Catastrophic mass mortality of benthic invertebrates in the NW Mediterranean Sea: do *Vibrio* infections play a role?

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Introduction

In the temperate North-Western (NW) Mediterranean Sea unprecedented and extensive mortality episodes occurred in 1999 and 2003, affecting several species of benthic invertebrates from different phyla (sponges, cnidarians, molluscs, ascidians, bryozoans) on several hundred kilometres of shoreline from the Bay of Genoa in Italy to the Bay of Marseilles in France (Cerrano *et al*, 2000). It is generally agreed that global warming may be linked to the occurrence of such catastrophic events in the Mediterranean Sea (Garrabou *et al*., 2009). The increase in temperature causes high respiratory demand, and secondly, it decreases the amount of food and nutrients available due to 'thermal stratification'. In “long” and “hot” summer, benthic suspension feeders experience a prolonged energetic constraints, which determine a stressed physiological state leading to mortality in late summer and early fall (Coma *et al*, 2009). In this scenario, opportunistic microbial infections are suspected to play a role although this role has yet to be clarified. Using the model coral *Paramuricea clavata*, which is one of the most affected organisms, we addressed the hypothesis that *Vibrio* infections can play a significant role in promoting the occurrence of mass mortality events in the NW Mediterranean Sea, and that the magnitude of this role can be directly linked to climate change variables.

Material and Methods

Microbiological and environmental analysis in the NW Mediterranean Sea

Twenty-two sampling trips were carried out from June 2006 to September 2007 at an experimental marine station in the NW Mediterranean Sea. The most-probable-number technique coupled with standard PCR (MPN-PCR) was used to enumerate the number of culturable *Vibrio* spp. and *V. coralliilyticus* in seawater. Chlorophyll-a in seawater was measured fluorometrically. Average sea surface temperatures from 0-20 m (SST) were also recorded.

Enumeration, isolation and phylogenetic analysis of Vibrio spp.

During mortality episodes occurred at Tavolara island (NW Mediterranean Sea) in October 2008 and in the marine reserves of Portofino (NW Mediterranean Sea) and Capo Mortola (NW Mediterranean Sea) in September 2009, branch fragments of diseased, healthy and recovering *P. clavata* (5 cm length) from different colonies were collected by scuba divers. Crude lysate of coral tissue was directly spread-plated onto thiosulphate citrate-bile salt sucrose agar (TCBS) and bacterial colonies were isolated in pure culture. DNA was extracted from 1 g (wet weight) of coral tissue and

real-time PCR for the enumeration of *Vibrio* spp. was performed using a LightCyler instrument 1.5 (Roche Diagnostics, Mannheim, Germany). More than 100 bacterial isolates were subcultured and identified by phenotypic analysis and sequencing of the 16S rRNA gene.

Infection experiments

For laboratory infection experiments, apical fragments of healthy *P. clavata* primary branches were collected in April 2009 by SCUBA divers at a depth of 35 m in the Marine Protected Area of Portofino (NW Mediterranean Sea) and transferred in laboratory aquaria. In a first set of experiments aimed at evaluating the pathogenic potential of selected *Vibrio* strains, infections were performed under different temperature conditions matching those observed in the environment (18°C, 22°C, 24°C). For each temperature condition, groups of replicate aquaria (20 l) were inoculated with selected *Vibrio* isolates collected during mortality episodes as well as control strains at a final concentration of 10^5CFU ml^{-1}. In addition a second set of experiments aimed at evaluating the minimum infectious dose of pathogenic *Vibrio* strains, a set of aquaria were kept at 22°C and inoculated with different concentrations (form 10^4 to 10^1 CFU l^{-1}) of the experimental isolates. All experiments lasted 15 days.

Results and Discussion

Results from a 16-month *in situ* study at an experimental marine station in the NW Mediterranean Sea showed that the occurrence of *P. clavata* mortality episodes were concomitant to a condition of prolonged high sea surface temperatures, low chlorophyll content and the presence of culturable *Vibrio* spp. in seawater. The analysis of the culturable *Vibrio* community associated to *P. clavata* population during mortality episodes revealed that these bacteria were consistently more abundant in diseased organisms with concentration up to two-fold higher than those found on the healthy corals. The 16S rDNA sequencing and phenotypic characterization of several *Vibrio* isolates associated to diseased and healthy *P. clavata* colonies showed a close homology of the majority of the strains with *V harvey*, *V crassostrea* and *V. coralliilyticus* the latter only identified in diseased organisms. *V. coralliilyticus* was consistently found in association with *P. clavata* and other invertebrates (corals and molluscs) hit by the events both at Tavolara island, Portofino and Capo Mortola in the NW Mediterranean Sea.

Pathogenicity tests performed in aquaria at different temperatures and environmental conditions simulating those observed in the environment during the occurrence of the events (*e.g*. food depletion) showed that representative *Vibrio* strains isolated from diseased *P. clavata* colonies were all able to induce necrosis in a few days (6–8 days). In particular, an isolated strain identified by phenotypic test and multilocus sequence typing as *V. coralliilyticus* showed the highest virulence towards *P. clavata* colonies and satisfied Koch's postulates. Virulence of this strain increased at increasing temperature, in a range of temperature values consistent to those observed in the field. Experiments testing susceptibility to this pathogen by other invertebrate species hit by mortality episodes as well as molecular genotyping of several collected strains are currently ongoing in our laboratory.

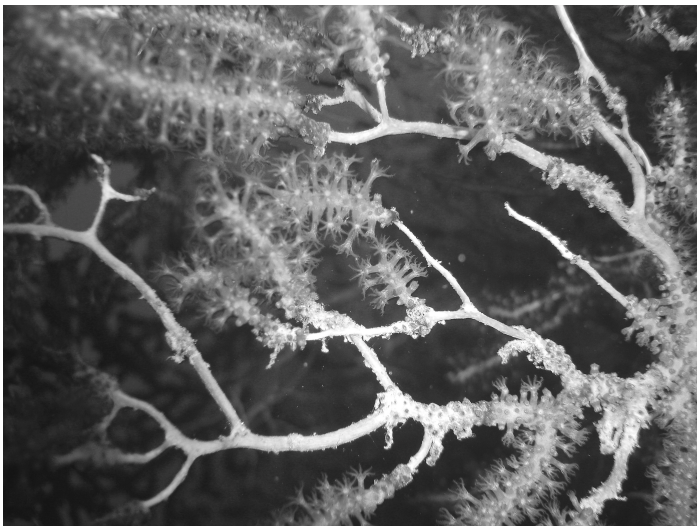

Figure 1: Red sea fan (*Paramuricea clavata*) showing disease signs of patchy tissue loss exposing bare areas of the skeletal axis observed in the North Western Mediterranean sea during mass mortality events

The occurrence of mortality events in the NW Mediterranean reported in this study was restricted to late summer and early fall (e.g. September to October) (Figure 1). Using field data, we observed that during this period, which coincides with the mortality episode, culturable *Vibrio* spp. concentrations were not among the highest recorded in the season. In addition, although microbial pathogens, such as *V. coralliilyticus,* were found in seawater during early autumn, this microorganism was also detected in early and middle summer months, which never experienced the occurrence of mortality episodes. These observations suggest that the occurrence of *Vibrio* linked diseases in natural *P. clavata* populations and the mechanisms regulating their occurrence must be better considered under a multiple etiology perspective of the coral disease. In a conceptual multi-etiology model, based on our data and literature data, temperature and time, which translate into a long and hot summer period, are the primary triggering factors which positively affect *Vibrio* concentrations in seawater and negatively influence food supply (*e.g*. low food due to 'thermal stratification') (Coma *et al*, 2009).

It is concluded that *Vibrio* infections play a role in triggering mass mortality events in the NW Mediterranean Sea and that their severity is likely to increase under a global warming scenario.

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