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Refining the Occurrence of Viral Encephalopathy and Retinopathy, Photobacteriosis, and Vibriosis in Connection with Seawater Physicochemical Parameters: A Five-Year Case Study

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Abstract

The marine environment and its physicochemical parameters play a pivotal role in the context of fish farming. Fundamental physicochemical factors of water, including temperature (T°C), salinity (S psu), dissolved oxygen (D.O. mg/l), and pH, exert a profound influence on the physiology of farmed aquatic animals and the composition of microbial communities. This study aimed to establish correlations between documented disease occurrences in sea bass intensive rearing and the prevailing physicochemical parameters of water. The objective was to analyse the significance of these parameters in disease outbreaks and develop a predictive model for the outbreak risk of key diseases. To this end, real-time physicochemical data were collected daily over five years (2011-2015) from two fish farms (A and B) located in the Gulf of Argostoli, island of Cephalonia, Western Greece. The focus was on incidences of Viral Encephalopathy and Retinopathy (VER), Photobacteriosis caused by *Photobacterium damselae* subsp. *piscicida* (PHDP) and Vibriosis caused by *Vibrio anguillarum* serotype O1 (VAO1). Statistically significant correlations were found between increasing water temperature and salinity and the occurrence of VER, PHDP, and VAO1 diseases. The reduction of dissolved oxygen, when combined with other parameters, synergistically triggered the manifestation of these three diseases. The study's findings were discussed within the context of their potential to predict disease outbreaks based on fluctuations in thephysicochemical parameters of water and anticipated climate change scenarios.

Keywords: physicochemical parameters of water; fish diseases; *Dicentrarchus labrax*.

Introduction

Intensive fish farming is linked to the emergence of novel pathogenic microorganisms and the occurrence of infectious disease outbreaks. The stress induced by intensive fish farming has been reported to impact fish immunity, as stress-related hormones released during such events can affect the functioning of the immune system (Ortuno *et al.*, 2001). Stress can also result from sudden fluctuations in the physicochemical parameters of water. The key parameters in marine fish farming, including temperature (T), salinity (S), dissolved oxygen (D.O.) and pH, are crucial, and changes in such parameters can disrupt the normal functions of both the host and the pathogen, potentially leading to disease or mortality (Boeuf *et al.*, 2001).

Temperature is a primary factor influencing metabolism in poikilothermic organisms, regulating food intake levels, and interacting with external environmental factors (D.O., S, food availability) as well as internal biological factors (age, genetic code), thereby influencing fish development (Portner & Peck, 2010). High water temperatures can reduce oxygen levels due to decreased oxygen solubility and an accelerated rate of biological processes that consume more oxygen. Temperature also affects the pH value of body fluids (blood, cytoplasm), with changes in body temperature being accompanied by changes in pH values. For most organisms, a decrease in the pH values of extracellular and intracellular fluids is noted with rising temperature. Ndong *et al.* (2007) demonstrated a decline in the immune system capacity offish exposed to heat stress resulting from temperature changes. Tilapia (*Oreochromis mossambicus*) reared at 23°C exhibited a better blood profile and lower susceptibility to disease compared to fish reared at 17°C. Additionally, fish raised under more stable temperatures were less affected by diseases than those exposed to temperature fluctuations (Varsamos *et al.*, 2006). Temperature, especially in shallow waters, lakes, or reservoirs experiencing increased daily temperature cycles or occasional temperature shocks during water replacement activities, can act as a stress factor (Person-Le Ruyet *et al.*, 2004; Sampaio & Rosa, 2019).

Temperature and salinity exert a notable influence on the production of melatonin in the seabass epiphysis, subsequently impacting food intake, activity, metabolism, and reproduction (López-Olmeda *et al.*, 2009). High salinity diminishes the solubility of oxygen in water, affecting aquaculture animals by regulating osmotic pressure, ion balance, and energy consumption. Abrupt changes in salinity can induce a transient (up to 80%) increase in the metabolic rate of fish, persisting for up to 10 hours post-salinity change (Abou Anni *et al.*, 2016).

Dissolved oxygen (D.O.) is indicative of water quality in coastal regions and plays a crucial role in maintaining ecosystem balance (NLWRA, 2002). Water renewal in fish cages is vital for oxygen replenishment, removal of metabolic by-products, and facilitating the aerobic decomposition of unused food. Limited oxygen availability (<40% saturation) in sea-cages with high biomass levels leads to diminished appetite, food conversion, and consequently reduced fish growth (Makridis *et al.*, 2018). In intensive farming units, dissolved oxygen serves as a restrictive production capacity factor under the conditions prevailing in the study area. Any reduction in oxygen levels below the required thresholds induces significant stress, impacting both fish growth and the immune system's ability to combat infections (Sampaio & Rosa, 2019).

Active acidity of water (pH) is a critical factor ensuring water quality and enhancing production. It influences numerous biological and chemical reactions and often serves as an indicator of pollution (Vryonis, 2013). High CO2 concentrations and subsequent pH decreases have altered oceanic chemical processes (Beman *et al*., 2011). Seawater acidification affects microbial diversity, primary productivity, and trace elements in oceans, indirectly influenced by the oxygenation levels at different depths, impacting pH and the formation of calcium compounds that are crucial for building bones and shells in aquatic organisms (Sampaio & Rosa, 2019). All these parameters of water, through abrupt changes or sustained fluctuations, can act as predisposing factors for disease outbreaks, by disrupting the physiological status of fish, inducing stress, and influencing microbial communities, thereby promoting the multiplication of pathogenic organisms (Lucas *et al.*, 2019).

Viral Encephalopathy and Retinopathy (VER) that is caused by b-nodaviruses is one of the globally prevalent diseases affecting farmed fish (Toffan *et al.*, 2017a; Toffan & Panzarin, 2020). Disease prevention in fish farms involves implementing preventive hygiene measures and adopting biosecurity management practices to deter pathogen entry into rearing systems (Shetty *et al.*, 2012). Vaccination is also a key preventive strategy (Bakopoulos *et al.*, 2018). In the Mediterranean basin, VER is now endemic, with documented occurrences in both farmed and wild fish (Toffan *et al*., 2017b). In intensive fish farming areas, the virus can be transmitted by wild fish acting as carriers (Krkošek, 2017). B-nodaviruses exhibit significant resilience and survive for extended periods in seawater at low temperatures (4^{o}C to 15^{o}C), retaining infectivity under a range of pH values from 3 to 7 (Frerichs *et al.*, 2000).

Photobacteriosis (PHDP) is another significant marine fish disease, which is caused by the Gram-negative bacterium *Photobacterium damselae* subsp. *piscicida* (Phdp), accounting for high pathogenicity in various marine fish species without host specificity (Bakopoulos *et al.*, 2015; Pham *et al.*, 2021). The pathogenicity of PHDP is contingent on the presence of virulence genes (Lattos *et al.*, 2022) and is more prevalent at higher water temperatures (>23^{o}C), salinity (20-30psu), and in contaminated (e.g. polluted) water (Korun *et al.*, 2005; Essam *et al.*, 2016; Eissa *et al.*, 2021).

Vibriosis (VAO1), a haemorrhagic septicaemia with high mortality, is another major disease affecting farmed marine fish, caused by the Gram-negative bacterium *V. anguillarum*. Vibrio species are present in water and fish gut microflora, spreading around fish farms through natural fish populations. The infection spreads through contact between sick and healthy fish, particularly under conditions favouring disease development (Hickey *et al.*, 2018).The objective of this study is to correlate recorded disease incidences in the field with the prevailing physicochemical parameters of waters during intensive rearing of sea bass. The study aims to analyse the statistical significance of these parameters for disease outbreaks and build a predictive model for assessing the risk of outbreak of the most crucial diseases in farmed fish.

Materials and Methods

Location

The study was conducted in the Gulf of Argostoli, island of Cephalonia, a region characterized by diverse anthropogenic activities, including fishing, aquaculture, tourism, and coastal shipping, particularly during the summer months. Three floating fish farms are located in the eastern and north-eastern parts of the Gulf, known as Mid-East (Fig. 1) (Spinos *et al.*, 2012). The largest of these fish farms, labelled as A and B, play a central role in the study. Fish farm A, in the south, covers an area of 0.26 km^2, featuring 70 fish cages. On the other hand, fish farm B, in the north, spans an area of 0.39 km^2, comprising 45 fish cages. The fish farms primarily rear *Dicentrarchus labrax* (sea bass), *Sparus aurata* (sea bream) and *Argyrosomus regius* (meagre). For the period 2011-2015, the annual capacity for *D. labrax* was 570 tons at fish farm A and 690 tons at fish farm B.

Notably, fish farm A is situated in waters 4 meters deeper than fish farm B. The two farms are approximately 4 km apart, with differences in sea bed composition (Spinos *et al.*, 2012) and domain currents (Conides *et al.*, 2006) between them. Furthermore, fish farm A is more exposed to anthropogenic activities due to its proximity to the ports of Argostoli and Lixouri. Although fish farm B has a larger annual capacity, it rears a smaller number of fish, but these fish are larger compared to those of fish farm A.

Fig. 1: Study area.

Data collection

Daily records of water temperature (T, ^oC), salinity (S, psu), dissolved oxygen (D.O., mg/l), pH, and the presence or absence of three fish diseases (VER, PHDP, VAO1) were obtained from fish farms A and B during the period spanning from January 1, 2011, to December 31, 2015. A binary coding system was used for the data, with [1] indicating the presence of disease and [0] indicating its absence. For the assessment of mortality, the number of deceased fish at the study site during the specified period was documented. The percentage mortality rate for each fish farm was calculated using the formula: Mortality%=(Number of Dead fish Number of fish in the cage)×100 Mortality%=(Number of fish in the cage Number of Dead fish)×100.

Water temperature data were collected using waterproof auto-recorders (Onset HOBO® UA-001-08 Waterproof Pendant 8K Temperature Data Logger, U.S.A.) strategically positioned at the centre of the floating fish farms, at positions A and B. The recording interval was set to 1 hour. Salinity, dissolved oxygen, and pH were recorded daily at the centre of each cage at both farms using portable digital instruments (WTW®Cond197i, Germany). All measurements were taken at a water depth of 1-2 meters.

Identification of pathogens

The microorganisms causing diseases were identified according to the following methods:

a) Betanodavirus Detection:

The presence of Betanodavirus in brain and eye samples taken from diseased fish was identified using RT-PCR, following the protocol outlined by Dalla Valle *et al*. (2000). In brief, tissues were homogenized, and supernatants were collected and sterilized (0.45-micron filters) in Leibovitz medium (L-15, Gibco) containing 5% foetal calf serum, L-glutamine, and a 2% standard antibiotic/antimycotic solution. These supernatants were then used to seed monolayers of the SSN-1 cell line. Isolated supernatants from cell cultures exhibiting cytopathic effects (CPE) were collected, and total RNA was extracted using the QIAamp Viral RNA kit (Qiagen, Germany). The cDNA synthesis and amplification were performed using the “One Step RT-PCR” kit (Qiagen, Germany) as per the manufacturer’s instructions. One Step real-time RT-PCR reactions were executed with the Quantitect Probe RT-PCR kit (Qiagen), using 10µL of purified RNA. The reactions included 600 nM of each primer, 400 µM of dNTPs, 5× QIAGEN OneStep RT-PCR Buffer, and 2 µL of QIAGEN OneStep RT-PCR Enzyme Mix (Qiagen). The reaction protocol comprised 30 min at 50°C, 15 min at 95°C, and 40 cycles of denaturation/extension for 15s at 94°C, 60s at 60°C, and 60s at 72°C, followed by 10 min at 72°C. All reactions were performed in technical triplicate using a Rotor Gene Q PCR Detection System.

b) PHDP Isolation:

PHDP was isolated from spleen or kidney samples taken from diseased fish using standard microbiological techniques and the API20E system (Bakopoulos *et al.*, 1995; Topic Popovic *et al.*, 2007).

c) VaO1 Identification:

VaO1 was identified using the Aqua rapid-Va kit (*V. anguillarum* from BIONOR A/S, Skien, Norway) following the manufacturer's recommendations.

Efforts were made to correlate the collected and processed data with the annual losses of the total seabass population atthe specific units.

Data analysis

Moving average by step 15 days on both data sets (physicochemical: MAF_{15} and diseases presence/ absence: MAD_{15}),and the short-term variability of each physicochemical parameter (Pr) by the standard deviation of 15 consecutive days (SD_{Pr15}), were estimated. This standard deviation was attributed to the 8th day of this temporal window (following the approach used in the moving average computation). In order to identify seasonal pattern, on the new time series data set (X_t: MAF_{15}, MAD_{15}, SD_{Pr15})an harmonic regression model (HREG) was adjusted to the X_t time series:

$$X_t = c + \sum b_{1i}\, cos(\omega_\iota t) + b_{2i}\, sin(\omega_\iota t),$$

Wherea, b_{1i}, b_{2i} are coefficients estimated by least squares regression techniques, $\omega_i$is the frequency of a cyclic component (Diggle, 1990) for i=1 (annual component: ω_1=0.002738 cpd) and i=2 (semi-annual component: ω_2=0.005476 cpd). The frequencies and waves (cosine: cos and sine: sin) used in the final model were selected through stepwise variable selection. In order to identify differences of X_t between the two fish farms, on the HREG residuals t-test was applied (t-test; P=0.05) (Zar, 1999).

The presence / absence of disease (PD_t) relationships on day t in relation to the water parameters (WP) the following equation was described:

$$PD_t = \frac{1}{1 + e^{-WP_t}}$$

where,

$$WP_t = -\ln\left(\frac{1 - PD_t}{PD_t}\right) = k + \sum\nolimits_{j=1}^{8} \sum\nolimits_{i=1}^{8} b_{j,i} X_{j,i}$$

3

where 'k', 'b' are coefficients, 'j'concerns the water parameters (X: T, S, D.O. and pH) and their SD and 'i' is time lags: t-10 days: i = 1, t-20 days: i = 2, t-30 days: i = 3 and t-40 days: i = 4.

The significant predicted variables ($WP_{j,i}$) used in the final model were selected by the step-wise variable selection method (*F*-to-remove, $P \leq 0.05$) (Zar, 1999). The Variance Inflation Factor (VIF) was used to assess the level of collinearity of the explanatory variables of the multiple regression analysis. High or low VIF values represent a high or low level, respectively, with a standard collinearity interrupt value of VIF = 10. A VIF value close to 1 means that the variables are not statistically significant ($r \approx 0$) (Hair *et al*., 1998). In this study, the cut off value of the VIF value was set at 2. Finally, the relative explanatory significance of the selected variable was evaluated by controlling the changes in the values of the coefficient of determination (R^2). The analyses were performed with the SPSS 17.0 statistical software package (SPSS Inc., Chicago IL, USA).

To evaluate the performance of the model for each disease, two percentages were calculated: a) the correctly predicted data about the presence of the disease in relation to the total actual data (recorded data)about the presence of the disease and b) the correctly predicted data about the presence of the disease in relation to the set of predicted data on the presence of the disease.

Results

During the study, data were collected in the field, analysed and processed in order to assess the effect of environmental parameters on the occurrence of diseases at fish farms located in the Gulf of Argostoli. Table 1 summarizes the outbreaks of diseases at the studied fish farms and shows that they are characterised by seasonal periodicity. In addition, a number of mixed cases of diseases were observed, such as PHDP and VAO1 (2013-2014) as well as VER and PHDP (2015), which were distinguished from the clinical findings of the fish and microbiological sampling. It appears that percentage mortality rates at location B were lower than at location A.

All-time series displayed an annual and a semi-annual cycle (Table 2), indicating that the variables follow a constant annual pattern (Fig. 2).

The arithmetic coefficients of the HREG models fitted to the daily values, using the estimated frequencies as independent variables, and stepwise variable selection are shown in Table 2. The r^2 value of the HREG models ranged from 0.05 for standard deviation of salinity (here after SSD) to 0.97 for Temperature (T). The t-test on the HREG residuals of physicochemical variables showed statistical differences between farms (Table 2: $P<0.05$) while on the HREG residuals of presence/absence, diseases showed non-statistical differences between farms (Table 2: $P>0.05$).

The highest values of temperature were recorded at the end of summer (August) and the lowest values at the end of winter (February). The lowest values of salinity and pH were recorded in the winter and the highest in the summer; finally, the lowest values of dissolved oxygen were recorded in the summer and the highest in the winter, through out the study period.

Graphs A, B, C (Fig. 3) show the appearance of the

Table 1. Mortality (%)associated with diseases in fish farms located at two sites in the Gulf of Argostoli, Cephalonia,

		Mortality (%)	
Date of diseases occurrence	**Disease**	**Farm A**	**Farm B**
01/07-31/08/2011	PHDP	7.0	3.3
01/06-31/07/2012	PHDP	8.0	1.0
13/09-30/11/2012	VER	32.0	4.0
01/07-31/07/2013	PHDP & VAO1	15.0	7.0
01/09-07/11/2013	VER	10.0	2.0
01/07-31/07/2014	PHDP & VAO1	7.0	3.0
17/10-15/12/2014	VER	11.0	4.5
01/01-31/05/2015	VAO1	1.5	1.8
14/08-09/11/2015	VER & PHDP	17.7	4.8

Table 2. Statistically significant coefficients of the harmonic multiple regression (HREG) models for physicochemical variables, presence/absence of diseases, and the annual and semi-annual frequency residuals between farms. *N=3652, where T=temperature, TSD=standard deviation of T, S=salinity (psu), SSD=standard deviation of salinity, O=dissolved oxygen (mg/ml), OSD=standard deviation of oxygen, pH=pH, pHSD=standard deviation of pH, VAO1=Vibriosis, PHDP=Photobacteriosis, VER=Viral Encephalopathy & Retinopathy.*

Period (T_i) in days, $xi=(2\pi t/T_i)$								
	T	**365.25**		**182.62**			**t-test results**	
Xt	a	Cos(x_1)	Sin(x_1)	Cos(x_2)	Sin(x_2)	r^2	P	Fish farm
T	19.88	-3.26	-4.59	-0.45	0.10	0.95	0.000	B<A
TSD	0.66	-0.21	-0.10	0.13		0.28	0.000	B>A
S	38.54	-0.56	-0.34	0.19	0.19	0.67	0.000	B<A
SSD	0.19	-0.04		0.01		0.05	0.730	B=A
O	6.06	0.25	0.36	0.02	-0.02	0.96	0.000	B>A
OSD	0.05		-0.01	0.00	-0.02	0.22	0.390	B=A
pH	7.76	0.02	0.05	0.02	0.01	0.70	0.000	B<A
pHSD	0.01	-0.002	-0.002	0.001	0.003	0.12	0.890	B=A
VAO1	0.12	-0.03	0.10	0.03	0.05	0.07	0.910	B=A
PHDP	0.25	-0.19		0.08	0.08	0.15	0.950	B=A
VER	0.16	0.06	-0.28	-0.17	-0.08	0.46	0.960	B=A

three diseases VAO1, PHDP, and VER, according to the actual data collected. The results show that in the months of the year when the water parameters changed (T, S, D.O. and pH) the occurrence of diseases at fish farms was affected.

Multiple regression analysis showed that the R^2 model of disease presence / absence potential ranged from 0.11 for VAO1 to 0.41 for VER (Table 3). The likelihood of presence / absence of VER is negatively correlated with temperature fluctuations during the 10-day lag [SD_T (t-10)] and with a 20-day lag [SD_T (t-10)], with the value of salinity at a delay time of 10 days S (t-10) and positively with the value of temperature at a delay time of 30 days T (t-30). Temperature was the main explanatory variable at a delay time of 30 days (R^2ch = 0.34), followed by salinity at a delay time of 10 days (R^2ch = 0.04). The likelihood of the presence / absence of PHDP was positively related to temperature variability during classification and to the salinity value at a 10-day delay. Salinity was the main explanatory variable at a delay time of 10 days (R^2ch = 0.12), while R^2 was 0.17. According to the model applied in this study, the probability of the presence / absence of

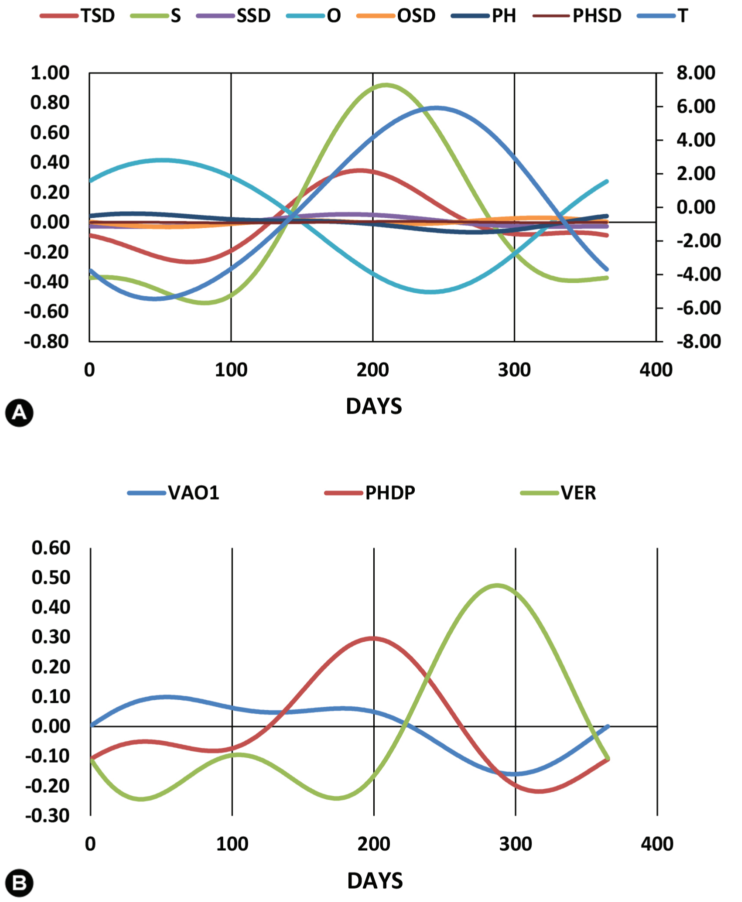

Fig. 2: **A** - Annual variation (HREG) of physico-chemical parameters based on mean value parameters (T=temperature °C), TSD=standard deviation of T, S=salinity (psu), SSD=standard deviation of salinity, O=oxygen (mg/ml), OSD=standard deviation of oxygen, pH, pHSD=standard deviation of pH). **B** - Presence/absence of diseases (t0= 1/1, step 1 day) (VAO1=Vibriosis, PHDP=Photobacteriosis, VER=viral encephalopathy & retinopathy).

Table 3. Statistically significant coefficients (k and bi) of the multi-regression model for the possibility of disease presence/absence on the dataset of 2011-2015. R^2represents the coefficient of determination. Brackets indicate the change in R^2 after the inclusion of the corresponding explanatory variable.

		Diseases	
Variable	**VER**	**PHDP**	**VAO1**
K	26.32	-56.6	-49.7
		bi	
$SD_{T(t-10)}$	-1.04(0.01)	2.19(0.05)	
$S_{(t-10)}$	-0.95(0.04)	1.44(0.12)	
$SD_{T(t-20)}$	-1.08(0.02)		
$T_{(t-30)}$	0.62(0.34)		
$SD_{T(t-40)}$			-1.37(0.02)
$S_{(t-40)}$			0.79(0.01)
$O_{(t-40)}$			3.22(0.07)
$SD_{O(t-40)}$			11.24(0.01)
R^2	0.41	0.17	0.11

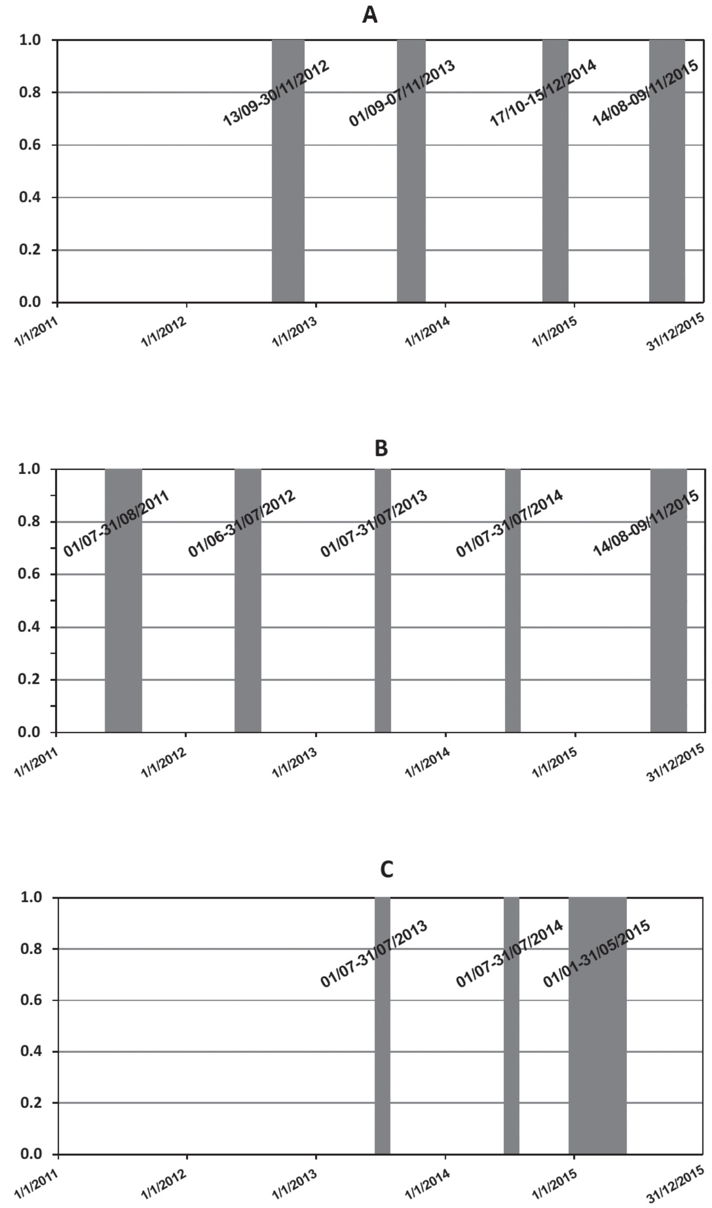

Fig. 3: Presence/absence of fish diseases in the years 2011-2015. (A: VER - Viral Encephalopathy & Retinopathy, B: PHDP - Photobacteriosis, C: VAO1 - Vibriosis). For the presence of disease, the value was [1], and for absence, it was [0].

VAO1 is positively related to salinity, dissolved oxygen and the variation of dissolved oxygen sorting time and negatively related to temperature fluctuation during the 40-day interval between outbreaks. The main explanatory variable was dissolved oxygen (R^2ch = 0.07) (Table 3).

Figures 4 and 5 display the documented presence or absence of three diseases and the corresponding estimated probabilities at the two fish farms under investigation. The patterns of estimated probabilities of disease presence or absence between the two locations exhibit significant similarities. Instances where no disease was recorded may be attributed to either vaccination practices implemented by the facilities or the absence of data. These figures illustrate the correlation of temperature (T), salinity (S), dissolved oxygen (D.O) and pH with the likelihood (forecast model data) and the actual recorded data in the present study, taking under consideration the occurrence of three diseases at fish farms A and B.

Analysis of the recorded data reveals that an increase in temperature (T) correlates with mortality due to VAO1 and PHDP diseases, while the occurrence of VER is associated with a decrease in temperature following a particularly hot summer. A slight increase in salinity (S) is recorded, favouring the occurrence of VAO1 and PHDP diseases, but not VER. Reduced values of dissolved oxygen (D.O.) are conducive to the onset of VAO1 and PHDP diseases, whereas VER is more likely to occur when D.O. increases. The recorded pH values (7.70-7.89) in the study area are lower than the average pH of ocean water (8.1) (Maia *et al.*, 2022). Lower pH values have been reported to contribute to the occurrence of diseases in marine environments (Sampaio & Rosa, 2019; Lucas *et al.*, 2019). This observation is supported by the patterns depicted in Figures 3b and 4.Overall, the figures and data

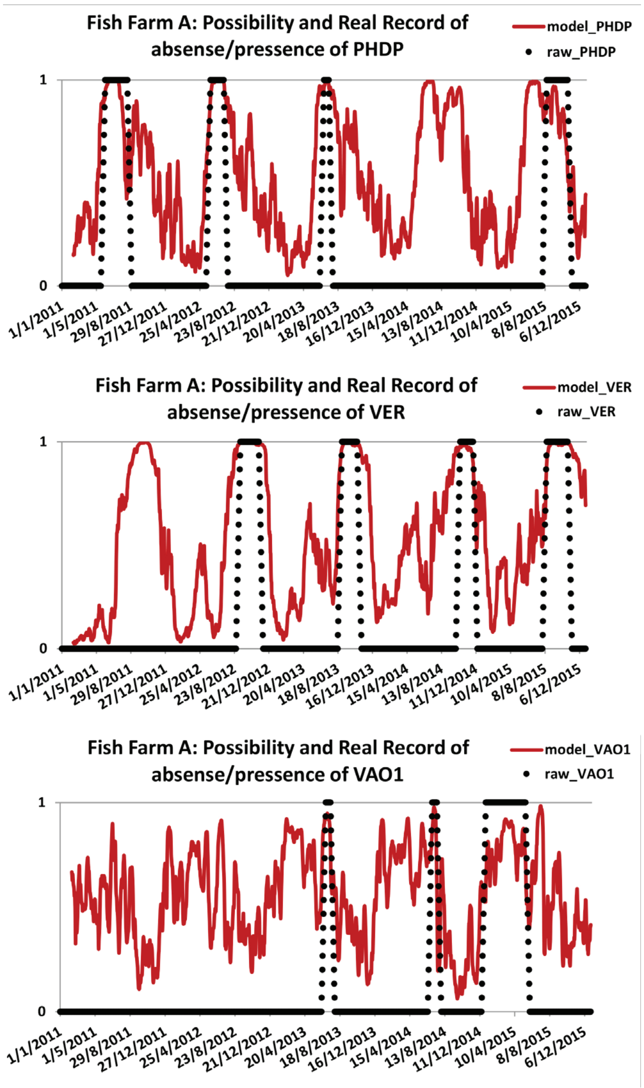

Fig. 4: Estimated time series of the probability of absence/presence (0 to 1) based on the model (depicted by a line), alongside on-site records of absence/presence (represented by dots) for three diseases at Fish Farm A.

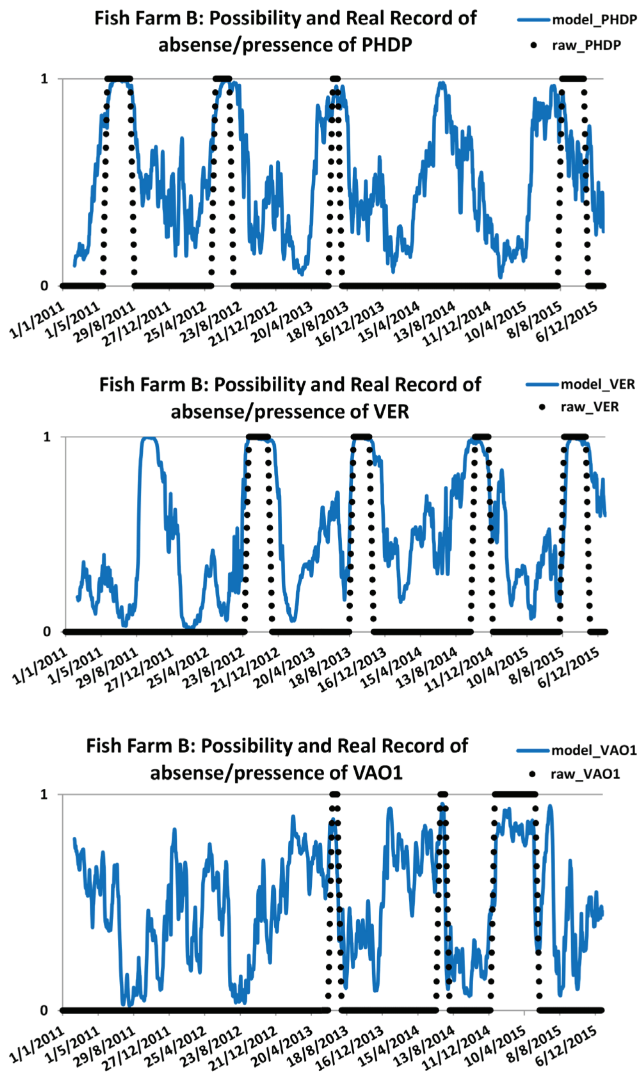

Fig. 5: Estimated time series of the probability of absence/presence (0 to 1) based on the model (depicted by a line), alongside on-site records of absence/presence.

analysis provide valuable insights into the relationships between environmental parameters and the prevalence of diseases at the examined fish farms.

The model accurately predicted Betanodavirus-induced VER disease at a potential presence level greater than 0.85 in 93.5% of the actual disease presence data. Furthermore, diseases caused by PHDP and VAO1 at a presence level greater than 0.75 were correctly predicted at 76.6% and 65.9%, respectively, compared to the actual disease presence data. The overall correct predicted presence of the models for VER, PHDP, and VAO1 was 58.29%, 39.49%, and 36.95%, respectively, as detailed in Table 4.

In Figures 4 and 5, the patterns of the estimated probability of disease presence/absence between the two regions reveals ignificant similarity, reflecting the proximity of the studies and the resemblance of water parameters (Table 2, Fig. 3). The model accurately predicted VER caused by Betanodavirus, as well as PHDP and VAO1 diseases (Table 4). This suggests that the models predict higher attendance values than the actual recordings. Notably, there is a positive correlation of the temperature 40 days earlier with the probability of VER occurrence and a negative correlation with salinity 10 days earlier, considering short-term temperature variability at 10 and 20 days before.

Additionally, there is a positive correlation between the temperature 40 days prior to appears the disease and the probability of PHDP occurrence, coupled with a negative correlation with salinity 10 days earlier, along with short-term temperature variability at 10 and 20 days before. Similarly, a positive correlation is observed between the temperature 40 days earlier and the probability of VAO1 occurrence, accompanied by a negative correlation with salinity 10 days earlier, along with short-term temperature variability at 10 and 20 days before. The positive influence of temperature 40 days before the onset of VER, PHDP, and VAO1 diseases on their probability of occurrence, coupled with the negative impact of salinity 10 days prior to onset for all three diseases, as well as the influence of temperature variability 10 and 20 days before disease onset, is likely attributed to the structural and

Table 4. Assessment of model performance. PD=presence/absence of disease. The sample sizes are as follows: A1: 552, A2: 417, A3: 281/B1: 590, B2: 544, B3: 426/C1: 947, C2: 1056, C3: 760.

Predicted presence/absence according cut off PD value	Pathogen detection	Predicted pathogen presence	Correct prediction of Real presence records %	Total correct prediction of presence records %
		VER		
absence (PD<0.85)	38	0		
presence (PD>0.85)	552 (A_1)	947	93.56 (100*A_1/B_1)	58.29(100*A_1/C_1)
Total	590 (B_1)	947 (C_1)		
		PHDP		
absence (PD<0.75)	127	0		
presence (PD>0.75)	417 (A_2)	1056	76.65 (100*A_2/B_2)	39.49(100*A_2/C_2)
Total	544 (B_2)	1056 (C_2)		
		VAO1		
absence (PD<0.75)	145	0		
presence (PD>0.75)	281 (A_3)	760	65.96(100*A_3/B_3)	36.97(100*A_3/C_3)
Total	426 (B_3)	760 (C_3)		

physiological characteristics of pathogenic microorganisms and the physicochemical parameters of the studied microenvironment.

Discussion

The examination of physicochemical parameters over the five-year period (2011-2015) revealed significant insights. The average minimum temperature in the study area ranged from 11.9°C in February to an average maximum of 27.5°C in August, in line with the anticipated seasonal pattern that is typical of Mediterranean (temperate) coastal ecosystems. Mean salinity measured was 38.01 psu, while pH fluctuated between 7.70 and 7.89. Dissolved oxygen exhibited a mean value of 6.06 mg/l and varied seasonally within the range of 4.8-7.2 mg/l. These parameter values fall within the normal range for seawater in the study area. While they appear conducive to productive growth rates (Fytianos, 1996; Spinos *et al.*, 2018), the recorded lower values during the study period, particularly for dissolved oxygen and pH, could negatively impact the physiology of farmed organisms, induce stress, and potentially contribute to the onset of diseases.

The analysis of on-site data demonstrated that temperature exhibited the highest correlation with the probability of occurrence for all three diseases (VER, PHDP, and VAO1) in the study area (Fig. 3). It appears that temperature operates synergistically with other factors, such as ocean currents, fish farm depth, freshwater inflow in the eastern part of the Gulf of Argostoli, and on-growing management practices. Moreover, temperature influences the physiology and infectivity of aquatic microorganisms, emerging as a crucial predisposing factor for seasonal diseases in farmed seabass. These findings are in line with Bellos *et al.*, (2015) who state that temperature-seasonality is a predominant factor in Greek aquaculture, influencing disease outbreaks associated with the studied bacteria. Additionally, in accordance with Doblin & Van Sebille (2016), sea currents play a critical role in the manifestation of diseases.

The Impact of Salinity on Disease Susceptibility

An increase in water salinity correlates with a higher likelihood of developing any of the three diseases. An increase in water salinity prompts increased energy expenditure for osmoregulation. Studies on turbot (*Scophthalmus maximus*) and butterfish (*Scatophagus argus*) indicate that acute changes in salinity can trigger reactive dysregulation of the immune response. When coupled with additional stressors such as sudden increases in temperature and/or dissolved oxygen fluctuations, such conditions may render fish more susceptible to infectious diseases. Consequently, these findings shed light on how abrupt environmental changes can initiate disease outbreaks, potentially leading to critical declines in both cultured and wild fish populations (Choi *et al.*, 2013; Huang *et al.*, 2015).

Seasonal Patterns and Environmental Factors in Disease Occurrence

VAO1 and PHDP infections predominantly occurred during the summer months, while VER infections were observed after the summer and prolonged high temperatures extending into early to mid-autumn (Spinos *et al.*, 2013). For PHDP, dissolved oxygen emerges as a critical and determining factor, with the disease typically appearing in spring and lasting until autumn at temperatures ranging from 18 to 20°C (Magarinos *et al.*, 1992; Alicia *et al.*, 2005). PHDP exhibits a seasonal prevalence, predominantly infecting fish during summer months when temperatures exceed 23°C (Korun *et al.*, 2005; Essam *et al.*, 2016; Matanza *et al.*, 2018; Eissa *et al.*, 2021). In this study, VAO1 was observed with temperature in the range of 18-27°C, PHDP in the range of 19-27°C, and VER in the range 18-28°C.

It should be noted that as water temperature rises, the oxygenation capacity of water diminishes. The shift from body temperature is likely related to anoxic pressure (Varsamos *et al.*, 2006), as supported by other authors (Hadj-Kacem *et al.*, 1986; Pörtner & Peck, 2010; Sampaio & Rosa, 2019). Rapid temperature increases from 17°C to 26°C can alter the haematology of *Dicentrarchus labrax*, inducing hypoxic conditions. Temperature fluctuations can significantly impact the immune system, leading to the production of high cortisol levels and decreased serum IgM levels, associated with increased sensitivity when exposed to pathogenic microorganisms causing PHDP (Varsamos *et al*., 2006). PHDP can thrive at temperatures between 15-32.5°C, with the optimal temperature for bacterial multiplication identified as 22.5°C.

Seasonal Occurrence of VER and Influencing Factors

In the course of this study, VER incidents were observed during the autumn months and extended into December. This was attributed to prolonged high temperatures persisting from late summer to mid-autumn, creating favourable conditions for the manifestation of VER in previously infected fish. Symptoms onset and the subsequent high mortality rate coincided with a decrease in water temperature to 26-27°C, following the development of the virus in infected fish during the period of high temperatures, ultimately leading to the collapse of the organisms. This observation is in line with the findings of Le Breton *et al*., (1997) and a similar event in 2012 in Western Greece, where the disease resulted in substantial mortality in fish farms (Spinos *et al*., 2013).

Determinants of VER Appearance in Seabass

As evidenced in this study, the primary determinants influencing the appearance of VER in farmed seabass are temperature and salinity. Specifically, the occurrence of VER is favoured by the synergistic effects of increased temperature and salinity, coupled with evaporation and a lower rate of water renewal in the Gulf of Argostoli during the warmer months. The optimal growth temperature for the Red Grouper Nervous Necrosis Virus (RGNNV) genotype, the betanodavirus, which is responsible for seabass infections in the Mediterranean, is between 25-30°C, unlike other genotypes showing pathogenicity at lower temperatures, such as the Atlantic Halibut Nervous Necrosis Virus (AHNNV) (Lopez-Jimena *et al.*, 2010).

Betanodaviruses exhibit increased infectivity at water pH values of 3-7. Inactivation occurs after four days at 37°C and after three months at 25°C when maintained in cell culture, but infectivity is retained after 12 months at 15°C (Frerichs *et al*., 2000). Multiplication of betanodaviruses is inhibited at temperatures of 30-35°C, rendering them unable to multiply at 37°C (average human body temperature), hence posing no threat to humans who consume infected fish (Hata *et al.*, 2007). In freshwater, the virus appears to be less stable and undergoes inactivation after six months (Frerichs *et al.*, 2000). The data collected within the framework of this study indicates higher mortality rates for VER compared to the other diseases studied.

Factors Influencing VAO1 Appearance

The on-site data collected during this study highlights the significance of water pH as a crucial factor for the appearance of VAO1, with observed cases being associated with a reduction in pH, despite low pH fluctuations. The bacterium responsible for VAO1 exhibits maximum and minimum populations in summer and winter, respectively (Larsen, 1984; Lages *et al.*, 2019). Incidences of VAO1 have been documented during winter at temperatures below 15°C (Grisez & Ollevier, 1995). The disease typically manifests when water temperature exceeds 10-12°C, with key contributing factors being changes in water temperature, stress induced by fish handling, and adverse weather conditions. VAO1 is more prevalent in areas characterized by high fish densities and environmental imbalances, including low oxygen levels, pH fluctuations, high feeding rates, temperature fluctuations, and the presence of parasites (Ellis, 1989; Frans *et al.*, 2011; Austin & Austin, 2016).

The correlation identified in this study between actual data indicating a pH drop and the occurrence of VAO1 disease aligns with previous research suggesting that *Vibrio anguillarum* is more adept at causing infections at lower pH levels than in normal seawater. Under favourable environmental conditions, *V. anguillarum* can become highly pathogenic for fish. Instances of infection in the Mediterranean Sea appear to exacerbate when water temperature ranges between 18-20°C, with significant mortality observed from the initial days of an epidemic. Prior studies have reported species of Vibrio causing diseases beyond *V. anguillarum*, such as *V. alginolyticus*, *V. parahaemolyticus*, and cases of *Photobacterium damselae* subsp. *damsela* (formerly *V. damsela*) in North Africa (Heenatigala & Fernando, 2016).

Climate Change Implications

Considering climate change scenarios projecting a substantial increase in seawater temperature and acidification (decrease in pH), the correlations established in this study suggest an intensification of epidemics for all three diseases, either individually or in combination. The antibodies created through fish immunization against *V. anguillarum* primarily target pathogenic O-antigens, specifically lipopolysaccharide (LPS) (Schrøder *et al.*, 2006).

Antibody Response and Energy Efficiency

Lipopolysaccharide (LPS) acts as a T cell-independent antigen, enabling B lymphocytes to produce antibodies without requiring pre-treatment with immune system cells, unlike proteins, which are T cell-dependent antigens. As a result, an immune response against LPS demands less energy in fish compared to protein antigens. In poikilothermic organisms like fish, where metabolism and energy production are proportionally influenced by temperature, this efficiency is particularly noteworthy (Spinos *et al.*, 2017).

pH Values and Disease Occurrence

Despite the observed decrease in pH values, their range recorded during this study did not exhibit a statistically significant correlation with the occurrence of the three diseases in the model. This finding underscores the complexity of disease dynamics and suggests that other factors play a more dominant role in disease manifestation.

Comparison of Fish Farms and Disease Incidence

Comparing the two different locations of the fish farms, cumulative mortality due to diseases was lower at location B compared to location A (Table 1). Fish farm B experienced less intense disease outbreaks, possibly due to differences in hydrological characteristics and management practices. Fish farm A, located at a higher average depth of 4m, exhibits distinct bottom composition and currents compared to fish farm B. Additionally, fish farm A is more affected by anthropogenic activities, being closer to the ports of Argostoli and Lixouri. Notably, fish farm B has a smaller annual capacity with fewer fish reared, but the fish are larger on average compared to fish farm A, making them more resistant to diseases (Spinos, 2019).

Model Improvement Considerations

The study focuses on a model highlighting the connection between physicochemical parameters and disease occurrence in farmed seabass, emphasizing the duration of their influence before disease manifestation. To enhance the model's performance, a low R^2 is essential. This can be achieved by (a) incorporating all possible parameters related to the event and disease recording, (b) implementing a more precise recording system (capturing the onset of symptoms rather than waiting for economically significant mortalities), and (c) considering the potential impact of vaccinations coinciding with disease occurrence or leveraging past vaccination experiences to predict disease outcomes. All three aspects contribute to refining the model's accuracy and predictive capabilities.

Key Findings of the Statistical Analysis

The statistical analysis of real data reveals that for the occurrence of VAO1, pH is a relatively important parameter but not statistically significant by standard measures. Conversely, temperature is the decisive factor for both PHDP and VER, with salinity also playing a crucial role in PHDP and VER. Additionally, a decrease in dissolved oxygen triggers the emergence of all three diseases. The analysis shows that PHDP and VAO1 exhibit lower predictability (R^2 approximately 0.17 and 0.11, respectively) compared to VER (R^2 approximately 0.93). The performance evaluation conducted for the model indicates that while the overall correct forecast of presence is more predictable than the actual recorded values, the forecasting performance is relatively lower for PHDP and VAO1 compared to VER.

Coincidence of Actual Incidence Data and Model Predictions

The statistical processing and analysis demonstrate that the actual incidence data for all three diseases are in line with the model's predictions of the probability of occurrence (Fig. 3). The evaluation suggests that the model is more accurate in predicting the presence of diseases collectively rather than individually.

Complexity of Disease Dynamics

According to a model published by Ögüt (2001), the appearance of diseases in farmed fish results from a complex interplay of environmental factors, host characteristics, and pathogenic microorganisms. Managing environmental factors (temperature, oxygen, pollution), host-related factors (fish species, age, immunity, fish density, genetic background), and characteristics associated with pathogenic microorganisms (degree of virulence) is crucial for disease control. While the system is inherently complex, not all parameters exert the same impact on disease outcomes. Certain parameters, identified as "key" factors, play a pivotal role in disease dynamics. Focusing on these key factors could significantly enhance the system, reducing losses and the prevalence of disease-causing pathogens. The authorizing a prognostic stochastic model studied the extent to which stress due to heavy fish loads in fish farms contributes to the occurrence and impact of infections in the fish farm.

In a study carried out by Alaliyat *et al.* (2019) on fish farms in various Norwegian fjord regions, a model was applied to assess disease occurrences. Their results revealed that the risk of infection in the vicinity of an infected area is influenced by both the type of pathogen and fish density at that location. The risk increases with higher densities of pathogens or fish. The concentration of pathogens demonstrated an exponential decrease with rising water temperature, based on cold-water fish and pathogen species. In the fish cages located in the infected area, the concentration increased with faster currents or higher fish density.

However, the reverse relationship between temperature and pathogen load observed in the study by Alaliyat *et al.*, (2019) differs from the findings of the current study where high temperature emerged as a significant factor in disease incidence. This divergence may be attributed to the different pathogens involved considering, in particular, the difference between the cold-water pathogens of the Alaliyat *et al.* (2019) study and the temperate or warm-water pathogens of the current study.

Pathogens tend to move swiftly with strong currents, which may slow down the infection process at the locally infected site but accelerate their transportation to nearby areas. The direction of the current is a critical factor, as pathogens primarily move along with the currents (Doblin & Van Sebille, 2016).

In both studies, models were developed to predict the occurrence and impact of infections on fish farms. Comparing prediction percentages between these studies and the present one, the effectiveness of our model in predicting three diseases (PHDP, VER, and VAO1) using key physicochemical parameters [T(^{o}C), S (psu), D.O. (mg/l), and pH] is substantiated.

Conclusion

This study has successfully developed a predictive model for forecasting the risk of outbreaks associated with three prevalent infectious diseases -VER, PHDP, and VAO1 - commonly observed in the Mediterranean region. The investigation focused on correlating physicochemical parameters of water, namely temperature (T), salinity (S), dissolved oxygen (D.O.) and pH, measured at marine fish farms in Greece with the occurrence of these diseases. Increase in temperature emerged as a key factor influencing the onset and outbreaks of all three diseases and has an impact on both the marine environment and the pathogens themselves. An increase in salinity was found to correlate with a higher probability of outbreaks for any of the three diseases. A decrease in pH was specifically associated with the onset of Vibriosis, emphasizing the relevance of this parameter to disease dynamics. The reduction in dissolved oxygen demonstrated a synergistic effect with other parameters (temperature, salinity, pH) and acted as a trigger for the appearance of all three diseases. Notably, the actual incidence data for VER, PHDP, and VAO1 are in line with the model's predictions for the incidence of these diseases. This convergence between observed and predicted data demonstrates the efficacy of the model in accurately forecasting the occurrence of VER, PHDP, and VAO1based on the examined physicochemical parameters.

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