Host-microbiota interactions shed light on mortality events in the striped venus clam *Chamelea gallina*

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Abstract
Mass mortalities due to disease outbreaks have recently affected a number of major taxa in marine ecosystems. Climate- and pollution-induced stress may compromise host immune defenses, increasing the risk of opportunistic diseases. Despite growing evidence that mass mortality events affecting marine species worldwide are strongly influenced by the interplay of numerous environmental factors, the reductionist approaches most frequently used to investigate these factors hindered the interpretation of these multifactorial pathologies. In this study, we propose a broader approach based on the combination of RNA-sequencing and 16S microbiota analyses to decipher the factors underlying mass mortality in the striped venus clam, *Chamelea gallina*, along the Adriatic coast. On one hand, gene expression profiling and functional analyses of microbial communities showed the over-expression of several genes and molecular pathways involved in xenobiotic metabolism, suggesting potential chemical contamination in mortality sites. On the other hand, the down-regulation of several genes involved in immune and stress response, and the over-representation of opportunistic pathogens such as *Vibrio* and *Photobacterium* spp. indicates that these microbial species may take advantage of compromised host immune pathways and defense mechanisms that are potentially affected by chemical exposure, resulting in periodic mortality events. We propose the application of our approach to interpret and anticipate the risks inherent in the combined effects of pollutants and microbes on marine animals in today’s rapidly changing environment.

Keywords
bivalve, host-microbiota interactions, mass mortality, molluscs, pollutant-pathogen interactions, transcriptomics
1 | INTRODUCTION

For decades, the study of mortality events affecting marine species has been restricted to simplified experimental plans focused on investigating the response to environmental stressors through biomarkers or gene expression analyses. While recent technological advances (e.g., next-generation sequencing) have made it possible to obtain increasingly relevant information regarding the response to various environmental factors, even in nonmodel species, the interactions between hosts, their associated microorganisms and biotic and abiotic environmental factors have been minimally explored. These reductionist approaches have led to difficulties in deciphering multifactorial pathologies and mass mortality events affecting nonmodel species worldwide (de Lorgeril et al., 2018).

Currently, there remains a lot to elucidate about how the host and its microbial component can cooperate or interact in response to various environmental factors, how these interactions can undergo changes due to anthropogenic environmental stress and finally how infections can arise from opportunistic pathogens following a series of changes in both the host and its environment. This lack of knowledge concerns in particular nonmodel species of ecological and/or economic interest as they are increasingly affected by recurrent mass mortalities. The objective of the present study was to investigate mass mortality events occurring in the striped venus clam, Chamelea gallina, by applying a holistic approach able to concomitantly characterize host transcriptional profiles and the dynamics of their associated microbiota. The striped venus clam C. gallina (Linnaeus, 1758) is a bivalve venerid mollusk distributed throughout the Mediterranean and Black Sea (Moschino & Marin, 2006), inhabiting sandy bottoms from the lower shore to depths of approximately 15 m (Morello, Froglia, Atkinson, & Moore, 2005). Commercial landings of C. gallina have an important economic role along the central and northern Adriatic coasts of Italy (Moschino & Marin, 2006; Ramon & Richardson, 1992), and consumer interest in this clam species has recently increased (Orban et al., 2006). The number of natural beds of the striped venus clam in the Adriatic Sea has significantly declined over the last decade due to unexplained mortality events, forcing national and regional authorities to introduce new regulations to preserve juveniles by forbidding the fishery of clams less than 25 mm in diameter. Multiple factors have been proposed to be involved in venus clam mass mortalities (Del Piero, Piero, Fornaroli, & Balzo, 1998; Froglia, 2000), such as pollution (Visciano et al., 2015), alterations in water temperature and salinity (Ezgeta-Balić et al., 2011; Matozzo et al., 2012; Monari, Foschi, Rosmini, Marin, & Serrazanetti, 2011; Sobral & Widdows, 1997), summer phytoplankton blooms (Romanelli, Cordisco, & Giovanardi, 2009), increased predation, fishery pressure (Moschino, Deppieri, & Marin, 2003) and infectious diseases (Milan, Matozzo, et al., 2016; Milan, Palazzo, et al., 2016; Torresi et al., 2011). Among the most affected sites, the Abruzzo coast has experienced significant fluctuations in fishing yields over the last six years and has observed a severe demographic decline of the locally available C. gallina stock. A first study (Milan, Matozzo, et al., 2016; Milan, Palazzo, et al., 2016) focused on comparing gene expression profiles of C. gallina collected at different sampling times (from June 2014 to September 2014) along the Abruzzo coast in a control site (T7; Francavilla) and in a site subjected to mortality events (T4; San Vito) through a DNA microarray approach. This study highlighted (a) similar chemical concentrations of heavy metals, polycyclic aromatic hydrocarbons (PAHs) and pesticides (OCPs, OPPs) in sediments between investigated sites, suggesting that chemical pollution was not the singular primary cause of differences in mortality observed between sites T4 and T7, although Artemia franciscana toxicity tests showed higher mortality in T4; (b) consistent up-regulation of several genes involved in immune response at all sampling times; (c) reduced energy metabolism in T4 clams, likely related to metabolic depression affecting the energy budget available for immune response. The substantial number of overexpressed genes involved in the immune response strongly supported the hypothesis of a bacterial and/or viral infection as the major cause of clam mortality in T4 (Milan, Matozzo, et al., 2016; Milan, Palazzo, et al., 2016). Additional stressors such as transitory episodes of acute chemical contamination or reduced salinity could not be excluded as possible factors contributing to increased susceptibility to infection. Since the summer of 2015, a dramatic reduction in landings was also reported in Francavilla (T7) by fishing cooperatives (mainly affecting juvenile individuals), resulting in a severe stock reduction that raised the concerns of fishermen and control authorities (personal communications by fishermen and control authorities).

Here, a novel approach combining the microbiome characterization (16S) and gene expression profiles (RNA-seq) has been applied to this case study. This holistic approach allows us to shed light on the potential stressors involved in mortality events, demonstrating the potential of this approach to investigate the responses of marine species to environmental stressors and to prevent risks associated with pollutant-pathogen interactions.

2 | MATERIAL AND METHODS

2.1 | Sampling of Chamelea gallina

Chamelea gallina specimens were collected from the following three sites: T7 (near Francavilla; 42°16’59”N, 14°29’41.6”E), T4 (near San Vito; 42°25’37.8”N 14°17’28.8”E) and Cupra Grottamare (CG; 43°2’3.026”N 13°51’53.894”E; Figure 1) considered as a “control” site. Sampling was performed at 0.25 mi (ca. 0.4 km) from the coast in collaboration with local fishing cooperatives. No dead C. gallina individuals were found in the CG control site, while in T4 and T7 sites, it was extremely difficult to find live individuals. Individuals were collected at commercial size (min. 25 mm) by hydraulic dredge. All individuals considered for microbiological, gene expression and microbiota analyses appeared to be in good health as confirmed by histological analyses (see below), with no apparent differences in reproductive status between the control site and sites subject to mortalities. The sampling took place in July (T4 and CG) and September (T4, T7 and CG) 2016. After fishing, clams were kept on ice until their
arrival at the laboratory, within 6 hr. At each sampling site and time, the digestive gland of approximately 80 clams was dissected, frozen in liquid nitrogen and stored at −20°C for gene expression and microbiota analyses. In addition, approximately 100 clams for each sampling site/time were transferred to the Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe) for microbiological analyses. All sample sites, sampling times and analyses performed are summarized in Table S1.

2.2 | Histological, microbiological and electron microscope analyses

The whole soft tissue of 30 specimens for each sampling time and site was fixed in Carson's solution for 48 hr. The sample was then reduced to obtain transverse whole-body sections. The tissue was dehydrated and embedded in paraffin, and 3 µm sections were stained with Harri's haematoxylin and eosin-phloxine. For each sample, a pool of 50 g (about 15 clams) containing flesh and intravascular liquid was transferred to a stomacher bag, then homogenized for 1 min. From the homogenate, 50 µl was directly plated in duplicate on marine salt agar for the total viable bacteria count, and another 50 µl was plated on thiosulfate-citrate-bile-sucrose (TCBS). Random colonies of Vibrio spp. from TCBS plates were identified to the species level through MALDI-TOF mass spectrometry. To do so, first, the plates were incubated at 22°C for 4 days. Preliminary identification of Vibrio spp. strains was performed on the basis of colony morphology on TCBS, oxidase test, sucrose fermentation and salt requirements. The suspected colonies were analysed with API 20E (modified adding 2.5% NaCl in the microtubes) and vibriostatic O129 (10 µg and 150 µg). To confirm the preliminary identification, strains were identified using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). MALDI-TOF target plates were inoculated into the spots by picking a freshly grown overnight colony and overlaid with 1 ml of 70% formic acid (Sigma-Aldrich). Each spot was allowed to dry and subsequently overlaid with 1 ml of matrix (α-cyano-4-hydroxycinnamic acid). Mass spectra were acquired using the MALDI-TOF MS spectrometer in a linear positive mode (Microflex LT, BrukerDaltonics). The MALDI BIOTYPER BDAL Library 5627 MSPs (v. 4.0.0.1) and the MALDI BIOTYPER software version 3.1 were used for bacterial identification. The similarity of patterns was represented as a score (≥2.000, identification at the species level; 1.700 to 1.999, identification at the genus level; <1.700, no reliable identification). Supernatants were put onto Formvar/carbon-coated grids, ultra-centrifuged for 15 min in a Beckman Air-fuge at 20 psi (125,000 g) using an A-100 rotor, stained with 1% phosphotungstic acid solution and subjected to direct transmission electron microscopy (TEM; Philips EM208S operating at 80 kV, at a magnification of 19,000–45,000).

2.3 | qPCR for total bacteria and Vibrio quantification

In order to define the total bacteria and Vibrio community load, DNA extraction was performed using DNeasy PowerSoil Kit (Qiagen) from the pools of C. gallina digestive gland employed for gene expression analyses and microbiota characterization (see below). In detail, each pool was composed of five individuals pooled together based on their DNA concentrations to obtain five pools for each site and each time point. After extraction, the quality of DNA was assessed by agarose gel electrophoresis 1%. Quantification of total 16S rDNA and 16S rRNA genes specific to the Vibrio genus were performed using quantitative PCR (qPCR). All amplification reactions were
analysed using a Roche LightCycler 480 Real-Time thermocycler. The total qPCR reaction volume was 10 μl and consisted of 2.5 μl DNA (10–50 ng/μl) and 7.5 μl LightCycler 480 SYBR Green I Master mix (Roche) containing 10 μM PCR primer (Eurofins). Total bacteria specific primer pairs were the 16S_Fw—TCTAGGGAGGCCAGCAGT and 16S_Rev—GAGTACAGGATCTTACCATTTGT, targeting the variable V3V4 loops for bacterial communities (Nadkarni, Martin, Jacobs, & Hunter, 2002). Total Vibrio specific primer pairs were Vib1-Fw GGGCTAAAGGCAGTACGGG and Vib2-Rev GAATTTCTACCCCCCTCTACAG (Siboni, Balaraju, Carney, Labbate, & Seymour, 2016), targeting the 16S rRNA of Vibrio genus. As a reference gene, we considered the universal 18S ribosomal RNA gene, using the specific primer pairs bivalve_uni_Fw CCGATAACGAACGAGACTC and bivalve_uni_Rev CACAGACCTGTTATTGCTC.

2.4 | RNA extraction and microbiota analyses

Total RNA was extracted from the digestive gland of individual samples with RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Each extraction cycle included a sterility control pool. Pools of total RNA were composed of five individuals pooled together based on their RNA concentrations to obtain five pools for each site and each time point. Samples collected in September 2016 were considered for both gene expression (RNA-Seq) and microbiota analyses (16S) using the same RNA pools. Microbiome analysis was also performed for samples collected in July in T4 and CG. A single sterility control pool was created by mixing 1 μl of each sterility control pool, and the resulting pool was included in the microbiome analysis. RNA integrity number (RIN) index was calculated for each sample using Agilent 2100 expert software. The RIN provides a numerical assessment of the integrity of RNA, facilitating the standardization of sample quality interpretation. In order to reduce experimental biases in RNA-Seq analysis due to poor RNA quality, only RNA samples with a RIN > 7 were further processed.

For microbiota analyses, 1 μg of RNA was retro-transcribed to cDNA using the Superscript IV kit (Invitrogen, Life Technologies). cDNA was diluted to 0.2 ng/μl and amplified in a 50 μl reaction including 5 μl diluted DNA and 1.5 μl of both reverse and forward primers (10 μM) that specifically target the V3–V4 gene region of the bacterial 16S rRNA as described by Milan et al. (2018). Libraries were then pooled together based on their concentrations, and the final pool was quantified using a Bioanalyzer 2100 (Agilent Technologies) and sequenced by GenomiX4Life s.r.l. with a Miseq Illumina 2x300 (Illumina). The microbiome sequencing generated 5 million reads, averaging about 170,000 reads per pool (sequences available in NCBI Sequence Read Archive SRA https://www.ncbi.nlm.nih.gov/sra; BioProject PRJNA494711). Raw reads were trimmed using CLC Genomic Workbench version 10.1.1 to eliminate adaptors and select high-quality sequences (https://www.qiagenbioinformatics.com/). The same software was used to merge forward and reverse sequences of the same fragment. Sequences were analysed with QIIME 1 (Quantitative insights into microbial ecology; Caporaso et al., 2010) to explore the microbial communities in each pool. First, merged reads were clustered by OTU (Operational Taxonomic Unit) based on their sequence similarity and a taxa assignment text file was created by blasting clusters to Greengenes 13_5 (http://green genes.secondgenome.com), a 16S full-length database with a minimum match value of 0.8. Data were normalized by random sampling based on the number of OTUs in the least represented pool. Rare sequences were filtered out of the final OTU list by applying a 0.005% cut-off. In order to analyse the microbial diversity in a single sample (richness), alfa-rarefaction data were used to calculate different metric statistics. β-diversity was calculated using the UNIFRAC method for comparing biological communities in order to estimate the variability of microbial communities between pairs of samples. The first statistical analyses on microbiota composition were performed using the software CALYPSO, version 8.20 (Zakrzewski et al., 2017) using the OTU table produced in QIIME. Data were normalized using the cumulative sum scaling (CSS + log) as explained in Paulson, Colin Stine, Bravo, and Pop (2013). Every group of samples was organized by Principal Coordinates Analysis (PCoA), and a two-way ANOVA was carried out in order to identify different taxa between sample groups (i.e., sites subjected to mortality events vs. control site). The OTU table produced in QIIME was also used for PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) in Galaxy (http://huttenhower.sph.harvard.edu/galaxy/; Langille et al., 2013) to carry out a functional analysis of detected microbial communities. The PICRUSt output was visualized with STAMP (Statistical Analysis of Metagenomic Profiles (http://kiwi.cs.dal.ca/Software/STAMP).

2.5 | RNAseq library preparation and sequencing

As mentioned above, RNA-Seq analyses were performed for samples collected in September in T4, T7 and CG sites (Table S1). The cDNA libraries were constructed starting from five pools for each sampling site using a SureSelect Strand-Specific mRNA Library (Agilent Technologies) according to the manufacturer’s protocol. Briefly, before fragmentation, oligo d(T) beads were used to purify poly(A) mRNA from total RNA. First-strand cDNA was synthesized from the fragmented mRNA using random hexamer primers, and the cDNA libraries were prepared in accordance with the Illumina protocol. After a purification step, the libraries were quantified with a Qubit Fluorometer (Invitrogen) and pooled together according to their relative concentrations. The concentration and quality of the pool was assessed by Agilent 2100 Bioanalyzer. Library pools were sequenced by HiSeq 4000 (Illumina) with a 150 bp paired-end approach (University of California) yielding a total of 780,364,422 reads (details are reported in Table S2, sequences available in NCBI SRA, BioProject PRJNA494495).

2.6 | Chamelea gallina transcriptome assembly

A FastQC report was used to perform an initial quality check of the raw sequencing data (Babraham Bioinformatics). Adapter trimming was
carried out on clc genomics workbench v.10.1.1. C. gallina transcriptome assembly was performed with clc genomics workbench version 10.1.1 with the following parameters: Minimum contig length = 250; Perform scaffolding; Auto-detected paired distances; word size: 18; Bubble size: 45; map reads back to contigs. A total of 266,178 transcripts were obtained (reported in File S1) and used as the reference transcriptome for RNA-seq reads mapping. Transcriptome annotation was performed by Blastx similarity search on Swissprot (Uniprot), Homo sapiens protein Ensembl database, Danio rerio protein Ensembl database, Crassostrea gigas protein Ensembl and Drosophila melanogaster protein Ensembl database (Evalue < 0.0001). Of 266,178 unique sequences, 64,315 (24.16%) showed at least one significant match. Details are reported in Table S3, while the annotation of each contig is reported in File S2. Trimmed RNA-Seq reads of each sample were then mapped against the reference transcriptome to obtain gene expression profiles of each pool.

2.7 | Gene expression analyses

Mapping of RNA-Seq reads against the reference transcriptome was performed in clc genomics workbench version 10.1.1 with the following parameters: mismatch cost 2, insertion cost 3, deletion cost 3, length fraction 0.8 and similarity fraction 0.8. For each pool, between 85.97% and 87.54% of the total reads were successfully mapped. Gene counts were then carried out using EdgeR to analyse differential gene expression (Robinson, McCarthy, & Smyth, 2010). Samples were grouped according to sampling area and were normalized using the Trimmed Mean of M-values (TMM) method. After normalization, only annotated transcripts were kept for subsequent analysis. Likelihood-ratio test (LRT) was carried out with edger to assess differentially expressed genes (DEGs), with significant log fold change (log FC) threshold set to >1 and false discovery rate (FDR) set to <0.05. A functional interpretation of differentially expressed genes was obtained by enrichment analysis using Database for Annotation, Visualization, and Integrated Discovery (DAVID ver. 6.8) software (Dennis et al., 2003; Huang, Sherman, & Lempicki, 2009), considering GO Biological Process (BP), Cellular Component (CC) and Molecular Function (MF) Database and KEGG pathways. DAVID retrieves the functional annotation of differentially expressed genes through enrichment analyses based on an integrated biological knowledge-base containing over 40 annotation categories. Since DAVID databases contain functional annotation data for a limited number of species,

TABLE 1  Summary of results obtained through microbiological analyses

<table>
<thead>
<tr>
<th></th>
<th>CG_Ju</th>
<th>CG_Se</th>
<th>T4_Ju</th>
<th>T4_Se</th>
<th>T7_Se</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viable counts data (CFU/g)</td>
<td>7.2 × 10⁴</td>
<td>1.5 × 10⁷</td>
<td>4.8 × 10⁷</td>
<td>2.3 × 10⁷</td>
<td>1.2 × 10⁷</td>
</tr>
<tr>
<td>Parasites</td>
<td>1/30 Nematopsis sp.</td>
<td>Rare Nematopsis</td>
<td>3/30 Nematopsis sp.</td>
<td>Rare Nematopsis</td>
<td>Rare Nematopsis</td>
</tr>
<tr>
<td>Virus</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Bacteria detection through MALDI-TOF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteria/total colonies investigated</td>
<td>6</td>
<td>7</td>
<td>10</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Vibrio diabolicus</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vibrio harvey</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Vibrio tubiashii</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Vibrio orientalis</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vibrio fortis</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vibrio alginolyticus</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td></td>
<td></td>
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<tr>
<td>Vibrio pomeroyi</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Vibrio rotiferanus</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
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<tr>
<td>Vibrio orientalis</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
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<tr>
<td>Vibrio campbelli</td>
<td></td>
<td></td>
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<td>1</td>
</tr>
<tr>
<td>Vibrio pelagius</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Relative quantification of total bacteria and total Vibrio load (qPCR)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacterial 16S relative quantification (2⁻ΔΔCT)</td>
<td>0.998 ± 0.713</td>
<td>9.904 ± 5.961</td>
<td>2.154 ± 2.07</td>
<td>24.339 ± 33.689</td>
<td>10.791 ± 18.774</td>
</tr>
<tr>
<td>Total Vibrio 16S relative quantification (2⁻ΔΔCT)</td>
<td>0.735 ± 0.369</td>
<td>0.493 ± 0.432</td>
<td>12.895 ± 9.306</td>
<td>1.009 ± 0.202</td>
<td>9.826 ± 18.791</td>
</tr>
</tbody>
</table>

Note: For each investigated site, Viable counts data (C. gallina homogenate), Parasites, “Virus,” “Bacteria/yeast” detections are reported. Identification of colonies in TCBS Agar through MALDI-TOF mass spectrometry is also reported: number of total investigations for each sampling site/time and the corresponding number of each Vibrio spp. identified are reported. Relative quantification of total bacteria and total Vibrio load obtained by qPCR are also reported. qPCR has been performed in the same pools of samples considered for gene expression and microbiota analyses. The 2⁻ΔΔCT here reported represent the mean value of the 5 pools analysed for each sampling time/site. CG, Cupra Grottamare; T4, San Vito; T7, Francavilla. “Ju” and “Se” indicate sample time: Ju, July; Se, September.
C. gallina transcripts were associated with sequence identifiers that could be recognized in DAVID (e.g., Swissprot and Zebrafish Gene IDs). This was carried out using dedicated BLAST searches performed with blastx. The assignment of a putative homologue to a larger number of clam transcripts was obtained using Swissprot (Uniprot). IDs corresponding to differentially expressed striped venus transcripts and to all genes represented in the C. gallina transcriptome were obtained and were then used in DAVID to define a "gene list" and a "background," respectively. A functional annotation was obtained for significant genes identified by each pairwise comparison (CG vs. T4; CG vs. T7; T4 vs. T7). DAVID settings: gene count = 2 and ease = 0.1.

3 RESULTS

3.1 Microbiological analyses and quantification of bacteria and Vibrio spp. by qPCR

The results obtained by microbiological analyses are summarized in Table 1. The viable count data indicate that the number of colony forming units (CFU) per bivalve (C. gallina homogenate) was higher than those detected during routine controls carried out by the authorities responsible for guaranteeing food safety ($10^3$–$10^4$ CFU/g; personal communication), in both sites subject to mortalities (T7 and T4) in July ($10^7$ CFU/g) and in the control site CG in September ($1.5 \times 10^7$ CFU/g). Based on histological examination performed on 30 individuals for each investigated site/time of sampling, the percentage of parasites detected in the clams was generally low and the species detected are usually not pathogenic to C. gallina. Bacterial infection and extent of necrosis were detected in only 2 of 30 analysed individuals collected in T7. Virus detection by electronic microscopy was negative. Random colonies of Vibrio spp. from TCBS plates were also identified to the species level by MALDI-TOF mass spectrometry. Although the number of identified species was limited, this analysis showed that sites subject to mortality (T4 and T7) were not characterized by any specific Vibrio spp. Relative quantification of total bacterial communities obtained by qPCR in C. gallina digestive gland confirmed the results obtained by viable count data (Table 1 and Figure S1). A significant increase in total bacterial abundance was observed in CG clams collected in September with a value similar to those detected in mortality sites T4 and T7. However, the relative quantification of total Vibrio spp. revealed higher Vibrio load in T4 and T7 compared with CG at both sampling times, suggesting that the increase in total bacteria communities revealed in CG_Se is not associated with an increased presence of potentially pathogenic Vibrio species.

3.2 Microbiome analyses

Similar to what previously found in the Manila clam, mussels and oysters (ClearyBecking(210,547),(792,869), Polónia, Freitas, & Gomes, 2015; King, Judd, Kuske, & Smith, 2012; Milan et al., 2018; Trabal et al., 2012), the overall composition of clam-associated bacterial communities showed a predominance of the phyla Proteobacteria (60%) and Tenericutes (33.4%), of the classes Alphaproteobacteria (42.8%) and Mollicutes (33.2%) and of the genus Mycoplasma. The overall distribution of phyla is reported in Figure S2. $\beta$-diversity was evaluated through ANOSIM among sampling times (July [Ju] vs. September [Se]), sites (three groups CG vs. T4 vs. T7), sampling time and sites (five groups, CG_Ju, CG_Se, T4_Ju, T4_Se, T7_Se). All comparisons
showed significant "time" and "site" heterogeneity among sample groups (p < .05) with higher statistical significance in the comparison between "sites" (p < .001).

The PCoA analysis confirmed the temporal and geographical heterogeneity among investigated sites and across sampling times and showed a clear separation between July and September in site T4. The control site (CG) appears separated from T4 and T7 independently of the sampling time (Figure 2). Overall, PCoA analyses highlighted temporal and geographical heterogeneity among investigated sites and across sampling times. Considering the temporal proximity of sampling activities (July and September 2016), these findings indicate that bivalve microbiota can be highly diverse and easily influenced by environmental factors (Cleary et al., 2015; Green and Barnes, 2010; Lokmer et al., 2016; Milan et al., 2018; Trabal Fernandez, Mazon-Suastegui, Vazquez-Juarez, Ascencio-Valle, & Romero, 2014).

To better understand the differences between sampling sites, a one-way ANOVA analysis was performed to identify significant differences in microbial communities between CG, T4, and T7. Four separate pairwise comparisons were carried out within each sampling time to avoid the confounding effect of seasonal variation: (a) CG_Ju vs. T4_Ju; (b) CG_Se vs. T4_Se; (c) CG_Se vs. T7_Se; (d) T4_Se vs. T7_Se. The complete list of significant taxa for each comparison is reported in File S3. The comparison between the microbial profiles of clams collected in July in control site CG and in the mortality site T4 showed the highest number of differentially represented taxa with (100 OTUs, p-value < .05). This result could be related to the higher "total colony counts" detected in T4 (10^7 CFU/g) compared with CG (10^4 CFU/g; Table 1). Among the 62 over-represented taxa in T4, 31 OTUs (50%) belong to Vibrio genera or members of the Vibrionaceae family, and 12 OTUs represent Photobacterium genera (19%). Unfortunately, only few OTUs were able to reliably identify species, such as Vibrio fortis (2), Vibrio shilonii (2), Vibrio ichtyoenteri, Photobacterium angustum, Photobacterium damselae, Photobacterium rosenbergii (3). Similar results were found when comparing the mortality site T7 to the control site CG in September, with the 30% of over-represented OTUs in T7 belonging to the Vibrio genus or Vibrionaceae family and 7 OTUs (13%) belonging to the Photobacterium genera. Finally, the pairwise comparison between the two sites subjected to mortality (in September) identified 34 and 11 over-represented OTUs in T7 and T4, respectively. Among them, T7 showed an over-representation of the Vibrio genus or Vibrionaceae family (12 OTUs; 35%), the Pseudoalteromonadaceae family (8 OTUs; 25%) and the Photobacterium genera (4 OTUs; 11%), while site T4 showed an over-representation of the Chlamydiales order (4 out of 11 over-represented OTUs).

Venn diagrams were then constructed to identify commonly differentially represented taxa in sites subjected to mortality events (T4 and T7) compared with the control site (CG; Figure 3). Six taxa, represented by the Mycoplasma genus (3), the Ehrlichia genus, the Rhodospirillaceae family and the Rickettsiales order were found consistently under-represented in mortality sites, regardless of sampling season (Figure 3c). A total of 38 commonly differentially represented taxa were obtained comparing T4 and CG in July and T7 and CG in September. Among them, a total of 28 OTUs were over-represented in both mortality sites, representing the Vibrio (14) and Photobacterium (6) genera, the Pseudoalteromonadaceae family (6) and the Aeromonadaceae family (2).

Functional analysis using PICRUST and STAMP was then carried out for each comparison. The full list of significant terms is reported in File S4. Over-representation of several pathways involved in "Xenobiotic biodegradation and metabolism," such as "Benzoate degradation," "Naphthalene degradation," "Biphenyl degradation," "Atrazine degradation," "Polycyclic aromatic hydrocarbon degradation," and "Polycyclic aromatic hydrocarbon metabolism" were highlighted.
degradation" and "Dioxin degradation" in the microbiota of samples collected in T4 and T7 was observed, as well as pathways related to "Aminoacid metabolism" and "Biosynthesis of secondary metabolites." ANOVA results were confirmed by the over-representation of "Vibrio cholerae pathogenic cycle" and "Vibrio cholerae infection" pathways in T4 and T7 in July and September while the KEGG pathways related to "genetic information processing" were over-represented in CG.

3.3 | Gene expression analyses

To identify persistent transcriptional changes among the investigated sites, pairwise comparisons through EdgeR were performed (log FC > 1; FDR < 0.05). Comparisons between sites affected by mortality events (T4 and T7) and the control site CG showed a high number of DEGs (1,040 and 705, respectively), while fewer transcriptional differences were found between T4 and T7 (157). The complete lists of DEGs (1,040 and 705, respectively), while fewer transcriptional regulated in T4 and T7, respectively) and of genes involved in "response to stress" (43 and 25 down-regulated genes in T4 and T7), "regulation of response to stress" (22 and 11 down-regulated genes in T4 and T7) and "positive regulation of response to stimulus" (32 and 8 down-regulated genes in T4 and and T7). The full lists of enriched Biological Process, Cellular Component, Molecular Function and Molecular Pathways for up- and down-regulated genes within each comparison are reported in File S6.

4 | DISCUSSION

In this study, we seek to decipher the complex host-microbiota interactions underlying the mass mortalities affecting the clam Chamelea gallina, providing a more complete representation of the factors potentially involved in the recent population decline. In our previous study, gene expression profiles of the striped venus C. gallina in a mortality site (T4) revealed the up-regulation of several genes involved in immune response. The results obtained suggested bacterial and/or viral infection as the major cause of clam mortality (Milan, Matozzo, et al., 2016; Milan, Palazzo, et al., 2016). Here, by using a combination of microbiota characterization (16S) and host-gene expression profile through next-generation sequencing (NGS), we provide a new hypothesis on the mechanism that may have impaired the physiological response of clams and favoured infection.

4.1 | Impacts of chemical pollutants in natural populations affected by mass mortality

Indirect evidence of potential exposure to chemical pollutants is provided by C. gallina gene expression profiles, which show a large number of up-regulated genes involved in xenobiotic metabolism and in the antioxidant defense system in sites T4 and T7 when compared to CG, such as CYP450 (Livingstone, Kirchin, & Wiseman, 1989; Stegeman, 1985; Zanette, Goldstone, Bainy, & Stegeman, 2010), SULT (Milan, Ferrareso, et al., 2013; Milan, Matozzo, et al., 2016; Milan, Palazzo, et al., 2016; Pessati et al., 2016), GSTs (Mezzelani et al., 2016; Milan, Matozzo, et al., 2016; Milan et al., 2015; Park, Ahn, Kim, Lee, & Shin, 2009; Xu, Pan, Liu, Wang, & Miao, 2010) and GPX (Cossu et al., 1997). Noteworthy, GADD45G, that is involved in DNA repair, cell cycle control and apoptosis (Fornace, Jackman, Hollander, Hoffman-Liebmann, & Liebmann, 1992), showed the highest fold changes in mortality sites when compared to the control site. This up-regulation has also been previously described in the Manila clam and in mussels exposed to ibuprofen and pyrene-contaminated materials (Avio et al., 2015; Milan, Pauletto, et al., 2013), further corroborating its involvement in counteracting genotoxic stress (Takekawa & Saito, 1998). Finally, three transcripts coding for putative NRF6 were also commonly up-regulated in sites T4 and T7. NRF6 plays a role in the uptake of a range of molecules including xenobiotic compounds from the intestine to surrounding tissues in the nematode Caenorhabditis elegans (Choy, Kemner, & Thomas, 2006; Choy & Thomas, 1999) and was found positively correlated...
with increased concentrations of PCBs, PCDD/Fs, HCB and PBDEs in the Manila clam (Milan, Ferrareso, et al., 2013, 2015).

Although it is a hypothesis that requires further validation, it is possible that the observed microbiome-transcriptome response is triggered by chemical pollutants originating from the freshwater conveyed to the coast by several rivers that flow into the Adriatic Sea (e.g., the Pescara, Foro, Arielli and Feltrino rivers; Figure 1). Waters from these rivers are transported southwards by the Western Adriatic Current, increasing the probability that southern sites along the coast become regularly contaminated by untreated urban sewage and industrial and agricultural pollutants. The Pescara river, the longest river in Abruzzo, collects runoff waters from a densely populated area that includes several urban settlements and industrial facilities. Historically, toxic waste was illegally discharged in this river in the industrial area of Bussi sul Tirino, where water flows down from the Apennine Mountains into the Pescara river. In 2007, Bussi sul Tirino was claimed to be the biggest illegal toxic waste dump in Europe, containing years’ worth of dangerous industrial waste responsible for contaminating almost 2 million tons of soil, as well as both the superficial and deep-water tables, with dozens of toxic compounds (Di Francesco, 2010; Piccoli et al., 2010).

In a previous study by Milan, Matozzo, et al. (2016) and Milan, Palazzo, et al. (2016), chemical pollution was ruled out as a primary trigger of clam mortality based on the chemical analyses and gene expression profiles of *C. gallina* in sites T4 and T7. In this study, the combination of transcriptomic and microbiota profiles and the documented high contamination load in river run-off suggest a different scenario, whereby xenobiotic exposure may instead play a crucial role in clam mortality. Three reasons may explain the discrepancy between this study and the one by Milan, Matozzo, et al. (2016) and Milan, Palazzo, et al. (2016). First, lack of transcriptional differences between T4 and T7 related to xenobiotics response in Milan, Matozzo, et al. (2016) and Milan, Palazzo, et al. (2016) might be ascribed to geographical proximity of the two sites, considered to be equally impacted by chemical stress (Figure 1). In this study, we introduced a new control site (Cupra Grottamare; CG) located north to the Pescara river mouth and therefore less affected by the river plume and discharge. In this site, no mass mortality events have ever been recorded in the control site CG. Comparisons among mortality sites and control site suggest that chemical contaminants affect clams in T4 and T7 with equal severity. Secondly, only a few compounds such as heavy metals, PAHs, and pesticides have been tested, to date, in chemical analyses of sediments and clams in the affected area (Milan, Matozzo, et al., 2016; Milan, Palazzo, et al., 2016; Visciano et al., 2015). Considering the complexity of chemical pollutants potentially contaminating the Pescara river waters, we cannot exclude that other, previously unassessed, chemical compounds may be present in marine sediments where clams live. Thirdly, chemical pollution in the areas affected by mass mortality might be episodic rather than constant, with acute peaks of exposures triggered by events such as abundant rainfall. Episodic exposure peaks, being transitory, would be more difficult to detect through periodic chemical analysis of the sediment and water column or by bioaccumulation analysis. Unfortunately, for several reasons chemical analyses to determine the concentrations of potential pollutants in the water column, sediments and biota could not be carried out on 2016 samples. That said, we believe that these analyses may not prove conclusive unless future sample collection and chemical analyses are carried out frequently and promptly after abundant rainfall.

In keeping with transcriptomic data, functional analysis of microbial communities also highlighted significant over-representation of several pathways involved in xenobiotic metabolism in clams from mortality sites. A similar pattern was observed in Manila clams collected in highly polluted sites (Milan et al., 2018). Likewise, other significant pathways (“Amino acid metabolism,” “Biosynthesis of secondary metabolites,” “Gene expression information processing”) showed a consistent behaviour in the two species. As previously suggested (Milan et al., 2018), exposure to pollutants might open the way to opportunistic infection leading to host-inflammation and enhanced protein turnover. In turn, the reduction in carbohydrate supply in inflamed tissues may select bacterial taxa that mostly rely on amino acid metabolism for energy production (Davenport et al., 2014).

### 4.2 Abundance of opportunistic pathogens in sites subjected to mass mortality

Overall, histological, microbiological and electron microscope analyses showed no differences between the investigated sites for the presence of viruses and parasites, while bacterial infection and extent of necrosis were found in only T7 site in 2 of 30 investigated individuals. Considering that at the time this manuscript was written (March 2019) no mortality had been found in CG while mortality persisted in T4 and T7 sites, the higher total number of bacteria detected in CG in September may be linked to seasonal variations such as rainfall and high summer temperatures (Tabanelli et al., 2017) and is likely not associated with increased presence of potentially harmful bacterial species. This is supported by results obtained through quantification of *Vibrio* spp. using quantitative qPCR, which showed a higher total *Vibrio* spp. load in T4 and T7 compared with the control site CG in both the July and September sampling times (Table 1). MALDI-TOF analyses showed different combinations of *Vibrio* spp. in each sampling site/time, while sites subject to mortality were not represented by specific *Vibrio* spp. However, MALDI-TOF analysis was applied to a limited number of colonies (from 6 to 10 per sampling site/time), and we should exercise caution in interpreting this result. Indeed, when 16S microbiota data are considered, pairwise comparisons revealed 38 common taxa differentially represented in T4 and T7 compared with CG. The dominant organisms belonged to *Vibrio* spp. (14 OTUs) and *Photobacterium* spp. (6 OTUs) while the prevailing significant pathways were “Vibrio cholerae pathogenic cycle” and “Vibrio cholerae infection”. Vibrios are ubiquitous marine bacteria with high metabolic versatility and genetic variability that confer a high colonization potential (Hazen, Pan, Gu, & Sobbecky, 2010; Le Roux, Wegner, & Polz, 2016; Reen, Almagro-Moreno, Ussery, & Boyd, 2006). *Vibrio* spp. have been suggested to be among the major
causes of mass mortality in oyster beds, mostly affecting larvae and juveniles and resulting in losses of up to 80%–100% of oyster production (Eistón, Hasegawa, Humphrey, Polyan, & Hase, 2008; Le Roux et al., 2016; Petton et al., 2015; Sugumar, Nakai, Hirata, Matsubara, & Muroga, 1998). The elevated abundance of Vibrio spp. in oyster beds has been recently confirmed by 16S microbiota analyses that demonstrated the implication of the Vibrio community either as infective agents or opportunistic colonizers in oyster mass mortality events (King et al., 2019). In our study, 50% and 30% of significantly over-represented taxa in T4 and T7, respectively were assigned to Vibrio genera or Vibrionaceae family. Species-level identification, however, was possible only for 7 OTUs, which were represented by Vibrio fortis, Vibrio ichthyoventeri and Vibrio shilonii, already proposed as potential pathogens in other marine species (Austin, Austin, Sutherland, Thompson, & Swings, 2005; Ding, Dou, Wang, & Chang, 2014; Ishimaru, Akagawa, & Muroga, 1996; Romalde, Diguez, Las, & Balboa, 2014; Rosenberg, Kushmaro, Kramarsky-Winter, Banin, & Yossi, 2009; Wang, Zhang, Qin, Luo, & Lin, 2016).

Potentially pathogenic Photobacterium species were found in high abundance in the microbiota of Vibrio-infected C. gigas (Lokmer & Mathías Wegner, 2015). Photobacterium is one of the oldest genera in the family Vibrionaceae and is commonly found in the marine environment and in the intestinal contents of marine animals. In the present study, members of the genus Photobacterium, such as Photobacterium damselae, were over-represented in T4 and T7. Photobacterium damselae is a pathogen of a variety of marine animals including cultured fish species (Labella, Manchado, et al., 2010; Labella, Sanchez-Montes, et al., 2010; Labella et al., 2006; Pedersen, Skall, Lassen-Nielsen, Bjerrum, & Olesen, 2009; Romalde et al., 2014). P. damselae has also been isolated in Octopus joubini (Hanlon et al., 1984) and commercial bivalve species such as Mytilus galloprovincialis (Lozano-León, Osorio, Nuñez, Martínez-Urtaza, & Magariños, 2003) and oysters (Richards, Watson, Crane, Burt, & Bushek, 2008).

Although multiple infections involving a virus cannot be excluded in C. gallina, the down-regulation in both mortality sites of putative Interferon-induced protein 44-like (IFI44), a key element for effective antiviral defense in bivalve species (Olicard, Renault, Torhy, Bennamoun, & Bourguignon, 2005; Pauleto et al., 2017; Renault, Faury, Barbosa-Solomieu, & Moreau, 2011; Segarra et al., 2014) and the significant down-regulation of several pathways typically involved in virus response suggests that viral replications should be excluded in C. gallina. Furthermore, the absence of viral detection in the histological analysis carried out in our study supports the premise that viral replication is not at the root of C. gallina mortality as recently proposed in C. gigas (De Longeril et al., 2018).

### 4.3 Interactions between pollutants and pathogens could play a key role in C. gallina mortality events

The abundance of opportunistic pathogens in clams from sites T4 and T7 might be explained by the disruption of host defense strategies, as suggested by transcriptional profiles. When compared to the control site, both mortality sites showed a significant down-regulation of several pathways and genes typically involved in immune defense, such as Macrophage-expressed gene 1 protein, Toll-Like Receptor 5 and Hemagglutinin/amebocyte aggregation factor among others (Benard et al., 2015; Fujii et al., 1992; Moreira et al., 2012; Renault et al., 2011). These results suggest that clams in sites T4 and T7 may have reached a threshold of tolerance that hinders any further response to environmental stress. Several studies suggest a mechanism in which an environmental trigger compromises the host-immune response, thus promoting viral or bacterial infections in bivalves. In a recent study, using 16S rRNA amplification sequencing, changes in the oyster microbiome were induced by water temperature increase to 25°C and included a significant increment of the abundance of the putative bacterial pathogens Vibrio harveyi and Vibrio fortis (Green et al., 2018). Therefore, a marine heat wave could potentially trigger a mass mortality event of C. gigas by boosting the replication of specific members of the oyster’s bacterial community which become able to overwhelm the immunological capacity of physiologically stressed oysters (Green et al., 2018).

Pollution, even at low concentrations, has documented dramatic effects on the physiology, immune response and ecology of bivalves (e.g., Morley, 2009; Rittschof & McClellan-Green, 2005) and in combination with infectious diseases can trigger synergistic responses that are difficult to disentangle (detailed in Morley, 2010). Loss of efficiency in phagocytosis and clearance rate of bacteria owing to pollution toxicity are documented in bivalves (Cheng, Hsiao, & Chen, 2004a, 2004b; Gagnaire et al., 2007; Galloway & Depledge, 2001; Parry & Pipe, 2004; Pipe & Coles, 1995; Pipe & Coles, 1995). With a similar mechanism, we hypothesize that high concentrations of pollutants in T4 and T7 could have disrupted host physiological homeostasis in C. gallina and consequently could have facilitated opportunistic pathogens such as Vibrio and Photobacterium genera in immune-compromised individuals.

This hypothesis needs to be validated through routine chemical analyses and an experimental design implementing clam translocations between control and mortality sites, followed by gene expression analyses, microbiota characterization and microbiological analyses.

### 5 CONCLUSIONS

Climate change, chemical contamination and the spread of emerging pathogens combined with fishery pressure are likely to have dramatic effects on marine ecosystems and aquaculture (e.g., Mohanty, Mohanty, Sahoo, & Sharma, 2010; Rizzi et al., 2016). These are major challenges for the shellfish farming industry and researchers for the years to come. Growing evidence on microbial compositions in marine organisms emphasizes the importance of host-microbial interactions in the response to environmental changes. In this study, we provide a revised hypothesis to explain mortality events occurring in C. gallina, demonstrating the potential of combining RNA-seq and
16S rRNA high-throughput sequencing to investigate the responses of marine organisms to changes in environmental conditions. This approach can represent a highly effective method for anticipating and interpreting the risks inherent in pollutant-pathogen interactions in rapidly changing environments.

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CONFLICT OF INTERESTS

The data of this study are original, and no part of this manuscript has been published or submitted for publication elsewhere. The authors declare no competing financial interest.

AUTHOR CONTRIBUTION

M.M., G.M., L.B., B.C. and T.P. conceived and designed the project. G.M., A.I., C.M., N.F., M.I. and L.G. performed C. gallina sampling. A.I., C.M., N.F., M.I. performed clam dissection and sample preparation. R.B. and G.A. performed histological, microbiological and electron microscope analyses. L.C., G.D.R. and B.C. performed laboratory experiments. L.C., M.S., G.D.R. and B.C. carried out 16S statistical analyses. M.M. performed gene expression analyses. G.D.R., S.I. and A.Z. performed qPCR. M.M., M.S., C.P., C.C., and L.B. wrote the manuscript and prepared the figures. All listed authors edited the final version of the manuscript. All authors read and approved the manuscript.

DATA AVAILABILITY STATEMENT

16S Sequence data are deposited in SRA database (BioProject PRJNA494711). RNAseq data sequencing files are available in NCBI Sequence Read Archive (SRA; https://www.ncbi.nlm.nih.gov/sra; BioProject PRJNA494495).

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