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# Life history of oysters influences *Vibrio parahaemolyticus* accumulation in Pacific oysters (*Crassostrea gigas*)

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## Summary

***Vibrio parahaemolyticus*** **infection in humans is associated with raw oyster consumption. Evaluation of *V. parahaemolyticus* presence in oysters is of most interest because of the economic and public health issues that it represents. To explore *V. parahaemolyticus* accumulation and depuration in adult *Crassostrea gigas*, we developed a GFP-tagged *V. parahaemolyticus* strain (IFVp201-*gfp*$^+$), as well as a rapid and efficient quantification method in *C. gigas* oysters haemolymph by flow cytometry. Impact of the life history of *C. gigas* on accumulation and depuration of *V. parahaemolyticus* IFVp201 was subsequently investigated. We found that naive oysters, i.e. grown in controlled facilities with UV treated seawater, accumulated significantly more IFVp201 than environmental oysters, i.e. grown in intertidal environment. We hypothesized that environmental oysters could have been immune primed, thus could limit *V. parahaemolyticus* accumulation. Meanwhile, both naive and environmental oysters had similar depuration rates.**

## Introduction

*Vibrio parahaemolyticus* is a halophilic bacterium widely inhabiting estuarine and marine environments. *Vibrio parahaemolyticus* is the first causative bacterial agent of acute gastroenteritis in Human worldwide and has been extensively studied to distinguish pathogenic from non-pathogenic strains. To date, three major virulence factors have been characterized: two haemolysins named Thermostable Direct Haemolysin (TDH) and TDH-Related Haemolysin, and effectors of the type III secretion system number II (Sakurai *et al*., 1973; Honda *et al*., 1988; Park *et al*., 2004). *Vibrio parahaemolyticus* strains have been isolated in seawater and in association with other marine organisms in Europe, Asia and North and South America (World Health Organization, 1999; Baker-Austin *et al*., 2010; Luo *et al*., 2017). Review of 48 scientific publications conducted from 2003 to 2015 highlighted that oysters showed the highest percentage of contamination by *V. parahaemolyticus* (63.4%) in comparison to other marine organisms such as mussels, clams, fish or shrimp (Odeyemi, 2016). Thus, analysis of the accumulation and persistence of potentially pathogenic *V. parahaemolyticus* in oysters is important to evaluate the subsequent risk for humans consuming these oysters especially since factors associated with efficiency of accumulation and depuration of Human-pathogenic *Vibrio* are not well known (Froelich and Noble, 2014). Thus, identification of conditions and factors that limit/promote *V. parahaemolyticus* accumulation and depuration in oysters could help to improve risk management practices. Furthermore, the Pacific oyster, *Crassostrea gigas*, is a real economic issue worldwide. Most of the oyster production in France (98%) is based on *C. gigas* representing 437 million of US$ for 84 760 tons in 2019 (FAO, 2021).

Bivalve molluscs, in particular oysters, are considered sentinels of the environment because of their sessile characteristic and filtration capacity. Hence, oyster microbiota reflects the diversity of microbial environment that changes

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in function of biotic and abiotic factors (Lokmer *et al*., 2016; Scanes *et al*., 2021). *Vibrio* spp. are an integral part of the marine bacterial diversity, explaining why they are frequently isolated from the microbiome of *Crassostrea* spp. oysters (Clerissi *et al*., 2020). Jones *et al*. (2014) showed that all oysters collected from environment during the proliferation period of *Vibrio* spp. (from May to September) were contaminated up to 4 log of most probable number per gram of oyster tissues of *V. parahaemolyticus* (Jones *et al*., 2014). Oysters can therefore represent an important ecological niche for *V. parahaemolyticus* and an issue for public health. This characteristic complexifies the study of particular *V. parahaemolyticus* strains in laboratory.

Numerous studies rely on the use of Green Fluorescent Protein (GFP) as genetic marker to track a specific *Vibrio* spp. strain in bivalve molluscs (Cabello *et al*., 2005; Travers *et al*., 2008; Aboubaker *et al*., 2013; Dubert *et al*., 2016). Flow cytometry (FCM) can subsequently be used for bacterial quantification, thus, simplifying the study of *Vibrio* spp. in marine organisms (Travers *et al*., 2008; Aboubaker *et al*., 2013) in comparison with standard microbiology procedures such as plate enumeration (PE). Introduction of exogenous DNA in *Vibrio* strain is generally performed by conjugation. This strategy, although efficient (but not in all strains), is time consuming, with a bi- or triparental mating between *E. coli* donor cells harbouring the plasmid of interest and the *Vibrio* transformants, sometimes with the use of an *E. coli* helper strain containing the conjugative machinery (Christensen *et al*., 2020). Recently, a rapid electroporation protocol was developed for *Vibrio harveyi* (Delavat *et al*., 2018). This transformation protocol allowed obtention of transformants after only 1–2 days, without the need of counter-selecting the *E. coli* strain(s).

In this study, we used the IFVp201 strain (environmental, *tdh*$^+$ *trh*$^+$), preliminary electroporated with a GFP-plasmid (IFVp201-*gfp*$^+$) to investigate accumulation and depuration in haemolymph of *C. gigas* oysters using FCM. The main objective of this study was to investigate the impact of life history of oysters on the accumulation and depuration of this *V. parahaemolyticus* strain. This was evaluated for naive oysters, i.e. grown in controlled facilities with filtered and UV treated seawater, and environmental oysters, i.e. grown in the intertidal environment.

## Experimental procedures

### *Bacterial strains and culture conditions*

The *V. parahaemolyticus* IFVp201 strain used in this study was isolated in 2009 from a sample of mussels (*Mytilus edulis*) in Poitou Charentes (France). This environmental strain was characterized as *tdh*$^+$ *trh*$^+$ (Lozach *et al*., in preparation) by qPCR, which are virulence genes widely present in clinical cases but rarely in environmental strains.

Wild type IFVp201 strain was grown overnight at 37°C on agar plates of Luria–Bertani containing 3% NaCl (LBS) and on LBS supplemented with 50 μg ml$^{-1}$ of trimethoprim (LBS50) for IFVp201-*gfp*$^+$ (see 'Electroporation protocol'). A single colony formed on LBS or LBS50 agar plate was transferred into the corresponding liquid medium (LBS or LBS50) and was incubated at 37°C overnight at 50–70 rpm. Optical density of overnight culture was measured at 600 nm (OD600) using a Spectrophotometer UV/visible Evolution™ (Thermo Scientific™) and culture was plated on LBS or LBS50 (incubated overnight at 37°C) to estimate the concentration of each cultured isolate.

Before experimentation in filtered and sterile seawater (FSSW, natural seawater filtered with 1 μm polypropylene mesh and treated with UV), IFVp201-*gfp*$^+$ overnight cultures were washed twice using buffered-physiological water (BPW, 0.4 g L$^{-1}$ of $K_2H_3PO_4$; 4.5 g L$^{-1}$ of $Na_2HPO_4$, $12H_2O$; 7.2 g L$^{-1}$ of NaCl) and analysed by FCM to determine bacterial concentration (log bacteria ml$^{-1}$).

### *Electroporation protocol*

The plasmid used in this study was pFD086 harbouring *gfp* gene and trimethoprim-resistance expression cassettes (Morot *et al*., 2021). IFVp201 strain was electroporated as described previously (Delavat *et al*., 2018). The positive clones were confirmed by PCR amplification on colony with the primers 181101 and 181102 (Morot *et al*., 2021), and with VP32 and VP33 (Lee *et al*., 1995) which target the pFD086 plasmid and the pR72H region for identification at the species level respectively. PCR amplifications were performed using a Taq DNA Polymerase Good Manufacturing Product grade (Roche Diagnostics) with the following thermal cycle: 5 min of initial denaturation at 95°C followed by 35 cycles at 95°C for 30 s, 56°C for 1 min and 72°C for 1 min 30 s. To verify GFP expression, colonies were placed under UV light to visualize fluorescence emission.

### *Estimation of plasmid stability in filtered and sterile seawater*

The ability of pFD086 to persist in IFVp201-*gfp*$^+$ was determined in FSSW. For this purpose, overnight cultures washed twice in BPW were diluted to obtain a final concentration of $10^8$ CFU ml$^{-1}$. This bacterial culture was suspended in FSSW up to a final concentration of $10^5$ CFU ml$^{-1}$. At 0, 24 and 48 h FSSW samples were plated onto LBS agar and incubated overnight at 37°C. Fifty isolated colonies were plated onto LBS50 and then onto LBS (overnight at 37°C). The stability percentage

was estimated as the number of colonies grown onto LBS50 divided by number of colonies grown onto LBS and multiplied by 100.

### *Growth kinetics of wild type and* Vp-gfp$^+$ *strains*

Overnight cultures of wild type IFVp201 and IFVp201-*gfp*$^+$ were carried out in LBS and in LBS50 respectively, at 37°C. The OD600 of the overnight cultures were measured and cultures of IFVp201 and IFVp201-*gfp*$^+$ were 100-fold diluted in LBS and in LBS50 respectively, then incubated at 37°C. Concentrations of IFVp201 and IFVp201-*gfp*$^+$ in the 100-fold diluted cultures were determined in triplicates by PE on LBS and LBS50 respectively and incubated at 37°C overnight. The OD600 of these diluted cultures were measured every hour for 8 h. The experiment was performed in triplicate.

The doubling time ($G$) corresponds to the time allowing the doubling of bacterial population during the exponential growth phase. Graphically, $G$ was calculated as the time between $OD600_t$ at a $t$ time and $2 \times OD600_t$.

### *Quantification of IFVp201-*gfp$^+$ *by FCM*

Overnight cultures of IFVp201-*gfp*$^+$ were used to establish the settings of quantification by FCM. Bacteria were identified and counted using a Cyflow Space (Sysmex-Partec, Munster, Germany). The bacterial counting was performed according to the number of cells detected in a volume of 200 μl of sample. They were detected by their green fluorescence (FL1 detector of the flow cytometer) and their relative size and granularity [Forward SCatter (FSC); Side SCatter (SSC)]. Concentrations were expressed in log bacteria ml$^{-1}$.

### *Preliminary assays of oyster contamination with IFVp201-*gfp$^+$

The first assay was performed to select the most appropriate matrix for IFVp201-*gfp*$^+$ quantification, i.e. haemolymph or oyster tissues, and to estimate the appropriate concentration of IFVp201-*gfp*$^+$ that could be used for the following experimentations of this study. Adult diploid *C. gigas* (approx. 30–40 g of total weight and 7 cm long) were bought from an oyster farmer located in the Bay of Brest (Pointe du Chateau, Finistère, France, 4°19′3″ W, 48°20′2″ N) in February 2020. Experimentation occurred less than 10 h after collection from their culture site. IFVp201-*gfp*$^+$ was inoculated at initial concentrations of $10^5$ CFU ml$^{-1}$ (5 log) and $10^7$ CFU ml$^{-1}$ (7 log) in 2 L of FSSW into two separate tanks. Four oysters ($n = 4$) were then placed into each tank and maintained overnight at room temperature (20°C–22°C). Control oysters ($n = 4$) not exposed to IFVp201-*gfp*$^+$ were kept in the same conditions. After the overnight accumulation, and using sterile tools and workstation, each oyster was shucked, and abductor muscles were cautiously cut with a scalpel to allow opening of the shell and avoid damaging of the pericardial cavity. Haemolymphs were collected into the pericardial cavity using a 25G needle and 1 ml insulin syringe. Collected haemolymphs (approx. 1–2 ml) were filtered on a 30 μm nylon mesh to remove aggregates and/or debris. All oyster tissues were shredded during $3 \times 30$ s at low speed using a Laboratory blender (Waring Blender). Analyses were performed at the individual level, and were carried out by FCM and by PE (on LBS50 and incubated overnight at 37°C) for both the haemolymph and oyster tissues.

This second assay was performed to validate experimental design. Wild diploid *C. gigas* (named E1), caught on the Atlantic coast of Charente Maritime (France), were maintained in mesh bags at La Floride (Ronce les Bains, Charente Maritime, France, 1°09′15″ W, 45°48′12″ N). These oysters were collected in February 2021 and transferred within 24 h at the Ifremer experimental platform at Bouin (Vendée, France). IFVp201-*gfp*$^+$ was inoculated in one 20 L tank of FSSW with initial concentration of $10^5$ CFU ml$^{-1}$. Initial concentrations of IFVp201-*gfp*$^+$ in FSSW were verified in triplicates by FCM in the tank. E1 oysters were placed in the contaminated tank ($n = 15$) and in a control 20 L tank of FSSW not exposed to IFVp201-*gfp*$^+$ ($n = 9$), and maintained at 19°C for an overnight balneation (17–19 h). Aeration was provided to all tanks. After the overnight balneation, IFVp201-*gfp*$^+$ concentration in seawater was determined in triplicates by FCM and oysters were transferred 1 h into 'fresh' FSSW to remove potential IFVp201-*gfp*$^+$ present on shells and in shell fluid. Haemolymphs were collected as described previously from one pool of three oysters from each batch and IFVp201-*gfp*$^+$ was quantified by FCM in each pool (two analytical replicates for each pool). Remaining oysters from the batch exposed to IFVp201-*gfp*$^+$ ($n = 12$) and from the control batch ($n = 6$) were transferred in 6 L tanks of FSSW and maintained at 19°C for 24 h of depuration. After this depuration, haemolymphs were collected from three pools and one pool of three oysters from the batch exposed to IFVp201-*gfp*$^+$ and from the control batch respectively. IFVp201-*gfp*$^+$ was quantified by FCM in each pool (two analytical replicates for each pool).

### *Challenge tests of naive and environmental oysters with IFVp201-*gfp$^+$

The following experiment was performed in order to evaluate the impact of the life history of oysters on *V. parahaemolyticus* accumulation and depuration. Experimental protocol is represented in Fig. S1.

For this experiment, two groups of adult oysters (approx. 30–40 g of total weight and 7 cm long) with three batches for each group were tested: naive oysters, i.e. grown in controlled inland facilities using sand-filtered and UV-treated seawater, and environmental oysters, i.e. grown in the intertidal environment. For the naive group, the three selected batches were hatchery-produced diploid oysters and were always maintained in controlled inland facilities at Ifremer. Two batches, called A1 and A2, were maintained in raceways at the experimental platform at Bouin (Vendée, France), and the third, called A3, was maintained in raceway at the experimental platform at La Tremblade (Charente Maritime, France). For the environmental group, two wild diploid stocks, E1 (as in the second assay) and E2 from Chaucre (Saint-Georges-d'Oléron, Charente Maritime, France, 1°23′44″ W, 45°58′56″ N), as well as one hatchery-produced diploid batch (E3) were used. E3 was bought to an oyster farmer that raised them near Bouin coasts (Port des Champs, Vendée, France, 2°2′54″ W, 46°57′54″ N). All six oyster batches were transferred at the experimental platform at Bouin within 24 h after collection and experimentations were carried out upon arrival at the platform in October 2021.

IFVp201-*gfp*$^+$ was inoculated in six 10 L tanks of FSSW with initial concentration of $10^5$ CFU ml$^{-1}$. Initial concentrations of IFVp201-*gfp*$^+$ were verified in triplicates by FCM in each tank. Each batch of oysters was individually placed in the contaminated tank ($n = 30$) and in six control 4 L tanks of FSSW not exposed to IFVp201-*gfp*$^+$ ($n = 15$), and maintained at 19°C for an overnight balneation (17–19 h). Aeration was provided to all tanks. After the overnight balneation, IFVp201-*gfp*$^+$ concentration in seawater was determined in triplicates by FCM and oysters were transferred 1 h into 'fresh' FSSW. Haemolymphs were collected as described previously from two pools and one pool of five oysters from each batch exposed to IFVp201-*gfp*$^+$ and each control batch respectively, and IFVp201-*gfp*$^+$ was quantified by FCM in each pool (two analytical replicates for each pool). Remaining oysters from batches exposed to IFVp201-*gfp*$^+$ ($n = 20$) and from the control batch ($n = 10$) were transferred in 10 and 4 L tanks of FSSW respectively and maintained at 19°C for 24 h of depuration. After this depuration, haemolymphs were collected from three pools and one pool of five oysters from each batch exposed to IFVp201-*gfp*$^+$ and each control batch respectively, and IFVp201-*gfp*$^+$ was quantified by FCM in each pool.

### *Statistical tests*

Data used to investigate reliability and agreement between FCM and PE represented a wide range of *Vp*-*gfp*$^+$ enumeration by FCM (in log bacteria ml$^{-1}$) and PE (in log CFU ml$^{-1}$) in different mediums and for different strains. Bland–Altman plot was used to explore the agreement between the two methods (Bland and Altman, 1986). The graphical representation shows the difference between enumeration by FCM and by PE for one sample in function of average enumeration between FCM and PE for one sample. The intraclass correlation coefficient (ICC) was used to explore the reliability that reflects both the degree of correlation and of agreement between the FCM and the PE methods. ICC values were calculated using a two-way mixed model with absolute agreement for single measurements. We rated ICC values as poor if 0–0.5, fair if 0.5–0.75, good if 0.75–0.9 and excellent if 0.9–1 (Koo and Li, 2016; R Core Team, 2019).

All statistical analyses were performed using RStudio 2021.09.0 + 351 'Ghost Orchid' Release (2021-09-20) for Windows. Significance level was set to $p < 0.05$. Prior further analyses, normality and variance homogeneity of the data were tested using Shapiro test and Levene test respectively. For the following analyses, quantification of IFVp201-*gfp*$^+$ in seawater (SW) and, in haemolymph after the overnight accumulation (ACCU) and after 24 h of depuration (DEPU) were analysed between naive and environmental oysters (CONDITION), and between each batch of naive and environmental oysters (BATCH). Comparisons of SW were carried out using non-parametric Kruskal–Wallis test and *post hoc* Dunn test in function of CONDITION and BATCH. Comparisons of ACCU were carried out using parametric one-way ANOVA and *post hoc* Tukey test in function of CONDITION and BATCH. Comparisons of DEPU were carried out using an ANCOVA with ACCU as covariable and *post hoc* Tukey test in function of CONDITION and BATCH. Comparisons of ACCU with DEPU were carried out using a parametric one-way ANOVA and *post hoc* Tukey test for each CONDITION and BATCH. This last analysis had for purpose to determine significance of depuration rates.

## Results

### *Construction and validation of IFVp201*-gfp$^+$ *transformants*

IFVp201 was electroporated with the pFD086 plasmid harbouring the *gfp* gene (Morot *et al*., 2021). Transformation efficiency was $1.2 \times 10^3$ CFU μg$^{-1}$ of DNA. Plasmid presence in the transformants was confirmed by PCR and by visualization of fluorescent colonies under UV light (not shown). Assays of plasmid stability in FSSW showed that pFD086 plasmid persisted in 100% of IFVp201-*gfp*$^+$ strain after an inoculation of $10^5$ CFU ml$^{-1}$

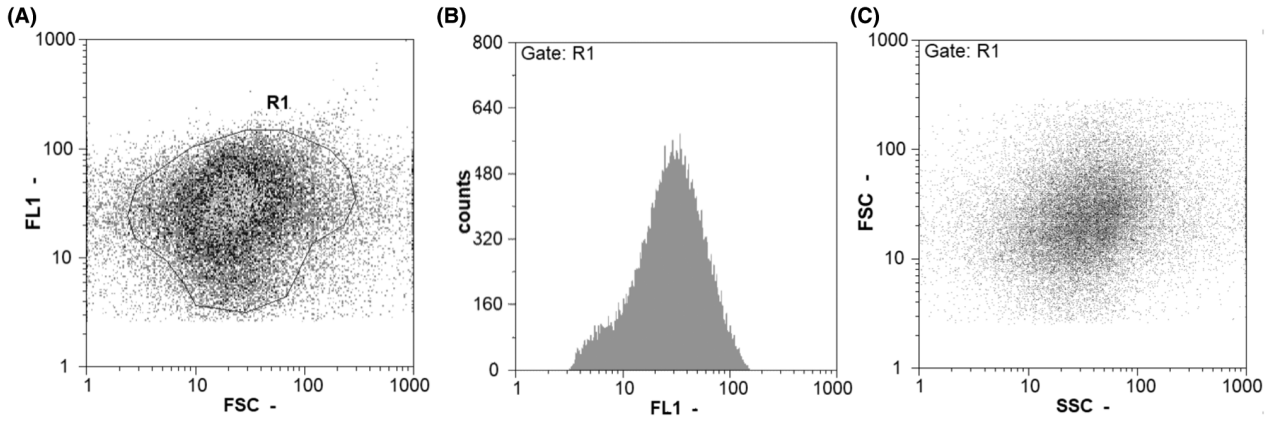


**Fig. 1.** Detection of IFVp201-*gfp*$^+$ by FCM. Results of overnight culture of IFVp201-*gfp*$^+$ in LBS diluted up to $10^5$ CFU m$^{-1}$ in buffered-physiological water based on OD600. Properties of green fluorescence (FL1, A and B), Forward Scatter (FSC, A and C) and Side Scatter (SSC, C) are shown.

in FSSW for 48 h. This result showed that the construction was stable in experimental conditions.

To determine the impact of pFD086 plasmid, growths of IFVp201-*gfp*$^+$ and IFVp201 strains were compared. Interestingly, OD600 of IFVp201 and IFVp201-*gfp*$^+$ of the overnight cultures were 1.41 $\pm$ 0.09 and 1.74 $\pm$ 0.04 respectively. However, concentrations in the 100-fold cultures before growth monitoring were 7.00 $\pm$ 0.13 log CFU ml$^{-1}$ and 7.00 $\pm$ 0.11 log CFU ml$^{-1}$ for IFVp201 and IFVp201-*gfp*$^+$ respectively. Only a minor difference was observed between the growths after 8 h of culture. The difference of doubling time between wild type IFVp201 and IFVp201-*gfp*$^+$ strains was 3 min (30 and 27 min respectively) thus showing that the pFD086 plasmid did not impact growth of IFVp201. Moreover, colonies size and aspect were similar for wild type and IFVp201-*gfp*$^+$ strains.

### *Quantification of IFVp201-*gfp$^+$ *culture by FCM*

Quantification by FCM was first set from a pure culture of IFVp201-*gfp*$^+$. A dot plot of FL1 against FSC was used to select the gate in which particles were identified as IFVp201-*gfp*$^+$, named R1 population (Fig. 1A). Dot plot of FSC against SSC, and histogram of count of particles against FL1 were used to confirm the identification of R1 population by gating results on R1 population (Fig. 1B and C). Importantly, no event was detected in the R1 gate for control LBS, control FSSW and for wild type IFVp201 strains (not shown).

### *Selection of oyster matrix (haemolymph or tissues) and quantification method (FCM or PE)*

Haemolymphs and oyster tissues were analysed for IFVp201-*gfp*$^+$ quantification by FCM and PE. For the control oysters not exposed to IFVp201-*gfp*$^+$ strain, 4.60 log event ml$^{-1}$ (in case of control oysters, we could not express the concentration in log bacteria ml$^{-1}$) were detected by FCM in R1 gate in oyster tissues, whereas no event was detected in haemolymph. The events detected in control oysters were associated to autofluorescence of oyster tissues, and if considered, they would impair bacterial quantification by fluorescence measurement in contaminated conditions. Consequently, only haemolymph was considered for IFVp201-*gfp*$^+$ enumeration by FCM. After an oyster exposition to 5 log of IFVp201-*gfp*$^+$, 3.32 log bacteria ml$^{-1}$ and 3.01 log CFU ml$^{-1}$ were enumerated in haemolymph by FCM and PE respectively. A similar slight overestimation was found for oysters exposed to 7 log of IFVp201-*gfp*$^+$ with 7.04 log bacteria ml$^{-1}$ and 6.93 log CFU ml$^{-1}$ by FCM and PE respectively. As a concentration of 5 log allowed a sufficient accumulation for further analysis, this concentration was used for the subsequent experiments using haemolymph as sample.

To assess the concordance of IFVp201-*gfp*$^+$ quantification by FCM in comparison to PE in several media (LBS, seawater, haemolymph and artificial seawater), a Bland and Altman graphical representation was carried out (Bland and Altman, 1986) (Fig. 2). The bias between FCM and PE was 0.44 log bacteria ml$^{-1}$ and the standard deviation was 0.27 log bacteria ml$^{-1}$. The 95% confidence interval (limits of agreement, in red dashed line in Fig. 2) for the bias was between 0.96 and $-$0.09 log bacteria ml$^{-1}$ that contained 20/22 (91%) of the values. The standard error of the limit was 0.10 log bacteria ml$^{-1}$. This analysis suggests that this overestimation was not significant because the 95% confidence interval included the '$y = 0$' axis. The ICC for the enumeration method was 0.975 with a 95%-confidence interval ranging between 0.387 and 0.994.

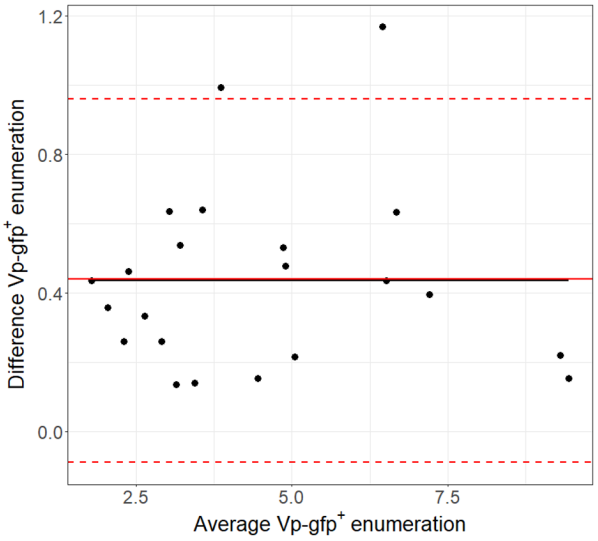


**Fig. 2.** Bland and Altman representation for IFVp201-*gfp*$^+$ enumeration in different media by FCM and PE. *X*-axis: average enumeration by FCM and by PE for one sample. *Y*-axis: difference between the enumeration by FCM and by PE for one sample. Red solid line: bias; red dashed line: limits of agreement/95% confidence interval.

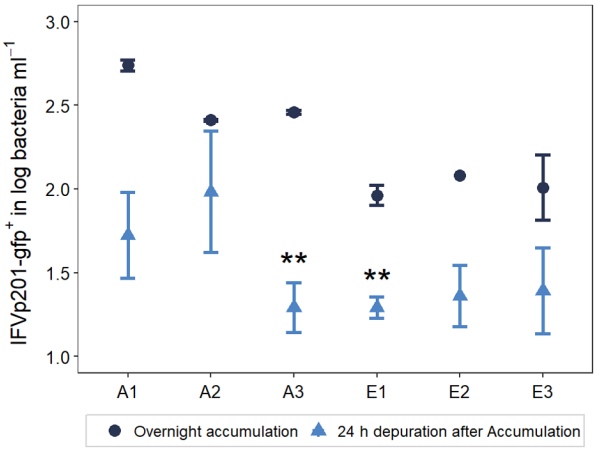


**Fig. 3.** IFVp201-*gfp*$^+$ quantification by FCM in haemolymphs of oysters after an overnight accumulation (black circles) and after 24 h of depuration (blue triangles). Oysters exposed overnight to $10^5$ CFU mL$^{-1}$ of IFVp201-*gfp*$^+$ then depurated for 24 h, A1-A2-A3: naive oysters, E1-E2-E3: environmental oysters. Data represent the mean of two pools of five oysters (two analytical replicates per pool) for overnight accumulation and the mean of three pools of five oysters (two analytical replicates per pool) for 24 h depuration ± standard error. Statistical analysis: one-way ANOVA and *post hoc* Tukey tests ($p < 0.05$).

In order to validate the experimental design and IFVp201-*gfp*$^+$ quantification by FCM, an assay was performed in February 2021 with E1 oysters. After the overnight accumulation, 3.22 log bacteria ml$^{-1}$ of IFVp201-*gfp*$^+$ were quantified in haemolymph. Concentration of IFVp201-*gfp*$^+$ after 24 h of depuration was 0.81 log bacteria ml$^{-1}$ thus the depuration rate of E1 was significant ($p < 0.001$). Those results confirmed the experimental design previously established.

### *Impact of the life history of oysters on accumulation and depuration of IFVp201*-gfp$^+$

In order to evaluate the impact of life history of naive and environmental oysters on accumulation (during 17–19 h) and depuration (during 24 h) of IFVp201-*gfp*$^+$, strains were determined in haemolymphs. Initial concentrations of IFVp201-*gfp*$^+$ in tanks (before oyster exposure) were 5.26 ± 0.03 log bacteria ml$^{-1}$, whereas no bacteria was detected in control tanks. After the overnight accumulation in oysters, concentrations of IFVp201-*gfp*$^+$ in seawater were 4.70 ± 0.26 and 4.58 ± 0.13 log bacteria ml$^{-1}$ for environmental and naive oysters respectively and no bacteria was detected in control tanks. No significant difference ($p = 0.749$) of concentration was observed between each tank before and after accumulation. Figure 3 shows IFVp201-*gfp*$^+$ concentration in haemolymph of oysters directly after the overnight accumulation and followed by 24 h of depuration.

Control oysters were all negative for IFVp201-*gfp*$^+$ by FCM. Batches of naive oysters (A-batches) accumulate significantly more IFVp201-*gfp*$^+$ than batches of environmental oysters (E-batches) ($p < 0.001$). Batches comparison showed that the difference of accumulation was only significant between A1 and E3 ($p = 0.0169$), and between A1 and E1 ($p = 0.013$), and on other fronts, between A1 and A2 ($p = 0.00265$) and between A1 and A3 ($p = 0.00419$). In contrast, IFVp201-*gfp*$^+$ concentrations after 24 h of depuration were not significantly different between A-batches and E-batches ($p = 0.55$). Finally, depuration rate (between the overnight accumulation and 24 h of depuration) was significant for E-batches ($p < 0.001$) and A-batches ($p = 0.00142$). Batches comparison showed that depuration rate was only significant for A3 ($p = 0.00878$) and E1 ($p = 0.00565$). Environmental oysters accumulated more but depurated equally to naive oysters.

## Discussion

Study of human-pathogenic bacteria in filter feeding bivalves is a major challenge for public health worldwide. *Vibrio parahaemolyticus*, first causal bacterial pathogen agent of gastroenteritis by seafood consumption, is a bacterium naturally present in marine and estuarine environments. The first shellfish implicated in these foodborne diseases are the cupped oyster (Odeyemi, 2016), *Crassostrea gigas* (Asia and Europa) and *Crassostrea virginica* (United States). Analysis of *V. parahaemolyticus*

accumulation and persistence in oysters and investigation of the conditions that favour or not accumulation and persistence are important to evaluate the risk for oyster consumers (Froelich and Noble, 2014).

The study of particular bacterial strains in laboratory requires the use of new methodologies in order to differentiate indigenous *Vibrio* spp. from *Vibrio* spp. experimentally accumulated in bivalve. The use of fluorescent molecules such as GFP was an interesting way to visualize and quantify *V. parahaemolyticus* accumulated in oysters in experimental conditions. In this study, an electroporation approach (Delavat *et al*., 2018) was used to produce the strain IFVp201-*gfp*$^+$ (Morot *et al*., 2021). To our knowledge, it is the first time that transformants of *V. parahaemolyticus* are obtained by an electroporation method. This method allowed fast production of transformants (2 working days) in contrast to conjugation (1 week), commonly used for marine bacteria (Cabello *et al*., 2005;Travers *et al*., 2008; Aboubaker *et al*., 2013). Electroporation efficiency for the IFVp201-*gfp*$^+$ was comparable to what was obtained with *Vibrio harveyi* in Delavat *et al*. (2018), i.e. around $10^3$ CFU $\mu g^{-1}$ DNA. Moreover, it is known that an exogenous plasmid could have an effect on bacterial physiology like bacterial growth. For example, Muturi *et al*. (2019) showed that GFP-tagged bacterial strains had lower growths than the wild type strains (Muturi *et al*., 2019). In our study, GFP-plasmid presence showed a minor difference between IFVp201 and IFVp201-*gfp*$^+$ growth, suggesting that GFP-plasmid did not have any noticeable impact on the growth of IFVp201-*gfp*$^+$.

The scope of application of FCM expands, allowing characterization of cell types, verification of cell viability and cell quantification. FCM was used in previous studies for the quantification of bivalve pathogens *Vibrio* spp. (Travers *et al*., 2008; Aboubaker *et al*., 2013). In our study, production of IFVp201-*gfp*$^+$ allowed us to develop a protocol for immediate quantification of bacterial culture or biological samples by FCM (GFP detection). FCM represented a real advantage in terms of time gained for experimentation. In our study, the reliability and agreement between FCM and PE were checked. On the one hand, Bland and Altman representation (Bland and Altman, 1986) showed that bacterial concentration calculated by FCM was slightly overestimated in comparison to PE. However, this overestimation was minimal and not significant at a concentration of $10^5$ CFU $ml^{-1}$. This difference could be explained by the fact that the quantification by FCM did not depend on bacterial culturability in contrast to PE. This was also considered by Gao *et al*. (2018) who showed that quantification of *Bifidobacterium longum* by FCM was four times higher than by PE (Gao *et al*., 2018). On the other hand, determination of the ICC (Koo and Li, 2016) showed that the reliability between the two methods was 'excellent' (ICC > 0.90). Thus, we showed that FCM was a good alternative to PE for *Vp-gfp*$^+$ quantification. In contrast to molecular approaches (e.g. qPCR), FCM quantified viable cells only. Indeed, the dead fluorescence-tagged bacteria not expressing the fluorescence (Lowder *et al*., 2000). On this basis, qPCR paired with propidium monoazide was developed to quantify only viable cells and proved to be a reliable for quantification of viable *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans* and *Fusobacterium nucleatum* in biofilm (Sánchez *et al*., 2014). Unlike molecular approaches, which need DNA extraction and could result in biological material loss, FCM allows immediate quantification of individual cells. The prerequisite of FCM use is the preliminary tagging of bacteria with fluorescent tag. As a result, FCM appears to be the simplest method for bacterial quantification in oysters exposed to fluorescent bacteria in an experimental setting.

Enumeration of *V. parahaemolyticus* from shellfish samples is commonly carried out with oyster tissues homogenates using cultural methods (NSSP, 2017). The use of FCM in our study was shown to be reliable and a promising method to replace cultural methods for experiments using fluorescence-tagged bacteria. In our study, auto-fluorescence of oyster tissues complicated the interpretation of FCM results, and haemolymph was an interesting alternative for quantification of IFVp201-*gfp*$^+$ by FCM during experimental infections of oysters. Indeed, Cabello *et al*. (2005) used a GFP-tagged *V. parahaemolyticus* strain (ATCC 17802) to study its fate and its capacity of proliferation in oysters (*Ostrea chilensis*). They showed that concentration of *Vp-gfp*$^+$ did not differ significantly between different individual tissues, including pericardium (Cabello *et al*., 2005). Furthermore, the presence of fluorescent particles in R1 population in tissues of control oysters showed that haemolymph was a better matrix for FCM analyses than oyster tissues. Concerning the IFVp201-*gfp*$^+$ initial concentration, although an exposure of 7 log showed a higher accumulation, 5 log provided a sufficient accumulation in haemolymph and was commonly used in literature for bacterial balneation with bivalves (Travers *et al*., 2008; Aagesen *et al*., 2013, 2018). This explained why we used the IFVp201-*gfp*$^+$ initial concentration of 5 log for bacterial quantification in haemolymph by FCM. Thus, we developed a reliable protocol to challenge oysters considering *V. parahaemolyticus* contamination, which could be adapted to other fluorescence-tagged bacteria infecting oysters or any other bivalve molluscs.

In order to evaluate the impact of life history of oysters on the accumulation and depuration of *V. parahaemolyticus*, environmental oysters and naive oysters maintained in secured structures were used. We

observed that naive oysters accumulated significantly more IFVp201 than environmental oysters. First, these results could be explained by the fact that filtration capacity of naive oysters could be less effective than the one of environmental oysters. However, no significant difference in IFVp201 concentration was observed in seawater after the overnight accumulation between naive and environmental oysters and each oyster batch. This result indicated that filtration capacities of the six batches of oysters were quite similar and thus, would not explain the accumulation differences between naïve and environmental oysters. Moreover, it was shown that the activity times of oysters linked to clearance rate and oxygen consumption (feeding time activity and respiratory activity) did not permit to distinguishing the oysters, when tested at 20°C, as a function of their life history trait (naive oysters vs. environmental oysters), their ploidy level (diploid and triploid) and their tolerance for OsHV-1 (Haure *et al*., 2021). Second, these results could be explained by the fact that naive oysters have never been exposed to environmental microflora, including *Vibrio* spp., contrary to environmental oysters. Indeed, Zhang *et al*. (2014) and Lafont *et al*. (2020) showed that a first exposure to agents (*Vibrio vulnificus* and OsHV-1 respectively) could lead to a development of a stronger immune response of the oyster for future reinfection (Zhang *et al*., 2014; Lafont *et al*., 2020). This phenomenon is called the innate immune priming. Furthermore, our results could also be explained by the oyster microbiota composition and diversity. Indeed, Lokmer *et al*. (2016) showed that bacterial community of oyster haemolymph varied between laboratory conditions and after field deployment (Lokmer *et al*., 2016). Thus, oyster haemolymphs from field condition were characterized by lower relative abundances of ε-, γ-proteobacteria (especially *Arcobacter* and *Vibrionaceae*) and Flavobacteria, and by higher relative abundances of α-proteobacteria, Mollicutes and bacterium related to Spirochaetes in comparison to oyster haemolymphs from laboratory condition. To our knowledge, characterization of haemolymph microbiota was never studied as a condition to favour or not bacterial accumulation or persistence unlike digestive gland microbiota which was shown to be linked to resistance or sensitivity to Pacific Oyster Mortality Syndrome (POMS). Indeed, previous studies showed that *Mycoplasmatales* (Clerissi *et al*., 2020) as well as *Vibrionaceae* (*Vibrio* and *Pseudomonas*) in digestive gland microbiota (King *et al*., 2019; Clerissi *et al*., 2020) were linked to higher sensitivity of oysters to POMS. Moreover, Offret *et al*. (2020) showed that oysters grown in controlled inland facilities at Ifremer (like naive oyster from our study) were characterized by high levels of *Mycoplasmatales* (Offret *et al*., 2020). Those results could explain why naive oysters accumulate more IFVp201 than environmental oysters from our study. Moreover, it was shown that oyster genetic take part in resistance or sensitivity of oysters against pathogens (Dégremont *et al*., 2015). In contrast, this was not studied for human pathogenic *Vibrio*. Indeed, it could be interesting to be taken into account for further analyses. Moreover, the presence of this microbiota could lead to niche competition with exogenous bacteria, i.e. bacteria experimentally accumulated. Srivastava *et al*. (2009) showed that treatment of *C. virginica* oysters, collected from environment, with tetracycline resulted in the decrease of more than 3 log CFU $g^{-1}$ of indigenous *V. vulnificus*. That treatment allowed the accumulation of *V. vulnificus* strain during experimental infection at approximately 4.5 log CFU $g^{-1}$ after a 24 h exposure at $10^6$ CFU $ml^{-1}$ which was comparable to experimental *V. vulnificus* accumulation in oysters collected during the winter months (Srivastava *et al*., 2009). In our study, oysters from intertidal environment could have indigenous *V. parahaemolyticus* that can prevent the accumulation of *V. parahaemolyticus* during experimental infection. Indeed, 60% of oyster samples ($n = 10$) were positive for the detection of *V. parahaemolyticus* by PCR in October 2019 in France (Copin *et al*., 2021). To verify this hypothesis, depuration of oysters before experimentation in water at low temperature and high salinity with variable efficiencies of depuration (Phuvasate *et al*., 2012; Phuvasate and Su, 2013) could be considered for further experimentations. Another way is the pre-treatment of oysters with antibiotics to eliminate indigenous bacteria (Srivastava *et al*., 2009).

In conclusion, we developed and validated the use of FCM as an alternative to PE for *V. parahaemolyticus* quantification in oysters using IFVp201-*gfp*$^+$ transformant. This strategy contributed to the investigation of the accumulation and depuration of *V. parahaemolyticus* in oysters, with a link with immune priming and microbiota of oysters. Further investigations are required on oyster microbiota using metagenomic to compare microbiota of naive and environmental and tentatively evaluate if there will be any correlations between higher/lower accumulation and diversities of microbiota. Moreover, it could be interesting to include season and physiological status of oysters in further studies of oyster microbiota, considering that it was shown that microbiota structure varied according to seasons (Lokmer *et al*., 2016). Finally, broader spectrum of *V. parahaemolyticus* strains, and combination of *in vivo* experimentation and genomic analyses of those strains could help to identify mechanisms implied or possibly implied in accumulation and colonization of oysters.

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## Author Contributions

Biological resources: B.P., F.D., L.D. Conception and design: M.S., D.H.H. Acquisition of data: M.S., D.H.H., C.L., M.P., S.L. Analysis and interpretation of data: M.S., D.H.H., L.D., M.P., D.P. Manuscript: M.S., D.H.H., L.D., D.P., F.D., C.L.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Fig. S1.** Experimental protocols with IFVp201-*gfp*$^{+}$ for the preliminary assay in February 2021 (A) and the challenge test with naive and environmental oysters in October 2021 (B).