Key Points:

- Optimal growth conditions for *Vibrio* spp. depend on the life stage: planktonic or biofilm formation
- Changes in pH and temperature in coastal areas may lead to a higher *Vibrio*-human interaction and influence adaptive responses
- pH effects must be included in *Vibrio* modeling efforts to predict *Vibrio* risk in zones with co-occurrence of *Vibrio* and harmful algal blooms

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Modeling pH and Temperature Effects as Climatic Hazards in *Vibrio Vulnificus* and *Vibrio Parahaemolyticus* Planktonic Growth and Biofilm Formation

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Abstract Climate-induced stressors, such as changes in temperature, salinity, and pH, contribute to the emergence of infectious diseases. These changes alter geographical constraint, resulting in increased *Vibrio* spread, exposure, and infection rates, thus facilitating greater *Vibrio*-human interactions. Multiple efforts have been developed to predict *Vibrio* exposure and raise awareness of health risks, but most models only use temperature and salinity as prediction factors. This study aimed to better understand the potential effects of temperature and pH on *V. vulnificus* and *V. parahaemolyticus* planktonic and biofilm growth. *Vibrio* strains were grown in triplicate at 25°, 30°, and 37°C in 96 well plates containing Modified Seawater Yeast Extract modified with $CaCl_2$ at pH's ranging from 5 to 9.6. AMiGA software was used to model growth curves using Gaussian process regression. The effects of temperature and pH were evaluated using randomized complete block analysis of variance, and the growth rates of *V. parahaemolyticus* and *V. vulnificus* were modeled using the interpolation fit on the MatLab Curve Fitting Toolbox. Different optimal conditions involving temperature and pH were observed for planktonic and biofilm *Vibrio* growth within- and between-species. This study showed that temperature and pH factors significantly affect *Vibrio* planktonic growth rates and *V. parahaemolyticus* biofilm formation. Therefore, pH effects must be added to the *Vibrio* growth modeling efforts to better predict *Vibrio* risk in estuarine and coastal zones that can potentially experience the cooccurrence of *Vibrio* and harmful algal bloom outbreak events.

Plain Language Summary Changes in temperature, salinity, and pH are increasing *Vibrio*-human interactions in coastal communities. Multiple efforts have been developed to predict *Vibrio* risk, mainly using temperature and salinity measurements. However, more comprehensive models are needed to help inform decision-makers on how to better design policies and create public health awareness. This study looks at how temperature and pH could affect the growth of the potential human bacterial pathogens, *V. vulnificus* and *V. parahaemolyticus*. *Vibrio* strains were grown in triplicate at different temperatures in acidic, neutral, and alkaline conditions (different pH ranges). The effects of temperature and pH were evaluated using randomized complete block analysis of variance, and the growth rates of *V. parahaemolyticus* and *V. vulnificus* were modeled using the MatLab Curve Fitting Toolbox. This study found different optimal conditions for free-living and aggregated *Vibrio* growth within and between species. In addition, this study showed that temperature and pH factors significantly impact *Vibrio* growth. Overall, the pH effects must be added to the *Vibrio* growth modeling efforts to have a more comprehensive model and to better predict *Vibrio* risk in climate change scenarios.

1. Introduction

Climate change is causing unprecedented ecological changes and altering infection patterns for diseases sensitive to environmental changes, such as *Vibrio* infections (Epstein, 2001; Mora et al., 2022; Wu et al., 2014). Some hazards resulting from climate change, such as warming, sea level rise, pH decline, floods, and abnormal weather patterns may lead to a potential increase in *Vibrio* infections (Trinanes & Martinez-Urtaza, 2021). The number of cases of vibriosis has been increasing during the past few decades worldwide, even in regions where environmental conditions had been considered adverse for *Vibrio* proliferation, especially in higher latitude locations (Baker-Austin et al., 2013, 2017, 2018; Newton et al., 2012; Vezzulli et al., 2013). In the United States, the

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Foodborne Diseases Active Surveillance Network (FoodNet, Tack et al., 2019) has reported increased incidences of *Vibrio* infections. For example, incidence in 2018 increased by 109% compared to 2015–2017, with similar increasing trends observed in previous years. In 2020, the incidence of *Vibrio* decreased by 15% compared to those in 2017–2019 due to the COVID-19 pandemic and the corresponding public health response that limited healthcare-seeking behaviors, healthcare delivery, and human exposure. This decrease in incidence was an abnormal trend, which is expected to change due to the abatement of COVID-19 related restrictions (Tack et al., 2020: Ray et al., 2021; Trinanes & Martinez-Urtaza, 2021). The increase in *Vibrio* spread and infection rates are thought to be a consequence of altered geographical constraints driven by warming seawater temperature, sea-level rise, and changes in salinity associated with climate change (Deeb et al., 2018). This poleward spread is contributing to the increase of human disease burden globally (Baker-Austin et al., 2013, 2018).

Vibrio spp. inhabit estuarine and marine environments and prefer relatively warm water (≥15°C) and low to moderate salinities, where seawater temperature modulates the abundance of *Vibrio* and salinity defines habitat suitability (FAO and WHO, 2020; Marinez-Urtaza et al., 2008; Thompson et al., 2004; Vezzulli et al., 2009; Wang et al., 2020). *Vibrio* spp. can persist in a free-living (planktonic) state in the water column or form biofilms on biotic and abiotic surfaces (Baker-Austin et al., 2018). Biofilm formation depends on many physical, chemical, and biological parameters and is considered a selective survival strategy for protection against stress, such as changes in temperature, pH variability, low nutrients, and antibiotics (Decho & Gutierrez, 2017; Harjai et al., 2005; Hoštacká et al., 2010; Jefferson, 2004; Yang et al., 2007). Also, some *Vibrio* spp., including *V. parahaemolyticus* and *V. vulnificus*, can enter a viable but non-culturable (VBNC) protective state under unfavorable conditions, where the bacterial metabolism becomes dormant and cells cannot grow under laboratory conditions (Colwell, 2000; Li et al., 2014). VBNC bacterial cells regain their culturability and virulence properties when the conditions become favorable, which may be triggered by changes in temperature and salinity (Oliver, 2010). Studies have demonstrated that VBNC *Vibrio* spp. can cause disease after resuscitation in their respective hosts (Baffone et al., 2003; Colwell, 1996; Sun et al., 2008) Also, another study showed that four *Vibrio* spp. expressed some virulence and toxin genes during the VBNC state (Vora et al., 2005).

Not all *Vibrio* strains are equally pathogenic and can be classified according to biotype and genotype. *V. vulnificus* strains are classified into three biotypes based on their biochemical characteristic: biotype 1 is responsible for human infections (Oliver, 2015), biotype 2 is primarily an eel pathogen (Amaro & Biosca, 1996; Tison et al., 1982), and biotype 3 is a hybrid of biotypes 1 and 2 that can cause wound infections and has been suggested to be geographically restricted to Israel (Bisharat et al., 1999; Zaidenstein et al., 2008). Strains within biotype 1 are commonly grouped into clinical (16S rRNA type B and *vcgC*) and environmental (16S rRNA type A and *vcgE*) genotypes (Nilsson et al., 2003; Rosche, Smith, et al., 2005; Rosche, Yano, & Oliver, 2005). Furthermore, the *vvhA* gene encoding hemolysin/cytolysin is used as a *V. vulnificus* species marker and has been associated with pathogenic strains. Other genes associated with pathogenic *V. vulnificus* strains are *rtxA1*, *vvpE*, *viuB*, *gbpA,* and pilin genes (*pilA* and *pilD*) (Gavin et al., 2017; Jang et al., 2016; Johnson et al., 1984; Natividad-Bonifacio et al., 2013; Panicker et al., 2004; Paranjpye & Strom, 2005). *V. vulnificus* regulates virulence gene expression by integration of signals during the course of infection. For example, in the early stages of infection in the upper intestine and bloodstream where glucose levels are high, IscR activates the up-regulation of *gbpA*, *prx3*, and *vvhA* and CRP up-regulates the *rtxA* for survival against stress, intestinal colonization, and dissemination through the host. In later stages of infection, CRP-mediated up-regulation of *plpA*, *vvhA*, and *vvpE* leads to inflammation and disease development (Choi & Choi, 2022). For *V. parahaemolyticus*, strains that carry thermostable direct hemolysin (*tdh*) and/or thermostable-related hemolysin (*trh*) genes are often considered pathogenic (Nishibuchi et al., 1992; Shirai et al., 1990). Non-cholera *Vibrio* species, such as *V. parahaemolyticus* and *V. vulnificus*, can cause infection by exposure to contaminated water or consumption of raw or undercooked contaminated seafood. Clinical manifestations include mild and self-limiting gastroenteritis and wound infections that can result in acute septicemia and death (Jones & Oliver, 2009).

Although environmental stressors such as temperature and salinity and *Vibrio* abundance and distribution data are used in current modeling to predict future *Vibrio* exposure risk (Semenza et al., 2017), other key factors have been suggested as contributing to the increase in *Vibrio* cases, such as demographic changes and population growth (Trinanes & Martinez-Urtaza, 2021). Population growth and development in coastal regions have been significantly higher compared to inland areas worldwide, generating pressures on coastal ecosystems due to anthropogenic pollution (Balk et al., 2009; Crossland et al., 2005; Neumann et al., 2015; Patterson & Hardy, 2008; Small & Nicholls, 2003).

The combination of climate hazards, demographic changes, and population growth in coastal areas has been suggested as key factors in the increase in *Vibrio-human* interactions (Archer et al., 2023; Froelich & Daines, 2020). But the potential for infection is more complex due to the variation in pathogenicity across species and even strains. Furthermore, little is known about how changing environmental parameters, such as pH and exposure to other potentially co-occurring biological hazards, such as harmful algal blooms (HABs), can shift or change *Vibrio* abundance, distribution, and pathogenicity in estuaries and marine environments. The limited studies that have addressed *Vibrio*-Phytoplankton interaction have shown a high correlation between *Vibrio* and phytoplankton abundance (Main et al., 2015; Rosales et al., 2022; Turner et al., 2009). These studies suggest that the correlation may be due to the combination of changes in environmental parameters and the production of algal exudate that activates *Vibrio* biofilm formation pathways. There is also a limited understanding of how changing ocean pH can affect the abundance of *Vibrio* species. Non-cholera *Vibrio* spp. have the capability to grow in a broad pH range from 5 to 10 and have been shown to develop resistance to acid inactivation in the VBNC state, adding to the complexity of the situation (Nowakowska & Oliver, 2013; Wong & Wang, 2004).

Changing environmental parameters in coastal areas, including temperature and pH, might led to the generation of new areas with ideal conditions for *Vibrio* growth. Future climate change scenarios project warmer, less saline, and more acidic coastal water. The ocean surface warming has accelerated in the last decade to $0.280 \pm 0.068°C$ per decade and is expected to increase more than 4°C by 2100. Meanwhile, the ocean pH has declined by 0.1 since the industrial revolution and is projected to decline by 0.1–0.4 pH units by the end of 2100 in the open ocean (Garcia-Soto et al., 2021). Coastal regions are more dynamic than the open ocean, with environmental parameters differing according to geographical locations, morphology, freshwater influx, and other environmental and anthropogenic pressures (Cloern et al., 2016). Biogeochemical processes in coastal zones can lead to seasonal pH variation and even daily changes higher than 1 pH unit, where daytime photosynthesis drives high levels of dissolved oxygen and pH and nighttime respiration drives decreased dissolved oxygen concentration and pH values (Baumann et al., 2015; Provoost et al., 2010; Raven et al., 2020; Wallace et al., 2014). Baumann and Smith (2018) used long-term monitoring data from the National Estuarine Research Reserve System (NERRS) to evaluate pH and oxygen fluctuation in 16 US nearshore sites and found that dissolved oxygen, as a result of metabolic processes, and salinity have a high correlation with pH fluctuations. Furthermore, in areas that have an extensive network of intertidal salt marshes, such as the ACE Basin and North Inlet NERRS sites (South Carolina, USA), pH fluctuations can be correlated with tidal and diurnal cycles. Dense cyanobacteria blooms in these dynamic ecosystems can also increase water column pH > 9 due to metabolically-driven decreases in dissolved CO_2 to less than 1 µmol per liter (Adams et al., 2022; Huisman et al., 2018). Changes in environmental parameters not only affect the growth and distribution of *Vibrio* species but may also alter their gene expression, resulting in enhanced virulence profiles (Billaud et al., 2022; Correa Velez & Norman, 2021; Pazhani et al., 2021; Williams et al., 2014). To understand the changing abundances of *Vibrio* spp. under natural conditions and to develop better models to predict future climate change impacts, it is necessary to determine how environmental stressors, such as pH and temperature, can affect *Vibrio* in their different forms of growth (planktonic and biofilm states). This study examined the potential effects of temperature and pH on non-cholera *Vibrio* planktonic and biofilm growth using *V. vulnificus* and *V. parahaemolyticus* as models of opportunistic pathogens. A better understanding of how clinical and environmental strains respond to coupled climatic hazards will aid in the development of more precise models to predict potential *Vibrio* exposure and increase awareness of health risk in coastal regions.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions

To examine the effect of pH and temperature on bacterial growth in planktonic and biofilm states, reference clinical and environmental strains of *V. vulnificus* and *V. parahaemolyticus* (Table 1) were grown at three temperatures (25°, 30°, and 37°C) and 11 pH's ranging from 5.0 to 9.6. The pH and temperatures were selected to encompass a range of natural conditions encountered by *V. vulnificus* and *V. parahaemolyticus*. The average sea surface temperature in warm coastal areas susceptible to *Vibrio* proliferation is between 25°C to 30°C, whereas a human host temperature is 37°C. *Vibrio* can survive in pH ranges from 5 to 10. The pH of the water column fluctuates between 7.2 and 8.1 under most environmental conditions, with photosynthesis-driven alkaline conditions (≥9.2) often occurring during cyanobacterial bloom events. The pH in human-host conditions is 6.5–7.5 in saliva and decreases to 2–6.5 in the gastrointestinal tract.

Table 1
Vibrio Strains Used in This Study

Strain ID	Type	Source	Location	Characteristics
V. vulnificus NBRC 15645 = ATCC 27562	Clinical	Human	Florida, USA Human blood	Type strain, 16S type B, biotype 1
V. vulnificus ATCC 33147	Environmental	Eel	Japan Diseased eel	16S type A, biotype 2
V. vulnificus NOAA48	Environmental	Water	South Carolina, USA ACE Basin, 28°C, 26 g/kg, pH = 7.8	16S type A
V. vulnificus NOAA155	Environmental	Water	South Carolina, USA Winyah Bay, 28°C, 10 g/kg, pH = 7.3	16S type B, *pilF* positive
V. parahaemolyticus ATCC 17802	Clinical	Human	Japan Shirasu food poisoning	tlh/trh
V. parahaemolyticus 48057 (BEI NR-21990)	Clinical	Human	Washington, USA Clinical case of food poisoning	tlh/tdh/trh, serotype O4:K12
V. parahaemolyticus C12	Environmental	Water	South Carolina, USA Winyah Bay, Oyster Landing, 22°C, 34 g/kg, pH = 7.6	tlh
V. parahaemolyticus 4.1PR	Environmental	Oyster	Cabo Rojo, PR Boquerón, 28°C, 35 g/kg, pH = 8	tlh

For free-living or planktonic growth, *Vibrio* strains were grown in 96 well plates containing three replicates of Modified Seawater Yeast Extract (MSYE; Oliver & Colwell, 1973) supplemented with calcium chloride (CaCl$_2$; 1.8 g/L), pH adjusted using 1M Sodium Hydroxide (NaOH) or 1M Hydrochloric acid (HCl), and a final salinity of 30 ± 0.5 g/kg. In each experimental condition, diluted 1:10 overnight fresh cultures (8 hr) were used as inocula. Optical density (OD600) of each replicate was measured hourly to determine bacterial growth over 24 hr using a Victor X3 plate reader (PerkinElmer, Waltham, MA, USA). Gaussian process regression was performed on background-subtracted OD data to model growth curves, and model-predicted ODs were used to estimate growth parameters for each treatment using AMiGA software (Midani et al., 2021). For biofilm formation assays, tissue culture-treated 96-well polystyrene microplates were used under each environmental condition without additional modifications.

2.2. Crystal Violet Staining Assay

The biomass of *V. parahaemolyticus* and *V. vulnificus* biofilms was estimated using crystal violet staining according to O'Toole (2011), with slight modifications. Briefly, after 0, 6, 12, 24, 36 hr of growth, planktonic cells were removed from the 96-well microplates before gently washing with 1× phosphate buffer saline (PBS) three times. After washing, 100% methanol (MeOH) was added to the plates to fix the biofilms to the plates. After 20 min of incubation at room temperature, MeOH was removed and the plates were allowed to air-dry to eliminate any MeOH residue. The biofilms were stained with 0.1% (wt/vol) crystal violet for 15 min at room temperature, and a second wash was performed three times using 1× PBS to remove the non-bound dye. The stained and washed biofilms were air-dried overnight, and 30% acetic acid was added to dissolve the bound crystal violet for 15 min. The solubilized crystal violet acetic acid solution was then transferred to a new 96-well polystyrene microplate and the optical density of each well was measured at 570 nm using a SpectraMax M3 plate reader (Molecular Devices, San Jose, CA, United States). These OD measurements were used to estimate the biofilm growth parameters for each treatment using AMiGA software.

2.3. Modeling and Statistical Analysis

The growth rates of *V. parahaemolyticus* and *V. vulnificus* obtained from the AMiGA analysis for each combination of factors (pH and temperature) were modeled using the interpolation fit function using the liner method in
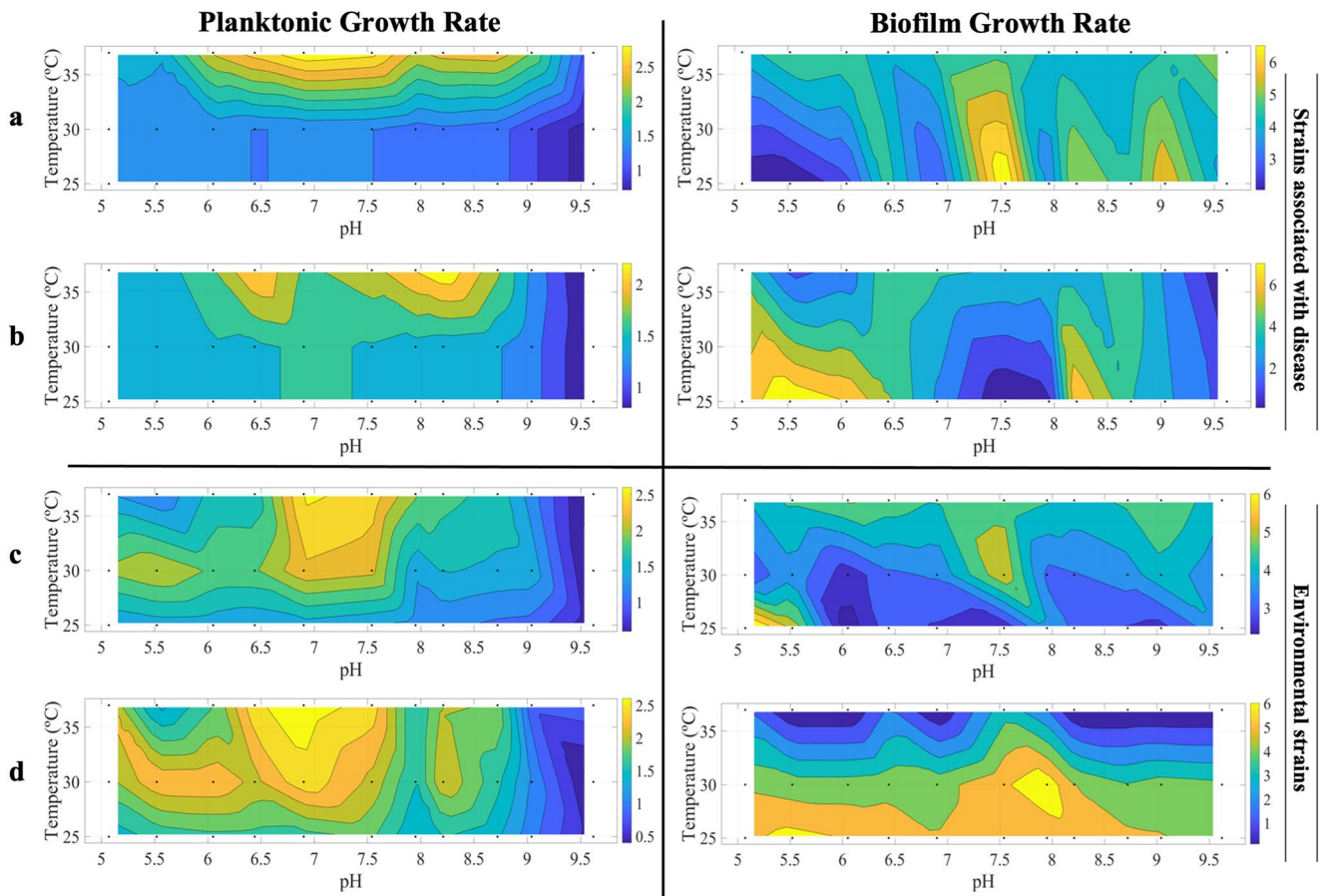
Figure 1. Modeling of bacterial growth rates in planktonic and biofilm stages of *V. vulnificus* strains at different temperatures and pH ranges. Strains associated with disease in humans and animals are shown in panels a (*V. vulnificus* NBRC 15645 = ATCC 27562) and b (*V. vulnificus* ATCC 33147). Environmental strains are shown in panels c (*V. vulnificus* NOAA 48) and d (*V. vulnificus* NOAA 155). Growth rates are calculated as $\left(\frac{d}{dt}\ln OD\right)$ with yellow representing higher growth rates and blue representing lower growth rates.

the MatLab Curve Fitting Toolbox (v. R2022b, 9.13.0.2049777). The interpolation method estimated the values between known data points, which involves the construction of a function f that matches given data values, yi, at given data sites, xi, in the sense that f(xi) = yi, all i. For the statistical analysis, a randomized complete block design analysis of variance (RCB-ANOVA) was performed using SPSS Statistics (v. 28.0.1.0) to evaluate the effects of temperature and pH on bacterial growth rate using the strain as a blocking factor. Ryan, Einot, Gabriel, and Welsch (R-E-G-W F) multiple comparisons of means were used to determine aposteriori differences among the factor combinations. The level of significance was set at $p < 0.05$. Interaction (profile) plots were generated using the general linear model, univariate function in SPPS software. The data used for both analyses are available at Mendeley data via https://doi.org/10.17632/xxkkkbx3hg.1.

3. Results

3.1. *Vibrio* Modeling Reveals That Optimal Growth Conditions Vary Between Planktonic and Biofilm States

Vibrio modeling using the interpolate method revealed that optimal pH and temperature conditions vary between strain and state of growth. *V. vulnificus* strains associated with disease in humans and animals showed optimal growth rates at 37°C and at pH's between 6.5 and 8.5 during planktonic growth (Figures 1a and 1b, left), with the highest growth rate at pH 7.1 for ATCC 27562 and pH 8.3 for ATCC 33147. In comparison, the environmental isolates of *V. vulnificus* (Figures 1c and 1d, left) exhibited growth throughout a wider range of conditions, suggesting greater adaptation to varying environmental conditions. Higher growth rates were observed at 36°C
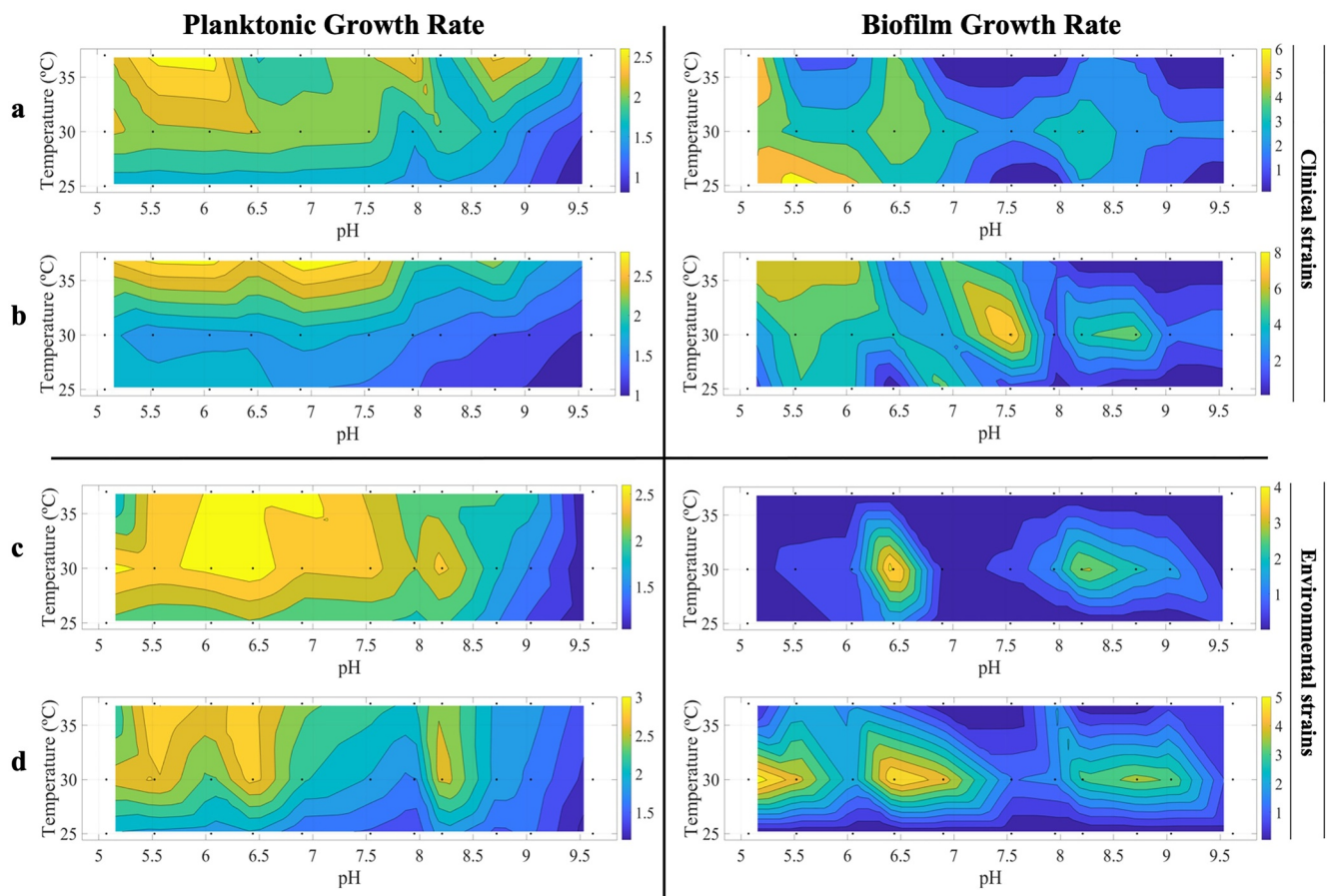
Figure 2. Modeling of bacterial growth rates in planktonic and biofilm stages of *V. parahaemolyticus* strains at different temperatures and pH ranges. Clinical reference strains are shown in panels a (*V. parahaemolyticus* ATCC 17802) and b (*V. parahaemolyticus* 48057). The environmental strains are shown in panels c (*V. parahaemolyticus* C12) and d (*V. parahaemolyticus* 4.1PR). Growth rates are calculated as $\left(\frac{d}{dt}\ln OD\right)$ with yellow representing higher growth rates and blue representing lower growth rates.

and pH 7.0 for the NOAA 48 strain and 32°C and pH 6.9 for the NOAA 155 strain. During biofilm growth, the optimal conditions in terms of pH and temperature were opposite from those observed during planktonic growth (Figure 1, right Panel). The greatest biofilm formation for *V. vulnificus* strains associated with disease were observed at 25°C and pH 7.5 for ATCC 27562, and 26°C and pH 5.4 for ATCC 33147. Similar patterns were observed for the environmental strains, where optimal growth conditions were 25°C and pH 5.2 for the NOAA 48 strain and 30°C and pH 7.9 for the NOAA 155 strain.

All *V. parahaemolyticus* strains in planktonic growth (Figure 2 left panel) exhibited increased growth at temperatures between 36.6–36.8°C and pH ranging from neutral to acidic: 7.1 (strain 48057), 6.4 (C12), 5.9 (17802), and 5.7 (4.1PR). In contrast, optimal biofilm formation for all strains was observed at lower temperatures, from 25.2–30°C and pH ranges similar to those of planktonic cells (Figure 2 right panel). Modeling *V. parahaemolyticus* growth during both planktonic and biofilm mode of growth showed similar strain-level patterns based on pH, where higher biofilm biomass developed in pH's ranging from neutral to acidic. However, different temperature optimums were observed for clinical and environmental strains, whereas the planktonic environmental strain patterns showed higher growth rates throughout a wider temperature range (26°C–37°C) compared with the clinical strains, suggesting better adaptability to changes in temperature.

3.2. pH and Temperature Have Significant Effects on *Vibrio* spp. Planktonic Growth Rates

A randomized complete block design model multifactor analysis of variance (RCB-ANOVA) was used to determine the effect of pH (11 levels) and temperature (3 levels) on growth rates of *V. vulnificus* and *V. parahaemolyt-*

Table 2
Randomized Complete Block ANOVA Results for Effects of Temperature and pH in Vibrio spp

Vibrio species			Sum of squares	df	Mean square	F value	Pr (>F)	Partial eta squared
Planktonic Growth	*Vibrio vulnificus*	Strain (block)	1.917	3	0.639	7.608	<0.001[a]	0.060
		Temperature	17.004	2	8.502	101.239	<0.001[a]	0.360
		pH	51.746	10	5.175	61.619	<0.001[a]	0.631
		Temperature [a] pH	11.118	20	0.556	6.620	<0.001[a]	0.269
		Error	30.232	360	0.084			
	Vibrio parahaemolyticus	Strain (block)	5.083	3	1.694	16.706	<0.001[a]	0.122
		Temperature	25.424	2	12.712	125.346	<0.001[a]	0.411
		pH	45.407	10	4.541	44.774	<0.001[a]	0.554
		Temperature [a] pH	4.884	20	0.244	2.408	<0.001[a]	0.118
		Error	36.509	360	0.101			
Biofilm Growth	*Vibrio vulnificus*	Strain (block)	24.523	3	8.174	2.158	0.093	0.022
		Temperature	84.573	2	42.286	11.161	<0.001[a]	0.071
		pH	39.560	10	3.956	1.044	0.406	0.034
		Temperature [a] pH	82.672	20	4.134	1.091	0.358	0.069
		Error	1113.890	294	3.789			
	Vibrio parahaemolyticus	Strain (block)	358.521	3	119.507	28.322	<0.001[a]	0.191
		Temperature	282.231	2	141.116	33.443	<0.001[a]	0.157
		pH	232.738	10	23.274	5.516	<0.001[a]	0.133
		Temperature [a] pH	115.935	20	5.797	1.374	0.132	0.071
		Error	1519.034	360	4.220			

Note. Growth Rate. Independent factors: temperature, pH; Dependent variable: growth rate; Block factor: strain.
[a]Significant: $p < 0.05$.

icus strains using strain as the blocking factor (4 levels) as shown in Table 2. Each factor combination has three replicates. For *V. vulnificus* planktonic growth, the RCB-ANOVA indicated that temperature and pH had a significant effect on the bacterial planktonic growth rate ($F_{2,360} = 101.239$, $p < 0.001$; $F_{10,360} = 61.619$, $p < 0.001$). There was also a significant block effect (strain, $F_{3,360} = 7.608$, $p < 0.001$) and a significant interaction between temperature and pH ($F_{20,360} = 6.620$, $p < 0.001$). A similar trend was observed in *V. parahaemolyticus* planktonic ANOVA results, temperature ($F_{2,360} = 125.346$, $p < 0.001$) and pH ($F_{10,360} = 44.774$, $p < 0.001$) and showed a significant effect on the growth rate. The interaction factor between temperature and pH and the blocking factor effect on growth rate was also highly significant ($F_{20,360} = 2.408$, $p < 0.001$; $F_{3,360} = 16.706$, $p < 0.001$).

Additionally, the RCB-ANOVA results of *V. vulnificus* biofilm growth indicated that temperature had a significant effect on growth rate ($F_{2,294} = 11.161$, $p < 0.001$). However, pH, the interaction factor (temperature * pH), and blocking effects were not significant ($F_{2, 294} = 1.044$, $p = 0.406$; $F_{20,294} = 1.091$, $p = 0.358$; $F_{3,294} = 2.158$, $p = 0.093$). For *V. parahaemolyticus* biofilm formation, the RCB-ANOVA revealed that temperature ($F_{2,360} = 33.443$, $p < 0.001$) and pH ($F_{10,360} = 5.516$, $p < 0.001$) had a significant effect on biofilm growth. There was a significant block effect (strain, $F_{3,360} = 28.322$, $p < 0.001$), but the interaction between temperature and pH was not significant ($F_{20,360} = 1.374$, $p = 0.132$).

Figure 3 illustrates the results of interaction plots for *V. vulnificus* and *V. parahaemolyticus* strains showing planktonic and biofilm growth rates. RCB-ANOVA suggests a significant interaction on planktonic growth rates in both *Vibrio* species and a significant effect of independent factors. These results indicate that the response to pH differed depending on the incubation temperature. The lines in the planktonic interaction profiles (Figure 3, left panel) were not parallel and converged, showing interaction. In *V. vulnificus* (Figure 3, panel a, left), the plot indicates interactions at pH < 6.0 and >9.0, where the pH drives the interaction. In comparison, temperature influences the interaction between pH 6.4 and 9.0, where higher temperatures showed higher growth rates. In the *V. parahaemolyticus* interaction plot (panel b, left), pH has a greater effect on the interaction at pH < 5.5 and
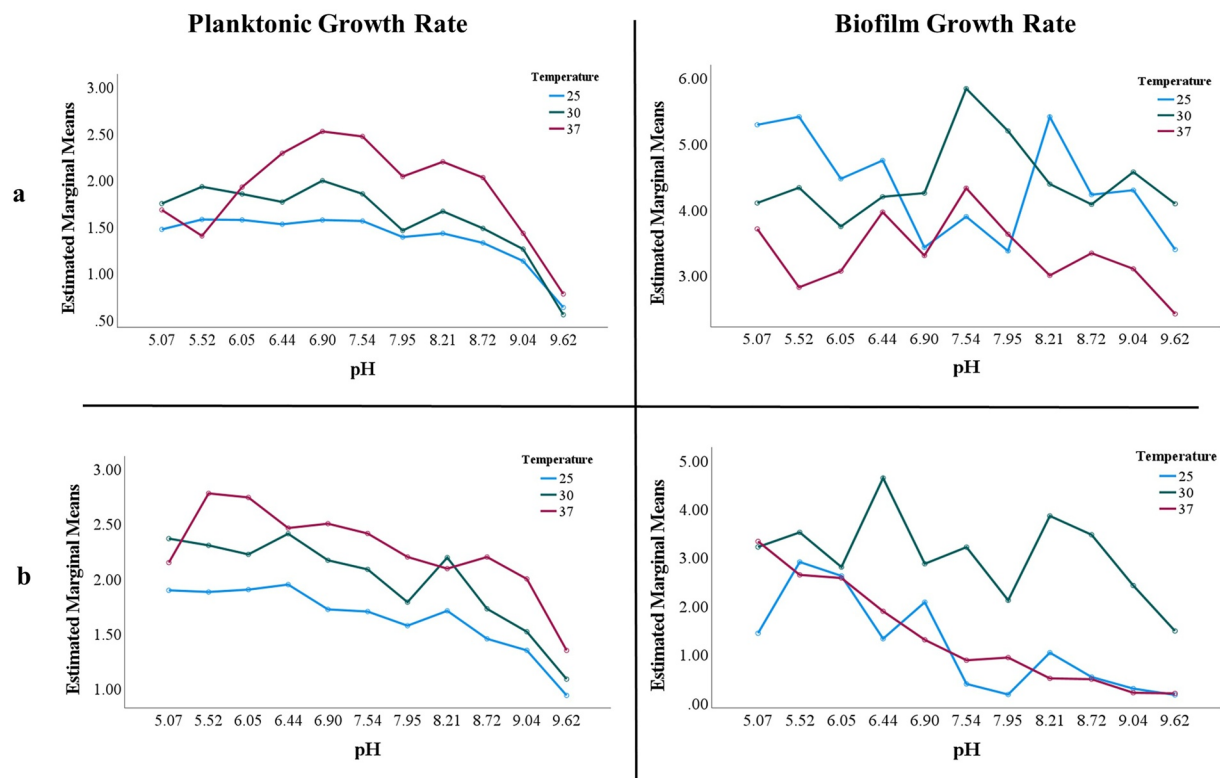
Planktonic Growth Rate

Biofilm Growth Rate

Figure 3. Interaction plots of the estimated marginal means of growth rate in planktonic and biofilm stages of *Vibrio* spp. at different temperatures and ranges of pH. Panel a shows *V. vulnificus* strains, and Panel b shows *V. parahaemolyticus* strains. The line colors represent the different temperatures: 25°C (blue), 30°C (green), and 37°C (red). The estimated marginal means of the growth rate are expressed in $\frac{d}{dt} \ln OD$.

>8.2. The temperature contributes more to the interaction in pH between 5.5 and 8.0, where higher temperatures exhibited higher growth rates.

Vibrio biofilm growth patterns showed no significant interaction, but the lines converge at some points due to the significance of the independent factors (Figure 3, right panel). RCB-ANOVA indicated that temperature significantly affected *V. vulnificus* growth rates; this is illustrated in the interaction plot where the blue line (25°C) converges with other temperatures at multiple points (Figure 3, panel a, right). A similar trend was observed in the *V. parahaemolyticus* profile plot, wherein temperature and pH significantly affected the growth rate, but this interaction was not significant. The red line (37°C) intercepted with the green (30°C) and blue (25°C) lines throughout multiple points (different pH values), which explained the significance of the Individual factors and the difference in response between conditions.

4. Discussion and Conclusions

Climate change is contributing to the successful emergence of human pathogenic diseases, including *Vibrio* infections (Edelson et al., 2022; Landrigan et al., 2020). *Vibrio* are expanding their geographical distribution toward the poles, and their abundances have increased during the past decade (Baker-Austin et al., 2018). At the same time, global temperature changes, increased eutrophication, and elevated pCO_2 have also enhanced cyanobacterial HAB growth rates, resulting in more frequent HAB occurrence within ecosystems that are conducive to *Vibrio* proliferation (O'Neil et al., 2012; Paerl & Paul, 2012; Suikkanen et al., 2013; Visser et al., 2016). Within these estuarine and coastal zone ecosystems, pH changes have become more frequent and driven, in part, by the increasing occurrence and metabolic activities of HABs, where localized pH can fluctuate from acidic (pH < 6) to alkaline levels (pH > 9) (Adams et al., 2022; Zepernick et al., 2021). High biomass blooms cause increases in pH (during daylight) due to the rapid cellular intake of CO_2, resulting in an advantage to cyanobacteria, which possess carbon-concentrating mechanics that provide a competitive advantage to growing in low CO_2 and high pH

(Sandrini et al., 2016; Wells et al., 2020). While multiple models have been developed to predict future risks of *Vibrio* outbreaks, the models do not consider pH or the pH changes in the water column due to co-occurrence of HABs as factors in *Vibrio* growth (Dickinson et al., 2013; Froelich et al., 2013; Semenza et al., 2017).

The results of the present study suggest that pH effects should be added to the *Vibrio* growth modeling efforts to better predict *Vibrio* risk in estuarine and coastal zones. The pH of these zones can be influenced by multiple environmental factors, including anormal weather patterns, influx of natural or man-made chemical nutrients, and co-occurrence of *Vibrio* and HAB outbreak events. Different optimal growth conditions in terms of temperature and pH were observed for planktonic and biofilm non-cholera *Vibrio* growth within and between species generating different modeling patterns (Figures 1 and 2). In addition, this study found that *Vibrios* could have multiple optimal growth conditions that depend on the mode of growth and their interaction with different stressors, including temperature and pH. These findings also suggest that *Vibrio* may express adaptive responses, switching between planktonic to biofilm and vise-versa to resist temperature and pH stressors, potentially increasing bacterial survival under climate change scenarios and increasing *Vibrio*-human interactions. Future transcriptomic studies are needed to understand the *Vibrio* adaptative responses and metabolic pathways expressed under different climate conditions.

Vibrio modeling showed that the bacterial response to acidic and alkaline conditions could vary between strains and species. The ability of *Vibrio* spp. to adapt to pH changes is an essential factor to consider in bacteria-host interactions. Previous studies have documented that *V. vulnificus* can adapt to acidic conditions and become acid resistant by breaking down lysine to cadaverine, which is regulated by the *cadBA* operon (Rhee et al., 2002, 2005). Cadaverine can also act as a superoxide radical scavenger that provides tolerance to oxidative stress (Kang et al., 2007). The link between acid and oxidant stress tolerance may enhance bacterial survival in transitioning between the environment to a human host. Studies have demonstrated that pre-exposure to slightly acidic environments increases *V. vulnificus* acid tolerance and may increase resistance to other stresses (Bang & Drake, 2005). The expression of cross-protective mechanisms in *V. vulnificus* is often regulated by the sigma factor RpoS (σ^S) and, after nutrient starvation, can induce cross-protective effects against oxidative stress (Rosche, Smith, et al., 2005; Rosche, Yano, & Oliver, 2005). Studies have also reported the expression of cross-protection in *V. parahaemolyticus* strains. For example, *V. parahaemolyticus* showed enhanced survival at lower pH (4.4) after exposure to mildly acidic conditions (pH 5.5) and showed cross-protection against low salinity and temperature (Wong et al., 1998). *V. parahaemolyticus* also developed cross-protection after exposure to alkaline conditions (pH 9.0), where adapted cells were found to increase resistance to heat, crystal violet, deoxycholic acid, and hydrogen peroxide (Koga et al., 2002). Furthermore, the production of Kanagawa hemolysin by *V. parahaemolyticus* has been related to lower pH ranges, where the hemolysin production increased (Cherwonogrodzky & Clark, 1981). These studies, in addition to the present work, suggest that *V. vulnificus* and *V. parahaemolyticus* can adapt, survive, and grow under a broad pH range from pH 5 to 9.5.

This study also shows that temperature and pH factors significantly affect the planktonic growth rate of *Vibrio* spp. and the formation of *V. parahaemolyticus* biofilms (Table 2, Figure 3). Previously, the effect of temperature and pH was evaluated in *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *V. cholerae*, where increased pH significantly affected biofilm formation in all species and strains tested (Hostacka et al., 2010). A recent study reported that *V. vulnificus* biofilm production in clinical strains is higher than in environmental isolates at 24°C, with the highest biofilm production in all strains observed at pH 5.5 and 24°C as compared to 30° and 37°C (Çam & Brinkmeyer, 2020). However, Çam and Brinkmeyer (2020) reported that the *V. vulnificus* biofilm growth rate was lowest for environmental strains at pH 5.5, whereas the clinical strains showed no difference between pH 5.5, 7.5 and 8.5, suggesting tolerance to acidic and alkaline conditions. In the case of *V. parahaemolyticus*, greater biofilm formation has been documented at 25°C compared to 15°C and 37°C (Song et al., 2017). Furthermore, another study showed greater biofilm formation and production of exoprotease and autoinducer-2 in food and food contact surfaces at temperatures between 25°C and 37°C (Han et al., 2016).

The relationship between temperature and growth rate has been reported in multiple studies examining V. *vulnificus* and *V. parahaemolyticus*, where increased temperature resulted in increased planktonic growth (Kim et al., 2016; Mudoh et al., 2014; Sheikh, John, et al., 2022; Sheikh, Najiah, et al., 2022; Sullivan & Neigel, 2018). The increase in *V. parahaemolyticus* growth at higher temperatures has been correlated with a shorter generation time, a shorter lag time (Kim et al., 2012), and a faster growth rate (Fernandez-Piquer et al., 2011). In *V. vulnificus*, this positive effect of temperature was also correlated with a higher growth rate and a short lag time

at 22°C and 30°C (Wang & Gu, 2005). Another study reported a positive correlation between temperature and growth under conditions from 11°C to 36°C, where the optimum growth rate, the shortest lag time, and the highest density were observed at 36°C (Kim et al., 2012). *Vibrio* incidence also has been positively associated with temperature after HABs during warmer months. Greenfield et al. (2017) suggested that HABs in retention pond systems were associated with *V. vulnificus* and *V. parahaemolyticus* increases when the water temperature was >10°C. After two cyanobacteria bloom events, the *Vibrio* incidence increased from non-detectable to 6.82×10^2 copies/100 mL and 1.17×10^3 copies/100 mL of *V. parahaemolyticus* and 5.10 and 5.16×10^3 copies/100 mL of *V. vulnificus*. The findings outlined by Greenfield et al. (2017) support the adaptability of *Vibrio* spp. as suggested by our modeling where regardless of the strain variability, the bacteria may survive at different temperatures and pH ranges. These observations and our models suggest a potential to respond to environmental changes and exhibit a higher virulence profile. Higher temperatures can enhance *Vibrio* planktonic growth and serve as a selective pressure for strains with higher virulence potential, allowing for more effective host invasion (Vezzulli et al., 2020). Future studies are needed to assess the interaction between *Vibrio* and HABs and how changes in environmental parameters, such as changes in pH, can strengthen their co-occurrence and selection for pathogenic *Vibrio* strains in real-time environmental conditions.

Overall, the data suggest that non-cholera *Vibrio*, such as *V. vulnificus* and *V. parahaemolyticus*, are capable of adapting to temperature and pH changes in coastal zones and switching between growth modes, increasing their potential to survive under climate change scenarios. While multiple efforts have been developed to create models to predict *Vibrio* exposure and raise awareness of health risk, most models employ water temperature and salinity to predict the potential risk (Brumfield et al., 2021). This study revealed that pH also plays an important role in the adaptive response of *Vibrio* spp., which can increase the virulence potential of environmental isolates. For example, potential exposure to different pH ranges during HAB events, where water pH can fluctuate over diel and tidal cycles, combined with other climatic hazards, can lead to an increase in the distribution, abundance, and virulence of *Vibrio* spp., contributing to the increase of *Vibrio*-human interactions in coastal regions. Future studies are needed to assess simulated climate change conditions and multiple stressors to better understand how climate change can influence *Vibrio* outbreaks and to develop better models to predict future risk of exposure to *Vibrio* with enhanced virulence profiles. This study provides a new perspective that could be integrated into existing models to help decision makers inform those individuals whose risk of infection is high.

Conflict of Interest

The authors declare no conflicts of interest relevant to this study.

Data Availability Statement

The growth rate data used for modeling and analysis of variance in the study are available at Mendeley data via https://doi.org/10.17632/xxkkkbx3hg.1 (Norman & Correa Velez, 2022).

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