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Aquaculture

Abundance and spatial patterns over time of Vibrionaceae and *Vibrio harveyi* in water and biofilm from a seabass aquaculture facility

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Abstract:	<p>One of the major threats to the seabass (<i>Dicentrarchus labrax</i>) aquaculture sector is the development of vibriosis, a disease caused mainly by bacteria from the family Vibrionaceae, especially <i>Vibrio harveyi</i>. This disease can result in important economic losses or severe public health problems. Furthermore, bacteria can form biofilms on the walls of aquaculture tanks, leading to the persistence of potentially pathogenic bacteria on fish farms. Given the relationship between abundance in the surrounding environment and prevalence in fish, monitoring the presence of <i>V. harveyi</i> in rearing water and biofilms over time may help better understand the dynamics of the pathogen and factors that increase the risk of vibriosis outbreaks.</p> <p>In this study, the dynamics and spatial patterns of cultivable Vibrionaceae and <i>V. harveyi</i> in water and biofilms from rearing tanks and in the cold- and hot-water inlets supplying the farm were monitored for 7 months. Several potential seabass pathogens were isolated: <i>Photobacterium damsela</i>, <i>Vibrio alginolyticus</i>, <i>V. harveyi</i>, <i>Vibrio owensii</i> and <i>Vibrio rotiferianus</i>. However, <i>V. harveyi</i> remained the most problematic pathogen. Its relatively constant presence in water and biofilm samples throughout the sampling campaign represents a risk for vibriosis outbreaks. Moreover, the correlation between water temperature and the abundance of <i>V. harveyi</i> suggests that the risk fluctuates throughout the year. Obtaining qualitative data regarding the relative diversity of viable culturable Vibrionaceae and quantitative data on the abundance of <i>V. harveyi</i> over time in a seabass aquaculture facility is the essential first step for designing new solutions to manage vibriosis.</p>
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Dr. Peter Bossier
Disease section editor
Aquaculture

November 17, 2020

Dear Dr. Bossier,

I am pleased to submit an original research article entitled "**Abundance and spatial patterns over time of Vibrionaceae and *Vibrio harveyi* in water and biofilm from a seabass aquaculture facility**" for consideration for publication in Aquaculture. The text reports the result of a study conducted by scientists from the "UMR 1158 BioEcoAgro, Institut Charles Viollette (Univ. Littoral Côte d'Opale), Boulogne-sur-Mer, France".

This manuscript has not been published and is not under consideration for publication elsewhere. All of the authors approve of the manuscript. We have no conflicts of interest to disclose.

Nowadays, aquaculture is the main source of marine food since the world total marine captures continue to decrease. Diseases, such as vibriosis caused by Vibrionaceae and particularly *V. harveyi*, affect aquaculture production. Today, prevention lies in understanding the dynamic and the persistence of *V. harveyi* on farms and environmental factors that increase the risk of vibriosis outbreaks. Therefore, this study will help designing of suitable prophylactic control measures, instead of using curative methods such as antibiotics.

The authors hope that you will find this manuscript suitable for publication in Aquaculture. Thank you for your consideration.

Sincerely,

Cedric Le Bris, PhD
Assistant Professor, UMR 1158 BioEcoAgro, Institut Charles Viollette, USC Anses, équipe Biochimie des Produits Aquatiques (BPA), Université du Littoral Côte d'Opale, Boulogne-sur-Mer



Abundance and spatial patterns over time of Vibrionaceae and *Vibrio harveyi* in water and biofilm from a seabass aquaculture facility

Julia Mougin, Roxane Roquigny, Christophe Flahaut, Maryse Bonnin-Jusserand, Thierry Grard and Cédric Le Bris

Highlights

- The spatio-temporal dynamics of Vibrionaceae cultivable at 37 °C was assessed
- Abundance and spatial patterns of *V. harveyi* was studied in both biofilm and water
- Several pathogenic bacteria were regularly isolated from biofilm and rearing water
- The presence of *V. harveyi* in biofilms may facilitate its persistence in the farm
- A correlation between abundance of *V. harveyi* and temperature was detected

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4 1 **Abundance and spatial patterns over time of Vibrionaceae and *Vibrio harveyi* in water and**
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6 2 **biofilm from a seabass aquaculture facility**

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9 3 Julia Mougin^a, Roxane Roquigny^a, Christophe Flahaut^a, Maryse Bonnin-Jusserand^a, Thierry
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22
23 9 **Keywords:** Aquaculture, MALDI-TOF MS, *Vibrio harveyi*, Vibrionaceae, real-time PCR

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25
26 10 **Abstract**

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28 11 One of the major threats to the seabass (*Dicentrarchus labrax*) aquaculture sector is the
29
30 12 development of vibriosis, a disease caused mainly by bacteria from the family Vibrionaceae,
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32 13 especially *Vibrio harveyi*. This disease can result in important economic losses or severe public
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34 14 health problems. Furthermore, bacteria can form biofilms on the walls of aquaculture tanks,
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36 15 leading to the persistence of potentially pathogenic bacteria on fish farms. Given the relationship
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38 16 between abundance in the surrounding environment and prevalence in fish, monitoring the
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40 17 presence of *V. harveyi* in rearing water and biofilms over time may help better understand the
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42 18 dynamics of the pathogen and factors that increase the risk of vibriosis outbreaks.
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44 19 In this study, the dynamics and spatial patterns of cultivable Vibrionaceae and *V. harveyi* in water
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48 21 monitored for 7 months. Several potential seabass pathogens were isolated:
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50 22 *Photobacterium damsela*, *Vibrio alginolyticus*, *V. harveyi*, *Vibrio owensii* and
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54 24 constant presence in water and biofilm samples throughout the sampling campaign represents a
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4 25 risk for vibriosis outbreaks. Moreover, the correlation between water temperature and the
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6 26 abundance of *V. harveyi* suggests that the risk fluctuates throughout the year. Obtaining
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8 27 qualitative data regarding the relative diversity of viable culturable Vibrionaceae and quantitative
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10 28 data on the abundance of *V. harveyi* over time in a seabass aquaculture facility is the essential
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12
13 29 first step for designing new solutions to manage vibriosis.

16 30 **1. Introduction**

17
18 31 In the context of rapid world population growth and the steep decline in marine fisheries catches,
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20 32 the development of aquaculture is of significant interest for ensuring global food security and the
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22 33 conservation of wild marine stocks. In Europe, seabass (*Dicentrarchus labrax*) is currently one of
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24 34 the most farmed fish species and this high-value species is heavily traded (Bostock *et al.*, 2016;
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26 35 FAO, 2018). Bacterial diseases such as pasteurellosis (*Photobacterium damsela* subsp.
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28 36 *piscicida*), photobacteriosis (*Photobacterium damsela* subsp. *damsela*), tenacibaculosis
29
30 37 (*Tenacibaculum* spp.), and vibriosis (*Vibrio* spp.) represent severe threats to the seabass
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32 38 aquaculture sector (Buchmann *et al.*, 2009; Muniesa *et al.*, 2020). They may not only result in
33
34 39 mass mortality events and highly negative economic effects, but are also potential zoonotic
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36 40 sources and may pose a risk for human health (Austin, 2010).

37
38 41 Among them, vibriosis, caused by certain species of *Vibrio*, stand as the most significant disease,
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40 42 affecting seabass at all stages of production chain (Korun and Timur, 2008; Le-Breton, 1999;
41
42 43 Muniesa *et al.*, 2020; Pujalte *et al.*, 2003). This disease is characterized by anorexia, erratic
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44 44 swimming behavior, exophthalmos, darkening of body color, pale gills, hemorrhages, ulcers, and
45
46 45 skin lesions (Korun and Timur, 2008). Several *Vibrio* species have been reported in seabass
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48 46 infections: *Vibrio alginolyticus*, *Vibrio anguillarum*, *Vibrio harveyi*, *Vibrio ordalii*,
49
50 47 *Vibrio parahaemolyticus* and *Vibrio vulnificus* (Ghittino *et al.*, 2003; Kahla-Nakbi *et al.*, 2006;
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52 48 Khouadja *et al.*, 2013; Korun and Timur, 2008). *Vibrio anguillarum* has been the main significant
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4 49 seabass pathogens for decades (Vázquez and Muñoz-Cueto, 2014). Commercially available
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6 50 prophylactic strategies, such as vaccines, have thus been extensively developed and effectively
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9 51 applied (Dadar *et al.*, 2017). However, *V. harveyi* has recently been identified as an emerging
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11 52 major concern for seabass aquaculture and, to date, there are no specific, effective prophylactic
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14 53 control measures (Dadar *et al.*, 2017; Vendramin *et al.*, 2016).
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16 54 *Vibrio harveyi* is a Gram-negative bacterium, ubiquitous in the marine environment, particularly
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18
19 55 in temperate and tropical waters. This species can be found in a free-swimming state, in biofilms
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21 56 attached to biotic and abiotic surfaces, or in symbiotic and host-pathogen interactions
22
23 57 (Montánchez and Kaberdin, 2019). Importantly, *V. harveyi* can be pathogen for a wide range of
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25
26 58 cultivated hosts, including crustaceans, mollusks or fishes (Austin and Zhang, 2006). Its
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29 59 occurrence is likely to increase due to global warming and may lead to further *V. harveyi*-
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31 60 associated infections and have devastating effects for the aquaculture sector (Vezzulli *et al.*,
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33 61 2015). Interestingly, vibriosis outbreaks in seabass farms exhibit strong seasonality and are more
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35
36 62 likely to occur from June to November, when the water temperature is maximal (Pujalte *et al.*,
37
38 63 2003). Warmer temperatures may indeed increase the abundance of viable *V. harveyi* in the
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40
41 64 surrounding environment and thus increase the prevalence of *V. harveyi* in fish (Kim and Lee,
42
43 65 2017). Importantly, contamination of fish usually occurs through contact with contaminated
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45 66 water via the digestive tract or skin (O'Toole *et al.*, 2004). Although the development of a disease
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48 67 arises from the contamination of fish, the abundance of the pathogen in the surrounding
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50 68 environment may be an indicator of the risk of vibriosis. Other environmental factors such as
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53 69 salinity, fish density, pH, or the susceptibility and physiological state of the host, may have an
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55 70 impact on the development of vibriosis (Maulvault *et al.*, 2018; Prayitno and Latchford, 1995;
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58 71 Snieszko, 1974; Vazzana *et al.*, 2002). Moreover, biofilm formation on the walls of rearing tanks
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60 72 may enhance the risk of vibriosis outbreaks. Biofilms can facilitate resistance to antibiotics, and

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4 73 water sanitizers and may induce the persistence of potentially pathogenic bacteria in the
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6 74 aquaculture facility (Bourne *et al.*, 2006; Karunasagar *et al.*, 1996). Thus, the release of
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9 75 *V. harveyi* from biofilms in the rearing water or the contact of fish with the walls of aquaculture
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11 76 tanks may increase the prevalence of bacteria in fish and increase the risk of vibriosis outbreaks.
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14 77 Therefore, monitoring the abundance of *V. harveyi* in the surrounding environment, such as in the
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16 78 rearing water and biofilms, may be an indicator of the *V. harveyi* contamination of seabass and
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18
19 79 thus of the probability of vibriosis outbreaks. Moreover, greater understanding of the correlation
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21 80 between the abundance, dynamics, and spatial patterns of *V. harveyi* and environmental biotic
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24 81 and abiotic factors might help to control vibriosis in aquaculture facilities.
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26 82 Here, we report the dynamics and spatial patterns of cultivable Vibrionaceae and *V. harveyi*,
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29 83 monitored in 2018 from April to October (months when vibriosis is most likely to strike). The
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31 84 study was conducted on a semi-closed seabass farm, located in Northern France. The bacterial
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34 85 abundance and relative diversity were determined from water and biofilm in rearing tanks, and in
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36 86 the cold- and hot-water inlets supplying the farm. The dynamics of cultivable Vibrionaceae was
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39 87 assessed by culturing them on selective media at 37°C. Presumptive cultivable Vibrionaceae were
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42 88 then identified using matrix-assisted laser desorption/ionization-time of flight mass spectrometry
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45 89 (MALDI-TOF MS) using an in-house database, called Luvibase, previously and specifically
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48 90 designed to identify *Vibrio* species from the Harveyi clade (Mougin *et al.*, 2020b). The
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51 91 abundance of *V. harveyi* was estimated using a *mreb*-targeted real-time PCR (Mougin *et al.*,
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53
54 92 2020a). Environmental parameters and fish density and/or mortality were recorded through the
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57 93 sampling campaign to identify the environmental factors that best correlate with bacterial
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60 94 abundance. Overall, the objective was to gain a greater understanding regarding the dynamics
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63 95 over time and spatial pattern of potentially pathogenic bacteria for seabass. This understanding
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65 96 can help design prophylactic measures to better manage vibriosis outbreaks.

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97 **2. Material and methods**

98 **Sampling site**

99 This study was conducted from April to October 2018 in Aquanord Ichthus, a fish farm located in
100 Gravelines, France along the southwestern coast of the North Sea. Because the aquaculture
101 system is situated near a nuclear power plant, the site is monitored annually in order to detect any
102 abnormalities in the development of the natural marine environment (Antajan *et al.*, 2019).

103 The farm is an intensive semi-closed system managed by a private company. This company
104 currently raises seabass (*Dicentrarchus labrax*) and seabream (*Sparus aurata*) during the on-
105 growing phase in flow-through tanks constructed from concrete. The water supply is regulated
106 via two water inlets: a “cold-water inlet” from the natural marine environment and a “hot-water
107 inlet” from the cooling system of a nuclear power plant nearby (Fig. 1). Water flow into the
108 rearing tanks is regulated using a valve to maintain uniform water quality and temperature
109 through the year. Overall, from mid-July to mid-September, the rearing tanks are supplied by the
110 cold-water inlet only. During the rest of the year, the rearing tanks are supplied by both cold- and
111 hot-water inlets. Fish are fed daily and aeration is provided to maintain an optimal level of
112 dissolved oxygen (DO). Farmers use a commercial vaccine against *V. anguillarum* and an annual
113 autogenous vaccine against *V. harveyi*. The latter is developed annually from pathogens isolated
114 from the spleens of moribund fish during previous outbreaks.

115 Three similar rearing tanks were chosen for this study. The water capacity of each tank was 75
116 m³, with a water column of 1.3 m and a holding capacity of around 30 000 seabass. Average fish
117 stock density was 48.13 kg.m⁻³, with an average fish weight of 200 g. During the sampling
118 campaign, the aquaculture facility provided weekly biomass and mortality data from each rearing
119 tank.

120 **Sample collection and environmental parameters**

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4 121 Two types of samples, water and biofilm, were collected from three tanks containing seabass and
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6 122 from the cold- and hot-water inlets. Water samples were collected weekly from tanks and hot-
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9 123 and cold-water inlets. Biofilm samples were collected every two weeks from tanks. Both types of
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11 124 samples were harvested as previously detailed in Mougín *et al.* (2020a). Briefly, a volume of 1 L
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14 125 of water was collected in a sterile bottle in triplicate, from both water inlets and from the three
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16 126 rearing tanks, just below the surface of the water. Pieces of concrete, made of the same material
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19 127 used to build the tanks and placed 20 cm below the surface of the water, were collected in
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21 128 triplicate from the three rearing tanks (Appendix A). Samples were maintained on ice during
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23 129 transport to the laboratory. A total of 465 water samples and 144 biofilm samples were thus
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26 130 collected during the sampling campaign.

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28 131 During sample collection, water temperature, pH, salinity, and conductivity were measured in
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31 132 each tank and in both inlets, within 50 cm of the water surface using a multi-parameter probe
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33 133 (Odeon Open X, Aqualabo, Champigny-sur-Marne, France). DO concentration was also
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36 134 measured in rearing tanks only using a Handy Polaris DO meter (OxyGuard, Farum, Denmark).

37 38 135 **Sample preparation**

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40 136 Samples were processed as previously described in Mougín *et al.* (2019) and Mougín *et al.*
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42
43 137 (2020a). Briefly, water samples were filtered through 0.45 µm pore- size nitrocellulose filters
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45 138 (Sartorius, Göttingen, Germany). Filters were then immersed in 25 mL of Luria-Bertani broth
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48 139 supplemented with 20% NaCl (LBS) and 20% glycerol, and stored at -80 °C until analysis.
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50 140 Biofilms from the concrete samples were collected using a homemade sterile stainless steel jig by
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53 141 swabbing with two 2.34 cm² swabs. The swabs (150C, Copan, Brescia, Italy) were then
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55 142 immersed in 3 mL of LBS with 20% glycerol and stored at -80 °C until analysis. Subsequently,
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58 143 both types of samples were defrosted at room temperature and vortexed for 3 min to dislodge
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60 144 bacteria from the filters and swabs. Filters and swabs were removed the LBS-glycerol solution

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4 145 and the liquid was centrifuged at 8000×g for 10 min. The cell pellets were then dissolved in 3 mL
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6 146 of physiological water (0.9% NaCl) and centrifuged at 8000×g for 10 min. Supernatants were
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9 147 removed and the cell pellets were dissolved in 2 mL of physiological water. Samples were then
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11 148 split into two subsamples. One subsample of 1800 µL was used to quantify *V. harveyi* using real-
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14 149 time PCR. The other 200 µL subsample was used for relative quantification and identification of
15
16 150 presumptive cultivable Vibrionaceae by plating on selective agar media and by MALDI TOF MS.

18 151 **Quantification of *V. harveyi* using real-time PCR**

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21 152 The 1800 µl subsample was centrifuged at 8000×g for 10 min and the supernatant was removed.
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23 153 Total DNA was extracted from the cell pellet, using the DNeasy PowerBiofilm kit (Qiagen,
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25
26 154 Hilden, Germany) in accordance with manufacturer's protocol, selected according to Mougin *et*
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28 155 *al.* (2020a). Relative quantification of *V. harveyi* based on a standard curve was performed
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31 156 according to the previously optimized real-time PCR protocol in Mougin *et al.* (2020a), using
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33 157 *V. harveyi*-specific primers targeting the *mreB* gene (synthesized by TIB MOLBIOL
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35
36 158 Syntheselabor GmbH, Berlin, Germany). Briefly, pure DNA of the collection strain *V. harveyi*
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38 159 LMG 4044 was extracted using the DNeasy Blood&Tissue kit (Qiagen, Hilden, Germany). Purity
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41 160 was verified on a DeNovix DS-11 spectrophotometer (UV spectroscopy, Clinisciences, Nanterre,
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43 161 France) and the quantity was determined using a Qubit 3.0 fluorometer (dsDNA High-Sensitivity
44
45 162 assay), (Invitrogen, Carlsbad, CA, USA). Then, standard curves were generated from a series of
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48 163 10-fold dilutions of pure DNA of the collection strain *V. harveyi* LMG 4044. For each assay, the
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50 164 calibration curve was plotted using the means of the threshold cycle (C_T) values from two
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53 165 replicate series versus the logarithm of each dilution factor. Each extracted environmental DNA
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55 166 template was analyzed at a 10-fold dilution to avoid the effects of real-time PCR inhibitors.
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58 167 Genome copy equivalent (GE) concentrations were then estimated, taking the dilution factor into
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60 168 account. All quantitative PCR reactions were run on a LightCycler® 480 (Roche Diagnostics,

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4 169 France) with a positive control (LMG 4044 *V. harveyi* DNA), a negative control (CIP 70.67
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6 170 *V. campbellii* DNA), and a no template control (NTC), DNA-free. Melting curve analysis was
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9 171 performed to confirm the specificity of the fluorescent signals. Each extracted environmental
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11 172 DNA template was analyzed at least twice to ensure robust measurements.

13 14 173 **Quantification of presumptive Vibrionaceae and identification using MALDI-TOF MS**

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16 174 An aliquot of 100 µL from the second subsample was plated on thiosulfate-citrate-bile salts-
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19 175 sucrose (TCBS) agar (Biokar Diagnostics, Beauvais, France). The remaining 100 µL was serially
20
21 176 10-fold diluted in physiological water (10^{-1} , 10^{-2} , 10^{-3}) and 100 µL of each dilution was plated
22
23 177 onto TCBS agar. All plates were then incubated at 37 °C overnight. Presumptive cultivable
24
25
26 178 Vibrionaceae colony forming units (CFUs) were counted using a Scan 500 plate reader
27
28 179 (Interscience, Saint-Nom-la-Bretèche, France) and the concentration in each sample was
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30
31 180 estimated taking the dilution factor into account.

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33 181 Different colony morphotypes were screened on the basis of color (green or yellow), color shade,
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36 182 size, circumference (smooth or irregular) and presence/absence of a halo within 10 min after
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38 183 removal from the incubator. One colony of each different morphotype was randomly selected and
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40
41 184 incubated on LBS agar at 37 °C overnight. The morphological features of each selected colony
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43 185 were recorded. Pure bacteria were stocked in LBS broth supplemented with 20% glycerol at -
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45 186 80 °C until identification using MALDI-TOF MS.

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48 187 Subsequently, once defrosted, these bacterial isolates were subcultured on marine agar (MA),
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51 188 (Difco Laboratories, Detroit, MI, USA) and incubated for 24 h, at 37 °C. Then, a single colony
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53 189 was plated on MA (Difco Laboratories) and incubated 24 h, at 37 °C to ensure the purity of the
54
55 190 isolate. Preparation of bacterial extracts, target-loading and acquisition of MALDI-TOF MS
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58 191 profiles were performed as previously described in Mougín *et al.* (2020b). Briefly, the cellular
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60 192 proteins of each bacterial isolate were extracted from bacterial cells according to manufacturer's

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4 193 recommendations (Bruker France, Wissembourg, France). The extracts were stored at -20 °C
5
6 194 until use. Then, 1 µL of the supernatant from each protein extract was deposited onto a clean 384-
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8
9 195 target ground steel microtiter plate (Bruker France) in four replicates. After air drying, 1 µL of a
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11 196 10 mg.mL⁻¹ matrix solution of α -cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile and
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14 197 2.5% trifluoroacetic acid (Sigma-Aldrich, St. Louis, MO, USA) was overlain on each spot.
15
16 198 MALDI-TOF MS analysis was performed using an Autoflex SpeedTM (Bruker France), running
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19 199 Flexcontrol 3.4 software (Bruker France). The Bruker bacterial test standard (BTS, Bruker
20
21 200 France) was used to calibrate the mass spectrometer before each run, according to the
22
23 201 manufacturer's recommendations. Mass spectra were acquired in the positive linear ion mode
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25
26 202 across a mass/charge (m/z) ratio of 2000-20,000 Da. Mass spectra were acquired using the
27
28 203 manufacturer's automatic MBT_FC.par method. For each MALDI-TOF MS profile, mass spectra
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31 204 were obtained from 5000 laser shots in 1000 shot steps performed randomly from different areas
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33 205 of the spot.
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35 206 Each MALDI-TOF MS profile was first compared with the mass spectra profiles (MSPs) of the
36
37
38 207 Bruker BioTyper database ver.9.0.0.0 implemented with the Luvibase, a supplemental, in-house
39
40 208 *Vibrio* database (Mougin *et al.*, 2020b). The degree of concordance between experimental
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42
43 209 MALDI-TOF MS profiles and the MSPs was evaluated using a log-score calculated using the
44
45 210 MALDI BioTyper Compass Explorer ver.4.1 (Bruker France). A log-score exceeding 2.3
46
47
48 211 indicated a highly probable identification at the species level; a log-score between 2.0 and 2.3
49
50 212 indicated a probable identification at the species level; a log-score between 1.7 and 2.0 indicated
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52
53 213 a probable identification at the genus level only; and a log-score below 1.7 indicated no
54
55 214 significant similarities between MSPs included in the databases and the unknown profile.
56

57 215 **Statistical analysis**

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4 216 All statistical analyses were performed using RStudio 3.5.2 software (R Core Team, 2013).
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6 217 Variation in bacterial abundance and environmental parameters were analyzed for each tank and
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8
9 218 inlet separately. A Kruskal-Wallis test for multiple comparisons and a Wilcoxon test for pairwise
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11 219 comparisons were used to analyze bacterial abundance between the three tanks and the hot- and
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13
14 220 cold-water inlets. A generalized linear mixed model with a random effect on time was used to
15
16 221 reveal correlations between selected environmental factors: temperature, pH, salinity,
17
18
19 222 conductivity, DO, fish data (i.e. biomass and mortality) and bacterial abundances. The models
20
21 223 were constructed using the function lme in the nlme package ver. 3.1-137 (Pinheiro *et al.*, 2018)
22
23 224 by excluding non-significant parameters or interactions one by one, starting with the interaction
24
25
26 225 terms. For all tests, a p-value of < 0.05 was considered statistically significant. All graphics were
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28 226 drawn using the ggplot2 packages ver. 2.2.1 (Wickham, 2016).
29

30 227 **3. Results**

31 228 **Water quality and fish density and mortality**

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33 229 The values of the parameters describing water quality and fish density and mortality recorded
34
35
36 230 from April to October 2018 are given in Table 1. In the cold-water inlet, the average temperature
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38 231 was 16.4 ± 3.5 °C and values fluctuated widely with a minimum of 9.1 °C, in April, and a
39
40
41 232 maximum of 21.1 °C, in August. The pH, salinity, and conductivity did not vary widely and were
42
43 233 7.9 ± 0.3 , 34.2 ± 0.4 and 51.9 ± 0.6 ms.cm⁻¹, respectively. In the hot-water inlet, average
44
45
46 234 temperature was 23.8 ± 1.3 °C and values did not fluctuate widely, with a minimum of 19.9 °C in
47
48
49 235 April and a maximum of 25.7 °C in September. The pH, salinity, and conductivity did not
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51
52 236 fluctuate widely either and were 7.9 ± 0.3 , 34.6 ± 0.5 and 52.5 ± 0.7 ms.cm⁻¹ respectively.
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54
55 237 Temperature, pH, salinity and conductivity were similar among rearing tanks; the values were
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57
58 238 thus averaged. Average temperature was 19.1 ± 1.0 °C, with a minimum of 17.9 °C in April and a
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60 239 maximum of 21.1 °C in August. The pH, salinity, and conductivity did not vary widely and were
61

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4 240 7.5 ± 0.3 , 34.4 ± 0.5 and 52.3 ± 0.5 ms.cm⁻¹ respectively. During the sampling campaign, batches
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6 241 of fish were removed and replaced once from Tank 1 and once from Tank 2, due to their growing
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8
9 242 size. The average DO concentration in the rearing tanks was maintained at 6.05 ± 1.2 mg.L⁻¹. The
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11 243 mortality rate was low throughout the sampling campaign and no vibriosis outbreak was recorded
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13
14 244 in the three chosen rearing tanks.

16 245 **Identification of presumptive Vibrionaceae isolates cultured on TCBS agar**

17
18 246 Across all the compartments, from April to October 2018, 1009 isolates were chosen on the basis
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20
21 247 of morphological traits on TCBS agar, after incubation at 37 °C for 24 h. Overall, 80.4%
22
23 248 (811/1009) of the isolates were collected from the three rearing tanks: 44.2% (446/1009)
24
25
26 249 originated from rearing water and 36.2% (365/1009) originated from biofilms. Only 11.5%
27
28 250 (116/1009) and 8.1% (82/1009) were collected from the hot- and cold-water inlets, respectively
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30
31 251 (Fig. 2.a).

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33 252 These isolates were then identified using MALDI-TOF MS with the Bruker BioTyper ver.9.0.0.0
34
35 253 database supplemented with our in-house *Vibrio* Luvibase. This analysis resulted in the
36
37
38 254 assignment of 91.2% (920/1009) of the isolates at the species level (log-score ≥ 2.0) and 8.8%
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40 255 (89/1009) at the genus level only ($1.7 \leq \text{log-score} < 2.0$) (Fig. 2.b). Among the 920 isolates
41
42
43 256 identified at the species level, 75.7% belonged to genus *Vibrio* and 24.3% to *Photobacterium*.
44
45 257 Within the *Photobacterium* genus, only *P. damsela* was isolated. Within the *Vibrio* genus, the
46
47
48 258 isolates were categorized into four clades, the Harveyi clade (97.3%), the Splendidus clade
49
50 259 (2.0%), the Orientalis clade (0.6%) and the Coralliilyticus clade (0.1%). The Harveyi clade
51
52
53 260 consisted of isolates assigned to *V. alginolyticus*, *V. harveyi*, *V. owensii*, *V. rotiferianus*,
54
55 261 *V. jasicida*, *V. mytili*, *V. campbellii* and *V. parahaemolyticus*. The Splendidus clade consisted of
56
57 262 isolates identified as *V. pelagius* and *V. fortis*. The Orientalis clade consisted of isolates assigned
58
59
60 263 to *V. brasiliensis*, and *V. sinaloensis*. The Coralliilyticus clade consisted of one isolate assigned
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4 264 to *V. coralliilyticus*. Overall, the most frequently identified species were *V. alginolyticus* (24.8%),
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6 265 *P. damsela* (24.3%), *V. harveyi* (24.2%) and *V. owensii* (15.8%) (Fig. 2.b). All other species
7
8
9 266 were very scarce.

10
11 267 The relative isolated and identified bacterial diversity was roughly similar between the
12
13
14 268 compartments and sample types (Fig. 2.c). Nevertheless, none of the species were isolated
15
16 269 systematically throughout the sampling campaign (Fig. 2.d). *Vibrio alginolyticus* was recorded
17
18 270 from May to October in the hot-water inlet, water tanks and biofilm, but was not recorded in June
19
20
21 271 and October in the cold-water inlet. The presumed pathogen *V. harveyi* was recorded from May
22
23 272 to September in all compartments. *Photobacterium damsela* was recorded from July to October
24
25
26 273 in the hot- and cold-water inlets and from May to October in the water tanks and biofilms.
27
28 274 Finally, *V. owensii* was recorded from May to October in water tanks and biofilms, but recorded
29
30
31 275 from July to September in the hot-water inlet, and in July and August only in the cold-water inlet.
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33 276 **Quantification of presumptive culturable Vibrionaceae isolates**

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35 277 Presumptive culturable Vibrionaceae CFUs grown on TCBS agar, after incubation at 37 °C for 24
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37
38 278 h, were enumerated and their abundance in each compartment and each sample was estimated.
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40 279 According to the statistical analysis, the average abundance of presumptive culturable
41
42
43 280 Vibrionaceae differed significantly between the cold- and hot-water inlets and water from rearing
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45 281 tanks (each p-value < 0.05). In contrast, there were no significant differences between the average
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47
48 282 abundances of the three rearing tanks, for either the water or biofilm samples (each p-value >
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50 283 0.05). Consequently, for the following analyses, for each sampling date, the average abundances
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52
53 284 detected in the three rearing tanks were pooled.

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55 285 In the cold-water inlet, the presumptive detected culturable Vibrionaceae, ranged from 3.37×10^2
56
57 286 to 4.85×10^3 CFU.mL⁻¹ (Fig. 3.a). The maximum abundance was detected from July to September,
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59
60 287 when the temperature recorded in this compartment was the highest, 19.8 ± 1 °C. A peak in
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3
4 288 abundance (3.37×10^2 CFU.mL⁻¹) was observed in late May at a temperature of 15.0 °C, and water
5
6 289 temperature continued to increase slightly (average +0.7 °C each week from April to June). No
7
8
9 290 bacteria were detected in October and April.

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11 291 In the hot-water inlet, the presumptive detected culturable Vibrionaceae followed more or less the
12
13 same pattern, ranging from 3.11×10^2 to 6.71×10^4 CFU.mL⁻¹ in abundance. Maximum abundance
14 292 was detected in late June when the temperature reached 24 °C. Then, abundance tended to
15
16 293 decrease slightly until mid-October, when a peak of 5.99×10^2 CFU.mL⁻¹ was detected at a
17
18 294 temperature of 24.9 °C. As in the cold-water inlet, a peak in abundance (6.43×10^3 CFU.mL⁻¹)
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20 295 was detected in late May at a water temperature of 24.7 °C in this compartment.

21
22
23 296 In the rearing tanks, the presumptive culturable Vibrionaceae detected in water ranged from
24
25 297 2.28×10^3 to 1.74×10^5 CFU.mL⁻¹. Presumptive detected culturable Vibrionaceae were enumerated
26
27 298 from mid-April until the end of the study. The abundance tended to increase slowly and
28
29 300 constantly with an average + 0.20 log per week until August, the warmest month of summer with
30
31 301 the maximum recorded temperature (21.7 °C). Subsequently, abundance decreased slightly in
32
33 302 September, and hit a peak of 2.50×10^4 CFU.mL⁻¹ in mid-October, when the temperature jumped
34
35 303 back to 19.1 °C. The presumptive attached culturable Vibrionaceae detected in biofilms ranged
36
37 304 from 2.51×10^5 to 2.03×10^7 CFU.cm⁻². Presumptive attached culturable Vibrionaceae were
38
39 305 enumerated from mid-May until the end of the study. As observed in the water samples,
40
41 306 abundance tended to increase slowly and constantly with an average + 0.27 log per week until
42
43 307 August, then decreased slightly. Interestingly, a peak in the abundance of 2.29×10^6 CFU.cm⁻²
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45 308 occurred with a peak in temperature in mid-October, similar to the pattern for the water samples
46
47 309 and the hot-water inlet.

57 310 **Quantification of *V. harveyi* using real-time PCR**

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4 311 Real-time PCR was used to estimate the abundance of *V. harveyi* detected in each compartment
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6 312 and each sample. According to the statistical analysis, the average abundance of presumptive
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8
9 313 culturable Vibrionaceae detected in the water from the cold- and hot-water inlets and rearing
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11 314 tanks were significantly different (each p-value < 0.05). In contrast, there were no significant
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13
14 315 differences between the average abundances observed in the three rearing tanks, for either the
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16 316 water or biofilm samples (each p-value > 0.05). Consequently, for each sampling time, the
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18
19 317 average abundances detected in the three rearing tanks were pooled together.
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21 318 Overall, the average estimated quantity of *V. harveyi* was higher in hot-water inlet than in the
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23
24 319 cold-water inlet. In the cold-water inlet, abundance of detected *V. harveyi*, ranged from 1.35 to
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26 320 8.57 GE.mL⁻¹ (Fig. 3.a). A few peaks were detected in late May, mid-June, early July, and mid-
27
28 321 September.
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31 322 Successive screenings for *V. harveyi* over 5 weeks demonstrated a rapid increase in abundance
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33 323 from mid-July to mid-August, when temperatures reached their highest values.
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35
36 324 In the hot-water inlet, abundance of detected *V. harveyi* ranged from 4.62 to 2.11×10² GE.mL⁻¹.
37
38 325 *V. harveyi* was first detected in mid-May when the temperature reached 25.1 °C for the first time
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40
41 326 in the year. Abundance increased slowly (average + 0.11 log per week) until early June when the
42
43 327 concentration reached 1.41×10² GE.mL⁻¹, and then decreased. The average abundance detected in
44
45 328 late June and mid-July was relatively high, with abundance decreasing slightly thereafter
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47
48 329 (average + 0.20 log per week), dropping to an absence of detection until a last peak of 4.62 of
49
50 330 GE.mL⁻¹ in mid-October when the temperature reached 24.9 °C.
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52
53 331 In the rearing tanks, detected *V. harveyi* abundance ranged from 3.72 to 9.51×10¹ GE.mL⁻¹ (Fig.
54
55 332 3.a). *V. harveyi* was first detected in April when the temperature reached 18.0 °C. The maximal
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57
58 333 abundances were recorded in mid-May (9.51×10¹ GE.mL⁻¹), mid-July (7.88×10¹ GE.mL⁻¹), and
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60 334 mid-October (7.65×10¹ GE.mL⁻¹), when the rearing water temperatures reached 19.0, 19.6 and
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4 335 19.1°C, respectively. Nevertheless, *V. harveyi* was detected in April when no *Vibrio* bacteria
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6 336 were recorded in the cold- and hot-water inlets. The abundance of attached *V. harveyi* detected in
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8
9 337 biofilms ranged from 1.45×10^3 to 3.25×10^3 GE.cm⁻² (Fig. 3.b). Attached *V. harveyi* were
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11 338 enumerated from mid-May (1.64×10^3 GE.cm⁻²) until mid-October (2.43×10^3 GE.cm⁻²), with no
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13
14 339 detection in early September or in early October.

16 340 **Influence of environmental factors on cultivable Vibrionaceae and *V. harveyi* dynamics**

17
18 341 Statistical analysis highlighted correlations between water temperature and the abundance of
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20
21 342 presumptive Vibrionaceae in the cold-water inlet (p-value=0.0065). A positive correlation was
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23 343 detected between water temperature (p-value=0.0005) and the abundance of presumptive
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25
26 344 Vibrionaceae in biofilms.

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28 345 Moreover, statistical analyses highlighted a positive correlation between water temperature and
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30
31 346 the abundance of *V. harveyi* in the cold-water inlet (p-value=0.0186) and in water from rearing
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33 347 tanks (p-value=0.0198). A positive correlation was also detected between water temperature and
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35
36 348 the abundance of *V. harveyi* in biofilms (p-value<0.0001).

38 349 **4. Discussion**

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40 350 The seabass aquaculture sector is being challenged by the emergence of recurring vibriosis
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42
43 351 outbreaks caused by *V. harveyi* (Vendramin *et al.*, 2016). The prevalence of the pathogen in fish
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45 352 is highly correlated with the abundance of the pathogen in the surrounding environment (Kim and
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48 353 Lee, 2017). Although the contamination of fish is not the only factor that can exacerbate the
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50 354 development of the disease, the abundance of *V. harveyi*, in rearing water and biofilm may be an
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53 355 accurate indicator of the risk of vibriosis outbreaks. Thus, deeper understanding of the dynamics
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55 356 and spatial patterns of *V. harveyi* in the aquaculture environment is needed.

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57 357 In this study, we assessed the temporal and spatial dynamics of Vibrionaceae, and the
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60 358 presumptive pathogen *V. harveyi* in particular, from April to October 2018 on a fish farm located

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4 359 in Northern France. Three rearing tanks were chosen from a facility frequently affected by
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6 360 vibriosis. In particular, vibriosis outbreaks, mostly caused by *V. harveyi*, were recorded in 2017
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9 361 on this fish farm. Furthermore, a few strains of *V. jasicida*, *V. rotiferianus*, *V. owensii*,
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11 362 *V. tasmaniensis*, and *V. gigantis* were also isolated from the spleens of moribund seabass
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13
14 363 (Mougin *et al.*, 2020a; Mougin *et al.*, 2020b). In 2018, vibriosis outbreaks caused by
15
16 364 *V. anguillarum* were recorded (Appendix B). According to the aquaculture facility operators,
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18
19 365 these latter outbreaks were due to a delay in vaccine delivery and were quickly and successfully
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21 366 managed. Some strains of *V. harveyi* were isolated from few moribund fish (Appendix B), but no
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24 367 *V. harveyi* vibriosis outbreak has been recorded on the fish farm, particularly in the three rearing
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26 368 tanks used during our monitoring study.

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28 369 A selective culture method was used during the survey (TCBS for 24 h at 37 °C) in order to
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31 370 mostly isolate Vibrionaceae belonging to the Harveyi clade, which includes the main pathogens
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33 371 for seabass. We are aware that not all Vibrionaceae are able to grow under these conditions and
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35
36 372 consequently, the diversity and the abundance of the presumptive Vibrionaceae detected could
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38 373 have been underestimated. Among potentially pathogenic bacteria for seabass, *P. damsela*,
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40
41 374 *V. alginolyticus*, *V. owensii*, *V. rotiferianus*, and *V. harveyi* were isolated during our sampling
42
43 375 campaign. The species *P. damsela* is divided into two subspecies with clinical and
44
45 376 epidemiological differences, both affecting seabass (Essam *et al.*, 2016; Vázquez and Muñoz-
46
47
48 377 Cueto, 2014). *P. damsela* subsp. *piscicida* (formerly *Pasteurella piscicida*) is known as the
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50 378 causative agent of a severe aquatic disease called pasteurellosis, and *P. damsela* subsp. *damsela*
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52
53 379 (formerly *Vibrio damsela*) is responsible for photobacteriosis commonly defined as sporadic
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55 380 pathologies (Buchmann *et al.*, 2009). Nonetheless, this latter disease had been recognized as an
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58 381 epizootic agent, transmitted by infected fish, and may cause public health problems (Rivas *et*
59
60 382 *al.*, 2013). In our study, presumably, only subspecies *damsela* was recorded, but the MALDI

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4 383 BioTyper database cannot discriminate between these two subspecies (Pérez-Sancho *et al.*, 2016).
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6 384 Given that no mortalities caused by *P. damsela* have been recorded, no further analysis was
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8
9 385 pursued. In the *Vibrio* genus, the Harveyi clade — especially *V. alginolyticus*, *V. harveyi*
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11 386 *V. owensii*, and *V. rotiferianus* were the most frequently isolated species on the fish farm.
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13 387 Importantly, these species have already been isolated from moribund seabass and are considered
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15
16 388 as potential pathogens on the farm. Nevertheless, *V. harveyi* remains the main pathogen
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19 389 responsible for recurrent vibriosis outbreaks.
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21 390 Interestingly, even though no vibriosis outbreak was recorded, isolates of *V. harveyi* were
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23 391 detected in the rearing tanks from water and biofilm, throughout practically the whole sampling
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25
26 392 campaign. The development of vibriosis occurs due to disruptions between host-pathogen-
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28 393 environment interactions (Snieszko, 1974; Toranzo *et al.*, 2005). Thus, many factors, such as the
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31 394 susceptibility and physiological state of the host, the pathogenicity and virulence of the pathogen,
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33 395 and the influence of environmental stress conditions, must be considered. At this fish farm, the
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35
36 396 absence of vibriosis outbreaks was attributed to the efficiency of the novel annual autogenous
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38 397 vaccine. However, in practical terms, this type of vaccine sometimes appears less efficient from
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41 398 one year to the next and must be regularly renewed, incurring high costs. Another hypothesis is
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43 399 that the strains of *V. harveyi* present on the farm during 2018 were not highly virulent. Our study
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45 400 focused on the abundance of all *V. harveyi* strains without distinguishing pathogens from non-
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47
48 401 pathogens. There is thus a possibility that the pathogenic isolates detected and collected in 2018
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50 402 are scarce.
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52
53 403 Environmental factors may also have an impact on the development of the disease. The
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55 404 correlation between the abundance of *V. harveyi* and water temperature indicates that the risk of
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58 405 vibriosis outbreaks may vary throughout the year. In the natural marine environment, previous
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60 406 studies have highlighted that the abundance of *Vibrio* spp. may follow seasonal variation in
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4 407 temperate regions (Johnson *et al.*, 2012; Martinez-Urtaza *et al.*, 2008). Water temperature and
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6 408 salinity are considered as the most effective predictors of temporal and spatial distributions.
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9 409 However, in a semi-closed aquaculture system, even though tanks are supplied with natural
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11 410 marine seawater, water quality is extensively monitored and maintained as constant as possible.
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14 411 Consequently, in our study, the environmental parameters monitored in rearing tanks (i.e. pH,
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16 412 salinity, conductivity, and DO) did not vary widely. Water temperature was generally constant,
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18
19 413 but the month of June and July showed temperatures above 20 °C. Increased prevalence of
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21 414 *V. harveyi* in oysters (*Crassostrea gigas* and *Crassostrea virginica*) was detected at temperatures
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23 415 above 20 °C (Pujalte *et al.*, 1999). Moreover, a previous study (Pujalte *et al.* (2003) reported
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25 416 increased prevalence of *V. harveyi* in European seabass and gilthead seabream during warm
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27
28 417 months. Consequently, the warmer months of summer may represent a higher risk for vibriosis
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30
31 418 outbreaks. Furthermore, temperature may not only influence the prevalence of the pathogen, but
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33 419 also the virulence and the physiological state of the host, disturbing the host-pathogen
34
35 420 relationship (Paillard *et al.*, 2004; Travers *et al.*, 2009). In the years ahead, the risk of vibriosis
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37
38 421 may increase with the rise of water temperature, due to global warming (Travers *et al.*, 2009;
39
40 422 Vezzulli *et al.*, 2015).
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42
43 423 In the rearing tanks, the abundance of presumptive cultivable Vibrionaceae appeared to follow a
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45 424 seasonal pattern, with the highest values in August and the lowest in April. On the contrary, there
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47
48 425 was no clear seasonal pattern in the variation of *V. harveyi* abundance. This difference can be
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50 426 attributed to the divergence between monitoring methods. The real-time PCR method is based on
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52 427 a DNA target and provides an estimation of the quantity of cultivable bacteria, viable but not
53
54 428 cultivable (VBNC) bacteria, and residual DNA released by dead bacteria. Quantification by plate
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56
57 429 count on selective media provides an estimation of the quantity of cultivable bacteria only, and
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59 430 does not allow recovery of VBNC bacteria. However, the quantification of VBNC bacteria in the

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4 431 rearing tanks is of significant importance. In particular, VBNC *V. harveyi* may conserve their
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6 432 pathogenic potential and infect a host when they break dormancy under favorable environmental
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9 433 conditions (Sun *et al.*, 2008). Moreover, VBNC bacteria are more resistant to antibiotics than
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11 434 cultivable bacteria (Ayrapetyan *et al.*, 2015). The major drawback of monitoring *V. harveyi*
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13
14 435 abundance using real-time PCR is the quantification of residual DNA of dead bacteria. A suitable
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16 436 solution, which could be used in a future study, is the use of propidium monoazide (PMA) real-
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18
19 437 time PCR (Mougin *et al.*, 2019; Nocker *et al.*, 2006). This method allows the estimation of both
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21 438 cultivable and VBNC bacteria, which may represent potential fish pathogens and zoonotic
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23
24 439 sources.

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26 440 Overall, the trend in the abundance of *V. harveyi* in the water of rearing tanks may follow the
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28 441 trend recorded in the water inlets. The water inlets may thus represent a source of contamination
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30
31 442 for the rearing tanks. Moreover, our work highlighted the presence of Vibrionaceae and
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33 443 *V. harveyi* in biofilms on the concrete walls of the rearing tanks. In particular, biofilms may be a
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35
36 444 reservoir of potentially pathogenic bacteria in aquaculture facilities (Bourne *et al.*, 2006). Their
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38 445 relative resistance to antibiotics and sanitizers make them more threatening to the aquaculture
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40
41 446 sector (Karunasagar *et al.*, 1996). In our study, attached cultivable Vibrionaceae were detected
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43 447 from day 42 of the sampling campaign and attached *V. harveyi* was detected from day 56. This
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45 448 delay in detection can be attributed to high water flow and fish movements in the rearing tanks. A
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47
48 449 better understanding of biofilm dynamics may help develop non-toxic anti-biofilm compounds or
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50 450 preventive anti-biofilm control measures. Quorum-sensing interfering agents hold promise for
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52
53 451 developing anti-biofilm therapies (Arunkumar *et al.*, 2020; Defoirdt, 2017). Previous studies have
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55 452 reported that the *Piper betle* ethyl acetate extract (PBE) or citrus limonoids may have anti-biofilm
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58 453 properties against *V. harveyi* (Srinivasan *et al.*, 2017; Vikram *et al.*, 2011). Their quorum-sensing
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60 454 interfering properties can be tested on the pathogenic isolates identified and collected from

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4 455 biofilms in this study. This approach may be the key to designing new control measures that can
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6 456 then be integrated in a preventive holistic strategy including hosts, pathogens, and the
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9 457 environment to control vibriosis.

11 458 **5. Conclusion**

13
14 459 In conclusion, the abundance of cultivable Vibrionaceae increased in the rearing tanks during the
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16 460 warm summer months. These bacteria may come from the water inlets, but biofilms may also be
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18
19 461 a reservoir for potential pathogenic bacteria for seabass. Importantly, several pathogenic bacterial
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21 462 species were isolated from rearing water and biofilm samples: *P. damsela*, *V. alginolyticus*,
22
23 463 *V. harveyi*, *V. owensii* and *V. rotiferianus*. Overall, *V. harveyi* is considered as the main pathogen
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25
26 464 on the fish farm and its constant presence represents a risk for vibriosis outbreaks. The correlation
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28 465 between its abundance and water temperature suggests that this risk may increase with the rise of
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30
31 466 water temperature, caused by global warming. Moreover, the probability of vibriosis outbreaks
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33 467 may be increased by the presence of *V. harveyi* in biofilms, which may facilitate resistance
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36 468 against antibiotics, water sanitizers, and environmental fluctuations.

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38 469 Future studies must now assess the pathogenicity of virulence of the isolates collected in this
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40
41 470 study. The pathogenic strains isolated from biofilms can be then used to study biofilm dynamics
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43 471 and to design and develop anti-biofilm control measures. Because *V. harveyi* affects not only
44
45 472 seabass, but also a wide range of crustaceans, mollusks, and fishes, these measures can be then
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47
48 473 adapted to other aquaculture facilities.

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50 474

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15

16 483 **7. References**
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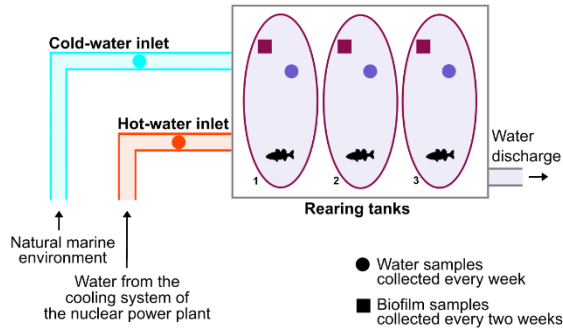
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626 Table 1. Means of parameters recorded from April to October 2018 in the aquaculture facilities.

Sampling months	Temperature (°C)			pH			Salinity			Conductivity (ms.cm ⁻¹)			Dissolved oxygen (mg.L ⁻¹)			Fish density (kg.m ⁻³)			Fish mortality per week (%)		
	Cold-water inlet	Hot-water inlet	Rearing tanks	Cold-water inlet	Hot-water inlet	Rearing tanks	Cold-water inlet	Hot-water inlet	Rearing tanks	Cold-water inlet	Hot-water inlet	Rearing tanks	Tank 1	Tank 2	Tank 3	Tank 1	Tank 2	Tank 3	Tank 1	Tank 2	Tank 3
April	10.2	21.4	18.1	8.1	8.0	7.8	33.8	33.8	34.1	51.5	51.6	51.9	7.2	5.8	7.1	28.3	54.5	31.8	0.02	0.13	0.04
May	13.2	24.0	18.8	8.1	8.0	7.8	34.2	34.6	34.4	51.9	52.6	52.2	7.1	7.0	5.8	37.2	28.5*	34.2	0.01	0.02	0.01
June	17.0	24.3	19.2	7.8	7.9	7.5	34.1	34.6	34.2	51.9	52.5	52.0	5.9	5.4	5.4	42.8	39.5	37.5	0.00	0.08	0.29
July	20.0	24.1	20.2	7.8	7.8	7.4	33.8	34.1	33.9	51.5	51.9	51.6	5.6	5.5	5.4	52.5	47.0	35.5	0.01	0.01	0.04
August	20.3	24.0	20.4	8.0	7.9	7.5	34.5	34.6	34.8	52.3	52.5	52.4	6.1	6.4	5.5	54.0	55.8	43.6	0.03	0.04	0.06
September	18.0	24.1	18.4	8.1	8.0	7.4	34.4	35.4	34.9	52.2	53.6	53.0	6.4	5.4	5.6	62.8	67.0	53.0	0.02	0.01	0.04
October	15.8	24.7	18.5	7.7	7.7	7.1	34.6	34.8	34.6	52.5	52.8	52.6	7.5	5.0	5.7	51.3*	74.8	59.0	0.06	0.01	0.04

*The on-growing batch of fish was removed and transported to a new rearing tank more suitable for their size, and a new batch of fish of smaller size was put in the rearing tank used in this study

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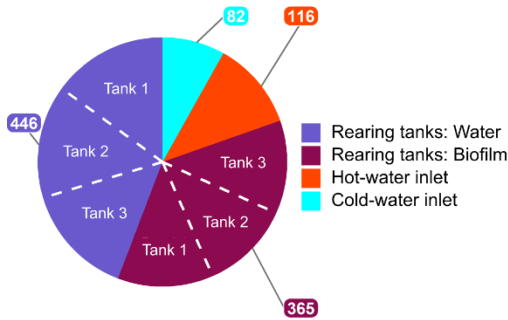
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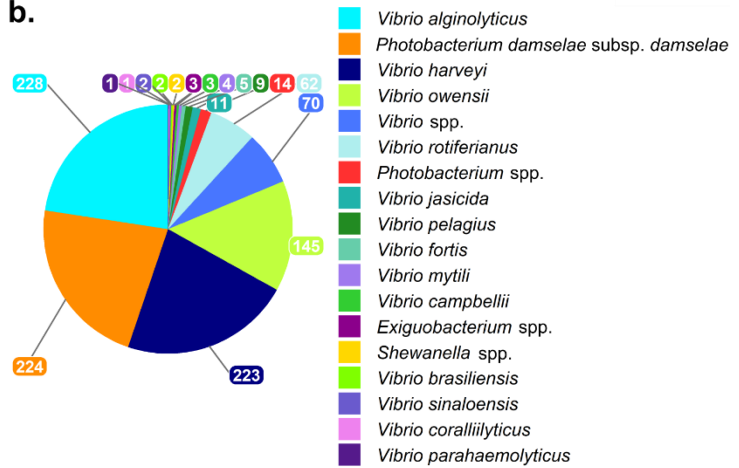
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Figure 1. Schematic representation of the sampling areas on the fish farm, illustrating the sampling compartments and types of samples collected. The water samples were collected in the rearing tanks, and in the cold- and hot-water inlets every week. The biofilm samples were collected in the rearing tanks every two weeks.

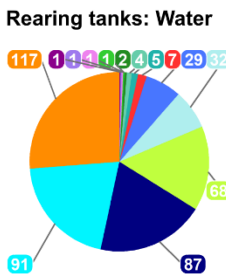
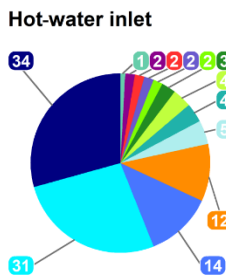
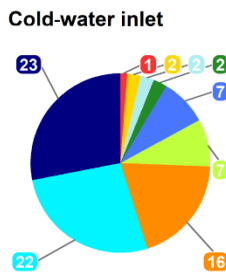
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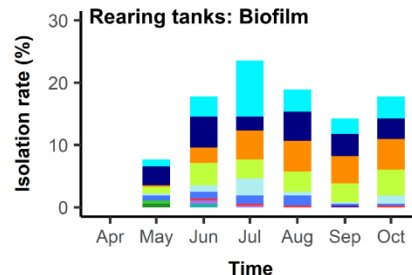
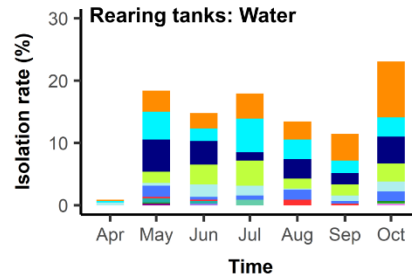
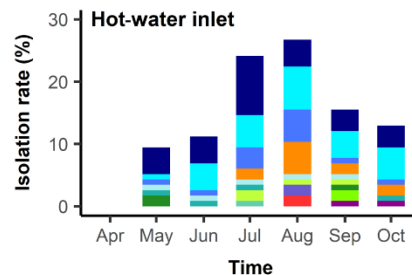
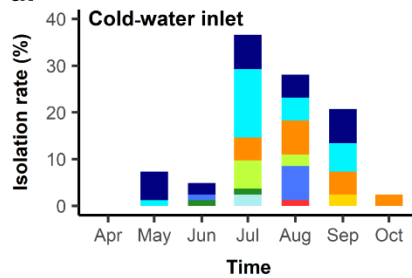
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- Vibrio harveyi*
- Vibrio alginolyticus*
- Photobacterium damsela subsp. damsela*
- Vibrio owensii*
- Vibrio spp.*
- Vibrio pelagius*
- Vibrio rotiferianus*
- Shewanella spp.*
- Photobacterium spp.*

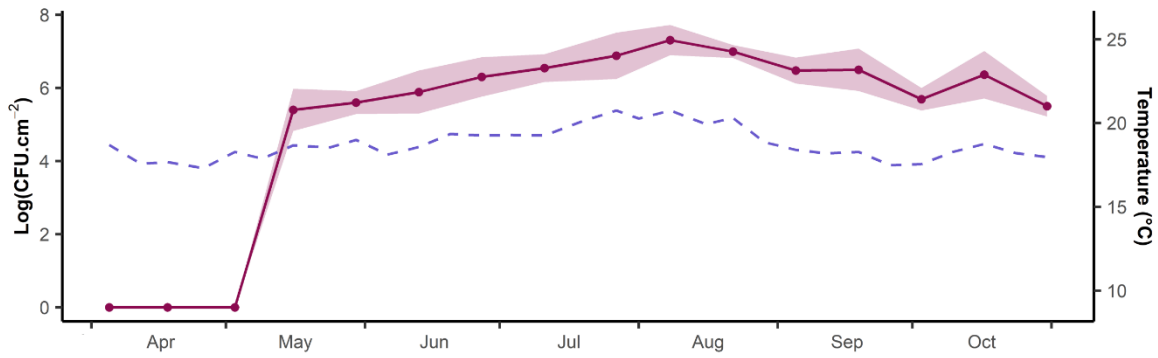
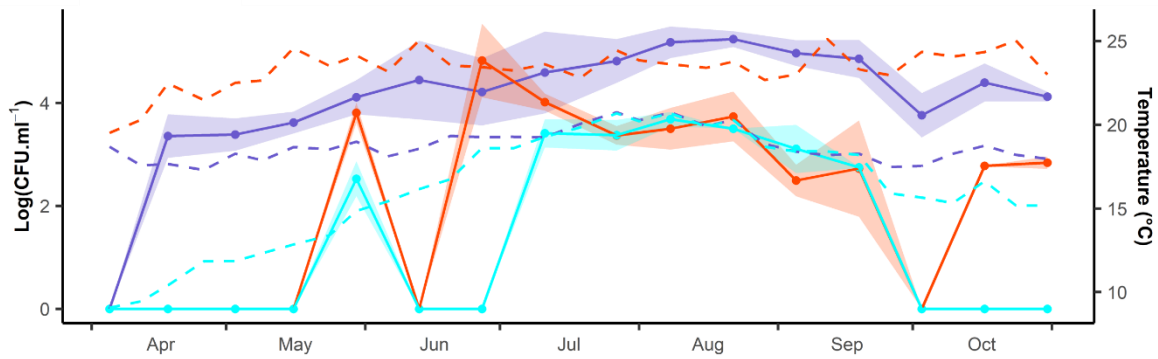
- Vibrio harveyi*
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- Photobacterium damsela subsp. damsela*
- Vibrio rotiferianus*
- Vibrio jascida*
- Vibrio owensii*
- Vibrio pelagius*
- Vibrio brasiliensis*
- Vibrio sinaloensis*
- Photobacterium spp.*
- Exiguobacterium spp.*
- Vibrio fortis*

- Photobacterium damsela subsp. damsela*
- Vibrio alginolyticus*
- Vibrio harveyi*
- Vibrio owensii*
- Vibrio rotiferianus*
- Vibrio spp.*
- Photobacterium spp.*
- Vibrio jascida*
- Vibrio fortis*
- Vibrio pelagius*
- Vibrio campbellii*
- Vibrio coralliilyticus*
- Vibrio mytili*
- Exiguobacterium spp.*

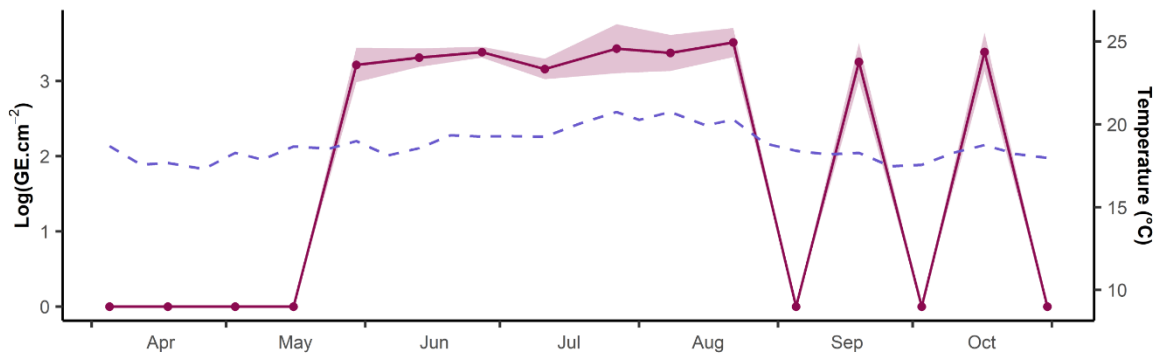
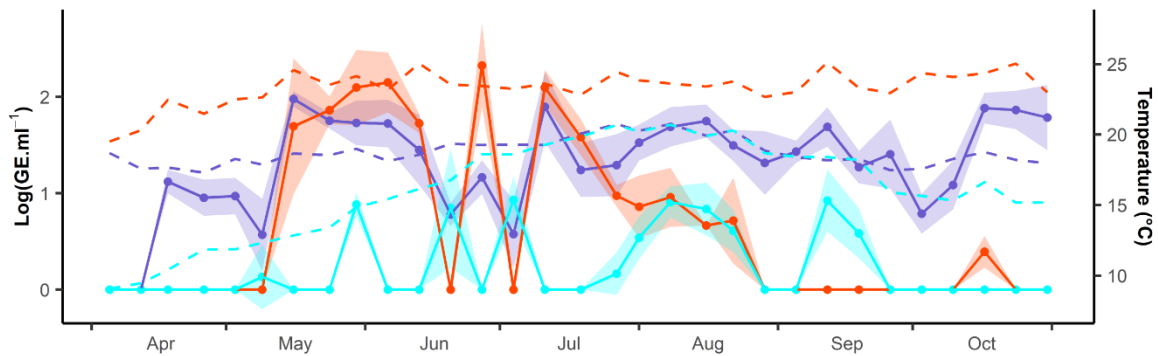
- Vibrio alginolyticus*
- Vibrio harveyi*
- Photobacterium damsela subsp. damsela*
- Vibrio owensii*
- Vibrio rotiferianus*
- Vibrio spp.*
- Photobacterium spp.*
- Vibrio mytili*
- Vibrio campbellii*
- Vibrio jascida*
- Vibrio pelagius*
- Vibrio parahaemolyticus*

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4 634 Figure 2. Qualitative analysis of the distribution and identification of the 1009 isolates (TCBS
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6 635 agar, 37 °C), using MALDI-TOF MS. a. Relative proportions of the isolates collected from the
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9 636 different sampling sites: rearing tanks and hot-/cold- water inlets and different sample types:
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11 637 water and biofilm. b. Relative proportions of identified species regardless of the sampling site and
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14 638 sample type. c. Relative proportions of identified species in the different sampling sites and
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16 639 sample types. d. Identified species in the different sampling sites and sample types throughout the
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19 640 sampling campaign, from April to October 2018. The key on the right indicates the color codes of
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21 641 the identified species, ranked from the most abundant (top) to the least abundant (bottom).
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4 **a. Presumptive Vibrionaceae - TCBS agar counts**
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31 **b. *Vibrio harveyi* - real-time PCR quantification**
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Concentration	Temperature
—●— Cold-water inlet	- - - Cold-water inlet
—●— Hot-water inlet	- - - Hot-water inlet
—●— Rearing tanks: Water	- - - Rearing tanks
—●— Rearing tanks: Biofilm	

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4 643 Figure 3. Fluctuation in abundance of a. presumptive culturable Vibrionaceae on TCBS agar,
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6 644 37 °C, and b. *Vibrio harveyi* in the cold- and hot-water inlets, water and biofilm samples from
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9 645 rearing tanks. For inlet samples, abundance values are the mean of three samples, collected
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11 646 simultaneously. For rearing tanks, abundance values are the mean of nine replicates,
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14 647 corresponding to the three replicates collected simultaneously from each of the three rearing
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16 648 tanks. The area around the curves represent the 95% confidence intervals for the abundance
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19 649 index. Water temperature in all compartments are also shown.

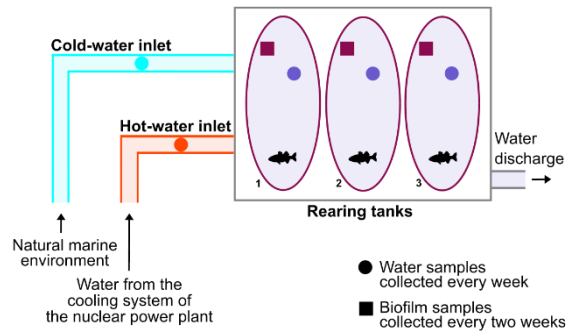


Figure 1. Schematic representation of the sampling areas on the fish farm, illustrating the sampling compartments and types of samples collected. The water samples were collected in the rearing tanks, and in the cold- and hot-water inlets every week. The biofilm samples were collected in the rearing tanks every two weeks.

Table 1. Means of parameters recorded from April to October 2018 in the aquaculture facilities.

Sampling months	Temperature (°C)			pH			Salinity			Conductivity (ms.cm ⁻¹)			Dissolved oxygen (mg.L ⁻¹)			Fish density (kg.m ⁻³)			Fish mortality per week (%)		
	Cold-water inlet	Hot-water inlet	Rearing tanks	Cold-water inlet	Hot-water inlet	Rearing tanks	Cold-water inlet	Hot-water inlet	Rearing tanks	Cold-water inlet	Hot-water inlet	Rearing tanks	Tank 1	Tank 2	Tank 3	Tank 1	Tank 2	Tank 3	Tank 1	Tank 2	Tank 3
April	10.2	21.4	18.1	8.1	8.0	7.8	33.8	33.8	34.1	51.5	51.6	51.9	7.2	5.8	7.1	28.3	54.5	31.8	0.02	0.13	0.04
May	13.2	24.0	18.8	8.1	8.0	7.8	34.2	34.6	34.4	51.9	52.6	52.2	7.1	7.0	5.8	37.2	28.5*	34.2	0.01	0.02	0.01
June	17.0	24.3	19.2	7.8	7.9	7.5	34.1	34.6	34.2	51.9	52.5	52.0	5.9	5.4	5.4	42.8	39.5	37.5	0.00	0.08	0.29
July	20.0	24.1	20.2	7.8	7.8	7.4	33.8	34.1	33.9	51.5	51.9	51.6	5.6	5.5	5.4	52.5	47.0	35.5	0.01	0.01	0.04
August	20.3	24.0	20.4	8.0	7.9	7.5	34.5	34.6	34.8	52.3	52.5	52.4	6.1	6.4	5.5	54.0	55.8	43.6	0.03	0.04	0.06
September	18.0	24.1	18.4	8.1	8.0	7.4	34.4	35.4	34.9	52.2	53.6	53.0	6.4	5.4	5.6	62.8	67.0	53.0	0.02	0.01	0.04
October	15.8	24.7	18.5	7.7	7.7	7.1	34.6	34.8	34.6	52.5	52.8	52.6	7.5	5.0	5.7	51.3*	74.8	59.0	0.06	0.01	0.04

*The on-growing batch of fish was removed and transported to a new rearing tank more suitable for their size, and a new batch of fish of smaller size was put in the rearing tank used in this study

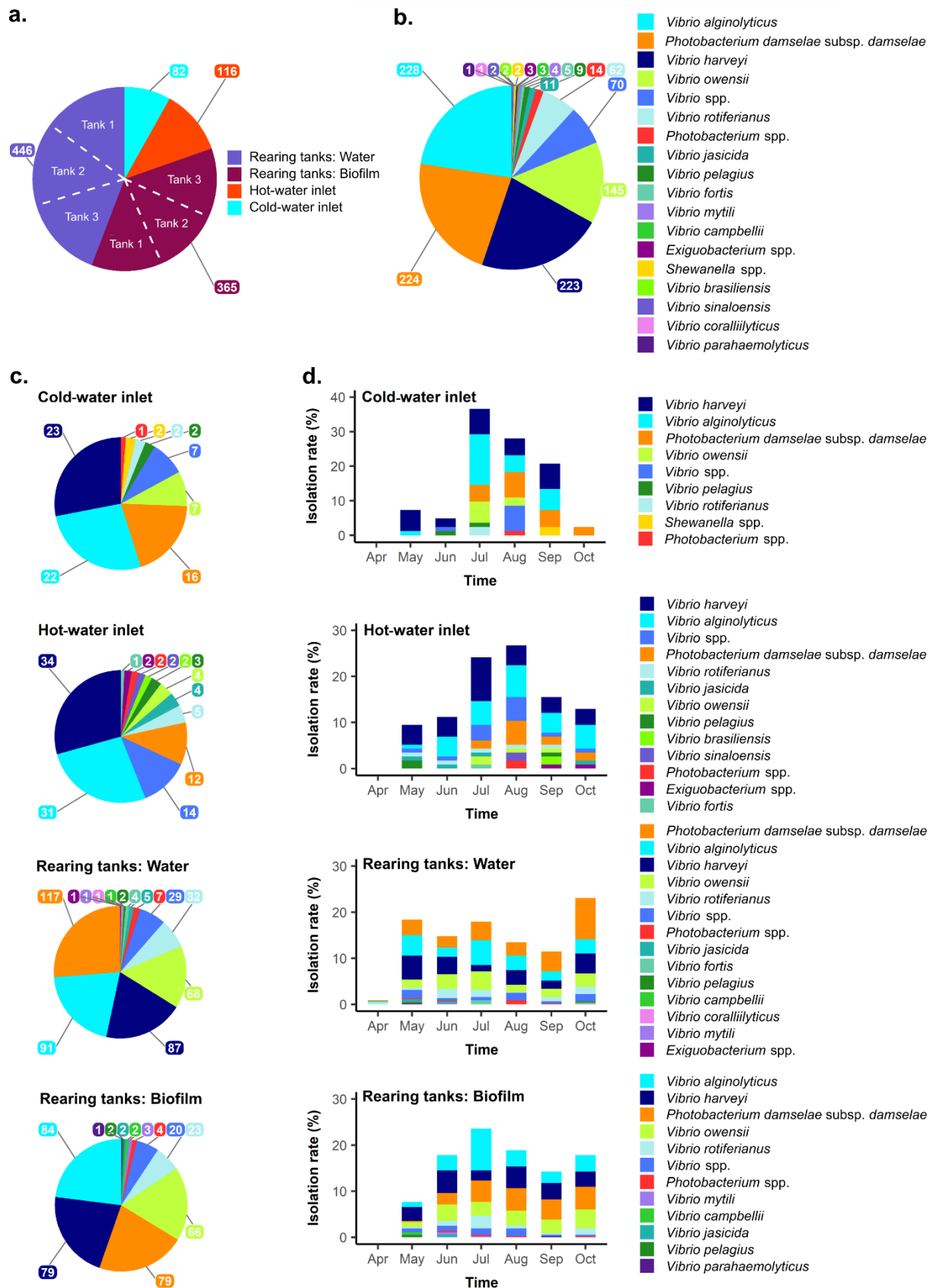


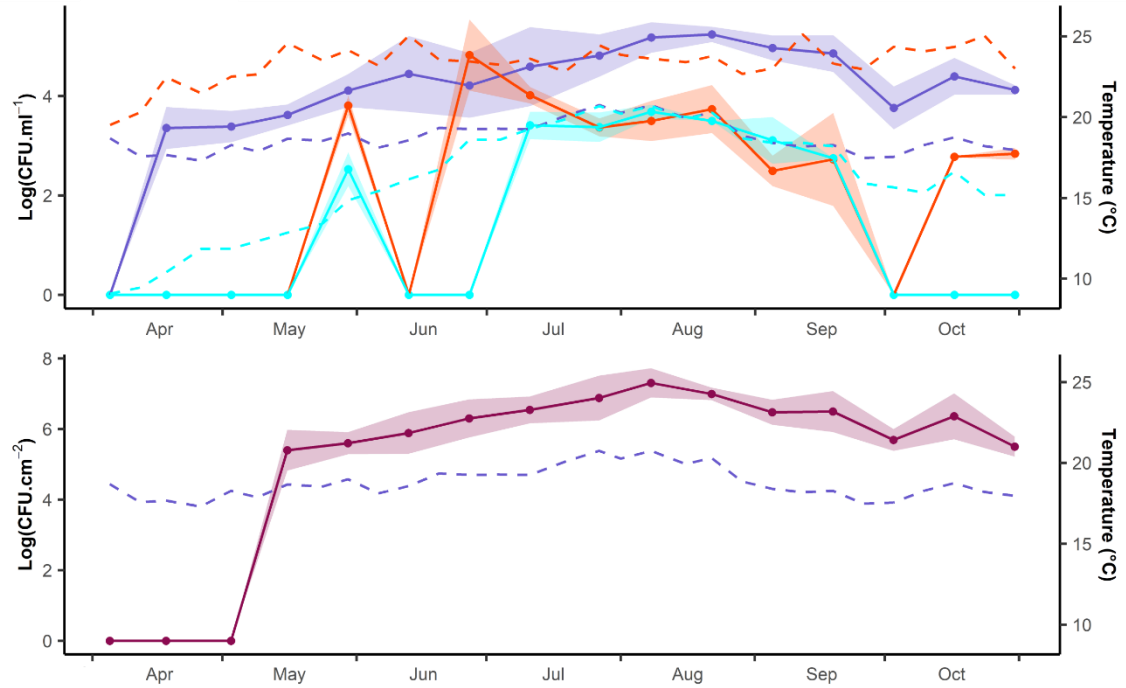
Figure 2. Qualitative analysis of the distribution and identification of the 1009 isolates (TCBS agar, 37°C), using MALDI-TOF MS. a. Relative proportions of the isolates collected from the different

sampling sites: rearing tanks and hot-/cold- water inlets and different sample types: water and biofilm.

b. Relative proportions of identified species regardless of the sampling site and sample type. c.

Relative proportions of identified species in the different sampling sites and sample types. d. Identified species in the different sampling sites and sample types throughout the sampling campaign, from April to October 2018. The key on the right indicates the color codes of the identified species, ranked from the most abundant (top) to the least abundant (bottom).

a. Presumptive Vibrionaceae - TCBS agar counts



b. *Vibrio harveyi* - real-time PCR quantification

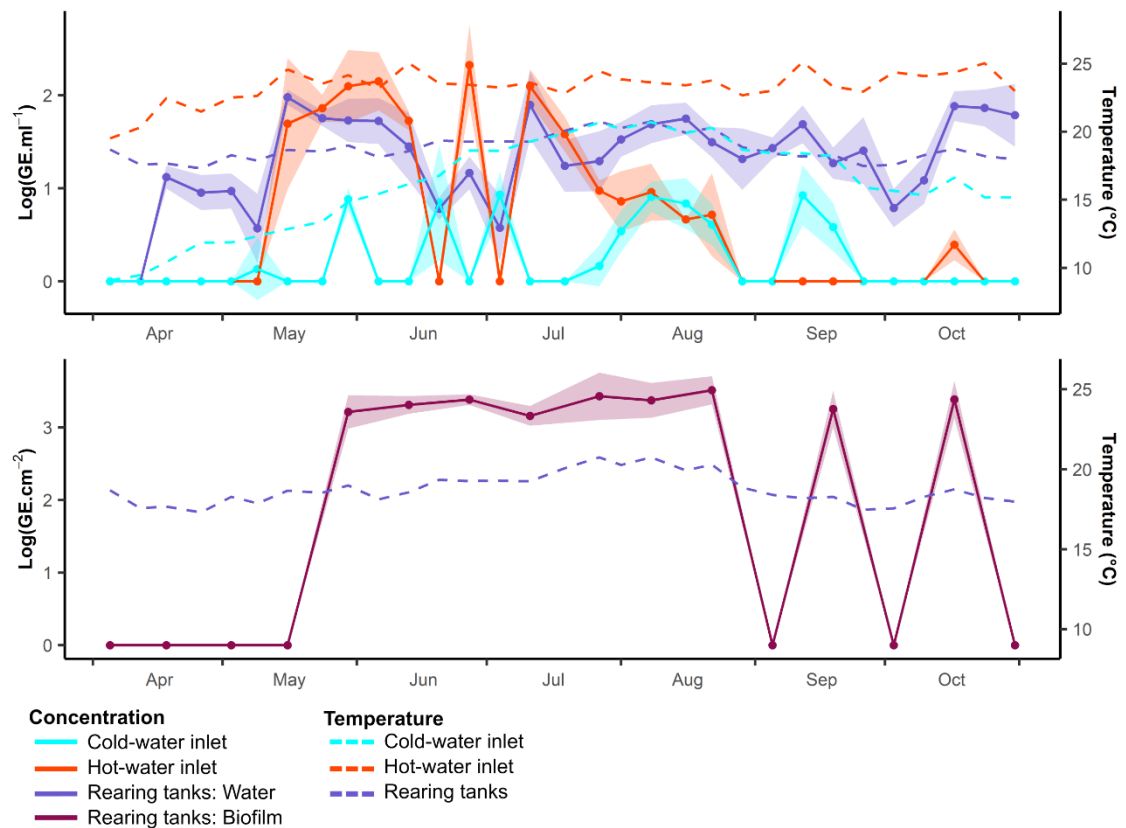


Figure 3. Fluctuation in abundance of a. presumptive culturable Vibrionaceae on TCBS agar, 37°C, and b. *Vibrio harveyi* in the cold- and hot-water inlets, water and biofilm samples from rearing tanks. For inlet samples, abundance values are the mean of three samples, collected simultaneously. For

rearing tanks, abundance values are the mean of nine replicates, corresponding to the three replicates collected simultaneously from each of the three rearing tanks. The area around the curves represent the 95% confidence intervals for the abundance index. Water temperature in all compartments are also shown.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:



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