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Analysis of the biofilm formation of *Vibrio parahaemolyticus* and *Vibrio cholerae*

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RESUMEN

Las biopelículas son comunidades microbianas en una matriz polimérica y asociadas con una complejidad de cambio fenotípico que confiere un entorno protector contra peligros externos, como productos químicos nocivos o depredadores. Comprender cómo las variables ambientales influyen en la formación de biopelículas y encontrar nuevas estrategias para su control se vuelve relevante para la industria camaronera y el manejo de *Vibrio* spp. *V. parahaemolyticus* y *V. cholerae* se encuentran entre los microorganismos más relevantes para las industrias debido a sus toxinas que causan pérdidas económicas y enfermedades transmitidas por los alimentos. En el presente trabajo evaluamos el impacto de tiempo, la temperatura y el inóculo inicial en el desarrollo de biopelículas de estas dos especies de *Vibrio* utilizando un diseño experimental multifactorial. Nuestros resultados mostraron que las mejores condiciones de crecimiento de biopelículas para *V. parahaemolyticus* fueron 24 h y 24 °C (valor de p <0,001), mientras que para *V. cholerae* fueron 72 h y 30 °C (valor de p <0,001) mediante ensayos de biomasa y viabilidad celular. Sin embargo, el inóculo inicial no tuvo ningún efecto significativo en los ensayos de biomasa y viabilidad celular para ambas especies de *Vibrio*. Las biopelículas de *Vibrio* se formaron por completo a las 24 horas, hubo una disminución del 34 % de las células vivas, el 43 % de la biomasa, 0,5 log en las tasas de viabilidad celular a las 48 horas y demostrando un nuevo crecimiento a las 72 horas. Además, con respecto a la variación de temperatura entre 24 y 30 ºC, ambas especies de biopelículas relacionadas con *Vibrio* no mostraron diferencias estadísticas al evaluar las células totales y las células vivas/muertas dentro de las biopelículas mediante análisis de microscopía de fluorescencia. Sin embargo, el inóculo inicial de 1E+8 UFC/mL (0,5 MacFarland) demostró un mayor número de células vivas y, en consecuencia, se observó un mayor número de células muertas para el inóculo inicial de 1E+7 UFC/mL (0,05 MacFarland; valor p <0,001) optimizando la evaluación de terapias alternativas contra biopelículas de *Vibrio*.

Palabras-clave: Biopelículas, ensayos de biomasa, *Vibrio parahaemolyticus*, *Vibrio cholerae*, microscopía de fluorescencia, unidades formadoras de colonias.

ABSTRACT

Biofilms are microbial communities embedded in a polymeric matrix and associated with a complexity of phenotypic shift in several genes exhibiting thus enhanced protective environment against external hazards such as harmful chemicals or predators. Understanding how environmental variables influence biofilm formation and finding new strategies for their control becomes relevant for the shrimp industry and the management of Vibrio spp. infections in shrimp production. V. parahaemolyticus and V. cholerae are among the most relevant microorganisms for shrimp industries due to their toxin-producing capacities that cause economic losses in farms and foodborne diseases in consumers. Therefore, in the present work, we evaluated the impact of temperature and initial inoculum in the biofilm development of these two Vibrio species over time using a multifactorial experimental design. Our main results showed that the best growth conditions of biofilm development for V. parahaemolyticus were 24 h and 24 °C (p-value <0.001), while V. cholerae biofilms were 72 h and 30 °C (p-value <0.001) through biomass and cell viability assays. Yet initial inoculum did not have any significant effect variation in the biomass growth and cell viability assays for both Vibrio species. V. parahaemolyticus and V. cholerae biofilms were fully formed at 24 hours, reaching a high decrease by 34% of live cells, 43% in biomass, 0.5 log in cell viability rates at 48 hours, and demonstrating regrowth at 72 hours. Furthermore, concerning temperature variation between 24 and 30 ºC, both Vibrio-related biofilm species did not show statistical differences when evaluating total cells and live/dead cells within biofilms through fluorescence microscopy analysis. However, the initial inoculum of 1E+8 CFU/mL (0.5 MacFarland) demonstrated a higher number of live cells and, consequently, a higher number of dead cells for the initial inoculum of 1E+7 CFU/mL (0.05 MacFarland) was observed (p-value <0.001) allowing to a better experimental set up for the evaluation of alternative therapies against Vibrio-related biofilms.

Keywords: Biofilms, Biomass assays, *Vibrio parahaemolyticus*, *Vibrio cholerae*, Fluorescence microscopy, Colony-forming Units.

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INTRODUCTION

The aquaculture industry according to the Food and Agricultural Organization (FAO) is one of the industries with the highest production growth with 9.4% per year (Arunkumar et al., 2020a). However, it has been affected by aquatic diseases mainly produced by various species of *Vibrio* (Sheikh et al., 2022), which generates economic losses for all crustacean-producing countries. According to Global Outlook for Aquaculture Leadership (GOAL) of 2019, Ecuador is the country with the largest production and export of shrimp in the world, and, in the last decade, it has had a rapid growth of approximately 16% (Anderson et al., 2019). Additionally, it represents 19% of the country's exports, generating 5 billion dollars in 2021. The shrimp industry is located in the coastal zone of the country, distributed mainly in 5 provinces, with Guayas being the province with the highest production (60%), followed by El Oro, Esmeraldas, Manabí, and Santa Elena (ISSUU, 2021).

Vibrio spp. is a curved rod gram-negative bacterium, facultative aerobic and facultative anaerobic rods, which has a single flagellum with which it moves (Roy et al., 2021). It is generally found in warm salty aquatic environments such as seawater and estuaries. This genus is found in different conditions and survives at 5-45 °C and achieves substantial growth when seawater temperatures are over 14-19 °C (Teschler et al., 2015; Wang et al., 2022). It is an opportunistic pathogenic bacterium for marine animals as well as for people (Odeyemi, 2016). It has three toxins toxic-release systems on its membrane that release three toxins upon entering a host, more exactly the species-specific thermolabile hemolysin (TLH), thermostable related hemolysin (TRH), and pathogenic thermostable direct hemolysin (TDH) (Song et al., 2017). In aquaculture, this pathogen can be a big issue causing a deadly disease for shrimp and also an opportunistic gastrointestinal infection in humans (Vestby et al., 2020).

In nature, *Vibrio* species can usually be found in multispecies communities that form biofilms, which gives them greater adaptability (Flemming et al., 2022). Biofilms are microorganism consortia

that are enveloped and protected by a polymeric matrix. They are the most common way in which you can find bacterial communities in nature. Biofilms are formed due to quorum sensing, which is a communication system used by bacteria that produce changes in gene expression driven by a message (c-di-GMP) (Loni Townsley and Yildiz, 2018). This causes a phenotypic shift with the reduction of the motor activity of the flagella and regulates the expression of surface proteins for the union and synthesis of the extracellular matrix (Abe et al., 2020).

The main diseases that affect shrimp are early mortality shrimp-acute hepatopancreatic necrosis disease (EMS - AHPND) and causes 100% of mortality, while vibriosis causes 70% of mortality, which damages the shrimp´s oral cavity and appendages (Sajali et al., 2019). These diseases are caused by pathogenic *Vibrio* species, the main ones being *Vibrio alginolyticus, V. parahaemolyticus, V. cholerae*, and *V. vulnificus* (Flemming et al., 2022). In marine biotic or abiotic surface under appropriate growth conditions, *Vibrio* sp. biofilms can function as a source of pathogenic bacteria with 10 – 1000 times more resistance against the hygiene treatment than planktonic counterparts. In study realized by Odeyemi (2016), the authors examined the prevalence of *V. parahemolyticus* based on 48 published studies and reported a 48.3% of its presence in shrimp, especially in uneven seafood surface areas where pits and edges provided protection for biofilm communities.

According to international standards, there are certain antibiotics such as chloramphenicol, tetracyclines, sulphonamides or sulfonamides, and quinolones, which cannot be used due to the presence of antibiotic residues in the shrimp that could be consumed by the consumers and therefore they are not allowed in the shrimp production (INEN, 2013; Roy et al., 2021). One of the strategies to control *Vibrio* species and their biofilm formation is through the use of probiotics in mature ecosystems with copepods (grazing) (Sheikh et al., 2022). In addition to preventing the growth and spread of *Vibrio* species due to competition, certain probiotic bacteria are also able to realize Quorum quenching, which is the inhibition of Quorum sensing and thus biofilm formation (Kewcharoen and

Srisapoome, 2019). However, when biofilms have already been established, these strategies don´t work well. Therefore, it is important to analyze the biofilm life cycle of the main pathogenic *Vibrio* species identifying the best growth conditions to further evaluate suitable treatments against *Vibrio*-related biofilms. So, the present work aimed to evaluate the biofilm formation of *V. parahaemolyticus* and *V. cholerae* and characterize their biofilm production through different *in vitro* conditions. This study analyzed biofilms of these *Vibrio* species in different temperature conditions (24 and 30 °C), during the time (24, 48, and 72 h), and different concentrations of initial inoculum (1E+7 and 1E+8 colony-forming unit (CFU)/mL) by biomass growth assays (optical density measurement at 630 nm using crystal violet (CV) staining and phosphate-buffered saline (PBS) suspension), CFU counting assay, fluorescence microscopy (FM) analysis, and scanning electron microscopy (SEM) analysis.

METHODS

Bacterial Isolates and Growth Conditions

Vibrio cholerae and *V. parahaemolyticus* isolates from Institute of Microbiology Universidad San Francisco de Quito (IM-USFQ), designated as IMUSFQ-VC112 and IMUSFQ-VP 87, respectively, were selected. Strains were stored at -80 °C, and, 24 hours before each assay, a new culture in Trypticase Soy agar (TSA) supplemented with NaCl 1% (w/v). After growth culture at 30 °C, bacterial cells were harvested and suspended in phosphate-buffered saline (PBS) to obtain a cellular density equal to 1E+8 colony-forming units (CFU) per mL using 0.5 McFarland turbidity standard and then a second dilution 1:10 was realized to obtain another concentration of 1E+7 CFU/mL for biofilm assays (see Supplemental Material).

Biofilm Assays

The two inoculums were evaluated in the present assays. Each inoculum of *V. cholerae* or *V. parahaemolyticus* was centrifugated at 10000 rpm, for 20 minutes and the pellet was resuspended in sterile Trypticase soy broth (TSB) supplemented with NaCl 1% (w/v). In each well of the 6-well plate containing a sterile coverslip, 3 mL of primary biofilm inoculum was added. Also, blank or sterility control was prepared on the same plate. Plates containing different inoculums were also incubated at two different temperatures (24 and 30 °C) and for different periods (24, 48, and 72 hours) under static conditions, replacing the medium in each well with 3 mL of fresh medium every 24 hours (Lohse et al., 2018). Each assay with a particular condition setting was performed with at least six assays and, in each assay, five biofilm samples were prepared.

We evaluated biomass formation using an optical density (OD) assay with crystal violet (CV) staining and phosphate-buffered saline (PBS) suspension (Atiencia-Carrera et al., 2022b). Briefly, each optical density assay is described below.

After a certain period of growth (24, 48, and 72 hours), the biofilm samples on coverslips were carefully washed four times with 3 mL of sterile PBS. Then, the coverslips were translated to a

clean 6-well plate and stained with 3 mL of crystal violet 1% (v/v) for 30 minutes, and the excess staining was carefully removed from the wells. 3 mL of alcohol 96% (v/v) was placed into each well for 5 minutes and then each coverslip was placed in a sterile plastic flask with 3 ml of alcohol 96% (v/v), and vortexed at maximum velocity for 15 minutes to ensure the biofilm remotion of the coverslip into the alcohol solution. Finally, 200 μL of each biofilm sample was placed in a 96-well plate and read in the ELISA Elx808 spectrophotometer (BioTek, Winooski, USA) at 630 nm.

Likewise, the second set of biofilm samples was carefully washed four times with 3 mL of sterile PBS. Then each coverslip containing the biofilm sample was placed in a sterile plastic flask with 3 ml of sterile PBS, and vortexed at maximum velocity for 15 minutes to ensure the biofilm remotion of the coverslip into the PBS solution. For each sample, 200 μL of the previous suspension was placed in a 96-well plate and read in the ELISA Elx808 spectrophotometer at 630 nm.

Colony-forming Units Counting

To enumerate culturable sessile cells, a colony-forming unit (CFU) counting assay was used. At least three individual PBS suspensions of each biofilm sample were used in a serial tenfold dilution, by adding 100 μL of sample to 900 μL of sterile PBS supplemented with NaCl 1% (w/v). The tested dilutions included (1E-5 –1E-7) and each dilution was plated on TSA supplemented with NaCl 1% (w/v) by triplicate results per biofilm sample. All plates were incubated for 24 hours at 30 °C, after which colonies were counted.

Fluorescence Staining

All time samples (24, 48, and 72 hours) were chosen to be analyzed by fluorescence staining. After each biofilm formation assay in 6-well plates, tree coverslips were transferred to a new and sterile 6-well plate. A working solution of fluorescent stains was prepared by adding 1.0 mL of SYTO® 9 stain and 10 μL of propidium iodide (PI) stain (FilmTracer™ LIVE/DEAD® Biofilm Viability Kit), mixed in the proportion 1:100 of PI/SYTO-9, into 10 mL of filter-sterilized water in a

foil-covered container. About 200 µL of the live/dead working solution was added onto each coverslip (biofilm sample) and all samples were then incubated for 15-30 min at room temperature, protected from light, before being rinsed with 200 µL of PBS. Finally, fluorescence microscopy (FM) was carried out using an Olympus BX50 microscope equipped with a 100x oil immersion objective. As previously described (Rosenberg et al., 2019), for counting purposes at least 12 images were taken per sample on the 22-mm diameter glass coverslip at random locations. These results were expressed as the number of cells ± standard deviation per cm^2 (N. of cells/cm^2 ± SD). The percentages of dead and alive cells within images were measured through ImageJ version 1.57 by Fiji (Schindelin et al., 2012) using the macros Biofilms Viability checker (Mountcastle et al., 2021).

Statistical Analysis

Normality data was analyzed using the Anderson-Darling, Ryan-Joiner, and Kolmogorov-Smirnov normality tests. The Johnson data transformation method appliedthe transformation from non-parametric to parametric data. A multivariate ANOVA analysis was performed. To compare the means between variables/conditions, the Tukey and Fisher tests were performed with an alpha of 0.05 ($\alpha = 0.05$). The values obtained with $p<0.05$ are considered significant. All data analysis was performed in Minitab version 20 (Minitab Statical Software, 2022).

RESULTS

Quantification of the *V. parahaemolyticus* and *V. cholerae* Biofilms and their Normality Assessment

The ability of *V. parahaemolyticus* strain VP 87 and *V. cholerae* strain VC 112 to develop a biofilm was determined by comparing biomass, cell viability, and total cell and live/dead cell counting assays (Figure 1 and Annex A). An assessment of normality was also applied to the obtained data, using the Anderson-Darling, Ryan-Joiner, and Kolmogorov-Smirnov normality test and the Johnson data transformation method. Subsequently, a reduction of the variable interaction model was performed, eliminating interactions that showed a p-value >0.05 (Table 1). Finally, the same statistical analysis was repeated in the new data set.

As shown in Figure 1, *V. parahaemolyticus* biofilms showed a higher ability to produce biomass and culturable sessile cells (CFU counting) during the first 24 h when compared to *V. cholerae* biofilms (p-value <0.001). However, at 72 h, *V. cholerae* biofilms had a higher ability to produce biomass and culturable sessile cells. Additionally, *V. parahaemolyticus* and *V. cholerae* biofilms showed a greater ability to produce biomass and culturable sessile cells at 24 °C when compared to 30 ºC (p-value <0.001). Normality tests showed normal distribution after the reduction of the model in biomass and viability tests, but a non-normal distribution in the total cell count test, so a data transformation by Johnson's method was performed. Consequently, a parametric statistical analysis was selected for future evaluation.

Comparison between Growth Conditions in Biofilms of *V. parahaemolyticus* and *V. cholerae*

Biomass and viable cells within the biofilm were quantified demonstrating statistical differences between species at different temperatures, times, and initial inoculums (Table 1). Multivariate ANOVA analysis showed a significant effect of the variables and the interactions with a p-value <0.05, to determine the biomass growth and biofilm viability in both *Vibrio* species (Figure

1). Both *Vibrio* biofilms had a significant increase in biomass and cell viability at 24 h of growth when compared to 48 h with a p-value <0.001 in both species by Tukey and Fisher tests, where the biomass and cell viability of *Vibrio* sp. biofilms significantly decreased by 43% and 0.5 log (Figure 1), respectively. Interestingly, *Vibrio* biofilms showed a significant increase in biomass and cell viability at 72 h (Anexo A). However, biomass and cell viability had a higher growth of *V. parahaemolyticus* biofilm at 24 °C, while *V. cholerae* biofilm showed a higher growth at 30 °C. It is worth mentioning that the initial inoculum was not significant for cell viability (p-value =0.45), but it was significant for biomass growth (p-value <0.001) with a slight augmentation of almost 10% at 1E+8 CFU (0.5 MacFarland) in both *Vibrio* species biofilms.

The statistical analysis of biofilm growth by variables comparison method with Tukey and Fisher tests showed a significant difference between *V. parahaemolyticus* and *V. cholerae* biofilms in the biomass assays with crystal violet staining (p-value <0.001; Table 2) but the biomass assays with PBS suspension and cell viability assay did not show any significant differences (p-values between 0.20-0.26). Likewise, in both species of *Vibrio*, Tukey and Fisher tests confirmed statistical differences in all biofilm samples during time in the biomass growth and cell viability assays, in particular between 24 and 48 hours (p-value 0.001). Contrasting with the previous Multivariate ANOVA analysis, Tukey and Fisher tests did not show any significant difference for the initial inoculum variation in the biomass growth and cell viability assays for both species (p-value 0.10 - 0.86). Although the temperature in the biomass assays did not evidence significant differences (p-value 0.51 - 0.99), Tukey and Fisher tests demonstrated a significant difference for the cell viability assays (p-value 0.02). It is interesting to observe that both *Vibrio* species evidenced similar biomass growth trends demonstrating their highest biomass growth and cell viability in the first 24 hours at 24 °C (Figure 2), where *V. cholerae* reached close to the same level of biomass at 72 h and 30 °C (Anexo A) when compared to the ideal conditions (24 h at 24 ºC).

Total Cell and Live/Dead Cells Count in Biofilms of *V. parahaemolyticus* and *V. cholerae*

Next, we analyzed the number of total cells and live/dead cells within biofilms among *Vibrio* species under different growth conditions. As shown in Table 2, the fluorescence microscopy (FM) analysis showed a significant effect between samples according to temperature, time, and initial inoculum in both *Vibrio* species. When comparing species, statistical differences were observed between *Vibrio* species. More exactly, the dead cells count at 48 h was statistically different by Tukey and Fisher tests (p-value <0.001), showing a decrease in both species of 34%. Over time *V. parahaemolyticus* showed a higher number of dead cells within the biofilm (around 4%) when compared to *V. cholerae*. Although no statistical differences were found between *Vibrio* species in the total cell and live cells count, both biofilms evidenced significant differences in their intrinsic growth through the total cell and live cell count between 24 and 48 h (p-value <0.001), and 48 and 72 h (p-value <0.001). Furthermore, concerning temperature variation between 24 and 30 ºC, both *Vibrio*-related biofilm species did not show statistical differences. Interestingly, in FM analysis, the initial inoculum of both *Vibrio* species for biofilm formation showed significant differences for total cell and live/dead cells, where the initial inoculum of 1E+8 CFU (0.5 MacFarland) demonstrated a higher number of live cells and consequently a higher number of dead cells for the initial inoculum of 1E+7 CFU (0.05 MacFarland) was observed (p-value <0.001). Consequently, it was also observed that *V. parahaemolyticus* and *V. cholerae* demonstrated a higher number of total cells and live cells within the biofilms at 24 h, 24 °C, and 1E+8 CFU (0.5 McFarland), as shown in Figure 3.

Although a thoughtful optimization of the FM analysis was performed during the present study, the pictures of live/dead cells within the biofilms of both species did not show the best clarity. However, the merged images showed a greater clarity of the biofilms' development, and the macros Biofilms Viability checker software provided a reliable assessment of the data set. Visual differences in the percentage of dead and alive cells were detected between *Vibrio* species during time, temperature, and initial inoculum. These differences are also validated by ANOVA statistical analysis

and the optimized growth conditions were provided by the Minitab statistical program~~,~~ based on Composite Desirability analysis (Figure 4) through the weighted geometric mean of individual desirability for responses. Minitab determines the optimal settings for the variables by maximizing the composite desirability (see Anexo B), in accordance with the previous statistical analysis (Table 2), showing a homogeneous distribution of dead cells within the biofilms of both *Vibrio* species (see merged images in Supplementary Figure S1).

Comparison of the four methodologies to assess biofilm development

After the statistical analysis of the data set, we compared the data accuracy of these different and classical methodologies for *Vibrio*-related biofilm characterization. Therefore, multivariate linear regression ANOVA was applied using CFU counting assays as a reference due to its well-known reliability, and R-squared values were observed as a goodness-of-fit measure for biofilm analysis. As expected, fluorescence microscopy analysis revealed the lowest R-squared values for both *Vibrio*-related biofilms (R^2 = 0.22 - 0.45), followed by CFU counting assays (R^2 = 0.83 - 0.85), biomass assays with CV staining (R^2 = 0.84 - 0.85), and finally biomass assays with PBS suspension (R^2 = 0.86 - 0.92). It is important to mention again that all assays were analyzed at least six assays with five biofilm samples, under the same conditions, to prevent data variability, bias, and manual errors by the researcher during biofilm analysis.

DISCUSSION

The ability to establish biofilms has given microorganisms an adaptive advantage in nature associated with resistance to environmental stress, antibiotics, evasion of immune systems, and horizontal gene transfer (HGT) mechanisms, giving them greater chances of survival and virulence. In *Vibrio* species, it has been observed that the ability to form biofilms intrinsically depends on numerous conditions (Yildiz & Visick, 2009), which also indicates that *Vibrio* biofilms can easily adapt to various environmental environments in nature (Karygianni et al., 2020). This study aimed to use multiple methods to compare the biofilm formation of *Vibrio parahaemolyticus* and *Vibrio cholerae* to find the best conditions for biofilm growth and thus realize further evaluation of antimicrobial treatments against biofilms in nature (Arunkumar et al., 2020). To the authors' best knowledge, this is the first study to analyze biofilms of these *Vibrio* species during temperature, time, and initial inoculum conditions by multiple classical methodologies, also evaluating the methodological accuracy of biofilm development.

Standard optical density measurement assays offer a quick way to analyze the biomass formed by *Vibrio* species during time, with low cost and a minimum of equipment. The present study demonstrated that PBS and CV assays have similar correlations with cell viability through the CFU counting methodology for *Vibrio*-related biofilms, as previously described in *Candida*-related biofilms(Atiencia-Carrera et al., 2022). Atiencia-Carrera and colleagues previously demonstrated the useful application of CV staining and PBS suspension in optical density (OD) assay for the measurement of biofilm formation. In concordance, our results evidenced that the additional procedural steps (such as staining, fixing, and washing steps) in CV staining lead to a loss of goodness-of-fit measurement in *Vibrio* biofilms.

As previously reported, both *Vibrio* species had different abilities to form biofilms (Díaz-Pascual et al., 2019; Kim et al., 2019; Karygianni et al., 2020). However, a thorough study of different experimental conditions in biofilm formation is needed to develop more efficient antibiofilm

treatments. Our results showed a discontinuous growth of biomass and viability in both *Vibrio* species, more exactly a significant decrease in biomass and viable cells at 48 h, which suggests the presence of stressful growth conditions during the biofilm life cycle in both *Vibrio* species and differing of the typical life cycle described in most bacterial biofilms (Ruhal and Kataria, 2021). Thus, *Vibrio* species possess a different biofilm formation cycle, since it depends mainly on time and temperature (Song et al., 2017). According to Arunkumar et al. and Wang et al. the initiation of biofilm formation in *Vibrio* species depends on its gene expression mechanisms and on the conditions in which it is found in nature (Arunkumar et al., 2020; Wang et al., 2022).

Moreover, when comparing the biofilm formation between the two *Vibrio* species, *V. parahaemolyticus* demonstrated a better ability to form biofilms in a shorter time and temperature, according to the results of biomass and viable cells. Our results coincided with the observations of previous studies (Roy et al., 2021; Shime-Hattori et al., 2006; Song et al., 2017), where it was reported that *V. parahaemolyticus* showed a greater growth within the first 24 hours and at temperatures between 24 °C and 25 °C. While *V. cholerae* was reported to establish well-consolidated biofilms at approximately 72 hours in previous studies (Díaz-Pascual et al., 2019; 2018; Matz et al., 2005). Regarding temperature conditions, a previous study demonstrated an improvement of biofilm growth in *V. cholerae* at higher temperatures than other *Vibrio* species (Loni Townsley & Yildiz, 2018), in particular, between 30 °C and 35 °C.

In the present study, the results of biomass and viable cells indicated that the initial inoculum was not significant for the formation of biofilms of the two *Vibrio* species, justifying the different concentrations of initial inoculums reported in various studies on biofilm formation through biomass and CFU counting assays (Karunasagar et al., 1996; Karygianni et al., 2020; Steinberg & Kolodkin-Gal, 2015). However, it is important to mention that there are no studies evaluating the formation of *Vibrio*-related biofilms with different initial inoculums, so it is difficult to have a more reliable comparison.

The fluorescence microscopy (FM) analysis collaborated with the highest production of biofilms by *V. parahaemolyticus* when compared to *V. cholerae*. FM analysis through syto-9 and propyl iodide (PI) fluorescence staining allowed a reliable evaluation of the live and dead cells within *Vibrio*-related biofilm (McGoverin et al., 2020), without the necessity to use DAPI staining (Vestby et al., 2020). According to the results obtained in this study, it was possible to observe that there is no difference between live and dead cells within the biofilms between *V. parahaemolyticus* and *V. cholerae*. However, *V. parahaemolyticus* biofilm reached a higher number of dead cells in a shorter time when compared to *V. cholerae*, suggesting a faster biofilm life cycle in these experimental conditions.

Although the initial inoculum did not show statistical differences in biomass and CFU counting assays, the concentration of the initial inoculum was significantly different between the amount of live and dead cells within *Vibrio*-related biofilms, obtaining a lower number of live cells and a higher number of dead cells when the initial inoculum is smaller. According to a study carried out by Wang and colleagues, *Vibrio* species can establish microcolonies (an early phase of biofilm development) with merely 50 cell aggregates (Wang et al., 2022) and these cell aggregates (microcolonies) can form a mature biofilm within the first 6 hours of growth culture, as previously reported by Xiao and colleagues (Xiao et al., 2020). Altogether, the results suggested that a lower concentration of *Vibrio* cells in the initial inoculum allows a faster biofilm formation leading *Vibrio* cells to quickly start the exponential phase of growth (Zhu & Mekalanos, 2003), increasing the dead cells mount and consequently inducing a premature dispersion phase of *Vibrio* biofilms into the outside environment (Wang et al., 2022). Meanwhile, a higher initial inoculum shows a greater number of cells that manages to encompass and form biofilm more easily and without the need to grow so rapidly (Teschler et al., 2015). Therefore, further studies with metabolic and gene expression assays should be performed to identify the phenotypic expression of *Vibrio* depending on the initial inoculum concentration.

Most studies evaluating *Vibrio*-related biofilms did not exceed 48 hours of biofilm development or different initial inoculums (Teschler et al., 2015; Yan et al., 2016; Song et al., 2017). Therefore, it is difficult to compare the results obtained in the present study on mature biofilms at 72 hours and the effect on biofilm development by different initial inoculums. Nonetheless, it was possible to analyze the formation of *Vibrio* biofilms under different conditions based on complementary reports from previous studies supporting the results obtained in the present study. Due to the emerging cause of *Vibrio*-related infections and diseases involving biofilms in shrimp and the loss of production in the industry, the characterization of *Vibrio* biofilms is currently important and further studies must be realized on alternative treatments to inhibit and eradicate *Vibrio* biofilms.

However, the present study has several shortcomings, such as the absence of analyzes based on metabolic or gene expression, flow cytometry, confocal microscopy, and quantitative polymerase chain reactions (qPCR) to assess differences between *V. parahaemolyticus* and *V. cholerae* biofilms development at in their diverse growth conditions. Also, only one specimen of each *Vibrio* species was evaluated in this study, although each strain belongs to a reference microbial collection culture (IM-USFQ).

CONCLUSIONS

The present study demonstrated the ability of *Vibrio parahaemolyticus* to more efficiently produce a biofilm at a lower temperature and time when compared to *Vibrio cholerae*, which requires a higher temperature and time. To the best of the authors' knowledge, this is the first study to simultaneously evaluate the biofilm development of these *Vibrio* species over time (up to 72 hours) under different temperature conditions (24 °C and 30 °C) and different concentrations of the initial inoculum (1E+7 and 1E+8 CFU/mL) through biomass, cell viability, and total cell and/or live/dead cells assays. The results obtained in this study showed that the best growth conditions of biofilm development for *V. parahaemolyticus* were 24 h and 24 °C, while *V. cholerae* biofilms were 72 h and 30 °C, with the initial inoculum concentration not being significant for the formation of biofilms through biomass and cell viability assays. Although both *Vibrio*-related biofilm species did not show statistical differences between 24 and 30 ºC when evaluating total cells and live/dead cells within biofilms through fluorescence microscopy analysis, the initial inoculum of 1E+8 CFU/mL (0.5 MacFarland) demonstrated a higher number of live cells. Further studies should use these experimental growth conditions to evaluate alternative treatments for biofilm inhibition and eradication assays.

Tables

Table 1 . Summary of results of reducing model of ANOVA statistical analysis of biomass, viability, total cells counting assays in *Vibrio parahaemolyticus* and *Vibrio cholerae*.

ANOVA reduced model abstract										
	Biomass CV A630					Biomass PBS A630				
Variables	**DF**	**SC adjusted**	**MC adjusted**	**F-Value**	**P-Value**	**DF**	**SC adjusted**	**MC adjusted**	**F-Value**	**P-Value**
1	1.00	49793.00	49792.90	112.60	0.00	1.00	765.00	765.00	1.12	0.29
2	1.00	9.00	9.40	0.02	0.88	1.00	458.00	458.00	0.67	0.42
3	2.00	37248.00	18623.90	42.11	0.00	2.00	394890.00	197445.00	288.31	0.00
4	1.00	15694.00	15693.90	35.49	0.00	1.00	13125.00	13125.00	19.17	0.00
1*2	1.00	30402.00	30402.10	68.75	0.00	1.00	59018.00	59018.00	86.18	0.00
1*3	2.00	16472.00	8235.80	18.62	0.00	2.00	238057.00	119029.00	173.81	0.00
1*4	1.00	137.00	137.10	0.31	0.58	1.00	23472.00	23472.00	34.27	0.00
2*3	2.00	99851.00	49925.50	112.90	0.00	2.00	49754.00	24877.00	36.33	0.00
2*4	1.00	527.00	527.30	1.19	0.28	1.00	923.00	923.00	1.35	0.25
3*4	2.00	18767.00	9383.30	21.22	0.00	2.00	45155.00	22577.00	32.97	0.00
1*2*3	2.00	45124.00	22561.80	51.02	0.00	2.00	134686.00	67343.00	98.33	0.00
1*2*4	-	-	-	-	-	1.00	6832.00	6832.00	9.98	0.00
1*3*4	-	-	-	-	-	2.00	12338.00	6169.00	9.01	0.00
2*3*4	-	-	-	-	-	2.00	26082.00	13041.00	19.04	0.00
1*2*3*4	-	-	-	-	-	2.00	41229.00	20614.00	30.10	0.00
Error	127.00	56162.00	442.20	-	-	-	-	-	-	-
Lack of fit	7.00	5523.00	789.00	1.87	0.08	-	-	-	-	-
Pure error	120.00	50640.00	422.00	-	-	120.00	82180.00	685.00	-	-
Total	143.00	370186.00	-	-	-	143.00	1128965.00	-	-	-
	Viability CFU Log/ml					**Live Cells cm^2**				
Variables	**DF**	**SC adjusted**	**MC adjusted**	**F-Value**	**P-Value**	**DF**	**SC adjusted**	**MC adjusted**	**F-Value**	**P-Value**
1	1.00	0.12	0.12	6.12	0.02	1.00	0.02	0.02	0.02	0.88
2	1.00	0.63	0.63	31.14	0.00	1.00	1401.00	14009.00	1.54	0.22
3	2.00	0.60	0.30	14.72	0.00	2.00	57704.00	288522.00	31.70	0.00
4	1.00	0.01	0.01	0.58	0.45	1.00	22723.00	227234.00	24.97	0.00
1*2	1.00	74667.00	746672.00	368.64	0.00	1.00	0.25	0.25	0.27	0.60
1*3	2.00	0.04	0.02	1.04	0.36	2.00	6749.00	33745.00	3.71	0.03
1*4	1.00	0.06	0.06	3.00	0.09	1.00	7290.00	72897.00	8.01	0.01
2*3	2.00	10657.00	0.53	26.31	0.00	2.00	38045.00	190226.00	20.90	0.00
2*4	1.00	0.08	0.08	4.11	0.05	1.00	7315.00	73152.00	8.04	0.01
3*4	2.00	0.81	0.41	20.10	0.00	2.00	2196.00	10979.00	1.21	0.30
1*2*3	2.00	0.69	0.34	16.93	0.00	2.00	16604.00	83018.00	9.12	0.00
1*2*4	1.00	0.09	0.09	4.60	0.03	-	-	-	-	-
1*3*4	-	-	-	-	-	2.00	16148.00	80738.00	8.87	0.00

2*3*4	-	-	-	-	-	-	-	-	-	-
1*2*3*4	-	-	-	-	-	-	-	-	-	-
Error	126.00	25521.00	0.02			701.00	638033.00	0.91		
Lack of fit	6.00	0.05	0.01	0.36	0.90	5.00	2488.00	0.50	0.54	0.74
Pure error	120.00	25066.00	0.02	-	-	696.00	635545.00	0.91	-	-
Total	143.00	142264.00	-	-	-	719.00	814369.00	-	-	-
	Dead Cells cm²					**Total Cells cm²**				
Variables	**DF**	**SC adjusted**	**MC adjusted**	**F-Value**	**P-Value**	**DF**	**SC adjusted**	**MC adjusted**	**F-Value**	**P-Value**
1	1.00	830.80	830.81	64.46	0.00	1.00	1818.00	18177.00	2.59	0.11
2	1.00	8.20	8.25	0.64	0.42	1.00	1699.00	16988.00	2.42	0.12
3	2.00	765.30	382.64	29.69	0.00	2.00	50120.00	250598.00	35.71	0.00
4	1.00	2419.80	2419.77	187.73	0.00	1.00	3696.00	36964.00	5.27	0.02
1*2	1.00	116.30	116.31	9.02	0.00	1.00	0.02	0.02	0.03	0.87
1*3	2.00	810.80	405.42	31.45	0.00	2.00	12317.00	61585.00	8.78	0.00
1*4	1.00	5.60	5.57	0.43	0.51	1.00	4844.00	48436.00	6.90	0.01
2*3	2.00	134.00	67.01	5.20	0.01	2.00	26037.00	130187.00	18.55	0.00
2*4	1.00	408.90	408.88	31.72	0.00	1.00	7601.00	76006.00	10.83	0.00
3*4	2.00	828.00	414.00	32.12	0.00	2.00	9025.00	45123.00	6.43	0.00
1*2*3	2.00	56.80	28.38	2.20	0.11	2.00	10246.00	51231.00	7.30	0.00
1*2*4	1.00	33.70	33.75	2.62	0.11	-	-	-	-	-
1*3*4	2.00	714.60	357.28	27.72	0.00	2.00	13492.00	67460.00	9.61	0.00
2*3*4	2.00	133.20	66.61	5.17	0.01	-	-	-	-	-
1*2*3*4	2.00	100.20	50.08	3.89	0.02	-	-	-	-	-
Error	696.00	8971.30	12.89			701.00	491957.00	0.70		
Lack of fit	-	-	-	-	-	5.00	2526.00	0.51	0.72	0.61
Pure error	-	-	-	-	-	696.00	489431.00	0.70	-	-
Total	719.00	16333.20	-	-	-	719.00	632666.00	-	-	-

Legend. Variables, 1: Species; 2: Temperature; 3: Time; and, 4: Initial Inoculum. A normalization test and data transformation were performed to obtain normalized data for a parametric test of biofilm growth. A multivariate ANOVA statistical analysis was performed to evaluate biofilm formation in TSB plus 1% NaCl differences between statistical values of multiple variables. The degrees of freedom (DF) indicate the number of observations free to vary, the adjusted sum of squares (SC) indicates the total sum of the variation or deviation contributed by the variables, the adjusted mean square (MC) indicates the variation that exists between the variables, the F value indicates the significance that a variable contributes to the system and the relationship between the variables (the higher the F value the greater the significance), and the p-value equal to or less than 0.05 indicates that the null hypothesis is false and variables are significant for biofilm growth.

Table 2. Summary of the statistical analysis and intervariable correlations by Tukey and Fisher methods in biofilm formation of *V. parahaemolyticus* and *V. cholerae*.

Tukey and Fisher Pot Variables Comparison Plot											
		Biomass CV A630					Biomass PBS A630				
Variables		Mean	Mean Difference	Significant difference		P-Value	Mean	Mean Difference	Significant difference		P-Value
Species	VP	0.10	0.01	VP - VC	Yes	0.00	0.08	0.00	VP - VC	No	0.20
	VC	0.08					0.07				
Temperature	24°C	0.09	0.00	24°C - 30°C	No	0.99	0.07	0.00	24°C - 30°C	No	0.51
	30°C	0.09					0.07				
Initial Inoculum	0.05	0.10	0.01	0.05 - 0.5	No	0.10	0.07	0.00	0.05 - 0.5	No	0.33
	0.5	0.09					0.07				
Time	24h	0.10	0.01	24h - 48h	Yes	0.00	0.08	0.02	24h - 48h	Yes	0.00
	48h	0.08		48h - 72h	No	0.18	0.06	0.01	48h - 72h	Yes	0.00
	72h	0.09	0.01	24h - 72h	Yes	0.03	0.07	0.01	24h - 72h	No	0.54
		Viability CFU Log/ml					Live cells cm^2				
Variables		Mean	Mean Difference	Significant difference		P-Value	Mean	Mean Difference	Significant difference		P-Value
Species	VP	7.72	0.06	VP - VC	No	0.26	2.98E+6	0.01	VP - VC	No	0.88
	VC	7.77					2.95E+6				
Temperature	24°C	7.81	0.13	24°C - 30°C	Yes	0.02	3.10E+6	0.09	24°C - 30°C	No	0.25
	30°C	7.68					2.84E+6				
Initial Inoculum	0.05	7.74	0.02	0.05 - 0.5	No	0.86	3.54E+6	0.36	0.05 - 0.5	Yes	0.00
	0.5	7.75					2.48E+6				
Time	24h	7.83	0.16	24h - 48h	Yes	0.02	3.97E+6	0.68	24h - 48h	Yes	0.00
	48h	7.67	0.07	48h - 72h	No	0.52	3.25E+06	0.48	48h - 72h	Yes	0.00
	72h	7.74	0.09	24h - 72h	No	0.26	2.02E+06	0.20	24h - 72h	Yes	0.03
		Dead cells cm^2					Total cells cm^2				
Variables		Mean	Mean Difference	Significant difference		P-Value	Mean	Mean Difference	Significant difference		P-Value
Species	VP	9.09E+5	2.15	VP - VC	Yes	0.00	4.26E+6	0.10	VP - VC	No	0.13

	VC	5.05E+5					3.86E+6				
Temperature	**24°C**	7.04E+5	0.21	24°C - 30°C	No	0.41	4.26E+6	0.10	24°C - 30°C	No	0.15
	30°C	6.64E+5					3.86E+6				
Initial Inoculum	**0.05**	1.10E+6	3.67	0.05 - 0.5	Yes	0.00	4.36E+6	0.14	0.05 - 0.5	Yes	0.03
	0.5	4.03E+5					3.77E+6				
Time	**24h**	1.00E+6	2.24	24h - 48h	No	0.00	5.51E+6	0.64	24h - 48h	Yes	0.00
	48h	5.68E+5	0.12	48h - 72h	Yes	0.93	2.89E+6	0.37	48h - 72h	Yes	0.00
	72h	5.49E+5	2.12	24h - 72h	No	0.05	4.18E+6	0.28	24h - 72h	Yes	0.00

Legend. ―The Tukey and Fisher methods of intervariable comparison create confidence intervals for all pairwise differences in the means of the levels of the variables. A p-value less than or equal to 0.05 indicates whether there is a significant difference between the pairs of variable levels.

Figures

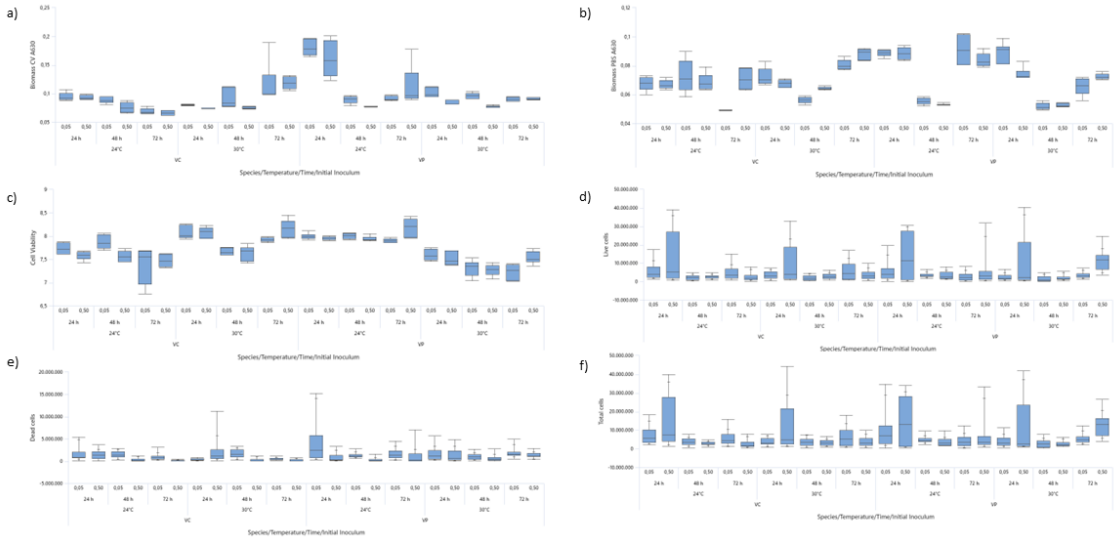

Figure 1. Box plot of the evaluation of the biofilm formation by *Vibrio parahaemolyticus* and *Vibrio cholerae* under different growth conditions.

Evaluation of biofilms formed by *Vibrio parahaemolyticus* (VP) and *Vibrio cholerae* (VC) during different temperatures (24 and 30 °C), time (24, 48, and 72 h), and initial inoculums of 1E+7 and 1E+8 CFU/mL (0.05 and 0.5 MacFarland). The biofilm characterization was evaluated by biomass, cell viability, and total cell and live/dead cells count assays. The biomass of the biofilm was assessed by two optical density measurement methodologies, while viable cells within the biofilm were analyzed by colony-forming counting assays. All data results were plotted for each species and condition, the overlaid boxplots cover interquartile ranges above and below, the whiskers extend to extreme data points, and the black diamond represents the median (data shown in Supplementary Table 1). (a) Biofilm biomass by crystal violet (CV) staining method, (b) Biofilm biomass by phosphate-buffered saline (PBS) suspension method, (c) Biofilm viability by colony-forming (CFU) counting assays, (d) Biofilm quantification by live cell count with Live/Dead staining, (e) Biofilm quantification by dead cell count with Live/Dead staining, and (f) Biofilm quantification by total cell count with Live/Dead staining. All plots were obtained through the Minitab statistical program.

Figure 2. Statistical analysis of the growth conditions involved in the biofilm formation of *Vibrio parahaemolyticus* and *Vibrio cholerae*.

The variables determined for biofilm formation of *Vibrio parahaemolyticus* (VP) and *Vibrio cholerae* (VC) were temperature (24 and 30 °C), initial inoculums (0.05 and 0.5 McFarland), and time (24, 48 and 72 h). The biomass of the biofilm was analyzed by two methodologies measuring the optical density by absorbance at 630 nm with crystal violet and PBS suspension, while cell viability analysis was evaluated by colony-forming counting assays, and biofilm quantification was realized by total cell and live/dead cells assays. These results were analyzed using the Tukey and Fisher tests methodology for growth conditions comparison, where the bars show the mean of the variables for each result, in order to determine whether or not there is a significant difference between the variables (growth conditions). (a) Biofilm biomass by crystal violet (CV) staining method, (b) Biofilm biomass by phosphate-buffered saline (PBS) suspension method, (c) Biofilm viability by colony-forming (CFU) counting assays, (d) Biofilm quantification by live cell count with Live/Dead staining, (e) Biofilm quantification by dead cell count with Live/Dead staining, and (f) Biofilm quantification by total cell count with Live/Dead staining. * p <0.05; ** p <0.01; * * * p <0.001; **** p <0.0001, - non-significant.

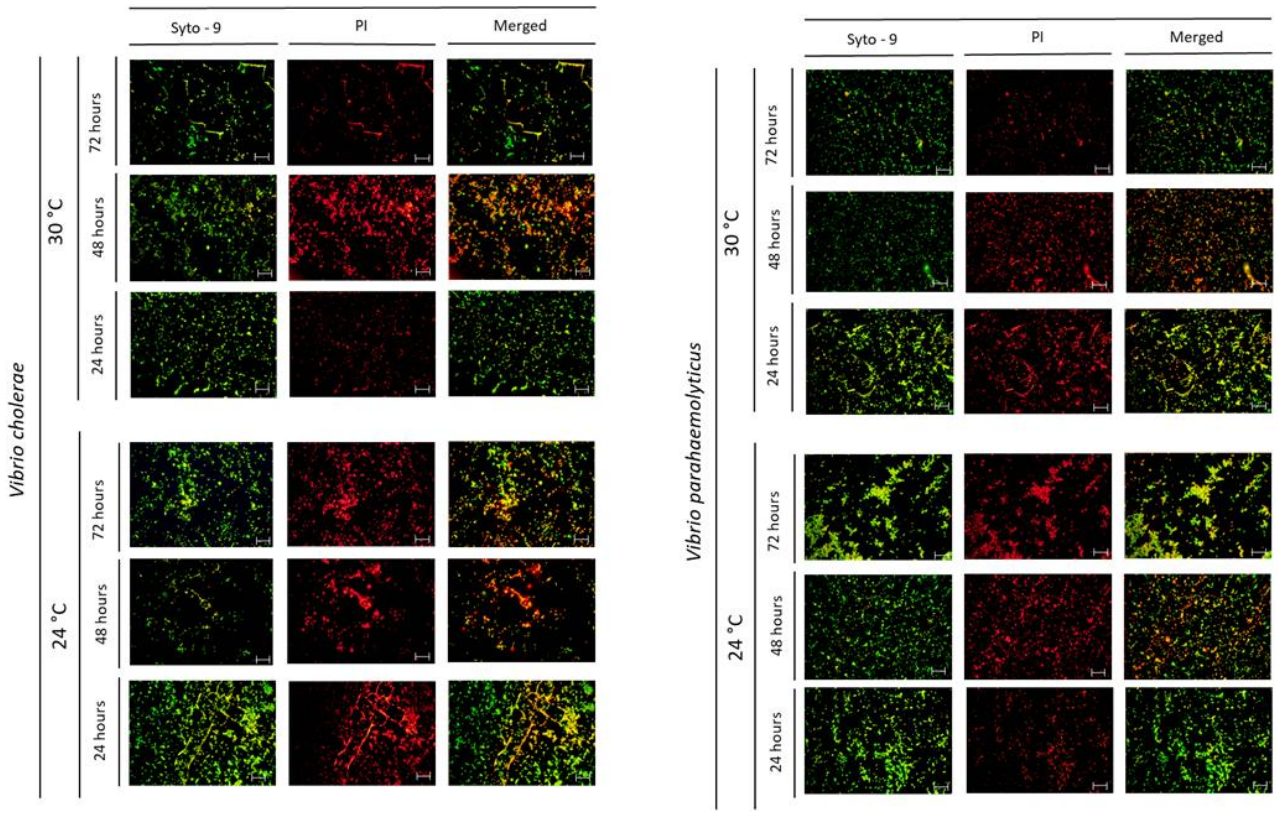

Figure 3. Illustration of fluorescence microscopy analysis in the biofilm formation of *Vibrio parahaemolyticus* and *Vibrio cholerae* at different growth conditions.

Biofilms of *Vibrio parahaemolyticus* and *Vibrio cholerae* at 24 and 30°C during time (24, 48, and 72 h) by fluorescence microscopy using Live/Dead staining assays. The illustrated biofilm samples with an initial inoculum of 1E+8 CFU/mL (0.5 McFarland) were used to compare the total number of live and dead cells. An Olympus BX50 microscope with 100X magnification was used, images were obtained with AmScope software, and images were merged with Fiji-ImageJ software.

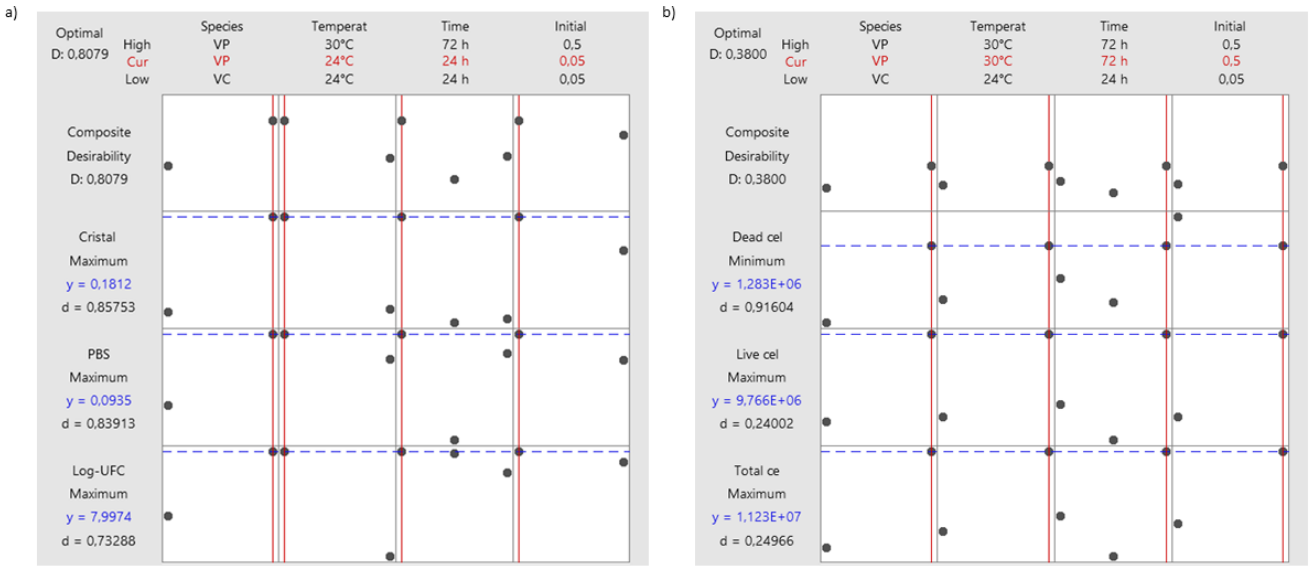

Figure 4. Composite desirability analysis of the growth conditions for biofilm formation of *Vibrio parahaemolyticus* and *Vibrio cholerae*.

The Minitab statistical program calculated an optimal solution that serves as the starting point for the plot, and the settings can be modified interactively to determine how different settings affect *Vibrio*-related biofilm growth responses. For biofilm formation, we sought to maximize the response of the variables with higher composite desirability. (a) Optimization of the biofilm formation of *V. parahaemolyticus* and *V. cholerae* through the biomass and cell viability assays, (b) Optimization of the biofilm formation of *V. parahaemolyticus* and *V. cholerae* through the total cell count assay.

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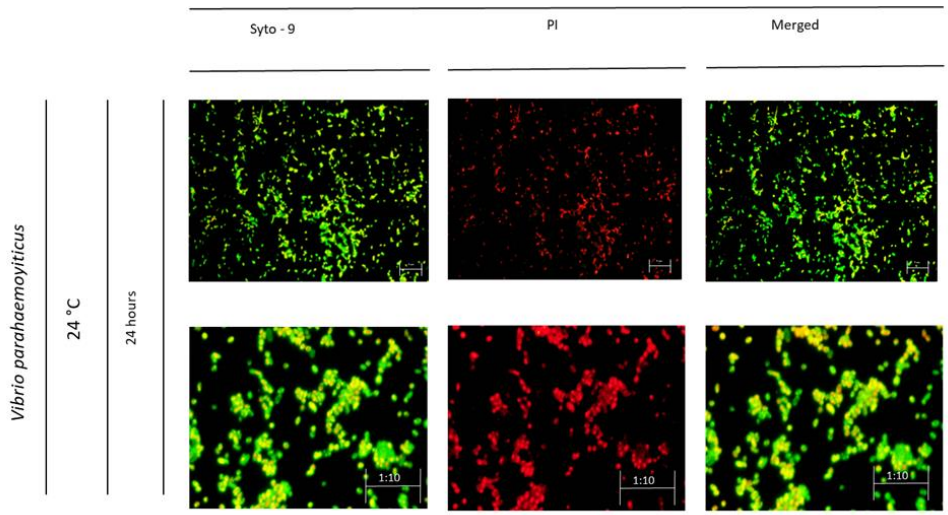
Appendix



Appendix 1: Fluorescence microscopy of the best conditions in biofilm formation by *V. parahaemolyticus* and *V. cholerae*.

Biofilms of *Vibrio parahaemolyticus* (VP) illustrated in the best growth conditions, (at (24 °C), (24 h), and initial inoculum of 0.5 McFarland) by fluorescence microscopy using Live/Dead Invitrogen staining. The original image was zoomed at 1:10 to observe the cells in the biofilm to compare the total live and dead cells. An Olympus BX50 microscope with 100X magnification was used, images were obtained with AmScope software, and images were merged with Fiji- ImageJ software.

Appendix 2*:* Statistical analysis obtained from *Vibrio parahaemolyticus* and *Vibrio cholerae* biomass, cell viability, and total cells count.

Species				***Vibrio parahaemolyticus***						***Vibrio cholerae***							***Vibrio parahaemolyticus***			***Vibrio cholerae***		
Measurement techniques				**Biomass PBS A630**		**Biomass CV A630**		**Viability CFU Log/mL**		**Biomass PBS A630**		**Biomass CV A630**		**Cell Viability CFU Log/mL**			**Fluorescence Microscopy**					
Time	**Temperature**	**Initial Inoculum (McFarland)**	**Assays n°**	**Mean (SD)**	**Median [Min - Max]**	**Mean (SD)**	**Median [Min - Max]**	**Mean (SD)**	**Median [Min - Max]**	**Mean (SD)**	**Median [Min - Max]**	**Mean (SD)**	**Median [Min - Max]**	**Mean (SD)**	**Median [Min - Max]**	**Total samples**	**Total mean cm ^{2} (SD)**	**Live mean cm ^{2} (SD)**	**Dead mean cm ^{2} (SD)**	**Total mean cm ^{2} (SD)**	**Live mean cm ^{2} (SD)**	**Dead mean cm ^{2} (SD)**
24h	24°C	0.05	6	0.09 (0.00)	0.09 (0.09 - 0.09)	0.20 (0.05)	0.18 (0.15 - 0.26)	8.04 (0.03)	7.99 (7.9 - 8.36)	0.07 (0.01)	0.07 (0.06 - 0.07)	0.10 (0.01)	0.09 (0.09 - 0.11)	7.74 (0.11)	7.73 (7.61 - 7.88)	15	9.29E+6 (8.97E+6)	5.35E+6 (4.63E+6)	3.94E+6 (4.41E+6)	7.15E+6 (1.83E+7)	5.53E+6 (3.95E+6)	1.62E+6 (1.52E+6)
		0.5	6	0.09 (0.41)	0.09 (0.08 - 0.09)	0.16 (0.03)	0.16 (0.123 - 0.201)	7.96 (0.00)	7.95 (7.91 - 8.00)	0.07 (0.00)	0.07 (0.06 - 0.07)	0.10 (0.01)	0.09 (0.09 - 0.10)	7.58 (0.09)	7.59 (7.43 - 7.68)	15	1.51E+7 (1.38E+7)	1.42E+7 (1.34E+7)	8.88E+5 (9.01E+5)	1.51E+7 (1.31E+6)	1.37E+7 (1.35E+6)	1.41E+6 (1.01E+6)
	30°C	0.05	6	0.09 (0.01)	0.09 (0.08 - 0.10)	0.10 (0.01)	0.098 (0.095 - 0.112)	7.60 (0.18)	7.58 (7.46 - 7.75)	0.07 (0.00)	0.07 (0.07 - 0.08)	0.08 (0.00)	0.08 (0.08 - 0.08)	8.14 (0.24)	8.02 (7.94 - 8.45)	15	3.92E+6 (2.73E+6)	2.41E+6 (1.53E+6)	1.51E+6 (1.31E+6)	3.92E+6 (1.91E+6)	3.45E+6 (1.99E+6)	4.03E+5 (1.85E+5)
		0.5	6	0.07 (0.00)	0.07 (0.07 - 0.08)	0.09 (0.00)	0.089 (0.082 - 0.089)	7.51 (0.14)	7.456 (7.38 - 7.69)	0.07 (0.00)	0.07 (0.06 - 0.07)	0.07 (0.00)	0.07 (0.07 - 0.07)	8.08 (0.11)	8.10 (7.95 - 8.23)	15	1.35E+7 (1.42E+7)	1.22E+7 (1.34E+7)	1.33E+6 (1.28E+6)	1.13E+7 (1.18E+7)	9.29E+6 (9.07E+6)	2.05E+6 (2.44E+6)
48h	24°C	0.05	6	0.06 (0.00)	0.06 (0.05 - 0.06)	0.09 (0.01)	0.918 (0.079 - 0.097)	8.00 (0.06)	8.01 (7.92 - 8.07)	0.07 (0.01)	0.07 (0.06 - 0.09)	0.09 (0.01)	0.09 (0.08 - 0.09)	7.87 (0.15)	7.85 (7.70 - 8.07)	15	4.83E+6 (1.51E+6)	3.53E+6 (1.09E+6)	1.30E+6 (4.84E+5)	3.67E+6 (1.84E+6)	2.14E+6 (1.24E+6)	1.53E+6 (7.21E+5)
		0.5	6	0.05 (0.01)	0.05 (0.05 - 0.05)	0.08 (0.00)	0.077 (0.077 - 0.078)	7.95 (0.05)	7.93 (7.90 - 8.05)	0.07 (0.01)	0.07 (0.06 - 0.08)	0.08 (0.01)	0.08 (0.07 - 0.08)	7.57 (0.11)	7.56 (7.45 - 7.73)	15	3.72E+6 (2.04E+6)	3.39E+6 (1.90E+6)	3.23E+5 (3.37E+5)	2.95E+6 (9.24E+5)	2.62E+6 (9.29E+5)	3.33E+5 (3.43E+5)
	30°C	0.05	6	0.05 (0.00)	0.05 (0.05 - 0.06)	0.10 (0.01)	0.096 (0.091 - 0.104)	7.32 (0.03)	7.36 (7.04 - 7.53)	0.06 (0.00)	0.06 (0.05 - 0.06)	0.09 (0.02)	0.08 (0.08 - 0.12)	7.66 (0.08)	7.64 (7.59 - 7.76)	15	2.70E+6 (2.12E+6)	1.69E+6 (1.52E+6)	1.01E+6 (6.46E+5)	3.79E+6 (2.16E+6)	2.13E+6 (1.37E+6)	1.66E+6 (8.86E+5)
		0.5	6	0.05 (0.00)	0.05 (0.05 - 0.05)	0.08 (0.00)	0.078 (0.076 - 0.078)	7.27 (0.12)	7.28 (7.08 - 7.43)	0.06 (0.00)	0.06 (0.06 - 0.07)	0.08 (0.00)	0.08 (0.07 - 0.08)	7.64 (0.16)	7.70 (7.42 - 7.84)	15	2.78E+6 (1.54E+6)	2.12E+6 (1.44E+6)	6.60E+5 (5.98E+5)	3.19E+6 (1.54E+6)	2.93E+6 (1.56E+6)	2.56E+5 (3.37E+5)
72h	24°C	0.05	6	0.09 (0.14)	0.09 (0.07 - 0.10)	0.09 (0.00)	0.090 (0.090 - 0.178)	7.90 (0.04)	7.90 (7.85 - 7.96)	0.05 (0.00)	0.05 (0.04 - 0.05)	0.07 (0.01)	0.07 (0.07 - 0.07)	7.38 (0.39)	7.56 (6.76 - 7.69)	15	4.31E+6 (3.15E+6)	2.63E+6 (2.11E+6)	1.66E+6 (1.11E+6)	5.52E+6 (3.58E+6)	4.58E+6 (3.56E+6)	9.43E+5 (5.94E+5)
		0.5	6	0.08 (0.00)	0.08 (0.08 – 0.09)	0.11 (0.03)	0.097 (0.090 - 0.178)	8.19 (0.21)	8.21 (7.96 - 8.42)	0.07 (0.00)	0.07 (0.06 - 0.08)	0.07 (0.01)	0.06 (0.06 - 0.07)	7.47 (0.13)	7.47 (7.32 - 7.62)	15	7.21E+6 (8.96E+6)	6.21E+6 (8.30E+6)	9.95E+5 (1.45E+6)	2.71E+6 (2.03E+6)	2.60E+6 (2.08E+6)	1.25E+5 (1.21E+5)
	30°C	0.05	6	0.07 (0.01)	0.07 (0.06 - 0.07)	0.09 (0.00)	0.089 (0.087 - 0.095)	7.24 (0.03)	7.27 (7.03 - 7.41)	0.08 (0.00)	0.08 (0.08 - 0.09)	0.11 (0.04)	0.10 (0.09 - 0.19)	7.92 (0.05)	7.93 (7.86 - 7.98)	15	5.43E+6 (2.47E+6)	3.51E+6 (1.55E+6)	1.91E+6 (1.07E+6)	6.06E+6 (4.95E+6)	5.56E+6 (4.81E+6)	4.99E+5 (2.38E+5)
		0.5	6	0.07 (0.00)	0.07 (0.07 - 0.08)	0.09 (0.00)	0.092 (0.089 - 0.093)	7.54 (0.02)	7.36 (7.36 - 7.73)	0.09 (0.00)	0.09 (0.08 - 0.09)	0.12 (0.01)	0.12 (0.11 - 0.13)	8.17 (0.04)	8.17 (7.95 - 8.45)	15	1.28E+7 (5.93E+6)	1.15E+7 (5.54E+6)	1.36E+6 (5.75E+5)	4.05E+6 (2.63E+6)	3.84E+6 (2.51E+6)	2.15E+5 (2.36E+5)

Legend. Evaluation of the *in vitro* biofilm formation of two *Vibrio* species (*Vibrio parahaemolyticus* and *Vibrio cholerae*). At least six assays with quintuplicate biofilms samples were performed on different days. For the evaluation of the data, normality and data transformation tests were performed in order to obtain a parametric analysis of all the data with the Minitab program. The mean, standard deviation, and minimum and maximum range of the trials are shown in the table.

Appendix 3*:* Optimization of the growth conditions of the *Vibrio* species.

Biomass and Viability								
Condition	Species	Time	Temperature	Initial Inoculum	Biomass CV Abs 630 adjusted	Biomass PBS Abs 630 adjusted	Cell Viability CFU Log/ml adjusted	Composite desirability
1	VP	24 h	24 °C	0.05	0.18	0.09	8.01E+5	0.78
2	VP	24 h	24 °C	0.50	0.15	0.09	7.94E+5	0.69
3	VC	72 h	30 °C	0.50	0.11	0.09	8.16E+5	0.61
4	VC	72 h	30 °C	0.05	0.11	0.08	7.94E+5	0.53
5	VP	72 h	24 °C	0.50	0.10	0.08	8.15E+5	0.53
Total cells counting								
Condition	Species	Time	Temperature	Initial Inoculum	Dead cell cm^2 adjusted	Live cell cm^2 adjusted	Total cell cm^2 adjusted	Composite desirability
1	VP	72 h	30 °C	0.50	1.28E+6	9.77E+6	1.12E+7	0.38
2	VC	24 h	24 °C	0.50	1.12E+6	6.00E+6	8.82E+6	0.29
3	VP	24 h	24 °C	0.50	6.81E+5	5.16E+6	6.69E+6	0.26
4	VC	24 h	30 °C	0.50	1.10E+6	5.07E+6	6.76E+6	0.26
5	VC	24 h	24 °C	0.05	1.03E+6	4.74E+6	6.30E+6	0.24

Legend. The Minitab statistical program calculates an optimal solution that serves as the starting point for the plot, and the settings can be modified interactively to determine how different settings affect *Vibrio* biofilm growth responses using composite desirability is the weighted geometric mean of the individual desirabilities for the responses. Minitab determines optimal settings for the variables by maximizing the composite desirability. For biofilm formation, we sought to maximize the response of the variables with higher composite desirability.