Presence of *Vibrio mediterranei* associated to major mortality in stabled individuals of *Pinna nobilis* L.

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ABSTRACT

A major epizootic event attributed to *Haplosporidium pinnae* leading to 100% mortality of *Pinna nobilis* L. populations along Mediterranean coastlines started in the fall of 2016. As a result, a project to rescue 221 adult individuals of the endangered pen shell, *Pinna nobilis* was conducted in November 2017 in the two areas of the Spanish coast where the species was still abundant and apparently free from infection by *H. pinnae*: Port Lligat in the Costa Brava, and the Alfacs Bay in the Ebro Delta. For biosecurity reasons, the 106 individuals from the Ebro Delta were stabled at the IRTA facilities located next to Alfacs Bay, whereas the 115 individuals from Port Lligat were stabled in different institutions throughout the Spanish territory. Initial biopsies showed that individuals from the Ebro Delta were free of the parasite, whereas most individuals from Port Lligat were already parasitized and died in the following months. Individuals at IRTA were hold in five tanks and fed ca. 4% of their dry weight with a mix of three species of phytoplankton and fine riverine sediments (13% OM). Seawater was filtered through 10, 5 and 1 µm to ensure the absence of the parasite and disinfected with UV light. No individuals died during the 4 initial months of captivity, but two died in April-May at temperatures from 17 to 19 °C. A peak of mortalities occurred during the summer months and early fall (53%) with maximums coinciding with temperatures above 25 °C. Individuals were again analyzed by PCR and histology for the presence of *H. pinnae*, *Mycobacteria* sp., and other locally important pathogens of commercial bivalves (*Vibrio splendidus*, *V. aestuarianus* and Herpesvirus OsHV-1 microVar), and therefore considered as potential pathogens of pen shells. However, with the exception of 3 individuals that were positive for *Mycobacteria* sp., results were all negative for the studied pathogens. Microbiological culture and isolation of bacteria from three moribund individuals, sacrificed for study purposes, showed *V. mediterranei* as the dominant species, and further PCR analyses confirmed the presence of the bacterium in ten deceased individuals. Overall, our results suggest the *V. mediterranei* is an opportunistic pathogen of stabled individuals possibly subjected to stress from captivity, and

that antibiotic treatment (Florfenicol) combined with vitamins and mineral supplementation and reduction of water temperature (15 to 18 °C), can be used to mitigate (not to eradicate) the disease. Further research is needed to determine diets and stabling conditions that minimize captivity stress and prevent the emergence of the disease.

Keywords: captivity stress; pen shell disease; antibiotic treatment; temperature; vitamins; Mycobacteria

1. Introduction

The populations of the pen shell, *Pinna nobilis*, have been devastated over the last two years associated with the presence of the parasitic protozoan *Haplosporidium pinnae* (Catanese et al., 2018; López-Sanmartín et al., 2019). The disease was first detected in the Spanish Levantine coast and Balearic Islands in autumn 2016, and since then it has spread to most of the Mediterranean countries including France, Italy, Greece, Tunisia, Cyprus, and Turkey (García-March et al., in review). Mortality is considered to be nearly 100% in all affected populations (Vázquez-Luis et al., 2017), with only 4 individuals among a population of thousands currently remaining alive in the Balearic Islands (https://www.observadoresdelmar.es/). In Spain, only the populations of Fangar Bay in the Ebro Delta (several hundred individuals) and that of the Mar Menor in Murcia (several hundred individuals found after the recovery of the lagoon from collapse due to eutrophication; Ruiz and Bernadeu, 2016) have not been infected by the parasite for reasons that are not well understood, but could be related to more restricted water circulation and altered patterns of salinity compared to the open sea (Cerralbo et al., 2019; Cabanellas-Reboredo et al., 2019). Under this critical scenario for the conservation of the species, the Spanish government changed the "vulnerable" conservation status approved by the European Union (Habitats Directive Annex IV EEC 1992 and ASPIM Protocol Annex 2 of the Barcelona Convention) to "critically endangered" (BOE 251-14181). Moreover, the MAPAMA approved in November 2017 a rescue project of 221 pen shells from the Alfacs Bay population (100 individuals plus 6 extra) and from Port Lligat (115 individuals) to be maintained in different institutions. For biosecurity reasons, individuals from Alfacs Bay (showing neither mortality nor *H. pinnae* infection at the time of the rescue) were all hosted at the IRTA institution of Sant Carles de la Ràpita, located in the same area. The remaining 115 pen shells, were transferred to IFAPA (50 individuals), IEO-Murcia (50 individuals), IMEDMAR (10 individuals), and l'Oceanogràfic (5 individuals).

Although some isolated pen shells have been maintained for long periods of time under captive conditions within aquarium exhibits, given their protected status the species has never been stabled in large numbers such as those of commercial stocks. Captive individuals are maintained in stock densities that may significantly differ from those in their natural environment and may be exposed to physiological stress resulting in mortality events associated to depression of the immune response (Dickens et al., 2010). Under the appropriate environmental conditions, pathogen transmission in aquaculture tanks without physical barriers and among densely spaced individual hosts is suggested as a common pattern for outbreaks in aquaculture facilities (Krkošek 2010). In particular, bacteria of the genus *Vibrio* include widespread opportunistic pathogens with high genetic variability and metabolic diversity, which confers to them an elevated potential for colonization (Beaz-Hidalgo et al., 2010; Le Roux et al., 2016). Additionally, individuals may host multiple strains of a given *Vibrio* species that feature significant differences in their degree of virulence, thus complicating the diagnosis and the treatment of the disease (Lane and Birkbeck, 1999; Choquet et al., 2003). *Vibrio mediterranei* (a former synonym of *V. shiloi*) has been recurrently isolated from apparently healthy bivalve populations including clams, oysters, and mussels (Tarazona et al., 2014), without a clear association to disease, although important mortality events have been reported in other marine species such as the coral *Oculina patagonica* (Kushmaro et al., 2001). More recently, Rodríguez et al. (2018a) found that *V. mediterranei* was present in a number of organs and tissues of ailing and dead individuals of *P. nobilis*, but not in those of a healthy individual collected simultaneously. Yet, healthy juvenile individuals collected from the Alfacs Bay and screened for the presence of *V. mediterranei* also showed positive results in 60% of the cases without any apparent disease symptom (KA Andree, unpublished data). The potential virulence of *V. mediterranei* under certain conditions is also suspected after challenge experiments causing mortality in clam (*Ruditapes philippinarum*) and turbot

106 (Rodríguez et al., 2018b), suggesting that similar effects may arise in other host species under
107 adequate conditions (Vattakaven et al., 2006).

108 The presence of *Vibrio* species observed in bivalve populations appears to be strongly
109 associated to environmental characteristics such as high summer water temperatures and low
110 salinity across geographical areas (Paillard et al., 2004; Garnier et al., 2008), as well as to the
111 physiological condition of the host (Pruzzo et al., 2005). Hence, the enhanced ubiquity of *Vibrio*
112 species as etiological agents has been related to climate change and increasing ocean
113 temperatures (Paillard et al., 2004), although a palliative effect of acidification in cases of
114 bacterial infection has also been proposed for bivalve cultures (Prado et al., 2016). Further,
115 physiological stress due to nutritional imbalances, inappropriate food size or other dietary
116 features may also arise in stabled animals, thus impairing host condition and increasing the risk
117 of developing associated pathologies (Pettersen et al., 2010; Ragg et al., 2010). For instance,
118 up to 29 taxa of phytoplankton and several groups of zooplankton including copepods, bivalve
119 and gastropod larvae, and ciliates have been identified from the gut contents of *P. nobilis*
120 (Davenport et al., 2011). However, the implementation of such dietary diversity, possibly
121 optimal for the species, is virtually impossible to reproduce under conditions of captivity, with
122 most bivalve diets being reduced to combinations of 2 or 3 species of phytoplankton (FAO
123 2006).

124 The aim of this study is to describe a disease outbreak occurring in long-term stabled
125 individuals of *P. nobilis* within IRTA facilities (N= 106). Herein, different molecular assays and
126 microbiological isolation approaches/ methods aimed at identifying the putative causative
127 agent of pen shell mortality and the conditions under which the outbreak occurred, are
128 described. Simultaneously, we conducted different prophylactic measures aimed at infection
129 control and improving the overall host condition, and we provide a number of management
130 recommendations based on our experience.

131

2. Materials and Methods

2.1. Capture of individuals and description of collection area

A total of 106 individuals, all of them healthy in appearance (extended mantle and rapid close of the valves), were captured in a shallow area of Alfacs Bay (Fig. 1) at ca. 60-80 cm water depth, in late November 2017 as a part of the MAPAMA rescue project. All individuals were adults, of sizes ranging from 45 to 67 cm shell length. The area was selected based on previous available information about locally large pen shell densities (in the order of hundreds of individuals; Prado et al., 2014), and easiness of access. Since the area of collection was formed by a mosaic of vegetated (*Cymodocea nodosa* with sparse *Caulerpa prolifera*) and non-vegetated habitats, individuals standing on sandy patches were targeted in order to facilitate extraction and minimize the stress of the animal. Each individual was quickly extracted (30 to 60 seconds) by sand excavation with a pressurized water jet from a hose connected to a hydraulic pump located in a support vessel. Groups of 6-8 individuals were placed inside large plastic water containers of 150 L capacity, covered with a lid to avoid the loss of water during the boat trip, and transported to the IRTA dock adjacent to the IRTA wet lab facilities.

Later in January 2018, a total of 46 juvenile pen shells were observed in a shallow (ca. 10 cm depth) sand bar located in the same area of adult extraction and were also moved to indoor facilities, as a precautionary measure against the possible arrival of *H. pinnae*. Once in the lab, they were set within 50 L tanks and assigned different diets aimed at assessing nutritional requirements beyond this study (Prado et al., in prep.). However, mortality was also detected in juvenile tanks and one of the individuals showing disease symptoms was sacrificed for the purpose of bacterial isolation (see later).

2.2. Experimental setup

Individuals were located in five 3,000 L tanks at a density of 20-22 per tank, which provided an average of approx. 150 L for each animal. In each tank, pen shells were placed in an upright

158 position by means of a planar PVC grid structure supported by multiple legs and strings
159 arranged perpendicularly for separation and identification of individuals. All individuals were
160 carefully cleaned of epibionts upon arrival at IRTA with an aluminum scouring pad and this
161 practice repeated when necessary during the acclimatization period in order to minimize the
162 number of undesired organisms that could present competition for food offered to the pen
163 shells and/or present a risk as vectors of pathogens. All tanks were set in a flow through
164 system directly connected with natural sea water pumped from Alfacs Bay. Seawater was
165 filtered through 10, 5 and 1 µm to ensure the absence of the haplosporidian parasite (ca. 2.8
166 µm size according to Darriba, 2017), disinfected with UV light, and then passed through an
167 active carbon filter during the summer months in order to neutralize possible agricultural
168 chemicals present in Alfacs Bay during the rice cultivation period. Stabling conditions were
169 salinity 36-37 ppt, oxygen 5-8 mg/ L, pH 8-8.1, seasonal temperature (from 11.5 to 25.7 °C),
170 natural photoperiod, and variable dissolved nutrients (NO_3: 81.4 ± 12 µmo L^{-1}; NO_2: 2.23 ± 0.4
171 µmo L^{-1}, and NH_4: 3.7 ± 0.8 µmo L^{-1}). Nonetheless, given the significant mortalities registered
172 in the tanks during the month of August 2018 (see later), individuals were transferred to a
173 recirculating aquaculture system (RAS) at approx. 18 °C on the 10th of September, and then
174 moved again to the open water system when the sea temperature reached the same value on
175 the 27th of November. Similarly, when the temperature rose again individuals were transferred
176 again to the RAS at approx. 15 °C (a mortality event was occurring at 17 °C) on the 16th of May
177 2019. Individuals in the open water system were subjected to the natural photoperiod,
178 whereas in the RAS they were exposed to artificial light with a photoperiod fixed at 12: 12
179 (L:D).
180
181 *2.3. Diet formulation*
182 Individuals were fed with a mix of three species of microalgae (*Isochrysis* aff. *galbana* (T-
183 ISO), *Tetraselmis chuii*, and *Chaetoceros calcitrans*) and sediment rich in organic material (OM)

184 (average of 13.5%) throughout the captivity period. These microalgae were supplied as live
185 monocultures from IRTA's hatchery. Daily diet rations per tank consisted on an average of
186 $2.05 \cdot 10^{11}$ cells of T-ISO, $1.91 \cdot 10^{11}$ cells of *C. calcitrans*, and $2.30 \cdot 10^{10}$ cells of *T. chuii*, with 18 g
187 of riverine sediments <200 µm per day, supplied in two doses (morning and afternoon). The
188 dried weight (DW) of the overall daily ration corresponded to approximately 4% of the DW of
189 an adult individual having an average DW of 27 g (50 cm shell length). After each
190 phytoplankton cocktail dose, individuals were allowed to feed for about 3 hours and then the
191 water tap was opened, for progressive water renewal. From December 2019 onward, a
192 dispenser tank with a PVC drain channel was installed above the tanks in order to achieve a
193 more homogeneous food supply throughout the feeding time of 3 h, as before, prior to the
194 opening of the water valve for renovation.
195 The sediment was collected from the Tarragona water consortium (CAT) at the Ametlla del
196 Mar, which depurates sediments from the Ebro River. Sediments were filtered through a 200
197 µm filter and then autoclaved to avoid potential contamination with pathogens. Only the
198 fractions below this grain size were retained for feeding pen shells. Tanks were cleaned weekly
199 to eliminate the excess of sediments and feces.
200
201 *2.4. Analyses for detection of potential pathogens*
202 In mid January 2018, five pen shells chosen at random from each tank (i.e., a total of 25
203 individuals of the 106 collected from the Alfacs Bay) were subjected to a small mantle
204 sampling in order to ensure the absence of *H. pinnae* among captive individuals. Samples were
205 collected by pinching off a small piece of mantle using a 15-cm-long hemostat inserted
206 between semi-opened valves, and preserved in absolute ethanol until later DNA extraction
207 (Andree et al., 2018). In all instances (see later), extraction of DNA was conducted with the
208 Qiagen Blood and Tissue Kit (Qiagen, Valencia, CA, USA), following the manufacturer's
209 instruction and for all samples the A260/280 ratios were examined by spectrophotometry to

evaluate purity and concentration. Since a positive control for *H. pinnae* was unavailable at the moment of sampling we used instead a DNA sample positive for *Bonamia exitiosa* (Haplosporidia) from IRTA collection (Carrasco et al., 2012). The DNA isolated from each individual was subjected to PCR amplification using the generic haplosporidian primers (HAPF1-HAPR3) from (Renault et al., 2000) as indicated by Darriba (2017) for detecting the presence of a haplosporidan parasite in *P. nobilis*.

Analyses conducted on dead pen shells were aimed at the detection of shellfish pathogens associated to shellfish from the Mediterranean region. These included *H. pinnae* (reported in mortality events of *P. nobilis* from different Mediterranean areas by Catanese et al., (2018) and in the Alfacs bay in July 2018 (Carrasco, personal communication)), *Mycobacteria* spp. (found to cause massive pen shell mortality in the Southwestern Italian coast; Carella et al., 2019), and some well-described pathogens of the commercial oyster, *Crassostrea gigas*: *V. splendidus* and *V. aestuarianus*, and Ostreid Herpesvirus microvar (OsHV-1 µvar). These two types of Vibrio species as well as the OsHV-1 µvar have been previously found in commercial bivalves collected in Alfacs Bay and identified as responsible agents of massive mortalities in *C. gigas*, causing important economic losses (Roque et al., 2012; Andree et al., 2014; Carrasco et al., 2017; Carrasco et al., 2018).

Duplicate DNA analyses for *H. pinnae* were run in some of the samples in order to verify the absence of the parasite in those samples. A first set of 20 samples were run at IRTA using the same protocol and primers described above for the initial biopsies. The second larger set of 62 samples was run at the LIMIA, using the specific primers for *H. pinnae* (HPNF3/HPNR3) and PCR conditions described by Catanese et al. (2018) and López-Sanmartín et al. (2019).

For *Mycobacteria* spp. analyses (N= 62 samples) we used specific primers (mycgen-f/ mycgen-r) described by Böddinghaus et al. (1990) and PCR conditions indicated by Carella et al. (2019).

235 The presence of *V. splendidus* and *V. aestuarianus* was investigated simultaneously with a
236 Taqman duplex qPCR assay (N= 20 samples) using the methodology previously described by
237 Saulnier et al. (2017).
238 Finally, potential infection of pen shells by OsHV-1 (N= 20 samples) was assessed using
239 primers specific for the herpes virus DNA polymerase gene (Webb et al., 2007) and conditions
240 described in Carrasco et al. (2017).
241
242 *2.5. Histological analyses*
243 Individuals collected post-mortem (usually no more than 15 h, except for those deceased
244 during the weekend) were removed from their valves and divided in two equal longitudinal
245 sections that included all the different organs (mantle, gill, kidney, digestive gland, and muscle;
246 gonad was not developed during the summer reproductive period). One half was fixed in
247 absolute ethanol for molecular study, and the other half was preserved in Davidson solution
248 and kept for at least 48 h at room temperature. In addition, ailing individuals sacrificed for
249 bacterial isolation purposes were also fixed in Davidson solution for later evaluation.
250 Subsequently, small pieces of the different tissues were dehydrated in ascendant alcohol
251 series, cleared with X-free and embedded in paraplast blocks. Based on previous experience
252 gained from histological evaluation of *H. pinnae* (Catanese et al., 2019) and *Mycobacteria* sp.
253 (Carella et al., 2019) the tissue used for assessing the presence of the parasite was the
254 digestive gland and the surrounding connective tissue. Sections of tissue 3-4 µm thick were cut
255 with a Microm HM330 rotary microtome and stained with Mayer's haematoxylin and eosin
256 (MH&E) for routine light microscopic examination. Some additional sections were stained with
257 Mayer's hematoxylin-VOF (Gutiérrez, 1967) for easy detection of *Haplosporidium pinnae*
258 spores. Ziehl-Neelsen (ZN) staining was also performed for detecting acid-fast bacteria. The
259 slides were examined under a light microscope (Olympus DP20 video camera on an Olympus

BX51microscope) for possible presence of pathogens and detection of structural tissue damage.

2.6. Isolation and identification of bacterial strains

Two adults (9.13 and 9.18) and one juvenile pen shell (juv. 46) displaying severe disease symptoms (highly retracted mantle and slowness to inability in closing the valves, which was not evident in many instances) were sacrificed for the purpose of microbiological evaluation. This sacrifice was necessary to avoid the development of fast-growing opportunistic microorganisms immediately after death, which would have masked the presence of the pathogenic bacteria causing pen shell disease. For each individual, small pieces of the different pen shell tissues (mantle, gill, kidney, digestive gland, and muscle) were homogenized with sterile PBS buffer (ca. 2 g tissue/ 2 ml buffer). For the juvenile individual, which was closed at the moment of death, intervalval water was also obtained. Then, 100 µl of homogenate or intervalval water was used to inoculate one agar plate of Thiosulfate Citrate Bile Salts Sucrose (TCBS) media for the growth of *Vibrio* spp. and another plate with Tryptic Soy Agar (TSA) supplemented with 2.5% NaCl as a less selective media for the isolation of other bacterial genera. After incubation during 24 h at 23°C, Petri dishes were observed for bacterial growth and a total of 19 dominant types of bacterial colonies growing on each medium (colonies 1 to 10 from TCBS and colonies 11 to 19 from TSA) were selected, and all of them purified onto TSA with 2.5% NaCl. After 24 h, pure colonies were cryopreserved at -80°C in Tryptic Soy Broth (TSB) and 15% glycerol to be used for later DNA extraction.

Extraction of DNA was conducted on the 19 isolated colonies that were purified in TSA media. Amplification of the 16S rDNA was achieved utilizing primers 20F and 1500R (Weisburg et al., 1991). The resulting genomic fragment was approximately 1400 bp. Each PCR product was purified using the Qiagen PCR Purification Kit (Qiagen, Valencia, CA, USA), and then sent to a private company for bidirectional sequencing using the same primers as those in the original

amplification (Sistemas Genomicos, Valencia, Spain). The resulting sequence data were edited and trimmed using BioEdit ver 7.0.5.2 (Hall, 1999) to remove terminal primer sequence artifacts and cleaned of any aberrant base-calling before aligning the forward and reverse reads. Each sequence was then subjected to BLAST analysis to determine species identity of DNA products. Fifteen of nineteen reads resulted in the same identity. A phylogenetic tree was inferred from aligned sequence data using MEGA. Among taxa selected for comparison there were multiple Vibrio pathogens from marine organisms chosen. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). A discrete Gamma distribution was used to model evolutionary rate differences among sites (five categories (+G, parameter = 0.3354). The analysis involved 58 nucleotide sequences, each with 554 positions in the final dataset. All positions containing gaps and missing data were eliminated.

Further PCR analyses were conducted on five additional deceased pen shells from each tank (N= 25), aimed at assessing if the previously isolated bacterium was also present in the samples, although this was difficult due to the rapid growth of other bacteria after the death of the individual. Total genomic DNA was purified using the NucleoSpin® DNA tissue extraction kit (Macherey-Nagel) following the manufacturer's instructions a fragment of about 550 bp of the ribosomal 16S DNA gene was amplified using the primers Vib16sF (5'-GAACTGAGACACGGTCCAGACTCCT-3') and Vib16sR (5'- TGAGTTTTACATTTGCGACCGTACTCC -3'). PCR reactions were performed in a total volume of 20 µl containing: 10 µl of Kapa Taq Ready mix (KAPA Biosystems, Sigma), 8.2 µl of sterile water, 0.4 µl of each primer (stock 20 Mmol), and 1 µl of DNA at 50 ng/ µl. The thermal cycler was set with an initial denaturation at 94°C for 5 min, followed by 35 cycles of 94◦C for 30 sec, 62°C for 30 sec and 72°C for 45 sec, and a final extension at 72°C for 3 min. PCR products were separated on 1.5% agarose in TAE 1×buffer gels (w/v), stained with GelRed (Biotium) including a LowRanger 100-bp DNA ladder size standard (Norgen) and visualized on UV transilluminator. All PCR fragments were purified

using a *mi*-gel extraction kit (Metabion) and bi-directionally sequenced using ABI 3130 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA). Obtained sequences were edited and aligned using the BioEdit v7.2.5 software (Hall, 1999) and MEGA 6.0 (Tamura et al., 2013) and from these, the BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) sequence similarity search program was used to identify the species on the basis of the highest score.

2.7. Treatment with hydrogen peroxide

In the absence of positive results for *H. pinnae*, and while awaiting sequencing results from the isolated strains of Vibrio, a hydrogen peroxide treatment was applied to individuals showing disease symptoms during a peak of mortality occurring in August 2018 (see later). This methodology has been successfully used for reducing bacterial loads in adult fish and larvae (e.g., Roque et al., 2010) as an alternative to applying antibiotics. In a preliminary trial conducted on one healthy individual, we adapted the protocol described by Roque et al. (2010) for adult sea bass, consisting of exposure to 100 ppm of H_2O_2 during 30 min. The immediate response was the closing of the valves, with no further opening until the product was removed through water renewal. The individual was monitored during two days and displayed no abnormal behavior suggestive of evidence of toxicity. Hence, the trial was considered as non-noxious for the species and the same procedure was repeated in the entire tank unit housing 10 individuals with disease symptoms. However, since during the addition of the product individuals did not show the same reaction of valve closing, the treatment was reduced to 50 ppm during 30 min as a precaution.

2.8. Antibiotic treatments and supply of vitamins and minerals

An antibiogram was prepared using four antibiotic disks: florfenicol (FFC, 30 mg), oxytetracycline (OT, 30 mg), erythromycin (E, 30 mg), and norfloxacin (NOR, 10 mg). Three different strains of *V. mediterranei* isolated from kidney [IRTA 18-104, IRTA 18-108, IRTA 18-

111] were streaked on three Petri dishes containing Mueler-Hinton agar prepared with 2.5% NaCl. Then, one disk of the four different types of antibiotic was carefully placed onto the agar medium and incubated at 23°C during 24 h and the inhibition zone measured with metric ruler. The antibiotic with the larger inhibition zone (FFC, 30 to 34 mm), was chosen for pen shell treatments using soluble powder of 20% purity (AMPHEM, Pintaluba S.A.).

To determine the MIC, the three chosen strains of *V. mediterranei* were inoculated into 100 ml of Mueler-Hinton broth with 2.5% NaCl and different concentrations of FFC (400, 200, 100, 50, 25, 12.5, and 6.125 ppm). Only the positive control and the 6.125 ppm concentration presented evidence of bacterial growth, but a dose of 30 ppm of FFC was adopted to compensate for potentially lower bioavailability under tank conditions. Antibiotic treatments were conducted accordingly to IRTA internal protocols consisting on 1 h bath exposure (see also Stoskopf, 1993) followed by water renewal during alternate days to minimize possible stress due to changes in water level. This discontinuity in the exposure to the antibiotic may lead to an unsuccessful treatment and to an enhanced risk of developing antibiotic resistance, but we prioritized the well-being of individuals in the short-term. The first antibiotic treatment was conducted in six alternate days in late November after a successful preliminary trial. Further treatments were conducted in January and February 2019, and then again in April 2019, but at these times the antibiotic was supplied on alternate days during an entire month.

From the beginning of December 2018, a multivitamin complex (Multivitaminas Deliplus) containing 13 different types of vitamins and 6 minerals was also supplied at doses of 10 capsules per 20 individuals per day mixed with food rations.

2.9. Data analyses

Monthly trends in mortality were investigated with RM-ANOVA (repeated measures), using tanks as replicates and with no between-subjects effects. Given that most bacterial outbreaks follow a Gauss bell curve with temperature that often fails to provide meaningful insight of

significant patterns with RM analyses, cumulative mortalities were used instead for assessing trends. The LSD post hoc test was conducted to assess significant differences among monthly groupings. Data were also tested for RM-ANOVA assumptions of normality and sphericity. Since the Mauchly's Test of Sphericity indicated that the assumption of sphericity had been violated, a Greenhouse-Geisser correction was used instead.

The existence of a significant association between monthly mortalities and temperature was investigated with linear regression analysis. All analyses were conducted with the SPSS software.

3. Results

3.1. Initial Haplosporidium spp. screening, monthly mortality patterns and molecular results for potential pathogens

All biopsies of mantle tissue conducted during the month of January showed negative results for the presence of *Haplosporidium* sp.

The first individual died on the 30th of April 2018 at approx. 17 °C, but no more deaths occurred until the temperature increased to 19 °C later in May. After this time, mortalities increased to reach a peak during early fall 2018 (i.e., significant association between mortality and temperature; R^2= 0.53; p=0.003). The highest mortality (N= 10) occurred in August (Fig. 2a), after the experimental treatment using 50 ppm of hydroxide peroxide, with disease signs evident in one of the tanks. The treatment had a negative effect on all individuals, which displayed an immediate retraction of the mantle and inability to close the valves, dying within 24-48 hr (although all individuals presenting disease symptoms have ultimately died). Individuals were transferred to tanks under controlled temperature conditions at 18 °C in mid-September, but overall mortality rates did not decrease until November (Fig. 2a) coinciding with decreased temperatures and the treatment with 30 ppm of FFC during 6 alternate days. A minimum was reached later in January-March, also in agreement with lowest annual

390 temperatures and a 4-week treatment with 30 ppm of FFC. It is worth to note that in March,
391 water temperature raised again to 16ºC, similar to the months of November and December,
392 but unlike the previous months, mortality remained at its lowest. Yet, mortalities increased
393 again in April-May 2019 when temperature reached 17 °C and a third FFC treatment (also 4
394 weeks) was conducted, but the outbreak did not subside until individuals were transferred to
395 controlled temperature at 15 °C.
396 RM-ANOVA for cumulative mortality indicated significant time differences along the study
397 period, but with important variability among tanks (Table 1; Fig. 2b). These differences in tank
398 mortality patterns prevented coherent pair-wise post hoc comparisons (LSD method) across
399 study months, but evidenced a contagious process from a given ailing pen shell towards the
400 remaining individuals of the tank.
401 All PCR results were negative for the presence of *H. pinnae* from samples analyzed both at
402 IRTA and LIMIA. Equally, none of the individuals showed a positive result for infection by *V.*
403 *splendidus*, *V. aestuarianus* or OsHV-1, but three individuals from tank 11 that died in April
404 2019 were positive to *Mycobacterium* sp. by PCR. None of the 41 and 4 individuals that died
405 during the summer reproductive period (ca. June to Sept) of 2018 and 2019, respectively,
406 showed any evidence of gonad development.
407
408 *3.2. Histological analyses*
409 Histological examination of the few individuals recently dead (less than an hour) with
410 preserved digestive tissue showed interstitial stroma hyperplasia, possibly a sign of constant
411 lack of a required specific nutrient coupled with a chronic inflammatory condition upon
412 infection (Fig.3a). Moreover, the structure of the digestive gland showed digestive tubules with
413 narrower diameter and wider lumen than usually observed in normal digestive epithelia in an
414 absorptive phase (Catanese et al., 2018). Abundant small, bacilli-like bacteria were observed in
415 the connective tissue surrounding the digestive gland, accompanied with an inflammatory

response characterized by the presence of hemocytes and brown cells (Fig. 3b). In sacrificed individuals (individuals 9.13, 9.18, and juv. 46), the bacterial load was scarce and bacilli-like bacteria were difficult to appreciate within the tissues, although they were also present.

The three individuals from tank 11 that died in April 2019 also evidenced the presence of long, acid-fast positive bacteria (*Mycobacteria* sp.) during histological examination, thus confirming PCR positives. In two of the samples, some *Mycobacteria* sp. were found inside immune cells located in the connective tissue circumscribing the digestive gland, forming inflammatory nodules coupled with brown cells, resembling Carella et al. (2019) description. In the third sample, however, we observed a more advanced stage of infection with *Mycobacteria* sp. both inside inflammatory cells and free in the necrotic debris of the destroyed digestive gland (Fig. 4).

3.3. Identification of bacteria strains from dominant colony types

Bacteria colonies obtained from homogenized tissues of sacrificed individuals and intervalval water (TCBS media for the growth of most *Vibrio* spp.) were 1 mm in size, yellowish, and generally featured smooth, opaque margins, although some variants with irregular and/or translucent margins were also observed.

PCR results of the 19 samples from tissues and intervalval water from the 3 sacrificed individuals were all positive for the amplification of the 16S ribosomal RNA gene of *Vibrio* spp. BLAST analysis showed that 16/19 samples matched *V. mediterranei* strain 224 with 99% identity (accession HF541944 isolated from the bivalve *Donax* sp. in Valencia, Spain), two amplicons were not monospecific and results were incomplete or with overlapping reads, but partial unambiguous sequences were obtained that had high BLAST scores indicating identity with *V. owensii* and *V. harveyi*, and one sequence was identified as *Halomonas venusta* (see Table 2). The twelve longest unambiguous sequences obtained of 1400 bp for *V. mediterranei* have been uploaded to GeneBank under accession numbers: MK471357-MK471368. From

these sequences, 554 bp were used for comparative purposes and aligned with those from extant *Vibrio* spp. available in Genebank. Results from the phylogenetic analysis showed that all *P. nobilis* colonies were clustered in the *V. mediterranei*/ *V. shilonii* clade, whereas *V. harveyi*, *V. owensii* and *Halomonas venusta* were all clustered in separate clades (Fig. 5).

For deceased individuals (N= 25), we obtained a 16S rDNA PCR amplification in all the analyzed samples. Sequence analysis by BLAST revealed that the nucleotide sequences of ten individuals from all tanks were homologous to the 16S rDNA sequence of *Vibrio mediterranei*, showing a similarity of 100%. For other sequences, two showed similarity with nucleotide sequences of species present in GenBank and identified as "uncultured bacteria" (92-100% identity), and thirteen featured more than one species and were impossible to identify.

4. Discussion

The marine bacterium *Vibrio mediterranei* was the dominant species in the three sacrificed pen shells and its presence was also confirmed in ten deceased individuals despite the high bacterial load. Although individuals were possibly subjected to chronic captivity stress, this finding supports the idea that *V. mediterranei* might be an opportunistic pathological agent for *P. nobilis* (Rodríguez et al., 2018a) as well as for other marine species such as manila clam and turbot used as models in challenge experiments (Rodríguez et al., 2018b). During the first year of captivity, the mortality of *P. nobilis* started at water temperatures of 17 to 19 °C and peaked during the summer months when temperatures reached 25-26 °C, which also agrees with reported preferences for the growth of *V. mediterranei* (Vattakaven et al., 2006). Conversely, this seasonal pattern differs from that observed for the pen shell disease caused by *H. pinnae* (Catanese et al., 2018) which is currently spreading throughout the Mediterranean (Cabanellas-Reboredo et al., 2019). A strong seasonal association has also been observed with *H. pinnae*, but pen shell mortality by the parasite can be observed at water temperatures of only 13 °C (García-March et al., in review). Besides, molecular analyses were all negative for

468 the parasite, as well as for other common bivalve pathogens such as *V. splendidus* and *V.*
469 *aestuarianus*, and Ostreid Herpesvirus microvar (OsHV-1 µvar) (Roque et al., 2012; Andree et
470 al., 2014; Carrasco et al., 2017). Only three individuals were positive for the same strain of
471 *Mycobacterium* sp. recently reported associated to pen shell mortalities in the Tyrrhenian
472 coastline of Italy (Carella et al., 2019), suggesting that it was neither the cause of observed
473 mortalities.

474 Strains of *V. mediterranei* have been reported from multiple hosts (mussels, oysters, clams,
475 coral, fish and plankton) and habitats (seawater and sediment) across the Mediterranean,
476 Atlantic and Pacific (Tarazona et al., 2014) demonstrating that it is a widespread marine
477 bacterium, although the possible pathogenicity has not been clearly indicated. The species was
478 first described by Pujalte and Garay (1986) from seawater, plankton and sediment samples
479 from coastal areas in Valencia, Spain. Later, Kushmaro et al., (2001) observed bleaching
480 disease of the coral *Oculina patagonica* and indicated *Vibrio shiloi* as a causative agent, but this
481 species has been shown to be a synonym *V. mediterranei* based on an identical 16S sequence
482 and additional molecular analyses (Thompson et al., 2001; Tarazona et al., 2014). The
483 pathogenicity of *V. shiloi* has also been discussed and proposed as an opportunistic outbreak
484 following undetermined stress conditions (Ainsworth et al., 2008), as observed here for captive
485 individuals of *P. nobilis*.

486 Krkošek (2010) conducted a review on disease control in farmed and wild target species
487 and pointed out that sudden disease outbreaks may be linked to aquaculture conditions
488 exceeding host density thresholds, and that temperature and salinity are likely to affect these
489 threshold values. In this direction, our results suggest that stabling *P. nobilis* in large groups of
490 20-22 individuals per tank is not an adequate unit size to manage disease transmission and to
491 control possible outbreaks. Temperature appeared to be a key factor determining the intensity
492 of the outbreak, which peaked at summer temperatures above 25 °C (as indicated for the
493 optimum growth of *V. mediterranei* and breaking point during challenge experiments; see

494 Vattakaven et al., 2006; Rodríguez et al., 2018b) and showed winter minimums at identical
495 host densities. Yet, some low mortalities (ca. 1 individual per month), were also observed in
496 the winter of the second year in spite of antibiotic treatments and low temperatures,
497 suggesting that increased stabling time may affect the condition of individuals and their
498 capacity to fight disease. Among possible factors triggering captivity stress, differences in diet
499 quality and composition are central factors determining the overall condition of individuals
500 (Pettersen et al., 2010, Ragg et al., 2010). Pen shells displayed negligible growth rates of during
501 their captivity period (ca. 0 to 0.1 mm length), and significantly lower rates (by ca. 40%) have
502 been also observed in captive juveniles compared to those in the field (P Prado, unpublished
503 data). Although diet acceptance throughout the stabling period was considered adequate, a
504 deficiency in some nutritional component, compared to higher diet diversity in the wild
505 (Davenport et al., 2011) cannot be discarded, and could have favored the outbreak of *V.*
506 *mediterranei*.

507 Based on the 16S ribosomal RNA gene, the majority of the 16 bacterial sequences isolated
508 from *V. mediterranei* strains cultured from *P. nobilis* showed a 99% coincidence with the strain
509 224 from Tarazona et al. (2014), but with minor differences attributable to SNPs located in 5
510 loci (data not shown). Although sequences were obtained from only 3 different individual pen
511 shells, given the observed variability of 16S sequences and the possible influence of strain
512 types, the presence of possible additive/ synergistic effects on the degree of pathogenicity
513 among strains cannot be discarded (see Gay et al. 2004) and would require further study. In
514 this context, the *V. mediterranei* strain 224 (Tarazona et al., 2014) may have undergone
515 evolutionary changes since its isolation from clams (*Donax* sp.) in 1986, providing a broader
516 host range or changes in virulence, as already indicated for the increasing persistence of other
517 bacterial diseases (Pulkkinen et al., 2009). In fact, virulence severity can be altered with small
518 changes in DNA sequences, as indicated by the elimination of virulence in one Vibrio strain
519 following the deletion of a single nucleotide (Le Roux et al., 2016). Some emerging genotypes

could also be more virulent and successful, as suggested by preliminary information from phylogenomic analysis in *V. aestuarianus* showing that the functional units of pathogenesis are clones, whereas healthy organisms (different species of bivalves and zooplankton) host more variable strains (Goudenège et al., 2015; Le Roux et al., 2016). In addition of this intraspecific variability in genetic traits, the presence of a more complex microbial consortium with other species of bacteria has also been proposed for disease dynamics in bivalves. For instance, Le Roux et al. (2016) injected oysters with a virulent strain of *V. crassostreae* alone or in combination with a non-virulent strain of *Vibrio* sp. or *Shewanella* sp., and observed that when the virulent strain was supplied in a low dose with a high dose of non-virulent strains mortality rates were markedly increased. Our results from cultures of ailing individuals demonstrated that the main bacterial species was *V. mediterranei*, which was also present in a significant number of deceased individuals. However, other species were also present in lesser dominance in petri dishes with TCBS and TSA media, which could be involved in the pathogenesis of the disease. Further research is needed to investigate the pen shell microbiome, in order to determine whether the potential unit of pathogenesis is a group of different species, a consortium of strains of *V. mediterranei*, or a single clone.

In terms of management of the disease outbreak, the long-term maintenance of individuals in captivity conditions proved to be an arduous task. The strong association between mortality and increasing temperature indicates that closed circuits of water recirculation are a necessary facility to host individuals of *P. nobilis*. Overall, mortality during months or periods with water temperature from 15 to 18 °C was ca. 27 and 1.5 times lower, respectively in 2018 and 2019, than in months with higher water temperature. Such enhanced mortality during colder months of 2019 also suggests there is a contribution from chronic stress, which results in pathology when some physiological and behavioral processes are pushed beyond the normal capacity and become dysregulated (Dickens et al., 2010). For instance, exposure to chronic stress has been indicated to reduce the immune system in terms of hemocytes' concentration and

546 phagocytic activity in mussels (Bussell et al., 2008) and abalone (Hooper et al., 2007). Besides,
547 it can lead to disruption of the reproductive behavior (Berga, 2008) as observed with pen shells
548 failing to attain seasonal maturation. Although the addition of antibiotic (FFC) contributed to
549 minimizing the magnitude of the outbreak, *V. mediterranei* appears to remain latent within
550 individuals until optimal temperature conditions arrive or host condition becomes
551 deteriorated, as suggested by the persistence of 14 individuals that remain alive at our
552 facilities. The presence of multiple strains with different degrees of sensitivity to FFC could be
553 a plausible explanation for this persistence. For instance, Molina-Aja et al. (2002) tested
554 antibiotic resistance of *Vibrio* strains isolated from cultured penaeid shrimp and found that
555 more than 70% of the strains analyzed were resistant. Since the 16 different strains of *V.*
556 *mediterranei* isolated from only three individuals of *P. nobilis* had different 16S genotypes,
557 persistent mortalities suggest that among strains present there could exist some with certain
558 resistance to FFC and that alternation with other antibiotics or prophylactic measures such as
559 probiotic treatments (Kim et al., 2014) could be useful for controlling the disease. In contrast,
560 the use of hydrogen peroxide appeared to be a counterproductive treatment, causing acute
561 toxicity in pen shells even at lower doses than those used for fish species (Roque et al., 2010).
562 To conclude, *V. mediterranei* appears to be an important opportunistic emerging pathogen
563 in captive individuals of *P. nobilis* possibly associated to long-term captivity stress, whereas the
564 natural population of the Alfacs Bay has been shown to carry the bacteria without obvious
565 symptoms (KB Andree, unpublished data). Since *V. mediterranei* is a widespread marine
566 bacteria (Tarazona et al., 2014), likely to be present in pen shells from other Mediterranean
567 regions, maintenance of individuals in captivity should always involve the use of controlled
568 temperature conditions (15 to 18°C) and low stocking densities within tanks. These measures
569 should aid disease prevention by reducing adverse conditions that promote bacterial growth
570 (Vattakaven et al., 2006) and disease transmission. If, nonetheless, disease finally arises in
571 some tank units, the use of FFC can help to minimize mortalities, although the bacteria are

572 likely to remain latent in the other exposed individuals. Further research is needed to explore
573 the genetic diversity of *V. mediterranei* and to assess the effectiveness of a wide spectrum of
574 antibiotics as well as other alternative prophylactic treatments against the different strains.
575 Finally, it is necessary to establish husbandry protocols which better allow *ex situ* preservation
576 of this valuable species currently under a major threat of extinction.
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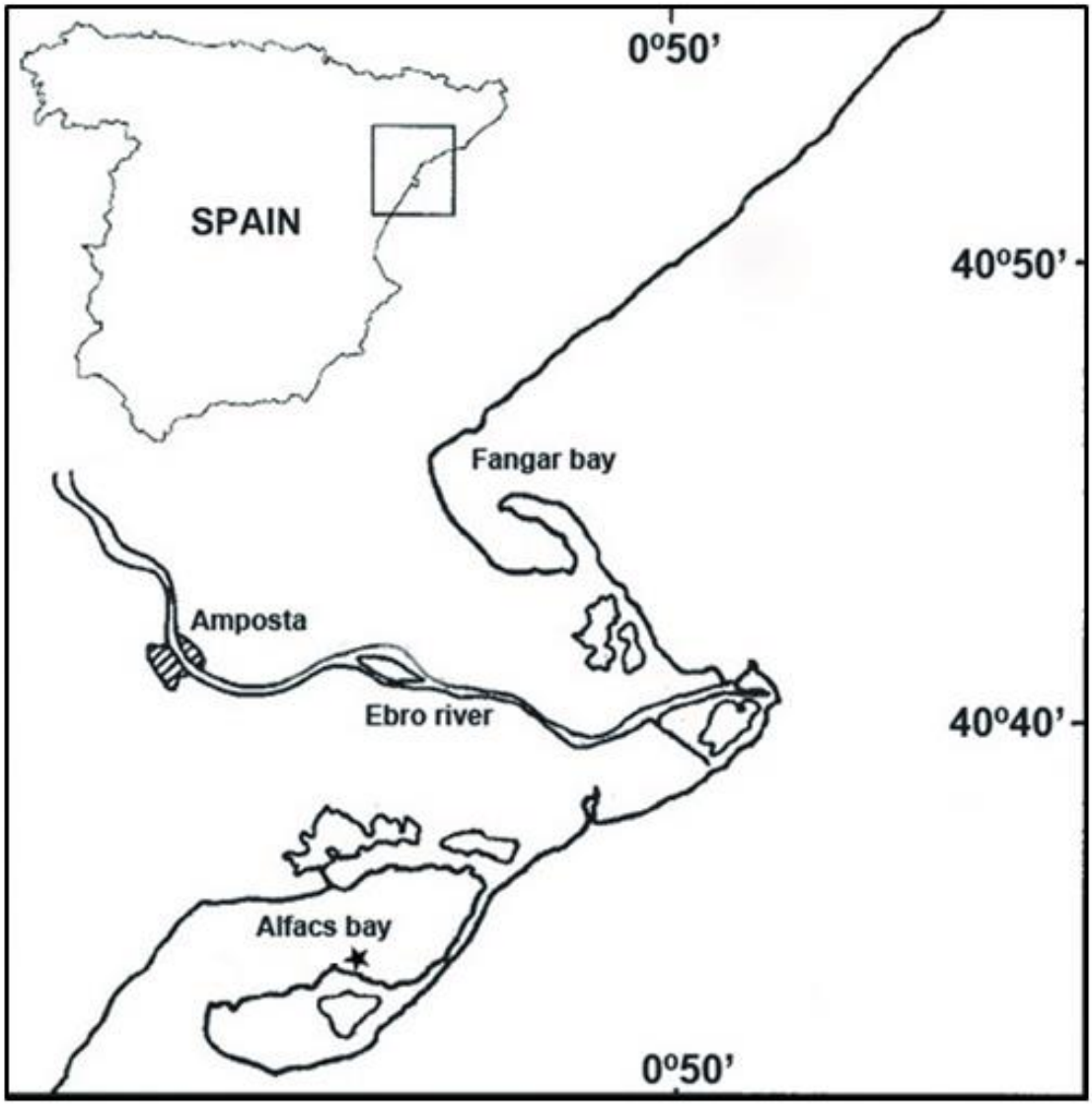
Fig. 1. Map of the Ebro Delta (NW Mediterranean) showing the collection site (★) of *P. nobilis* individuals in the Alfacs Bay.

Fig. 2. (A) Monthly patterns of pen shell mortality per tank. Patterns of temperature (Mean ± SE), and treatments with hydrogen peroxide, antibiotic (30 ppm FFC) and vitamins are also indicated. (B) Monthly patterns of cumulative mortality per tank during the study.

Fig 3. Histological sections of the digestive gland of captive pen shells. A) Aspect of the digestive gland area at low magnification, showing empty digestive tubules (DT) with wide lumen and abundant hypertrophic connective stroma (S). B) Higher magnification micrograph showing the presence of bacteria (arrowheads) within hemolymph vessels (V) of the connective tissue (CT) surrounding the digestive gland of death pen shells. Abundant hemocytes (*) coupled with brown cells (BC) can be observed. A-B) MH&E staining.

Fig. 4. Presence of acid-fast positive bacteria (arrowheads) free in the debris of the digestive gland and within intact immune cells. Ziehl-Neelsen staining.

Fig 5. Phylogenetic tree inferred from 16S rDNA sequences showing the molecular placement of bacterial sequences isolated from *P. nobilis* individuals. The maximum-likelihood method was used with the highest log likelihood (-1460.6859). Bootstrap values are shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.


0°50'
SPAIN
40°50'
Fangar bay
Amposta
Ebro river
40°40'
Alfacs bay
0°50'

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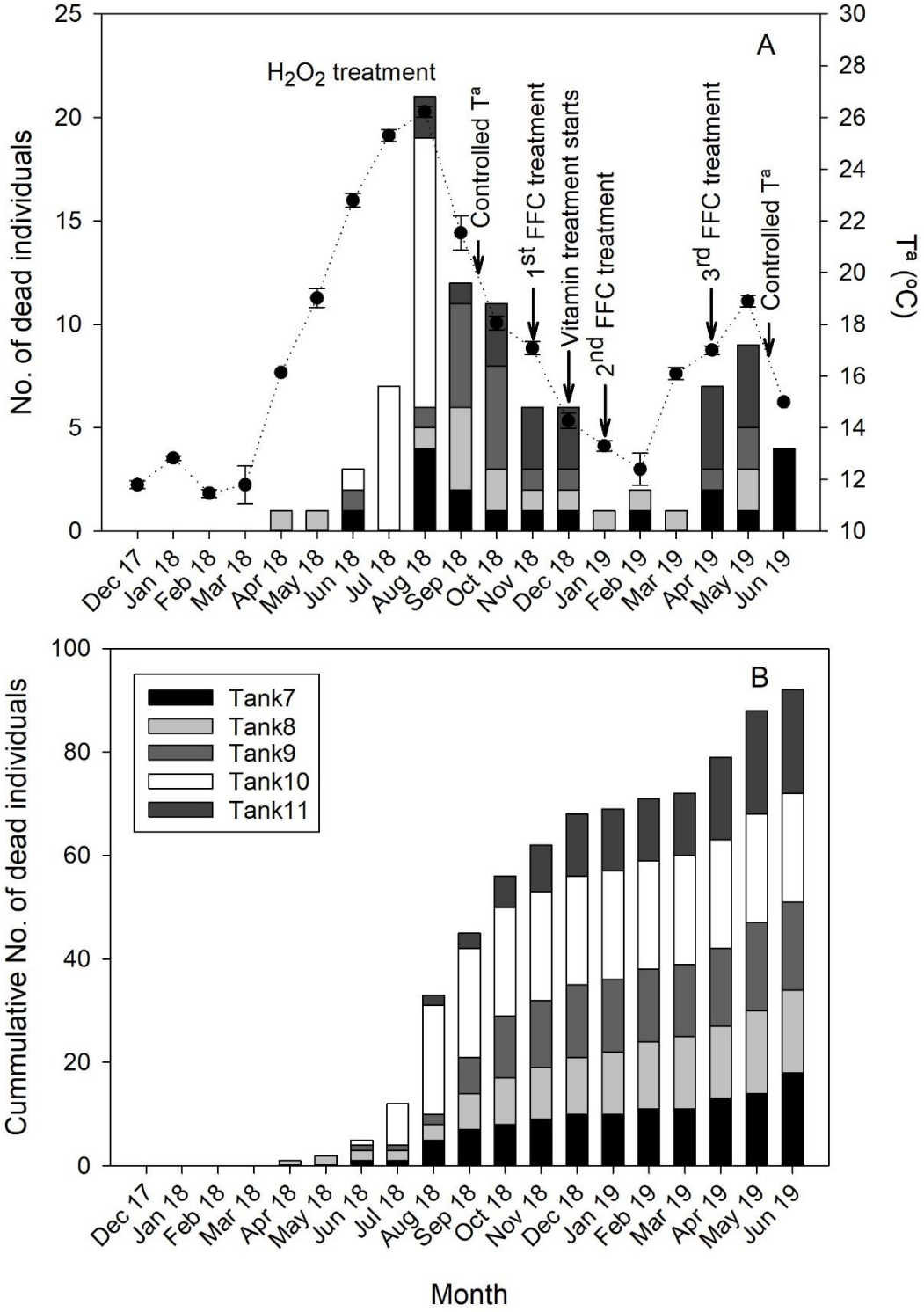
A
H_2O_2 treatment
Controlled T^a
1st FFC treatment
Vitamin treatment starts
2nd FFC treatment
3rd FFC treatment
Controlled T^a
No. of dead individuals
T_a (°C)
B
Tank7
Tank8
Tank9
Tank10
Tank11
Cummulative No. of dead individuals
Month
Dec 17
Jan 18
Feb 18
Mar 18
Apr 18
May 18
Jun 18
Jul 18
Aug 18
Sep 18
Oct 18
Nov 18
Dec 18
Jan 19
Feb 19
Mar 19
Apr 19
May 19
Jun 19

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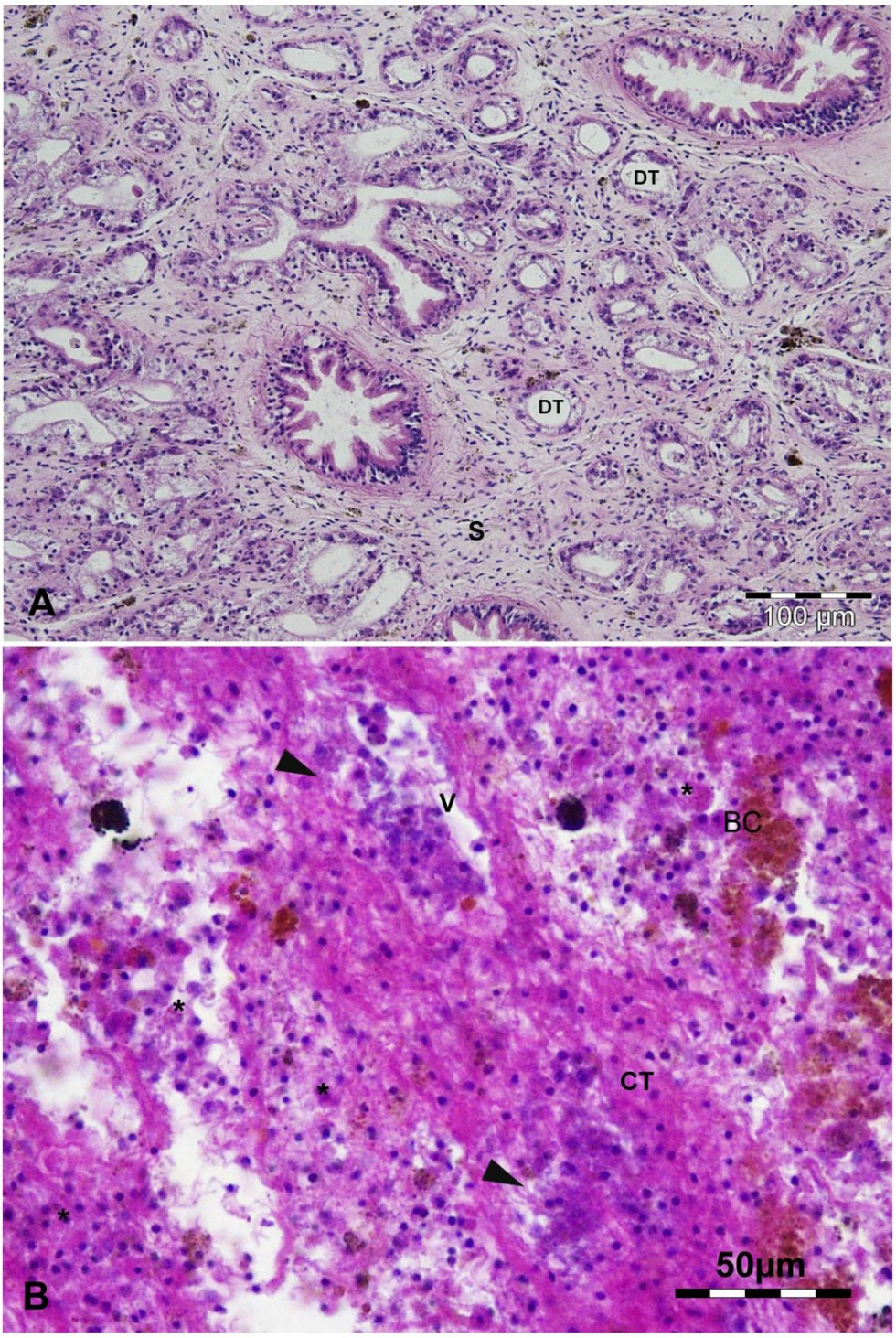

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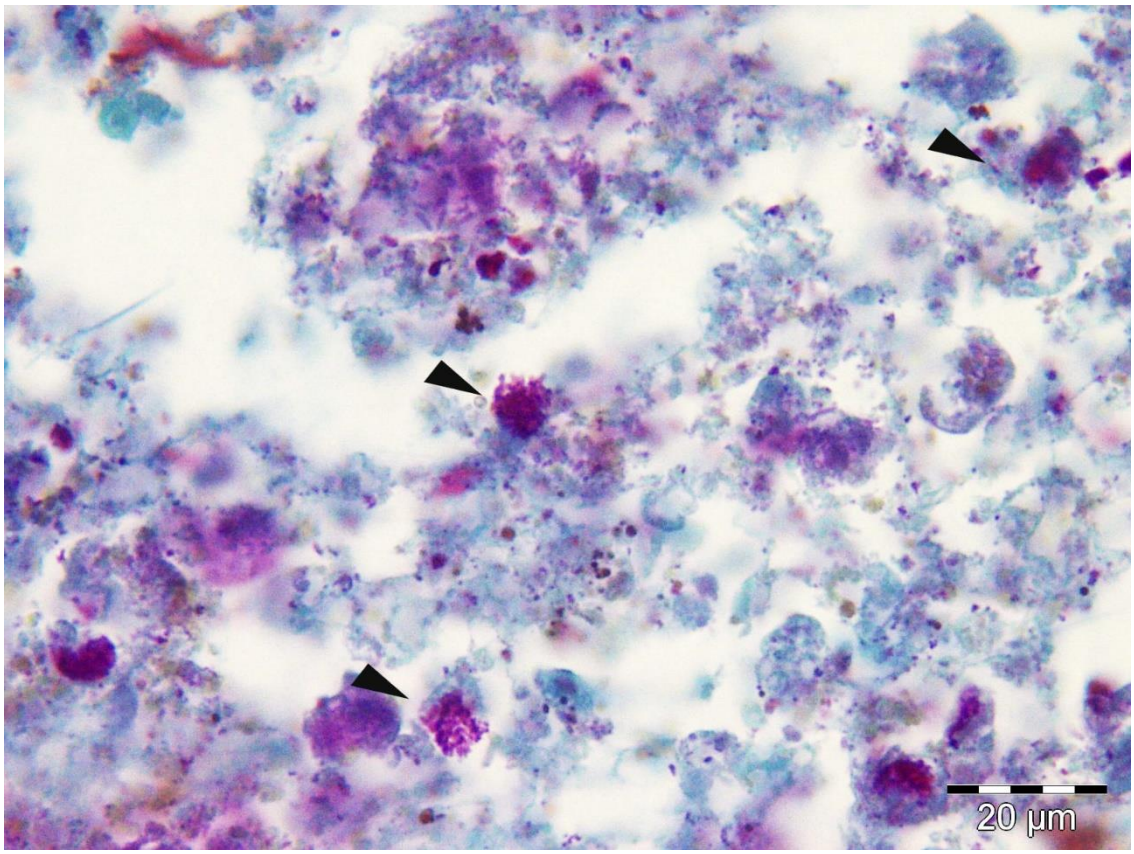
788 

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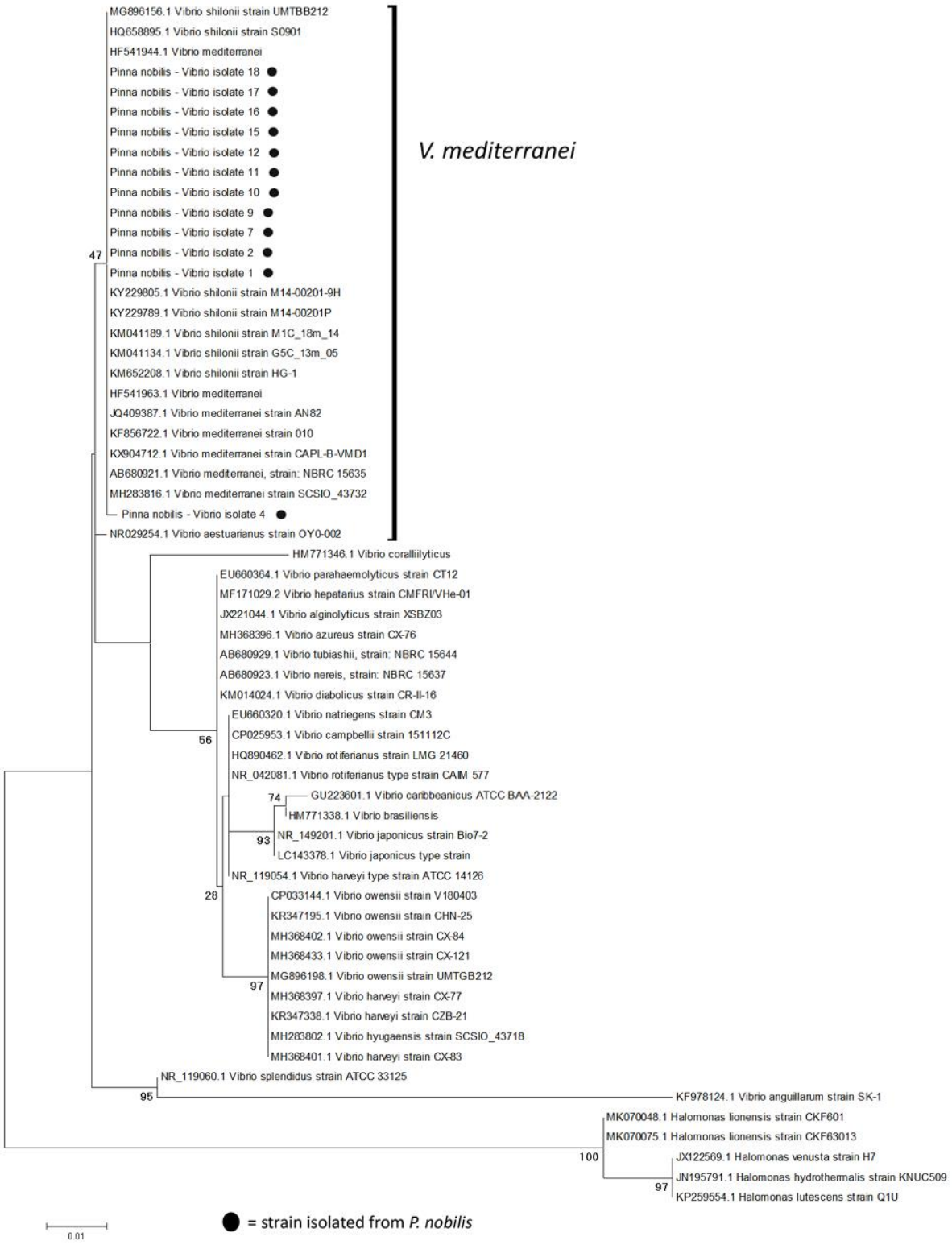
MG896156.1 Vibrio shilonii strain UMTBB212
HQ658895.1 Vibrio shilonii strain S0901
HF541944.1 Vibrio mediterranei
Pinna nobilis - Vibrio isolate 18
Pinna nobilis - Vibrio isolate 17
Pinna nobilis - Vibrio isolate 16
Pinna nobilis - Vibrio isolate 15
Pinna nobilis - Vibrio isolate 12
Pinna nobilis - Vibrio isolate 11
Pinna nobilis - Vibrio isolate 10
Pinna nobilis - Vibrio isolate 9
Pinna nobilis - Vibrio isolate 7
47
Pinna nobilis - Vibrio isolate 2
Pinna nobilis - Vibrio isolate 1
KY229805.1 Vibrio shilonii strain M14-00201-9H
KY229789.1 Vibrio shilonii strain M14-00201P
KM041189.1 Vibrio shilonii strain M1C_18m_14
KM041134.1 Vibrio shilonii strain G5C_13m_05
KM652208.1 Vibrio shilonii strain HG-1
HF541963.1 Vibrio mediterranei
JQ409387.1 Vibrio mediterranei strain AN82
KF856722.1 Vibrio mediterranei strain 010
KX904712.1 Vibrio mediterranei strain CAPL-B-VMD1
AB680921.1 Vibrio mediterranei, strain: NBRC 15635
MH283816.1 Vibrio mediterranei strain SCSIO_43732
Pinna nobilis - Vibrio isolate 4
NR029254.1 Vibrio aestuarianus strain OY0-002
V. mediterranei
HM771346.1 Vibrio coralliilyticus
EU660364.1 Vibrio parahaemolyticus strain CT12
MF171029.2 Vibrio hepatarius strain CMFRI/VHe-01
JX221044.1 Vibrio alginolyticus strain XSBZ03
MH368396.1 Vibrio azureus strain CX-76
AB680929.1 Vibrio tubiashii, strain: NBRC 15644
AB680923.1 Vibrio nereis, strain: NBRC 15637
KM014024.1 Vibrio diabolicus strain CR-II-16
EU660320.1 Vibrio natriegens strain CM3
56
CP025953.1 Vibrio campbellii strain 151112C
HQ890462.1 Vibrio rotiferianus strain LMG 21460
NR_042081.1 Vibrio rotiferianus type strain CAIM 577
74
GU223601.1 Vibrio caribbeanicus ATCC BAA-2122
HM771338.1 Vibrio brasiliensis
93
NR_149201.1 Vibrio japonicus strain Bio7-2
LC143378.1 Vibrio japonicus type strain
NR_119054.1 Vibrio harveyi type strain ATCC 14126
28
CP033144.1 Vibrio owensii strain V180403
KR347195.1 Vibrio owensii strain CHN-25
MH368402.1 Vibrio owensii strain CX-84
MH368433.1 Vibrio owensii strain CX-121
97
MG896198.1 Vibrio owensii strain UMTGB212
MH368397.1 Vibrio harveyi strain CX-77
KR347338.1 Vibrio harveyi strain CZB-21
MH283802.1 Vibrio hyugaensis strain SCSIO_43718
MH368401.1 Vibrio harveyi strain CX-83
NR_119060.1 Vibrio splendidus strain ATCC 33125
95
KF978124.1 Vibrio anguillarum strain SK-1
MK070048.1 Halomonas lionensis strain CKF601
MK070075.1 Halomonas lionensis strain CKF63013
100
JX122569.1 Halomonas venusta strain H7
97
JN195791.1 Halomonas hydrothermalis strain KNUC509
KP259554.1 Halomonas lutescens strain Q1U
0.01
= strain isolated from P. nobilis

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794 **Table 1.** Results of RM-ANOVA showing time effects (within subjects) for monthly patterns of
795 pen shells' cumulative mortality during the holding period. Since the Sphericity assumption
796 was not meet, the Greenhouse-Geisser correction was used. Significant results are indicated in
797 **bold**.

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Greenhouse-Geisser	Type III SS	df	Mean Square	F	*P*	
Time	4440.50	1.37	3241.41	31.34	**0.001**	
Error (Time)	5066.65	5.48	103.40			
Error (Tanks)	555.74	4	138.93			

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Table 2. Summary of BLAST results obtained for each of the 19 isolated colonies from the three sacrificed individuals of *P. nobilis*. The 16 samples of *V. mediterranei* matched the strain 224 (HF541944 isolated from the bivalve *Donax* sp. in Valencia, Spain) with 99% identity.

Sequence No.	Departure medium	Origin	Sequence Results	BLAST
1	TSA	Indiv. 1, tissue homogenate	OK	*V. mediterranei*
2	TSA	Indiv. 1, tissue homogenate	OK	*V. mediterranei*
3	TSA	Indiv. 1, tissue homogenate	mixed amplicon	*V. owensii*
4	TSA	Indiv. 1, intervalvar water	OK	*V. mediterranei*
5	TSA	Indiv. 2, tissue homogenate	incomplete	*V. mediterranei*
6	TSA	Indiv. 2, tissue homogenate	incomplete	*V. mediterranei*
7	TSA	Indiv. 2, tissue homogenate	OK	*V. mediterranei*
8	TSA	Indiv. 3, tissue homogenate	incomplete	*Halomonas venusta*
9	TSA	Indiv. 3, tissue homogenate	OK	*V. mediterranei*
10	TSA	Indiv. 3, tissue homogenate	OK	*V. mediterranei*
11	TCBS	Indiv. 1, tissue homogenate	OK	*V. mediterranei*
12	TCBS	Indiv. 1, tissue homogenate	OK	*V. mediterranei*
13	TCBS	Indiv. 1, tissue homogenate	OK	*V. owensii/ V. harveyi*
14	TCBS	Indiv. 1, intervalvar water	incomplete	*V. mediterranei*
15	TCBS	Indiv. 2, tissue homogenate	OK	*V. mediterranei*
16	TCBS	Indiv. 2, tissue homogenate	OK	*V. mediterranei*
17	TCBS	Indiv. 2, tissue homogenate	OK	*V. mediterranei*
18	TCBS	Indiv. 3, tissue homogenate	OK	*V. mediterranei*
19	TCBS	Indiv. 3, tissue homogenate	OK	*V. mediterranei*