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Growth performances in cupped oysters (*Crassostrea gigas*) during pre-fattening stages in the middle Adriatic Sea: Influence of pathogens and environmental factors

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ABSTRACT

In the last decades, demand for bivalves has significantly increased substantially worldwide. In the Adriatic Sea, offshore bivalve molluscs farming is almost entirely based on mussel farming although some companies started to diversify their production. Growth performance and interaction between host, pathogens and environmental factors were analyzed in different batches of cupped oysters (*Crassostrea gigas*), during the years 2018 and 2019. Our results show a significant growth rate of the oysters reared in the Adriatic Sea, but an increase of mortality in summer months. Histological analysis does not show the presence of specific pathogens, recording only a strong positivity with immunohistochemistry for *Vibrio* spp. in summer 2018, but not in 2019. The research of *OsHV-1* and *V. aestuarianus* shows negative results in all the time points. We demonstrated that there was a combined effect of Sea Surface Temperature, Chlorophyll-a, and *Vibrio* clade *splendidus* concentration on oysters' mortality. Despite the high mortality and the resulting economic loss during the summer months, oysters farming seems to be a feasible activity in the Adriatic Sea. In order to maximize production and the profitability of the farm, the use of new locations for product finishing could be suggested.

1. Introduction

The first growing phase of oysters requires particular attention, representing the phase that allows the spat, obtained from commercial hatcheries or collected in the wild, to grow up to a desired size, then the fattening phase starts. Pre-fattening phase can be performed on land-based farm, in valley, lagoon, or in open sea (Roncarati et al., 2017). In Europe, the nursery and/or pre-fattening phases traditionally take place in land-based facilities, often associated with hatchery, in reinforced concrete tanks or in floating systems placed in shallow and protected waters; more rarely in "Pearl net" or fine mesh lanterns placed in protected inland waters (Pouvreau et al., 2000). Usually, pre-fattening phase is not carried out in long line systems. In open sea, the management of the pre-fattening phase still has spaces to be optimized through the adoption of innovative systems. Since 2008, following the mortality due to OsHV-1 microvar, recorded on juvenile subjects, the availability of pre-fattened juveniles has considerably decreased, whereas prices have considerably increased (Roncarati et al., 2017).

In Italy, some hatcheries worked for short times in the past and natural collection of oyster spat is minimal. Consequently, since there is neither artificial reproduction nor spat uptake, only few pre-fattening plants are active. For the small juvenles, Italy depends almost entirely on imported pre-fattened, always coming from Northern European countries, with ever higher prices (Smaal et al., 2019).

Due to the absence of tides, in Italy farming techniques used on the Atlantic coast are not practicable. Consequently, the growing phase takes place both in inland waters and in open sea (long-line system). For both the cupped and flat oyster species, *Crassostrea gigas* and *Ostrea edulis*, containers of different types are used such as lantern nets, rigid plastic baskets with multiple superimposed levels or other plastic

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containers of different shapes, suspended on rows traditionally dedicated to bivalves (Roncarati et al., 2017). In Italy, as in Croatia and in some lagoons of the French Mediterranean coast, it is also used to cemente the oysters one by one on vertical peaks (Bonacic et al., 2017; Trocino et al., 2018).

In the Adriatic Sea, the cycle starts with spat from a mean weight around 0.15 g to 5–10 g (pre-fattening) until reaching the market size around 80 g mean weight after 12–24 months of cultivation (Fig. 1), which varies according to the temperature (latitude) and the sowing period (number of winter months in the cycle). During the cycle, the product undergoes at least three selections by size. In order to favour the growth rate of smaller oysters and to avoid juvenile stress, size grading is a practice advised during the nursery and pregrowing phase (Qu et al., 2009).

The selection of spat by size, at all levels of the nursery and pre-fattening cycle, represents a determining factor for the quality of the spat itself and to avoid aggregation, and then it is recommended all along the cultivation period. Grading is a well-established practice in commercial hatcheries (Wilson, 1981) because it is assumed that it improves the growth rate of smaller individuals, in the absence of larger and presumably dominant competitors (Jarayabhand and Newkirk, 1989).

From a biological point of view, the smaller animals obtained after grading could continue to grow at a slower rate than the larger animals, but if the environmental conditions were optimal, the small animals would be able to reach the dimensions of the heavier animals as a result of compensatory growth (Ricker, 1975) and are expected to grow at the same rate as larger animals. However, the studies concerning the effects of sizing on the growth of bivalves have given rather heterogeneous results (Robert et al., 1990), probably because the high variability of the growth rate between individual animals is such as to influence the efficiency of production by increasing handling and associated stress. A combination of intrinsic (genetics) and extrinsic (handling stress and environmental variability) factors are thought to be among the most common causes of growth variability within shellfish populations. In order to optimize production, density and a series of factors directly related to it must also be considered: among these, water quality, the production system and the type of farming have been particularly emphasized (Smaal et al., 2019).

Further factors not to be underestimated during the pre-fattening activity are the constant control and cleaning of the plants by the operators and the scrupulous attention to any signal that may presage a possible onset of pathologies, especially in active flow systems, which allow large quantities of biomass to be raised in a limited space and therefore the onset of any pathologies can spread very rapidly causing high economic damage (Petton et al., 2023). Recent trials on the recruitment efficiency of young oysters from the wild (mid Adriatic Sea), as possible source of initial stock to employ as starting stock to grow in longline farm, showed environmental parameters and good practices as traits affecting the success of the oyster cultivation (Roncarati et al., 2023; Turolla, 2023).

Fig. 1. Productive cycle of cupped oyster (*Crassostrea gigas*) in the middle of Adriatic Sea. After the hatching and nursing stages in North European hatcheries, spats are usually imported in Italy (0.15 g), for pre-fattening (from 0.15 to 10 g maximum) and fattening stages (10 g to 80 g).

Vibrio species are considered a threat for oyster farming worldwide. *Vibrio aestuarianus* is a major pathogen to adult oysters causing farmed oysters' mortality (Travers et al., 2017). *V. splendidus* clade is ubiquitous in marine coastal environments (Pérez-Cataluña et al., 2016) and includes strains (e.g., *Vibrio tasmaniensis* and *Vibrio crassostreae*) that have been associated with a multifactorial disease affecting spats and juveniles, triggered by herpes virus OsHV1 µVar (Segarra et al., 2010; Bruto et al., 2017; de Lorgeril et al., 2018;). The disease, known as Pacific oyster mortality syndrome, occurs when seawater temperature reaches 16–24 °C (Pernet et al., 2012; Pernet et al., 2012), i.e., from spring to autumn along the Mediterranean coast (Lopez-Joven et al., 2018). Therefore, detection of the *V. splendidus* clade is used in environmental surveys (Pernet et al., 2012). The knowledge about the ecology of farmed oysters pathogen remains scarce, however, phytoplankton concentration seemed to be positively associated with pathogenic *Vibrio* strains (Lopez-Joven et al., 2018). Chlorophyll a (chl-a) is a convenient phytoplankton biomass proxy as it is unique to plants and represents the principal pigment used by phytoplankton to capture light energy for natural oyster stocks growth and biomass production (Jakobsen and Markager, 2016).

This study was performed to assess the feasibility of cupped oyster pre-fattening phase in the middle Adriatic Sea, facing the main biotic and abiotic factors affecting the growth performances.

2. Materials & methods

The study was performed in a longline shellfish farm located in mid Adriatic Sea (43°26,042.7600 N–13°43,045.3300 E) as described in a previous study (Roncarati et al., 2023). Six batches (C11-C16) of cupped oyster (*Crassostrea gigas*) were monitored through two successive years (2018–2019). Each batch was composed by 50,000 oysters (diploid spat), obtained from a commercial hatchery in France. On arrival, each batch was named with the letter C (standing for *Crassostrea*), followed by a progressive number. Batches from C11 to C14 were farmed in the year 2018 and C15 and C16 in 2019. The animals were studied from the arrival to 10 months of farming, as the most critical period for their growth is the first summer of life (Dégremont et al., 2010; Dégremont, 2011). For the trial, oysters were introduced in net lanterns (5 trays; diameter: 50 cm; 2 × 2 mm mesh size; 145 cm total height), equipped with Velcro® closure, at the initial density of 1.5 oysters / cm^2. The net lanterns were suspended on the long-line, 4 m below the surface of the water. Oysters were permanently submerged. Eighty individuals from each productive cycle were randomly collected each season throughout the year (T0 = winter; T1 = spring: T2 = summer; T3 = autumn) and immediately processed. Oyster lanterns were immediately released back into the water to avoid stress for the molluscs. Growth performances and histopathological analysis were carried out to assess the health status of the oysters. Molecular analyses were performed to investigate the presence of specific pathogens (*Oyster Herpesvirus*-1µvar, *Vibrio* clade *splendidus* and *Vibrio aestuarianus*).

2.1. Growth performances and mortality rate

At each time point, from each batch, the weight of 80 live cupped oysters was recorded using a precision scale (CP224S, Sartorius Italy Srl, Varebo, MB, Italy). Width, length, and height were measured by means of a vernier calliper (Starrett, Athol, Massachusetts, US), and mortality was estimated by counting the shells of dead oysters in each group.

2.2. Histological and immunohistochemical analysis

For histopathological analysis, 10 cupped oysters were analyzed at

each sampling point. Samples were fixed in Davidson fixative for 48 h. Fixed tissues were sampled, reduced into tissue embedding cassettes dehydrated by gradually soaking in alcohol and xylene, and finally embedded in paraffin. For each paraffin-embedded specimen, 3 μm-thick sections were cut every 200 μm and stained with Haematoxylin-Eosin (HE) and Giemsa stain to detect the presence of any parasites and pathological alterations. Immunohistochemical analysis (IHC) was performed with an anti-*Vibrio* spp. polyclonal antibody (1:100, Bac-Trace®, LGC Sera Care, Milford, MA, USA). Tissues were analyzed using a light microscope DM2500 (Leica Microsystems, Wetzlar, Germany).

2.3. Molecular analysis

From each batch, at each time point, 30 specimens were analyzed for the presence of the pathogens mostly involved in oyster mortality: *OsHV-1*, *Vibrio* clade *splendidus* and *Vibrio aestuarianus*. After the opening of the shell, oysters were fixed in ethanol. DNA was extracted from 25 to 50 mg of homogenized gills and mantle of individual bivalves, as indicated in the manufacturer protocol of QIAamp® DNA Mini Kit (Qiagen GmbH, Hilden, Germany). The volumes of the reagents from the published protocol were scaled proportionally to fit in 1.5 mL tubes. To avoid excessive dilution, each sample's final elution was performed in 50 μL of elution buffer, then DNA were stored at −18 °C until analysis. After thawing, DNA integrity and quality were assessed by measuring the absorbance at 260 nm and 280 nm, and DNA contamination was excluded by A260/280 ratio (NanoDrop Lite Spectrophotometer, Thermo Fisher Scientific Inc., Waltham, MA, USA).

Presence of *OsHV-1* DNA was determined by QuantiFast SYBR® Green real time PCR Kit (Qiagen, GmbH, Hilden, Germany) with the primers described in Table 1. The positive samples were further analyzed by end-point PCR for ascertain the presence of a 12 bp deletion, which was reported in the variant μVar as well as in other micro-variants (upstream ORF4 of the reference OsHV-1 genome, GenBank: AY509523) (Segarra et al., 2010). Amplicons (expected size of 157 bp) were directly sequenced by ABI Prism 3130xl Genetic Analyzer (Life Technologies) at Istituto Zooprofilattico Sperimentale delle Venezie.

Quantitative PCR (qPCR) protocols (*V. splendidus* clade reaction mix:1× Brilliant III Ultra-Fast QPCR Master Mix, Agilent Technologies®; 0.8 μM of each primer; 0,2 μM of probe; 20 μL final volume. *V. aestuarianus* reaction mix: 1× Brilliant III Ultra-Fast QPCR Master Mix, Agilent Technologies®; 0,3 μM of each primer; 0,2 μM of probe; 20 μL final volume) and primers (Table 1) described by Saulnier et al. (2009) were employed for the detection and absolute quantification of *V. splendidus* clade and *V. aestuarianus* DNA. qPCR was performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) under the following conditions: activation enzyme 3 min at 95 °C; 40 cycles: denaturation 10 s at 95 °C, annealing 20 s at 60 °C for *V. aestuarianus* or 90 s at 60 °C for *V. splendidus*. The fluorescence is recorded at the end of each cycle with Texas Red / FAM filters. The quantity of molecular targets was estimated including, for each qPCR analysis, at least 5 dilutions in triplicate of a standard curve prepared using 1:10 serial dilutions of bacterial DNA suspension corresponding to a known amount of bacterial DNA genomes (extracted from bacteria culture). Pathogen quantification was expressed in genomes/μL with limits of quantification (LoQ) of 10 genomes/μL for *V.aestuarianus* and 100 genomes/μL for *V. splendidus*. Samples were considered negative for *Vibrio* when no amplification and no sigmoid curve were detected and positive when quantification levels were ≥ LoQ. Samples with quantification levels below LoQ were considered positive yet not quantifiable.

Table 1
Primers and probes used for real time, end point, and quantitative PCR in this study.

Target	Name	Sequence (5′-3′)	Reference
Os-HV1	HVDP-F	ATTGATGATGTGGATAATCTGTG	Webb et al., 2007
	HVDP-R	GGTAAATACCATTGGTCTTGTTCC	Pepin et al., 2008 Pepin, 2013
Os-HV1 μVar	C2	CTCTTTACCATGAAGATACCCACC	Segarra et al., 2010
	C6	GTGCACGGCTTACCATTTTT	
V. splendidus clade	16S SpF2	ATCATGGCTCAGATTGAACG	Saulnier et al., 2009
	16S SpR2	CAATGGTTATCCCCCACATC	
	16S probe	FAM-CCCATTAACGCACCCGAAGGATTG-BHQ1	
V. aestuarianus	dnaJ-F	GTATGAAATTTTAACTGACCCACAA	Saulnier et al., 2009
	dnaJ-R	TCAATTTCTTTCGAACAACCAC	
	dnaJ-probe	Texas Red-TGGTAGCGCAGACTTCGGCGAC-BHQ2	

2.4. Remote sensing data collection and analysis

Sea surface temperature (SST) and chlorophyll-a (chlor-a) measures were obtained from the database provided by Moderate Resolution Imaging Spectroradiometer (MODIS) instruments aboard NASA's Aqua satellite.

Sea surface temperature determination is based on MODIS-calibrated mid- and far-infrared (IR) radiances. It is an estimate of the warmth of the ocean's "skin" (top millimeter) at 1-km resolutions and consists of four fields, daytime (D1, D2) and night time (N1,N2), which are produced and calculated as daily, weekly, monthly, and yearly estimations (https://neo.gsfc.nasa.gov/view.php?datasetId=MYD28M, accessed on 3rd May 2022).

The near-surface concentration of chlor-a is estimated in mg/m^3, using an empirical relationship derived from in situ measurements of chlor-a and remote sensing reflectance in the blue-to-green region of the visible spectrum (https://oceancolor.gsfc.nasa.gov/atbd/chlor_a/, accessed on 3rd May 2022.).

Data from November 2017 to October 2019 were collected monthly. Chlor-a and SST were sampled using the point location of the oyster farm with QGIS (https://qgis.org/en/site/, version 3.16) command *sample raster values* (https://docs.qgis.org/3.4/en/docs/user_manual/processing_algs/qgis/rasteranalysis.html#sample-raster-values, accessed on 4th May 2022). The mean (± standard deviation) SST and chlorophyll-a were calculated for the three-month period before each date of sampling.

2.5. Statistical analysis

Cardinal data were assessed for normality using the Shapiro-Wilk test. All normally distributed cardinal variables were compared with repeated measure ANOVA (Analysis of Variance) and subsequent Holm–Sidak post-hoc test. When data were not normally distributed, statistical analyses were performed by means of a nonparametric approach: the Friedman test and subsequent Dunn's multiple comparison test were used to compare study time-points. For sea surface temperatures and chlorophyll-A, the Kruskal-Wallis and subsequent Wilcoxon's multiple comparison tests were used. The Chi-square (χ^2) test for trend was used to analyze the mortality frequency. Data were analyzed using the software GraphPad Prism 8 for MacOS, version 8.2.1 (GraphPad Software Inc., San Diego, CA, USA) and R version 4.1.3 (https://www.r-project.org). Differences were considered statistically significant when *p*-values were $P < 0.05$.

The effect of SST and chl-a on oysters' mortality was assessed using a mixed effect generalized linear model (GLM), Poisson family, which was fitted using the R package *lme4*. The employed model was:

$$ln(Y_j) = \beta_0 + \beta_1 SST + \beta_2 chla + \frac{1}{n_j} + u_j + e \quad (1)$$

where $ln(Y_j)$ was the natural logarithm of the count of dead oysters, β_0 was the baseline mortality, β_1 the effect of each SST degree increase on mortality, β_2 the effect of each chl-a mg/m^3 increase, $\frac{1}{n_j}$ the number of sampled oysters per batch which was introduced as an offset. A normally distributed batch-level effect, $u_j \sim N(0,1)$, was introduced to adjust for multiple observations derived from the same batch, along with a random error factor, *e*. Odds ratios for SST and chl-a were calculated as $exp(\beta_1)$ and $exp(\beta_2)$, respectively. The 95 % confidence intervals (95 %CI) were estimated using the Wald's method.

The effect of SST and chl-a on *Vibrio* spp. concentration was assessed using a mixed effect GLM, Gaussian family, using *lme4* R package. The model was:

$$\mu_{ij} = \beta_0 + \beta_1 SST + \beta_2 chla + u_j + e \quad (2)$$

Where μ_{ij} was the estimated *Vibrio* spp. concentration of the *i*-th specimen of the *j*-th batch, β_0 was the baseline concentration, β_1 was the effect of each SST degree increase on *Vibrio* spp. concentration, β_2 was the effect of each chl-a mg/m^3 increase. As in (1), to adjust for repeated measures within the same batch, a batch-level random effect, $u_j \sim N(0,1)$, was introduced, along with the random error factor, *e*.

Eventually, to estimate the effect of *Vibrio* spp. concentration on oysters' mortality, a mixed effect GLM, Poisson family, was fitted (3). It included the batch-level effect as well, to adjust for within-batch repeated measures.

$$ln(Y_j) = \beta_0 + \beta_1 SST + \beta_2 Chl\text{–}a + \beta_3 Vibrio + \frac{1}{n_j} + u_j + e \quad (3)$$

In (3), SST, chl-a, and *Vibrio* spp. concentration were standardized, in order to compare the magnitude of the effects.

3. Results

3.1. Growth performances and mortality rate

The growth of the oysters was monitored and analyzed. According to statistical analysis, the batches showed a significant growth during different sampling times (Table 2). At post-hoc multiple comparison, all batches showed significant growth between T0 and the other timepoints ($p < 0.0001$) for all four measured parameters (weight, width, length and height). T1 showed a significant differences with all the timepoints ($p < 0.01$), except for the batch C13 (width, length and height recorded in T1 were not significantly different with the subsequent timepoints) and for the batch 14 (all four growth parameter in T1 were not significantly different with the subsequent timepoints). T2 showed no significant difference with T3 in any batch and for any growth parameter.

3.2. Mortality rate

The mortality rate of the oysters was monitored and analyzed. According to statistical analysis, all the batches of oysters showed a significative increase in mortality during or after summer months (Fig. 2; **C11** $\chi^2 = 5.529$; $p = 0.0187$; **C12** $\chi^2 = 13.82$; $p = 0.0002$; **C13** $\chi^2 = 9.297$; $p = 0.0023$; **C14** $\chi^2 = 7.860$; $p = 0.0051$; **C15** $\chi^2 = 13.95$; p = 0.0002; **C16** $\chi^2 = 12.01$; $p = 0.0005$). In 2018, all the batches showed significant difference in mortality frequencies between T2 (average mortality rate in different batches from 31.2 % to 60 %) and the other timepoints (from 0 % to 2.5 %) ($p < 0.0001$). Batches of 2019 showed different results. For C15, the mortality in T2 (16.2 %) showed significant differences when compared to T0 and to T1 (both 0 %, $p = 0.0001$), and also in T3 (10.0 %) when compared to T0 and to T1 ($p = 0.0065$), indicating an incresing in mortality. In C16, the mortality in T2 (28.7 %) showed significant differences when compared to T0 (1.2 %) and to T1 (0 %) ($p < 0.0001$), and in T3 (mortality 8.7 %) when compared to T2 (28.7 %) (p = 0.002) and T1(0 %) ($p = 0.0136$). While, in 2018, mortality was only found in the summer sampling, in 2019, in addition to the summer period, mortality remained significant also in the autumn sampling.

3.3. Histological and immunohistochemical analysis

At T0, T1 and T3 all bathces revealed tissues in a physiological status but in summer sampling (T2), pathological conditions were observed. In 2018, these samples revealed a high prevalence (80 %) of atrophy of the tubules (tubule regression) in the digestive gland (Fig. 3A-B); tubular necrosis and frequently clusters of rod-cell bacteria only rarely adhered to the atrophic epithelium or free in the lumen (Fig. 3C), whereas a slight

Table 2
Mean values ± standard deviation of weight (g), width, length and height (cm) of cupped oysters (*Crassostrea gigas*) during pre-fattening phase (χ2r = results of Friedman statistics).

		T0 - Winter	*T1 – Spring*	*T2 - Summer*	*T3 - Autumn*	*Statistical results*
C 11	weight	0.1797 ± 0.0522	1.166 ± 0.493	3.018 ± 1.943	6.108 ± 7.706	$\chi^2_r = 189.9$; $p < 0.0001$
	width	0.9931 ± 0.1119	2.320 ± 0.5228	3.259 ± 0.9030	3.884 ± 1.661	$\chi^2_r = 183.2$; $p < 0.0001$
	lenght	0.8344 ± 0.1018	1.578 ± 0.2479	1.903 ± 0.3959	2.149 ± 0.6716	$\chi^2_r = 168.2$; $p < 0.0001$
	height	0.3531 ± 0.0618	0.7081 ± 0.1112	0.9619 ± 0.1931	1.165 ± 0.4181	$\chi^2_r = 168.2$; $p < 0.0001$
C 12	weight	0.1780 ± 0.0417	0.3703 ± 0.0942	2.246 ± 1.312	3.979 ± 5.377	$\chi^2_r = 200.0$; $p < 0.0001$
	width	0.9456 ± 0.1131	1.3490 ± 0.1817	2.973 ± 0.8851	3.201 ± 2.019	$\chi^2_r = 199.7$; $p < 0.0001$
	lenght	0.8638 ± 0.1250	1.136 ± 0.1654	1.792 ± 0.3154	1.847 ± 0.6226	$\chi^2_r = 193.0$; $p < 0.0001$
	height	0.4019 ± 0.0460	0.4844 ± 0.0745	0.9031 ± 0.1888	1.057 ± 0.7183	$\chi^2_r = 200.0$; $p < 0.0001$
C 13	weight	0.2502 ± 0.0445	1.675 ± 0.8152	2.654 ± 1.389	7.571 ± 10.5	$\chi^2_r = 178.4$; $p < 0.0001$
	width	1.1410 ± 0.1364	3.264 ± 0.8855	3.264 ± 0.8855	4.505 ± 2.609	$\chi^2_r = 169.9$; $p < 0.0001$
	lenght	0.9275 ± 0.091	1.958 ± 0.3623	1.958 ± 0.3623	2.118 ± 0.6622	$\chi^2_r = 164.0$; $p < 0.0001$
	height	0.4338 ± 0.0674	0.9825 ± 0.2173	0.9825 ± 0.2173	1.285 ± 0.4854	$\chi^2_r = 182.3$; $p < 0.0001$
C 14	weight	0.3019 ± 0.0787	1.458 ± 0.7355	2.110 ± 1.680	4.508 ± 6.019	$\chi^2_r = 149.1$; $p < 0.0001$
	width	1.196 ± 0.1449	2.591 ± 0.6515	2.960 ± 1.179	3.494 ± 1.798	$\chi^2_r = 147.5$; $p < 0.0001$
	lenght	1.036 ± 0.1047	1.669 ± 0.2386	1.773 ± 0.3479	1.919 ± 0.6025	$\chi^2_r = 147.6$; $p < 0.0001$
	height	0.4038 ± 0.0589	0.7269 ± 0.1378	0.7675 ± 0.2326	1.025 ± 0.5047	$\chi^2_r = 145.9$; $p < 0.0001$
C 15	weight	0.1795 ± 0.0428	0.7852 ± 0.5692	5.897 ± 3.698	8.860 ± 5.224	$\chi^2_r = 213.9$; $p < 0.0001$
	width	1.120 ± 0.1858	2.159 ± 0.7474	5.065 ± 1.734	5.468 ± 1.714	$\chi^2_r = 211.9$; $p < 0.0001$
	lenght	0.840 ± 0.0866	1.501 ± 0.3100	2.228 ± 0.5412	2.391 ± 0.5867	$\chi^2_r = 197.2$; $p < 0.0001$
	height	0.3638 ± 0.0579	0.5463 ± 0.1227	1.021 ± 0.3138	1.209 ± 0.4076	$\chi^2_r = 199.6$; $p < 0.0001$
C 16	weight	0.1971 ± 0.0499	1.155 ± 0.5869	5.410 ± 4.196	6.482 ± 4.608	$\chi^2_r = 203.1$; $p < 0.0001$
	width	1.045 ± 0.1179	2.300 ± 0.6543	4.007 ± 1.388	5.468 ± 1.714	$\chi^2_r = 203.5$; $p < 0.0001$
	lenght	0.8725 ± 0.1018	1.690 ± 0.3145	2.356 ± 0.4770	2.391 ± 0.5867	$\chi^2_r = 182.4$; $p < 0.0001$
	height	0.3863 ± 0.0611	0.7125 ± 0.1095	1.195 ± 0.7244	1.209 ± 0.4076	$\chi^2_r = 184.3$; $p < 0.0001$

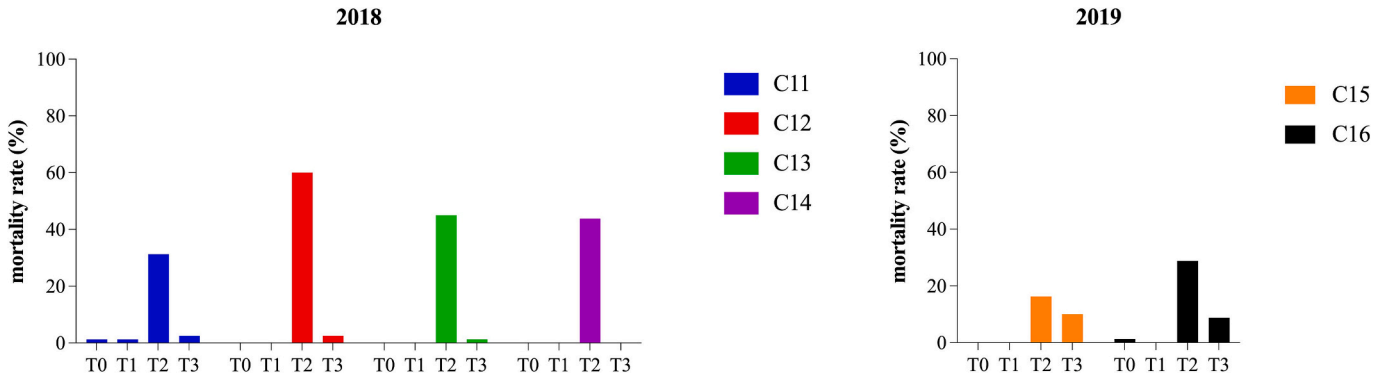

Fig. 2. Mortality rate (%) of different batches of cupped oyster (*Crassostrea gigas*) raised in middle of Adriatic Sea, during pre-fattening stage.

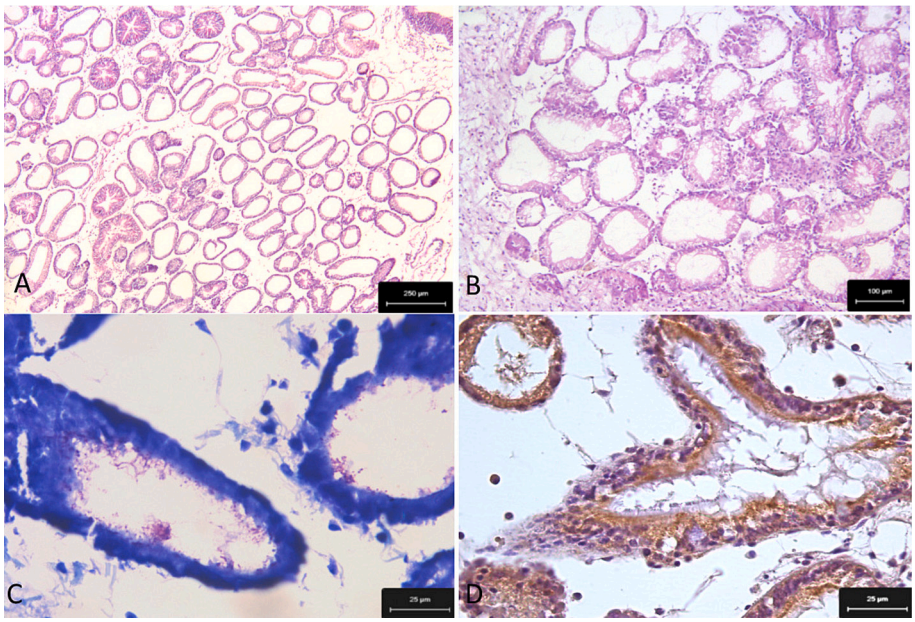

Fig. 3. Cupped oysters (*Crassostrea gigas*), batch C11, sampled at T2. A-B. Digestive gland, with marked tubular atrophy (HE). C. Digestive gland, clustered rod-cell bacteria adhered to the atrophic epithelium or free in the lumen (Giemsa stain). D. Digestive gland, tubular epithelium with numerous immunostained *Vibrio* spp. (IHC).

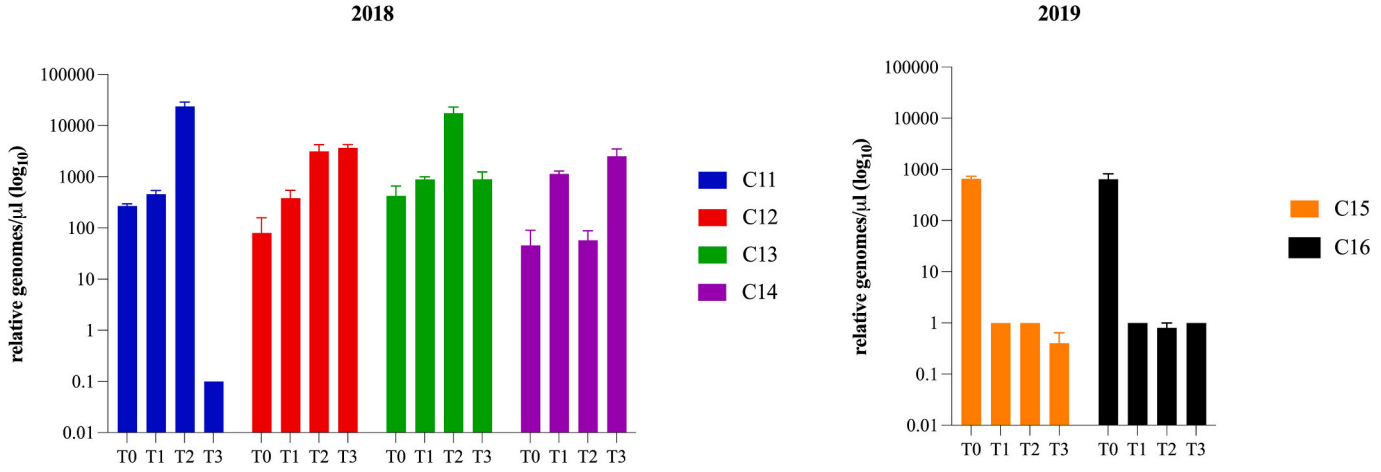

Fig. 4. *Vibrio splendidus* concentration values, expressed as number of relative genomes/µl (log_{10}), of different batches of Cupped oysters (*Crassostrea gigas*) raised in the middle of Adriatic Sea, during pre-fattening stage.

hemocyte infiltrate was present in the intertubular interstitium. In the same samples, IHC for *Vibrio* spp. revealed the presence of numerous stained bacteria, particularly in the tubular epithelium of the digestive gland and in the intestine (Fig. 3D). In 2019, samples revealed the same condition of atrophy and tubular necrosis, but Giemsa stain did not reveal the presence of bacteria and IHC for *Vibrio* spp. was negative.

3.4. Research of pathogens

No samples were found positive for *Oysters Herpesvirus-1* or *Vibrio aestuarianus*. *Vibrio* clade *splendidus* was found in all the batches analyzed, with a significant increase in the summer months (Fig. 4; **C11** $F = 19.87$; $p < 0.0112$; **C12** $\chi^2_r = 13.56$; $p < 0.0001$; **C13** $\chi^2_r = 10.68$; $p = 0.0055$; **C14** $F = 6.0$; $p = 0.0097$; **C15** $\chi^2_r = 13.62$; $p < 0.0004$; **C16** $\chi^2_r = 13.91$; $p < 0.0039$). At post-hoc multiple comparison, each batch showed significant differences in *V. splendidus* concentration values. In C11, all comparisons between timepoints give significant resultes ($p < 0.05$), except in T0 vs T1, with an increasing in T2 and a decrasing in T3. In C12, a significant increase was shown comparing T0 with T2 ($p = 0.0197$) and T3 ($p = 0.0087$). In C13, the only significant difference emerged with the increasing between T0 and T2 ($p = 0.0087$). In C14, T3 was significantly higher than T0 ($p = 0.0194$) and T2 ($p = 0.0194$). In C15, the only significant difference was obtained between T0 and T3 ($p = 0.0087$), and in C16, between T0 and T2 ($p = 0.0423$) (Fig. 4). While in 2018 *V. splendidus* showed an increase in its concentration during the year and especially in the summer months in all lots, in 2019 the opposite behavior was observed, as very low concentrations of the pathogen were found.

3.5. Sea surface temperature and chlorophyll-a

In both the years of study, the SST followed the expected trend, increasing from February to reach the peak in July–August, then decreasing to the minimum values of winter. However, in 2018, the SST rapidly increased over 16 °C in mid-March and persisted over that threshold until November. In contrast, in 2019, the temperature increase was gentler between February and May, when it reached 16 °C, then arose rapidly. The two years chl-a trends did not overlap. In 2018, it started high, then decreased slightly in February before rising rapidly to peak in spring (mid-April), whereas in 2019 lower values were recorded until an abrupt peak in June. Both in 2018 and 2019, chl-a dropped during summer months before increasing again during autumn (Fig. 5).

Table 3 reports mean values (± standard deviation) of SST and Chlorophyll-a.

As expected, sea surface temperature differed among seasons ($p = 0.0009$); in particular, T0 and T1 were significantly lower than T2 and T3. Also, chlorophyll-a was different ($p = 0.0079$), with T3 values significantly lower from each other sampling date (Fig. 6).

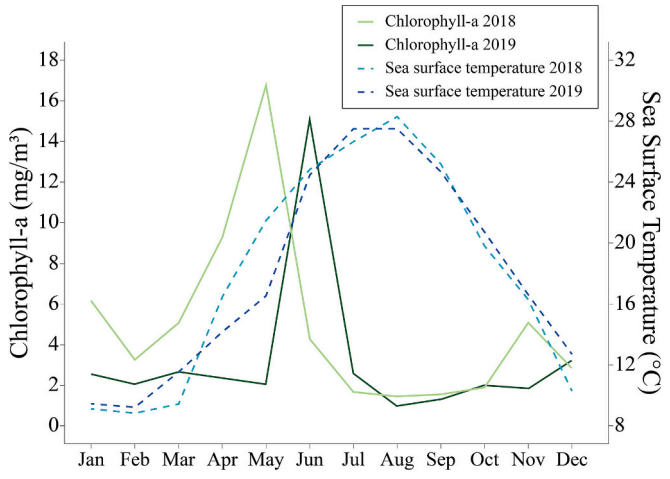

Fig. 5. Annual trend of Chlorophyll-a (mg/m^3), on the left, and sea surface temperature (°C), on the right: data from January 2018 to December 2019.

Table 3
Mean values ± s.d. of the three-month period before the sampling date. Data of sea surface temperature (°C) and chlorophyll-a (mg/m^3) are reported by year and aggregated.

Year	*T0 – Winter*	*T1 – Spring*	*T2 – Summer*	*T3 - Autumn*
SST (°C)				
2018	9.6125 ± 0.7177	11.5667 ± 4.198	24.3633 ± 2.57	24.9212 ± 4.3283
2019	11.9500 ± 3.6558	11.6433 ± 2.4793	22.8117 ± 5.6830	24.2867 ± 3.4437
Mean	**11.0150 ± 2.9069**	**11.6050 ± 3.0838**	**23.5875 ± 4.0364**	**24.3141 ± 3.4983**
Chlorophyll-a (mg/m^3)				
2018	4.2875 ± 2.6779	5.8378 ± 3.0818	7.5917 ± 8.1055	1.6579 ± 0.2483
2019	3.5031 ± 1.3927	2.3483 ± 0.2976	6.5831 ± 7.3987	1.4287 ± 0.5131
Mean	**3.8168 ± 1.7167**	**4.0930 ± 2.7362**	**7.0874 ± 6.9628**	**1.5432 ± 0.3817**

The oysters' mortality was associated with both SST (OR = 1.32, 95 %CI: 1.22–1.44) and chl-a (OR = 1.52, 95 %CI: 1.41–1.65). Also, *Vibrio* spp. concentration was associated with both SST and chl-a ($p < 0.001$). One SST degree increase determined on average a 305.0 genomes/μL increase in *Vibrio* spp. concentration (95 %CI: 157.1–452.8), while 1 mg/m^3 increase of chl-a determined on average a 1025.1 genomes/μL increase (95 %CI: 568.2–1499.5). The effect of SST (OR = 7.04, 95 %CI: 3.98–12.44) chl-a (OR = 2.69, 95 %CI: 2.23–3.24) on oyster mortality remained significant even including the *Vibrio* spp. concentration. On the contrary, *Vibrio* spp. concentration did not show a significant effect on oysters' mortality ($p = 0.07$).

4. Discussion and conclusion

This study considered the very early phase of farming (pre-fattening) after the introduction in the shellfish farm because this represents a critical phase due to the adaptation to new environment and housing. The growing of oysters exhibited a good trend in all the different rearing cycles. During the monitoring, all the batches showed a significant weight gain, demonstrating that raising oysters in the middle of Adriatic Sea is a possible and successful business. Although these productive results are satisfactory, the sustainability of the shellfish culture is different along the Western coast of the Adriatic Sea. The upper open coastal area of the Adriatic Sea, especially along the Emilia Romagna region, shows a productivity higher than that obtained in the waters along the Marche region because of the nutrient enriched waters coming from the Po River (Grant and Pastres, 2019). Anyway, the stocking density of bivalves, at which harvest is maximized without causing negative ecological impacts, must be considered alongside other parameters.

In our study, both in the years 2018 and 2019, a significant increase of mortality was observed in or after summer months. Some researchers, analyzing summer mortalities in different environmental sites, recorded a high mortality rate in September, when gonadal maturation occurred in adult triploid oysters, attributing to these specimens the low capacity of adaptation to environment, respect to diploids (Houssin et al., 2019). In our study, we used diploid specimens and the size recorded at the end of the pre-fattening phase (< 9 g) was very far from the phase of advanced gametogenesis, so we cannot evaluate the relationship between sexual development and mortality.

Histological analysis, performed in all the batches and all the time points of the 2-years monitoring, did not reveal the presence of specific pathogens or the presence of infections that can be implicated in the

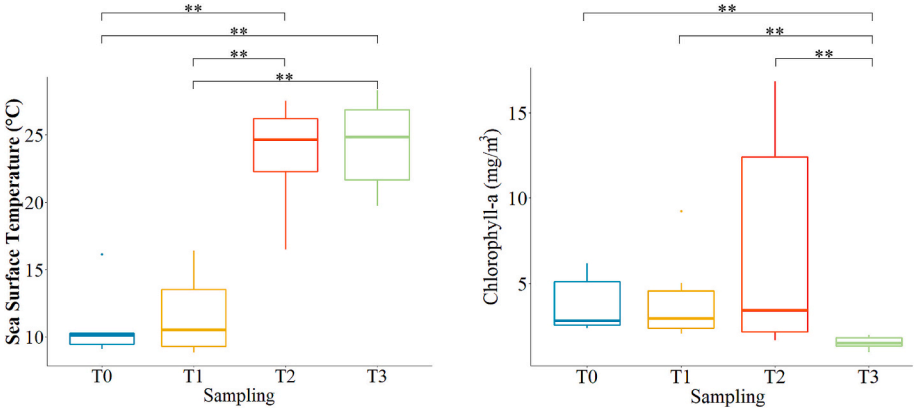

Fig. 6. Boxplots of sea surface temperature (°C) and chlorophyll-a (mg/m^3) by sampling date, whit the results of paired Wilcoxon tests [**:$p < 0.01$].

mortality. However, the high prevalence of atrophy of the tubules in the digestive gland, accompanied by tubular necrosis found in summer sampling, allowed us to evidence that tissues are affected in that period. Molecular analyses did not show the presence of *Oysters Herpesvirus-1* or *Vibrio aestuarianus*, often associated with outbreaks of summer mortality, but the presence of *Vibrio* clade *splendidus* was found in all the samplings, with a significant increase of the load in summer months of the year 2018 which confirmed that *Vibrio* clade *splendidus* includes strains pathogen to oysters. However, the role played by *Vibrio* clade *splendidus* in the mortality event remains to be clarified. Indeed, in 2019, a constant presence but with a low load was observed with persistent high mortality, which leads to thinking that other factors can influence the oysters' survival.

In this scenario, other factors should be considered. Abiotic and biotic factors are indeed important in oyster farming, a zootechnical activity in which it is impossible to supplement food or artificially modulate the environmental parameters, in order to improve productive performance. Several studies have ascertained that environmental factors affect the development of bivalve molluscs, including water temperature (Ramos et al., 2014; Yang et al., 2016; Ibarra et al., 2017; Lassoued et al., 2021; De Marco et al., 2023), water salinity (Paixão et al., 2013; Yang et al., 2016; Lee et al., 2017; Legat et al., 2017;), and the presence of suspended particulate organic matter (Guzmán-Agüero et al., 2013), as well as precipitation regime (Paixão et al., 2013). In 2018, the sea surface temperature rose above 16 °C early (mid-March), and it could possibly explain the high *Vibrio* clade *splendidus* infective load measured during the same year. The temperature remained over this threshold, which is considered optimal for *Vibrio* proliferation, until November. Also, in 2018, chl-a arose in early spring month, peaking in April instead of June, and persisted on higher levels throughout the year. Consistently, lower *Vibrio* loads were observed in 2019 when chl-a concentration was lower as well. Our findings confirmed the positive association between phytoplankton biomass and pathogenic *Vibrio* concentration described in a previous study conducted on a Mediterranean coastal area of France (Lopez-Joven et al., 2018), although biomass measuring methods and *Vibrio* species involved differed. Also, we demonstrated that there was a combined effect of SST, chl-a and *Vibrio* concentration on oysters' mortality, although *Vibrio*'s role is yet to be ascertained. This study contributed to some extent to fill the knowledge gap regarding *Vibrio* ecology in relation to oyster farming in the Mediterranean.

The growth rate of oysters has been put in relation to the different depths in the water column. In Mali Ston Bay (Croatia), the best growth performances were obtained at 3–5 m than at 13 m, where oysters were directly influenced by detritus and phytoplankton as consequence of the most favorable parameters as temperature, oxygen, chlorophyll concentration and particulate carbohydrates. The highest condition index was recorded in spring and summer of the first rearing year. This indicates that the farming cycle could be shortened to 18 months instead of 2 years (Zrnčić et al., 2007).

In France, research on the growth and survival rate of the cupped oyster showed that the total and soft tissue weight gain was higher in oysters grown above the bottom, while the mortality rate was significantly lower. Mortality distribution during the farming period was characterized by an increase during the summer months, likely due to spawning activities. Summer mortalities revealed a negative energy budget during the high maturity stage caused by reduced absorption deficiency. Oysters developed greater weight loss due to spawning, that led to a decrease in carbohydrate reserves, making them more susceptible to mortality (Soletchnik et al., 2002).

In oysters monitored during this study, growth curves and rates, in terms of total body weight, reflected the common pattern at temperate latitudes, where the growth from spring to early autumn is favored by the nutritional conditions and increasing temperatures, in contrast with the low food availability in colder months. In the second year, although benefitting of a short autumn peak in biomass availability (Oct-Nov), the high water temperature during autumn 2018 and winter 2019 could have affected the optimal conditions. It is well known that oysters are ectothermic organisms and changes in water temperature away from their optimum compromise the physiological processes that become less efficient and it is necessary to require more energy.

As reported in a recent study (Pereira et al., 2020), shell growth, condition index, lipid content and survival of flat oysters and Sydney rock oysters were all significantly reduced by elevated seawater temperature.

Oysters farming is a reliable activity in the Adriatic Sea, although the high mortality and the resulting economic loss during the summer months could suggest finding new locations for product finishing, in order to maximize production and the profitability of the farm. Since SST and chl-a, which proved to be the most relevant variables for oysters' mortality, are easily extracted for any location on earth, their use should be encouraged when searching for a suitable oysters' farming spot.

Ethics statement

The animal study was conducted according to the European Directive 2010/63/UE and the Italian Legislative Decree 26/2014.

Author contribution

LG, GEM and AR were responsible for the conception of the study. All authors performed raw data interpretation and wrote the manuscript. LG, GEM, NI and AR performed biometric and histological analysis. GA, FT, AV performed microbiological analysis. AMT, AB and A-RA studied environmental parameters and performed statistical analysis. All

authors discussed the results, reviewed the manuscript, provided critical suggestions and comments, and approved the final manuscript.

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CRediT authorship contribution statement

Livio Galosi: Writing – review & editing, Writing – original draft, Formal analysis, Data curation, Conceptualization. **Gian Enrico Magi:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation, Conceptualization. **Giuseppe Arcangeli:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Federica Tosi:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Alessia Vetri:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Adolfo Maria Tambella:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Alessandro Bellato:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Anna-Rita Attili:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Nicolaia Iaffaldano:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation. **Alessandra Roncarati:** Writing – review & editing, Writing – original draft, Resources, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

None.

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Data availability

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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