and low salinity levels are considered promoting factors for vibrios abundance (Hsieh *et al*., 2008; Oberbeckmann *et al*., 2012; Froelich *et al*., 2013; Takemura *et al*., 2014; Urquhart *et al*., 2016). According with these findings, in the present study the prevalence of total *V. parahaemolyticus* and total *V. vulnificus* resulted significantly higher in the warmer months (seawater temperature>16.45°C, P<0.05), but not at the lower values of salinity (< 27 psu, P>0.05), according to other studies, where salinity values resulted only marginally associated (Parveen *et al*., 2008) or unrelated (Deepanjali *et al*., 2005) with the abundance of *V. parahaemolyticus*.

The correlation with salinity and *V. vulnificus* abundance is still unclear, giving that notwithstanding medium or low salinity (between 5 and 25 ppt) are largely considered the optimal environmental conditions (Mahmud *et al*., 2008), *V. vulnificus* has been isolated from waters with salinities ranging from 1 to 34 ppt (Parvathi *et al*., 2004).

Unlike the total *V. parahaemolyticus,* in our study the prevalence of the subpopulation of potentially pathogenic *V. parahaemolyticus*, characterized by the presence of *tdh* and/or *trh*, resulted unrelated to seawater temperature, but it is important to outline that recently, a large number of clinical isolates revealed an unexplained number of strains lacking these genes, suggesting that *V. parahaemolyticus* may harbor other virulence factors (Ottaviani *et al*., 2012). Moreover it has been demonstrated that environmental isolates of *V. parahaemolyticus* lacking *tdh* and/or *trh* are also highly cytotoxic to human gastrointestinal cells (Raghunath, 2015).

Researches on this topic are ongoing, and therefore to ascertain the pathogenic potential of environmental strains, and the potential risk for consumers, more gene markers other

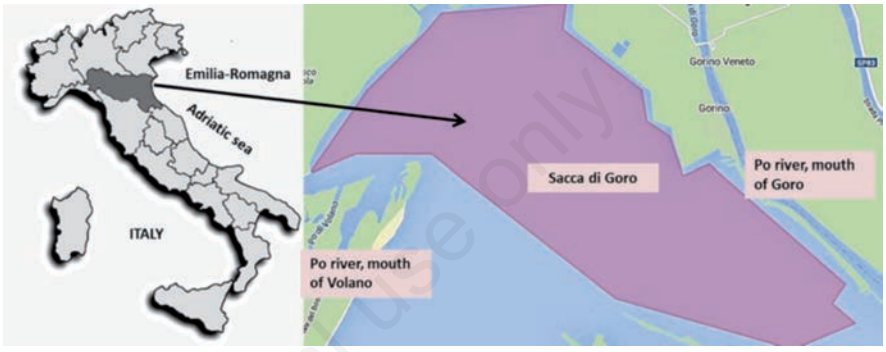

Figure 1. Sacca di Goro, Emilia-Romagna Region, Northern Italy.

Table 2. Summary data of seawater temperature, salinity and dissolved oxygen from 2007 to 2013.

	Temperature (°C)	Salinity (psu)	Dissolved oxygen (mg/L)
Mean	16.94	26.12	8.79
Median	16.45	27.07	8.24
Minimum	5.3	10.74	5.23
Maximun	28.73	37.62	14.49

Table 3. Number of clam samples analyzed per month from 2007 to 2015, Vibrio spp. load, number and percentage of samples positive for *Vibrio vulnificus* (total), *Vibrio parahaemolyticus* (total), and potentially pathogenic *Vibrio parahaemolyticus*.

Month of sampling	Samples (n)	*Vibrio* spp.* (mean±SD)	*Vibrio vulnificus,* total (%)	*Vibrio parahaemolyticus* Total (%)	*Vibrio parahaemolyticus* Potentially pathogenic
Warmer months					
April	7	4.87±0.25	1 (14.3)	0	0
May	6	4.75±0.85	0	1 (14.3)	1 (16.7)
June	10	4.60±0.68	5 (50)	10 (100)	2 (20)
July	6	4.70±0.47	4 (66.6)	3 (50)	1 (16.7)
September	15	4.74±0.63	1 (6.7)	7 (46.7)	0
October	12	4.61±0.74	1 (8.3)	7 (53.8)	1 (7.69)
Cooler months					
January	6	4.61±0.47	0	0	0
February	10	4.98±0.66	0	0	0
March	9	5.04±0.58	0	0	0
November	16	4.46±0.71	0	3 (17.6)	2 (12.5)
December	7	4.41±0.83	0	0	0
Total warmer months	56	4.71±0.10	12 (21.4)	28 (49.1)	5 (8.8)
Total cooler months	48	4.70±0.29	0	3 (6.3)	2 (4.2)

than *tdh* and/or *trh* could be investigated.

With respect to dissolved oxygen, it has been reported a negative correlation with *Vibrio* spp. abundance (Siboni *et al*., 2016), and *V. vulnificus* abundance (Pfeffer *et al*., 2003), and a positive correlation with *V. parahaemolyticus* abundance (Parveen *et al*., 2008). This, notwithstanding, during our study dissolved oxygen varied from a minimum of 5.23 to a maximum of 14.49 mg/L, neither *Vibrio* spp. load nor *V. vulnificus* (total) and *V. parahaemolyticus* (total and potentially pathogenic) prevalence resulted significantly related to this parameter.

Conclusions

Outbreaks of shellfish-associated infection have been reported worldwide, and among them *Vibrio* species lead the list of bacterial pathogens, therefore an understanding of the spatiotemporal dynamics of *Vibrio* population and its potential to cause disease outbreaks has become increasingly important (Oberbeckmann *et al*., 2012; Takemura *et al*., 2014). Moreover, this group of organisms is increasing in abundance as a consequence of environmental perturbations and climate change (Siboni *et al*., 2016). According to the European Environmental Agency, the rise of global sea surface temperature is one of the major physical impacts of climate change, and in coastal European seas it has increased 4-7 times faster over the past few decades than in the global oceans (Reid *et al*., 2011). This local increase in sea surface temperature has been linked to outbreaks of *Vibrio*-associated human illness caused by *V. cholerae* non-O1 and non-O139, *V. parahaemolyticus*, and *V. vulnificus* in several European countries. However, the lack of mandatory notification systems for *Vibrio*-associated illnesses prevents accurate estimates of the number of *Vibrio*-infections occurring in Europe (Le Roux *et al*., 2015). It is clear that for an appropriate risk assessment on *Vibrio*-infections in Europe, robust data on the prevalence of the potentially pathogenic species in seawater and seafood, particularly shellfish, are essential, and the knowledge of the impact of the environmental conditions allowing to their proliferation is of paramount importance to define predictive models.

The assessment of the environmental state of European surface waters comprises the collection and aggregation of a huge amount of information, to provide, among others, a basis for the identification and assessment of environmental threats at Regional and global levels (EEA, 2015). Unfortunately the monitoring of vibrios in the coastal areas is not considered among the EEA indicators, and for the control of the shellfish production areas (Regulation EC 854/2004; European Commission, 2004a) as well, consequently, only few data provided by field researches are currently available.

In this respect, our retrospective multi-year study, the first one realized in Italy, suggests that the prevalence of total *V. parahaemolyticus* and total *V. vulnificus* in clams is positively correlated to the seawater temperature, and their prevalence may be considered threatening to human health, also because the purge of these microorganism, through the purification process applied according to the European legislation in force (Regulation EC 853/2004; European Commission, 2004b), may be considered substantially ineffective (Serratore *et al*., 2014).

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