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Development of a PCR-free DNA-based assay for the specific detection of *Vibrio* species in environmental samples by targeting the 16S *rRNA*

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Abstract:

A novel PCR-free DNA-based assay was developed for the detection of *Vibrio* spp. A sandwich hybridization format using an immobilized capture probe and a labeled signal probe was selected, and combined with chemiluminescent method for the detection of the RNA target. In a first step, probes were validated using positive controls (PC). A linearity was observed between 0.1 nM and 2.5 nM of PC and detection limit was determined as 0.1 nM. In a second step, specificity was checked by using RNA extracted from a panel of 31 environmental bacterial strains. Detection limit of 5 ng μL^{-1} of total fragmented RNA was obtained, and the assay allowed a good discrimination between the 21 *Vibrio* and the 10 non *Vibrio* strains tested. Finally, the DNA-based assay was successfully applied to analysis of spiked and natural environmental samples. Stability and analysis time of the DNA-based assay were also investigated to optimize working conditions. We demonstrated that microplates can be coated beforehand with capture probe and stored at 4°C without any buffer in wells for at least 30 days. The use of the pre-made plates enables the assay to be completed in 2 hours. The developed assay appeared as an interesting tool to determine the presence of bacteria in environmental samples.

Key words: PCR-free; DNA-based assay; *Vibrio* detection; sandwich hybridization assay; chemiluminescence; environmental analysis

1. Introduction

Currently, the water resource is regularly monitored for its bacteriological quality through the detection of fecal indicator bacteria such as *Escherichia coli*, total coliforms and Enterococci using culture-based methods. Nevertheless, a large diversity of non fecal pathogenic bacteria is widely distributed in aquatic environments and is potentially harmful for humans and animals. Moreover, due to environmental stresses, microorganisms may enter into a viable but non culturable cell (VBNC) state becoming undetectable using cultural methods.This physiological state has been described for several pathogenic and non pathogenic bacteria, including species belonging to the *Vibrio* genus (Oliver 2005). This genus, composed of more than 100 species (Romalde et al. 2014), is ubiquitous and abundant in aquatic ecosystems, principally in marine and estuarine waters (Narracci et al. 2014). These bacteria can survive either in the water column, in sediments and also associated to plankton, mainly due to their capability of biofilm formation (Shikuma and Hadfield 2010). Their occurrence in aquatic environments is correlated with several parameters, water temperature and salinity being the most important (Lutz et al. 2013). Furthermore, several studies have demonstrated that *Vibrio* prevalence could be impacted by human activities (Okoh et al. 2015). Forty four years of monitoring in the Coastal North Sea revealed an increase in prevalence of vibriosis correlated with elevated water temperature as a consequence of global climate change (Vezzuli et al. 2012).

Among *Vibrio* species, twelve are known to be human pathogen, including *V. cholera*, *V. parahaemolyticus*, and *V. vulnificus* as the most notable and important for public health (Tarr et al. 2007). Several other species are considered as pathogens for aquaculture, such as *V. anguillarum*, *V. salmonicida* and *V. vulnificus* that infect fish, *V. penaeicida* and *V. nigripulchritudo* infecting shrimps and *V. splendidus* related strains and *V. aestuarianus* that are shellfish pathogens (Frans et al. 2011; Vezzulli et al. 2015; Walling et al. 2009; Goarant et al. 2006). During the last decade, food-borne infections caused by the ingestion of uncooked shellfish contaminated with human pathogen *Vibrio* have significantly increased, especially in the U.S, Canada, Asia and India. While the incidence of these bacteria is currently considered as relatively low in Europe, the potential risk associated with *Vibrio* infections must be taken seriously, due to their emergence in some European countries (Le Roux et al. 2015). These last years, with the development of aquaculture, vibriosis has become one of the major causes of biological loss, particularly in France for oyster production (Petton et al. 2015; Samain and McCombie 2008). Mass mortality outbreaks caused by *Vibrio*, particularly affect early larval stages and may lead to the decimation of entire populations, becoming one limiting factor for the viability of this economic activity (Thompson et al. 2004). However, even if these significant losses in aquaculture can be attributed to the presence of pathogenic agents in seawater, these pathogens are routinely detected in animals but rarely in harvesting waters. Therefore, anticipation of the biological resource contamination is not possible.

Current standardized methods for *Vibrio* detection and quantification are based on bacteria culture on specific agar media followed by colony counting. These methods are time consuming since 2–3 days are required to obtain the initial results, and at least 7 days are necessary to confirm positive results (Cai et al. 2006). Moreover, the ability of *Vibrio* to enter into VBNC state may lead to an underestimation of the viable cell number or a failure to isolate pathogens from the sample (Griffitt et al. 2011).

A wide range of techniques based on molecular methods have replaced the cumbersome microbiological analysis by targeting specific genetic markers. Within the last years, PCR-based methods have been set up for the

detection, quantification and differentiation of several bacteria (Oliveira et al. 2005; Frahm et Obst 2003; Clifford et al. 2012), and were applied to the identification of different *Vibrio* species, such as *V. vulnificus*, *V. cholerae*, *V. alginolyticus*, *V. coralliilyticus* and *V. aestuarianus* (Tall et al. 2012; Saulnier et al. 2009; Pollock et al. 2010; Garrido-Maestu et al. 2015).

More recently, DNA-based sensors have been proposed as an alternative and effective technique for the detection of DNA or RNA sequences specific to microorganism species (Paniel et al. 2013). These kinds of sensors offer several advantages compared to PCR methods. They could allow the direct detection of genetic markers bypassing the nucleic acid amplification step and thus reducing the drawbacks linked to PCR inhibitors frequently detected in environmental and food samples. Moreover, by incorporating oligonucleotide probes immobilized on a surface, DNA-based sensors have the potential to speed the detection of nucleic acid targets and to increase the specificity and sensitivity of the analysis. To the best of our knowledge, such analytical devices reported for the detection of *Vibrio* bacteria have been mainly developed for the pathogenic *V. cholerae* species (Low et al. 2012; Patel et al. 2013; Liew et al. 2015; Yu et al. 2015; Patel et al. 2015). Nevertheless, most of these DNA-based sensors targeted DNA amplified through a PCR reaction, limiting the interest of these assays.

In the present study, a novel PCR-free DNA-based assay for the detection of *Vibrio* spp. has been investigated. The developed analytical tool was based on a sandwich hybridization assay in which RNA, as the nucleic acid target, was bound between an immobilized capture probe and a labeled signal probe. The hybridization complex was then revealed by an enzymatic system, leading to a chemiluminescent signal proportional to the entrapped target. Targeting 16S *rRNA* will not only allow the detection of culturable *Vibrio* but also the detection of *Vibrio* cells in VBNC state (Coutard et al. 2005; Schauer et al. 2015). Designed probes were first validated with positive control (PC) and detection limit was determined. Then, nucleic acids extracted from a panel of bacteria were used to assess the specificity of the probes, and finally, the assay was tested with environmental samples. Stability and storage conditions of the assay were also studied.

2. Material and methods

2.1 Bacterial strains and culture conditions

Bacterial strains used in this study were provided by various bacterial culture collections (Microbial Observatory of the LaboratoireArago (MOLA, Banyuls-sur-Mer, France), American type culture collection (ATCC) and Collection de l'Institut Pasteur (CIP, Paris, France) **(Supplementary data - Table 1)**. All environmental strains have been previously identified from the 16S *rDNA*. Marine strains were grown at 25°C in Marine Broth medium 279110 (MB, Difco™ Becton, Dickinson and Co., USA). *E. coli*, *Citrobacter* and *Pseudomonas* strains were cultivated in Tryptic Soy Broth (TSB) (Biokar, France).

2.2 Total RNA extraction from pure cultures

Twenty one *Vibrio* and 10 non-*Vibrio* strains were used to evaluate the specificity of the assay. Each strain was grown overnight in appropriate medium at 25°C. In order to optimize the amount of extracted RNA, one milliliter of the overnight culture was added to 500 µL of fresh medium and incubated 4 hours at 25°C before total RNA extraction. Then, 1.5 mL of each culture was centrifuged at 8 000 x g for 5 minutes at 4°C. Total RNA was extracted with Tri-reagent (Sigma, France) as reported by Chomczynski and Sacchi (2006) and then, resuspended in 50 µL DNase-RNase free water. RNA integrity and concentration were checked using the Agilent 2100 Bioanalyzer. Then, RNA extracts were fragmented using a 10 X fragmentation buffer (100 mM $ZnCl_2$, 100 mM Tris HCl pH 7) (Gescher et al. 2008). Briefly, 1 µL of 10 X buffer was added to 10 µL of RNA and samples were incubated at 70°C for 15 min. Fragmentation reaction was stopped with 1 µL of EDTA 0.5 M pH 8 and samples were stored at -80°C until use.

2.3 Environmental samples collection and total RNA extraction

Analytical performances of the assay were tested with environmental samples naturally or artificially contaminated by *Vibrio* spp. Samples were collected at a French North-western Mediterranean lagoon dedicated to oyster culture (Leucate), and at a North-western Mediterranean marine open bay (SOLA) (42°29N, 03°08E) located ~ 500 m offshore of Banyuls sur-Mer city, France. A seawater sample (SW) was collected at SOLA and a phytoplankton sample (PK) was collected at Leucate.

Fifty liters of SW collected at SOLA were concentrated using an ultrafiltration cartridge (Hemoflow HF80S, Fresenius, Bad Homberg, Germany) with a 20 kDa molecular weight cut off. The cartridge was back-flushed with 1 L of elution buffer prepared with the ultra-filtrated water of the sampling site, 0.01% sodium hexametaphosphate (NaPP, Sigma, France) and 0.5% Tween 80 (Sigma, France). Twenty five milliliters of concentrate was filtered on 0.4 µm polycarbonate filters. Total RNA extraction was directly performed on those filters using the Tri-reagent method. Then, this total RNA extract, named SW RNA, was spiked with total RNA extracted from a pure culture of *V. splendidus* (CIP 102 893). Each spiked sample was composed of 2 µL of SW RNA in a final volume of 10 µL. SW RNA was spiked with different quantities of *V. splendidus* RNA to obtain final concentrations of *V. splendidus* RNA ranging from 0 to 100 ng μL^{-1}. A control was performed with different concentrations of *V. splendidus* RNA alone. All the samples were tested in the developed assay.

PK sample was collected at Leucate lagoon using a plankton net (50 µm) allowing the concentration of approximately 25 m^3 of water. The net was immersed for 5 minutes in water at a very low speed of 2 knots. The phytoplankton from the net was collected in polyethylene buckets and a total of 400 mL of concentrate was recovered after sampling. Ten milliliters of the concentrate were subsampled and filtered on 0.4 µm polycarbonate filters. Total RNA extraction was performed on those filters as described above. In parallel, in order to estimate the number of *Vibrio* cells in the PK concentrate, 100 µL of concentrate were spread at ten-fold dilutions on TCBS agar. Five replicates were performed for each dilution and the number of CFU mL^{-1} was determined after 24 h of incubation at 25°C.

For all environmental samples, RNA integrity and concentration were checked using the Agilent 2100 Bioanalyzer. Before analysis, RNA was fragmented as previously described and stored at -80°C.

2.4 Oligonucleotide probes design and use

Capture and signal probes were both designed to hybridize with two adjacent sequences located on the 16S *rRNA* of all *Vibrio* species. The probes were checked *in silico* for the specific recognition of all the members of *Vibrio* genus using the nucleotide Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi) against GenBank (http://www.ncbi.nlm. nih.gov/genbank/) and Silva (http://www.arb-silva.de/) databases. For the two probes, no perfect match was observed *in silico* with non *Vibrio* species. Probes biophysical properties were analyzed using the Oligonucleotide Properties Calculator software (http://www.basic.northwestern.edu/biotools/oligocalc.html). The 35 bp capture probe was biotinylated in 5', and the 35 bp signal probe was labeled with a digoxigenin (DIG) in 3' (Table 1). A 70 bp synthetic oligonucleotide corresponding to the complementary strand of both capture and signal probes was used as positive control (PC). Probes and PC were synthesized by Microsynth (Switzerland) and were diluted in DNase-RNase free water to obtain 100 µM stock solutions. Capture probe solutions were prepared at the appropriate concentration in 0.1 M Tris HCl, 0.3 M NaCl, pH 7.6 and were denatured by 3 min at 90°C, 2 min at 4°C and then 5 min at room temperature, before use.

2.5 DNA-based assay development for Vibrio spp. detection

NeutrAvidin was purchased from Thermo Fischer Scientific and all the buffer components from Sigma (France). Hammerstein-grade casein was purchased from MP Biomedicals (France) and anti-digoxigenin-horseradish peroxidase conjugate (Anti-DIG-HRP) was purchased from Roche (France). All solutions were prepared with diethyl pyrocarbonate-treated distilled water to inactivate RNase enzymes in prepared solutions.

Protocol was adapted from Paniel and Baudart (2013). MaxiSorp™ microplates (Nunc, Denmark) were coated with 100 µL of 1 µg mL^{-1} NeutrAvidin in HEPES 0.1 M, pH 7.2, for 1 h at room temperature. Then, the NeutrAvidin excess was eliminated by 3 washing steps using 1X PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4; pH 7.4).

Three strategies were then assessed. In the first one, pre-hybridization mix containing 25 µL of 4X hybridization buffer (4X HYB buffer: NaCl 0.3 M, Tris 80 mM, SDS 0.04%; pH 8), 10 µL of 1 µM denatured capture probe, 10 µL of 1 µM labeled signal probe, 10 µL of target (i.e PC or RNA or fragmented RNA) and 45 µL of ultra pure water was incubated for 10 min at 65°C. Then, one hundred microliters of this pre-hybrization mix were added to the wells and the microplate was incubated for 30 min at 46°C. Wells were washed 3 times with 1X HYB buffer. Unspecific sites were blocked with 1% Hammerstein casein in binding buffer (PBS 1X, 1 mM $MgCl_2$, 0.01% Tween 20; pH 7.4) for 45 min at room temperature and casein excess was removed by 3 washing steps using 1X HYB buffer.

In the second strategy, pre-hybridization mix containing 25 µL of 4X HYB buffer, 10 µL of 1 µM capture probe, 10 µL of target and 55 µL of ultra pure water was incubated for 10 min at 65°C. One hundred microliters of the pre-hybrization mix were added to each wells and the microplate was incubated for 30 min at room temperature. Wells were washed 3 times with 1X HYB buffer and unspecific sites were blocked as previously described. One hundred microliters of 0.1 µM signal probe were added to the microplate which was then incubated for 30 min at 46°C. Wells were then washed 3 times with 1X POP buffer (NaH_2PO_4 50 mM, NaCl 0.1 M; pH 6.45).

In the third strategy, one hundred microliters of 0.1 µM denatured capture probe were added to each wells and the microplate was incubated for 30 min at room temperature. Wells were washed 3 times with 1X HYB buffer. Unspecific sites were blocked as previously described. Pre-hybridization mix containing 25 µL of 4X HYB buffer, 10 µL of 1 µM labeled signal probe, 10 µL of target and 55 µL of ultra pure water was incubated for 10 min at 65°C. One hundred microliters of the pre-hybrization mix were added to the wells and the microplate was incubated for 30 min at 46°C. Wells were then washed 3 times with 1X POP buffer.

Then, the revelation of the sandwich hybridization was performed in the same way for the three strategies. One hundred microliters of anti-DIG-HRP conjugate at 50 mU mL^{-1} in PBS-T-BSA (PBS 1X, BSA 0.1%, Tween 20 0.05%; pH 7.4) were added to the wells and the microplate was incubated for 20 min at room temperature. Wells were washed using 1X POP buffer.

In this work, two detection methods were used. For colorimetric detection, 100 µL of ready-to-use 3,3′,5,5′-Tetramethylbenzidine substrate (TMB) (Sigma, France) were added to the wells, and after 15 min of incubation at room temperature optical density was measured at 630 nm using the Multiskan Ascent™. For chemiluminescent detection, 100 µL of luminol-H_2O_2 (Luminata forte™ ELISA HRP Substrate, Merck Millipore, France) were added to the wells and after 5 min of incubation at room temperature luminescence was measured using the Fluoro-Luminoskan Ascent™ FL (Thermo Scientific, France). For the specificity tests, all the values were standardized as followed: standardized value = measured value / 1 nM of PC value.

2.6 Storage and stability study of the DNA-based assay

To minimize experimental variations, all the solutions were prepared at day 0 and used until the end of the experiment. NeutrAvidin, capture probe and casein were successively added to the wells of several strip plates following the third protocol described above. Then, two storage conditions were tested. Strip plates were directly stored at 4°C with casein solution inside each well, or they were washed to eliminate the excess of casein and stored at 4°C without any buffer. For both conditions, a lid was placed on the top of each plate, which was then packed inside an aluminum foil. Then, hybridization and enzymatic revelation were carried out at day 0 as the reference point and at different times during 30 days (d1, d2, d3, d4, d7, d8, d10, d14, d16, d21, and d30) as previously described. Colorimetric detection was used. Results were expressed as: (value at d_x * 100) / value at d0 with d_x : day of the experiment (d1 to d30).

3. Results and discussion

3.1 Design strategy of the DNA-based assay

In this work, sandwich hybridization format was chosen to optimize the recognition of the target, and thus to improve the specificity of the assay. Three different hybridization strategies were investigated to determine the optimal working conditions. In the first strategy (Fig. 1a), pre-hybridization was carried out by mixing together all the components. Then, this mix was added to the microplate wells for NeutrAvidin binding. In the second strategy (Fig. 1b), the target was firstly pre-hybridized with the capture probe. This solution was then added to the wells for NeutrAvidin binding, and followed by signal probe addition. In the third strategy (Fig. 1c), capture probe was immobilized alone inside the wells. Pre-hybridization between the target and the signal probe was performed, and then this mix was added to the wells.

Comparison of the three strategies was performed using 50 nM of PC. For all experiments, the signals were at least 3 fold higher than the negative control without PC. The best response was achieved when the denaturated capture probe was immobilized alone in each well. This third strategy allowed a signal response 10 and 4 fold higher than the second and the first strategy, respectively (data not shown). These lower results could be explained by the steric hindrance of the biotinylated complex that could decrease the binding to NeutrAvidin, and highlight the importance of the binding step between the biotinylated capture probe and NeutrAvidin to optimize the signal response of our assay. According to those results, the third strategy (Fig. 1c) was selected for subsequent experiments.

3.2 Validation of the DNA-based assay

In order to assess the sensitivity of our system, a calibration curve was performed using PC. Chemiluminescent responses obtained for the different concentrations of PC from 0.1 nM to 25 nM are shown in Fig. 2. As expected, the intensity of the responses was proportional to the PC concentration. Moreover, the results showed that the chemiluminescent responses were linear over the range from 0.1 nM to 2.5 nM of PC (Fig. 2b). The correlation coefficient (R^2) value was 0.997, and the relative standard deviation (RSD) average of 8% indicated a good reproducibility of the assay. Due to the low RSD, the detection limit of the assay was determined, based on the threshold value of 3 times the background signal, as 0.1 nM corresponding to 2.17 pg μL^{-1} of PC.

After optimizing the experimental parameters with PC, assays were performed with 20 ng μL^{-1} of fragmented and non fragmented RNA extracted from three different species belonging to the *Vibrio* genus (*V. splendidus* ATCC 33125, *V. pomeroyi* CIP 108273 and *V. crassostreae* CIP 108327). For the three RNA samples, the obtained responses were 7 to 10 fold higher with fragmented RNA than with non fragmented indicating a positive effect of the fragmentation step on the RNA target (data not shown). For subsequent experiments RNA was always fragmented before analysis.

Various concentrations of total RNA from *V. splendidus*, used as reference strain, were assessed to validate the assay with nucleic acid target and to determine the optimal RNA concentration to be used for the specificity tests. As previously shown with PC, the intensity of the chemiluminescent responses was proportional to the total RNA concentration. Linear responses were observed between 1 and 100 ng $\mu L^{-1}$of total RNA, with a R^2 value of 0.995 (Fig. 3). Based on the threshold value of 3 times the background signal, the detection limit was determined as 5 ng μL^{-1} of total RNA which was 2 300 times higher than the detection limit observed with PC (2.17 pg μL^{-1}). This observation could be explained by the proportion of target. Indeed, the targeted 16S *rRNA* only represents a fraction of the total RNA. Moreover, instead of the PC which is a short 70-bp linear sequence, 16S *rRNA* is longer (1 500 bp) and can present some secondary and tertiary structures as hairpins that can interfere with probes hybridization.

For 20 ng μL^{-1}, the measured chemiluminescence was 4 fold higher than those measured for the detection limit value. Similar results were obtained for the two other *Vibrio* strains *V. pomeroyi* and *V. crassostreae* (data not shown). Thus, 20 ng $\mu L^{-1}$of total RNA is sufficient to clearly detect *Vibrio* RNA in the sample.

3.3 Specificity evaluation of the DNA-based assay

Regarding our previous results obtained with total RNA, a concentration of 20 ng μL^{-1} was retained to perform specificity tests. In order to standardize the chemiluminescent responses between experiments, the value obtained for 1 nM of PC was used as the reference.

Twenty one *Vibrio* strains belonging to seven different clades (Splendidus, Harveyi, Coralliilyticus, Mediterranei, Halioticoli, Rumoiensis and Anguillarum clades) and ten strains belonging to ten others bacterial genera were assessed (Fig. 4).

The results showed that a significant response was observed for the 21 *Vibrio* strains tested. The lower signal was obtained for *V. natriegens*, with a luminescence value 55 times higher than the background signal. All the other *Vibrio* strains tested gave higher signals, with a coefficient factor varying from 1.1 to 6.6 of the signal obtained for *V. natriegens*. The highest signal was observed for *V. gigantis*, with a luminescence value 243 times higher than the background signal.

For the 10 non *Vibrio* strains, observed chemiluminescence values were below the detection limit of the system for 4 strains. For the 6 others, the signal was therefore 2 to 10 times lower than the chemiluminescence value observed for *V. natriegens*, the *Vibrio* strain having the lowest signal. *Photobacterium lutimaris*, the closest relative species to *Vibrio* (Anzai et al. 2000), showed the highest signal. Interestingly, for all the non *Vibrio* strains showing a weak signal, no perfect match was observed *in silico* with the two probes. However, both signal and capture probes present mismatches at either the 3' or 5'-end. These observations are consistent with the results obtained by Letowski et al. (2004) demonstrating that probes hybridization could occur even in presence of mismatches located at the probe ends.

Among the 21 *Vibrio* strains tested, an important variation in the signal intensity can be noticed. We hypothesized that the variation of 16S *rRNA* operon number between the tested strains could explain these results. Then, the number of 16S *rRNA* operon was sought from *rrn* database (Wuyts et al. 2004) and collected

only for 4 strains of our study. Interestingly, the strains *V. splendidus* (CIP 107715), *V. alginolyticus*, *V. harveyi* and *V. natriegens* possess 8, 11, 8-11 and 13, 16S *rRNA* operons respectively but no correlation was observed with the signal responses since luminescence values of 0.713, 0.365, 0.417, and 0.154 were respectively obtained for these strains. Then, another hypothesis could be formulated to explain the obtained results. Since RNA was randomly fragmented with a fragmentation buffer, the length of the generated fragments cannot be controlled and can vary between *Vibrio* strains due to the differences of sequences. In similar works reported in the literature, specificity tests are routinely performed, but to the best of our knowledge, only on a limited number of species and signal variations observed between targeted species or strains were never explained (Low et al. 2012; Liew et al. 2015).

Nevertheless, we can conclude that a good discrimination between the 21 *Vibrio* strains and the 10 non *Vibrio* strains was observed, indicating a high specificity of the developed assay.

3.4 Storage and stability study of the DNA-based assay

To investigate the stability of the developed assay, a preservation test of the capture probe coated inside microplate wells was performed during 30 days. Storage conditions were assessed using 2 concentrations of PC, 50 nM and 250 nM. Assay responses over time were compared to the initial response value of day 0 (d0) (Fig. 5). For microplates stored at 4°C with casein in wells, signals were similar the first two days and then decreased from d2 to d7 for both target concentrations. The responses were then stable until d21 before sharply decreasing at d30. Since d3, responses were lower than the initial response. For both PC concentrations, maximal signal was observed at d2 and minimum signal at d30. Between these two days, a signal decrease of 77 % was observed for 250 nM of PC and for 50 nM of PC this decrease reached 93 %. Interestingly, for microplates stored at 4°C without casein, responses between d1 and d30 were a slightly higher than those measured at d0. For both PC concentrations, signals were stable over time. For 50 nM of PC, maximal signal was observed at d2 and minimal signal at d30 corresponding to a signal decrease of 32 % between these two days. For 250 nM of PC, maximal signal was obtained at d3 and minimal signal at d30 corresponding to a signal decrease of 26 %. Furthermore, for both PC concentrations, the measured values were also higher than those obtained at the same time for microplates stored with casein. We could hypothesize that an increase of casein adsorption on well surface could occur over time leading to a decrease in the measured signal from d2 to d30. The second storage condition appeared as the better way to preserve microplates. Indeed, the assay components were stable for at least 30 days and the signal stable over time. These results demonstrated that microplates can be coated beforehand, and stored at 4°C without casein until use allowing a reduction of 3 hours in experiment time. The use of pre-made plates enables the assay to be completed in 2 hours.

3.5 Environmental samples analysis

To our knowledge few studies have reported the application of DNA-based biosensors for environmental samples analysis. However, when biosensors are developed for in field application, this step is essential. For complex samples, the performances of such devices could be different from those obtained with bacterial strains growing under laboratory conditions due to matrix interferences. Thus, our developed assay was assessed with

two different environmental samples (seawater and phytoplankton) to evaluate the matrix effect, and to validate the suitability of the method for *Vibrio* detection.

First assay was assessed on SOLA SW sample. A previous analysis by a TaqMan qPCR targeting the 16S *rDNA* gene of *Vibrio* spp. was performed on total extracted DNA. No genome unit (GU) was detected in this sample, indicating that the concentration of *Vibrio* spp. estimated by qPCR was under the quantification limit of the assay (833 GU L^{-1} of SW) (personal data). In a second step, SW RNA was analyzed using the developed DNA-based assay. To test the matrix effect, a same quantity of SW RNA was spiked with known concentrations of total RNA extracted from *V. splendidus* ranging from 0 to 100 ng μL^{-1}, and the results were compared with those obtained with total RNA of *V. splendidus* alone (Fig. 6a). The analysis performed on SW RNA without addition of *V. splendidus* RNA showed a positive signal. The positive signal was linked to the presence of RNA from *Vibrio* in the sample. This genus is known to be a common inhabitant of the coastal marine areas, as demonstrated by a recent study that showed that *Vibrio* abundances at SOLA site ranged from 7 CFU L^{-1} to 2.7. 10^4 CFU L^{-1} over the year (unpublished work). Interestingly, SW RNA sample without spiked RNA was positive for *Vibrio* spp. analysis using the reported device but negative using qPCR. This negative result could be explained by the dilution procedure applied to extracted DNA before qPCR analysis, required to limit the matrix inhibitors effect, but leading to a decrease of sensitivity. The linearity of the chemiluminescent response was measured for the SW RNA spiked samples and also for the *V. splendidus* RNA alone, given a R^2 values of 0.997 and 0.989, respectively (data not shown). The signals measured in the spiked samples were 2.2 to 7.2 fold higher than those measured with the RNA from *V. splendidus* alone confirming the detection of environmental population of *Vibrio* already present in SW sample using the reported device.

PK samples from Leucate were also assessed since *Vibrio* bacteria are known to be tightly associated with phytoplankton (Huq et al. 2005, Turner et al. 2009), and use the phytoplankton extracellular matrix as nutrient sources (Thompson et al. 2004). As shown in Fig. 6b, a linear relationship was observed between the measured chemiluminescence and the total RNA concentrations of PK RNA. The R^2 value was 0.980, indicating that our system allowed the specific detection of *Vibrio* RNA from a total RNA sample extracted from an environmental sample, in the range of 10 to 100 ng μL^{-1}. However, important standard deviations were observed that could be linked to the sample matrix. These samples were collected by filtering 25 m^3 of seawater to concentrate phytoplankton. Phytoplankton can produce exopolysaccharides (Passow 2002) and some of these complex molecules could be extracted with RNA. Polysaccharides are well known as PCR inhibitors (Schrader et al. 2012). They have the ability to co-precipitate with nucleic acids, reducing the ability to resuspend the precipitated RNA (Butot et al. 2007). Furthermore, they can cross link with nucleic acids and change their chemical properties (Opel et al. 2010). In our case, polysaccharides may contribute to heterogeneous RNA solutions and may hide some hybridization sites. This could explain the differences observed between triplicates. By consequence, volumes of filtered water have to be optimized as well as the filtration method before analysis with the assay.

In order to facilitate evaluation of the assay performance, a detection limit corresponding to a number of cells per sample volume has been estimated for the PK sample. For this sample, *Vibrio* cell number has been determined by plate counting on TCBS agar as $6.17.10^4$ CFU mL^{-1} of concentrate. Thus, the *Vibrio* cell number in the 400 mL concentrate was $2.5.10^7$. Since the concentrate has been obtained by filtering 25 m^3 of water, the developed tool allows the detection of 1 000 CFU L^{-1}.

4. Conclusion

In this paper, a PCR-free DNA-based assay for the detection of *Vibrio* spp. in environmental samples was developed. The resulting assay showed an excellent specificity since a high discrimination was observed between the 21 *Vibrio* strains and the 10 closely related genera tested. The combination of sandwich hybridization format and chemiluminescent detection allowed a direct analysis of the extracted RNA, suppressing the PCR amplification step routinely performed in such analytic techniques. Detection limits of 0.1 nM and 5 ng μL^{-1}were obtained with synthetic probes and total RNA respectively, indicating a good sensitivity of the reported system.

To optimize experiment time, microplates were coated beforehand and various storage conditions were assessed. Storage at 4°C without casein not only showed that components were stable for at least 30 days, but also decreased the analysis time to 2 hours, allowing a rapid detection of *Vibrio* spp. using the developed device.

The results with spiked and natural environmental samples indicated that the proposed method appeared as an interesting tool to determine the presence of bacteria in aquatic samples. In future works, optimization of samples preparation and transfer towards an electrochemical format will be performed in order to improve *Vibrio* detection in aquatic samples. Furthermore, correlation between signal intensities and cell counts from environmental samples will be investigated.

Conflict of interest

The authors declare that they have no conflict of interest.

Complience with ethical standards

All authors agreed to be listed and have approved of the manuscript, its content and its submission to Environmental Science and Pollution Research. It has not been submitted or published elsewhere, whether partly or fully. All authors are in agreement with the ethical rules of ESPR.

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Captions (Table and figures)

Table 1 Synthetic DNA probes and positive control used for the developed DNA-based assay

Fig. 1 Schematic illustration of the sandwich hybridization assay. (a) first strategy ; (b) second strategy ; (c) third strategy

Fig. 2 Calibration curve (a) and linear part of the calibration plot (b) obtained in the presence of different concentrations of PC with the DNA-based assay using chemiluminescence detection. Error bars are standard deviations of the mean with n=3

Fig. 3 Calibration curve obtained for a range of total RNA concentration from *V. splendidus* with the DNA-based assay using chemiluminescence detection. Error bars are standard deviations of the mean with n=3

Fig. 4 Specificity test of the DNA-based assay using 20 ng μL^{-1} of total RNA extracted from several bacterial strains and using chemiluminescence detection. Both values obtained for each duplicates are shown. Red : *Vibrio* strains value 1 ; Pink : *Vibrio* strains value 2 ; Dark blue : non *Vibrio* strains value 1 ; Clear blue : non *Vibrio* strains value 2. * Non referenced strain, isolated from freshwater

Fig. 5 Stability study of the microplates coated with the capture probe, stored up to 30 days at 4°C (a) with casein and (b) without casein in wells. Hybridization, using 50 nM of PC (white bars) or 250 nM of PC (orange bars), and 0.1 µM signal probes, and enzymatic revelation using colorimetry detection were performed just after coating (day 0) or after storage for several days at 4°C (days 1 to 30). Values were expressed using day 0 as the reference point

Fig. 6 Environmental sample analysis using the DNA-based assay. (a) Matrix effect of SOLA samples was assessed by comparison of *V. splendidus* RNA analysis (black bars) with SW RNA analysis (white bars), alone (0), or spiked with different concentrations of *V. splendidus* RNA (10, 50, and 100 ng μL^{-1}) using chemiluminescence detection. (b) RNA extracted from Leucate PK samples were analysed using chemiluminescence detection. Error bars are standard deviations of the mean with n=3

Table

Table 1

Oligonucleotide name	Sequence (5'-3')	Modification
VibCap	TTGAGCCCCGGGCTTTCACATCTGACTTAATGAAC	5' Biotin
VibSig	ACCTGCATGCGCTTTACGCCCAGTAATTCCGATTA	3' Digoxigenin
PC	TAATCGGAATTACTGGGCGTAAAGCGCATGCAGGTG TTCATTAAGTCAGATGTGAAAGCCCGGGGCTCAA	None

Figure1

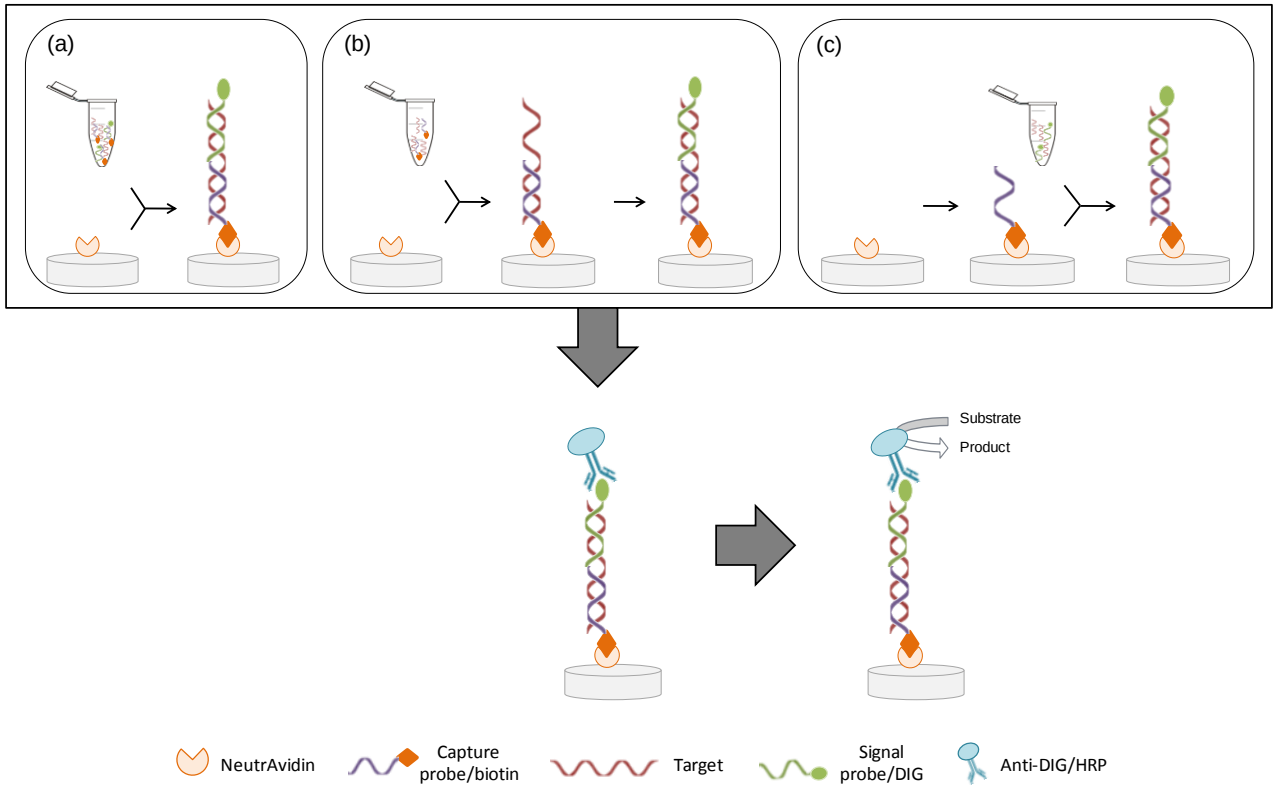

(a)
(b)
(c)
Substrate
Product
NeutrAvidin
Capture probe/biotin
Target
Signal probe/DIG
Anti-DIG/HRP

Figure2

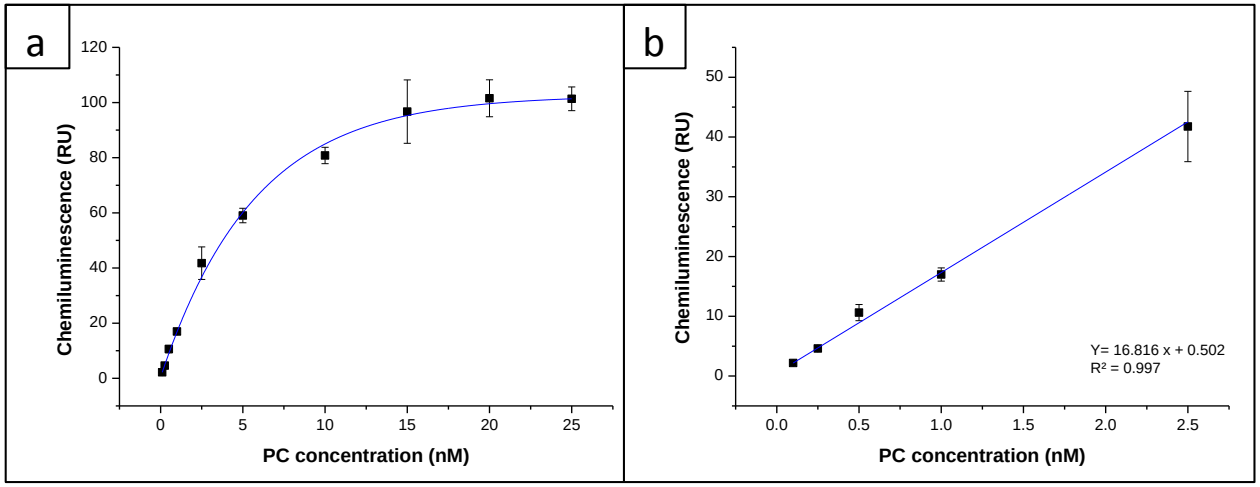

a
Chemiluminescence (RU)
PC concentration (nM)
b
Chemiluminescence (RU)
PC concentration (nM)
Y= 16.816 x + 0.502
R² = 0.997

Figure3

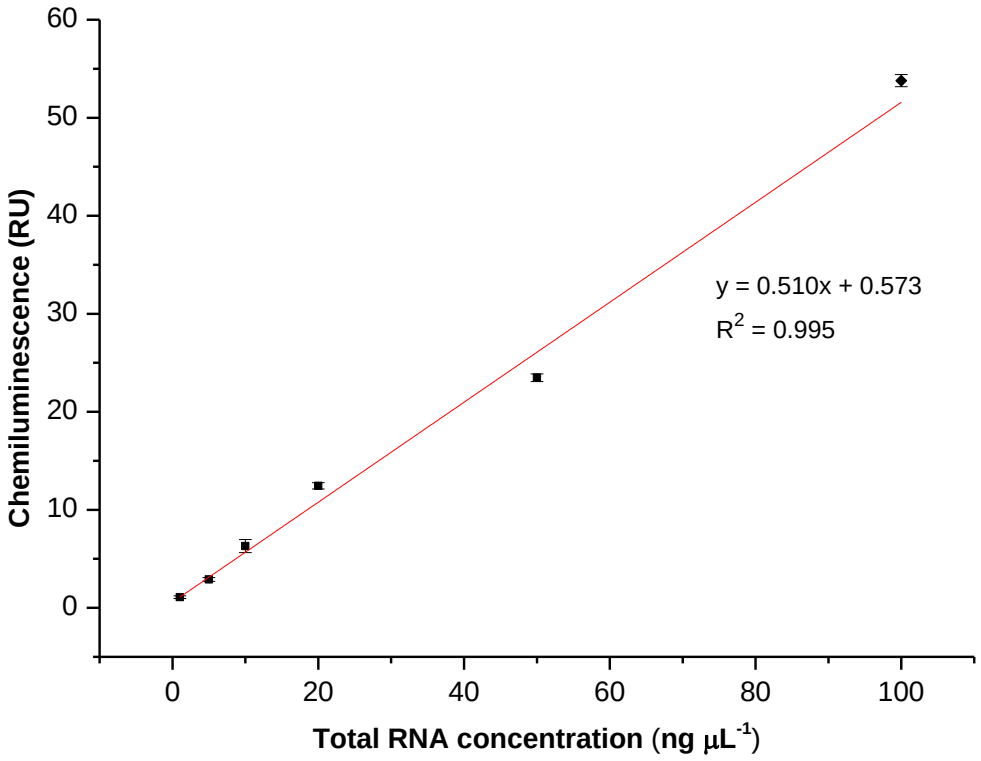

Chemiluminescence (RU)
Total RNA concentration (ng μL-1)
y = 0.510x + 0.573
R2 = 0.995
0
10
20
30
40
50
60
0
20
40
60
80
100

Figure4

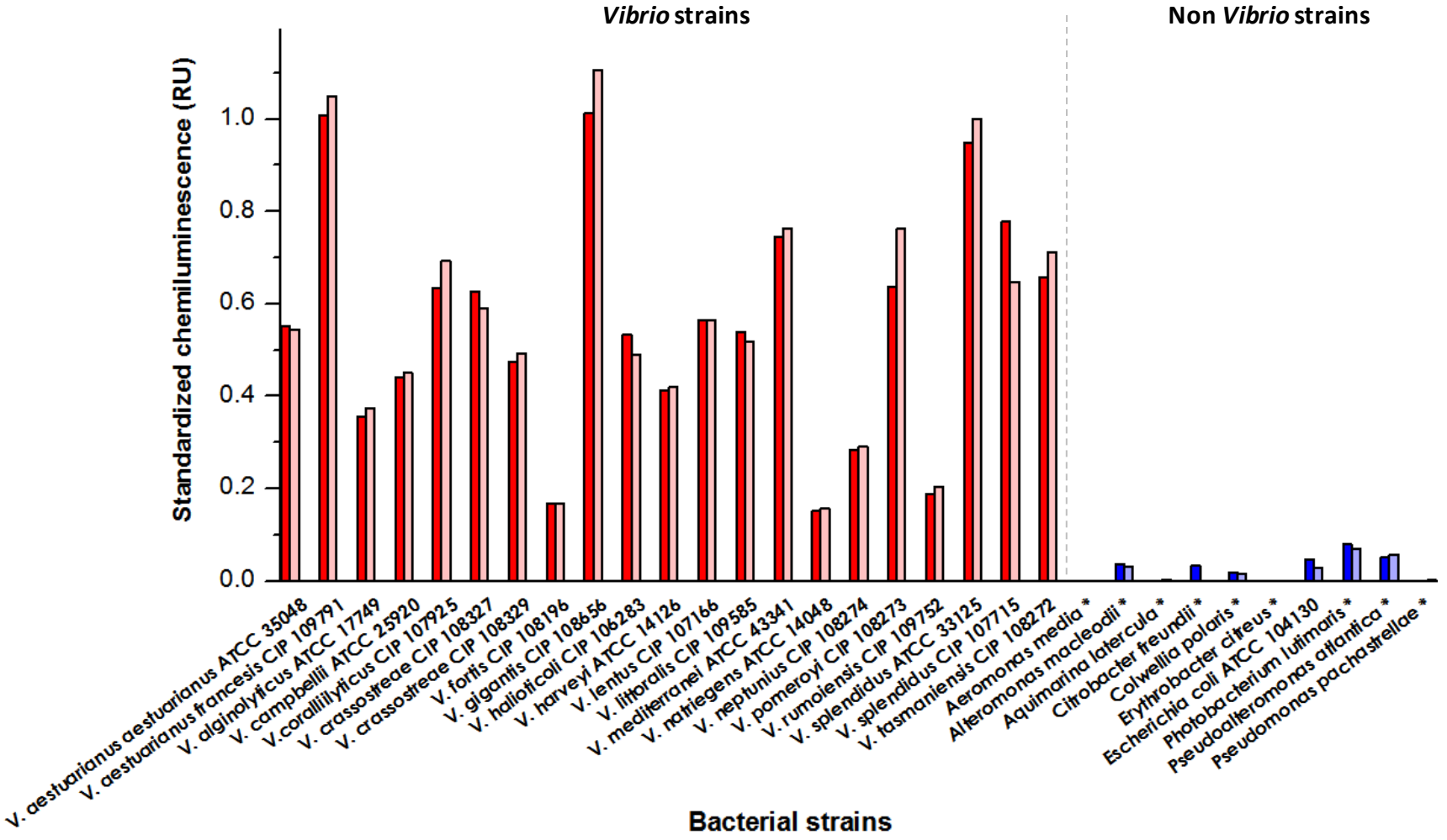

Vibrio strains
Non *Vibrio* strains
Standardized chemiluminescence (RU)
0.0
0.2
0.4
0.6
0.8
1.0
V. aestuarianus aestuarianus ATCC 35048
V. aestuarianus francesis CIP 109791
V. alginolyticus ATCC 17749
V. campbellii ATCC 25920
V.coralliilyticus CIP 107925
V. crassostreae CIP 108327
V. crassostreae CIP 108329
V. fortis CIP 108196
V. gigantis CIP 108656
V. halioticoli CIP 106283
V. harveyi ATCC 14126
V. lentus CIP 107166
V. littoralis CIP 109585
V. mediterranei ATCC 43341
V. natriegens ATCC 14048
V. neptunius CIP 108274
V. pomeroyi CIP 108273
V. rumoiensis CIP 109752
V. splendidus ATCC 33125
V. splendidus CIP 107715
V. tasmaniensis CIP 108272
Aeromonas media *
Alteromonas macleodii *
Aquimarina latercula *
Citrobacter freundii *
Colwellia polaris *
Erythrobacter citreus *
Escherichia coli ATCC 104130
Photobacterium lutimaris *
Pseudoalteromonas atlantica *
Pseudomonas pachastrellae *
Bacterial strains

Figure5

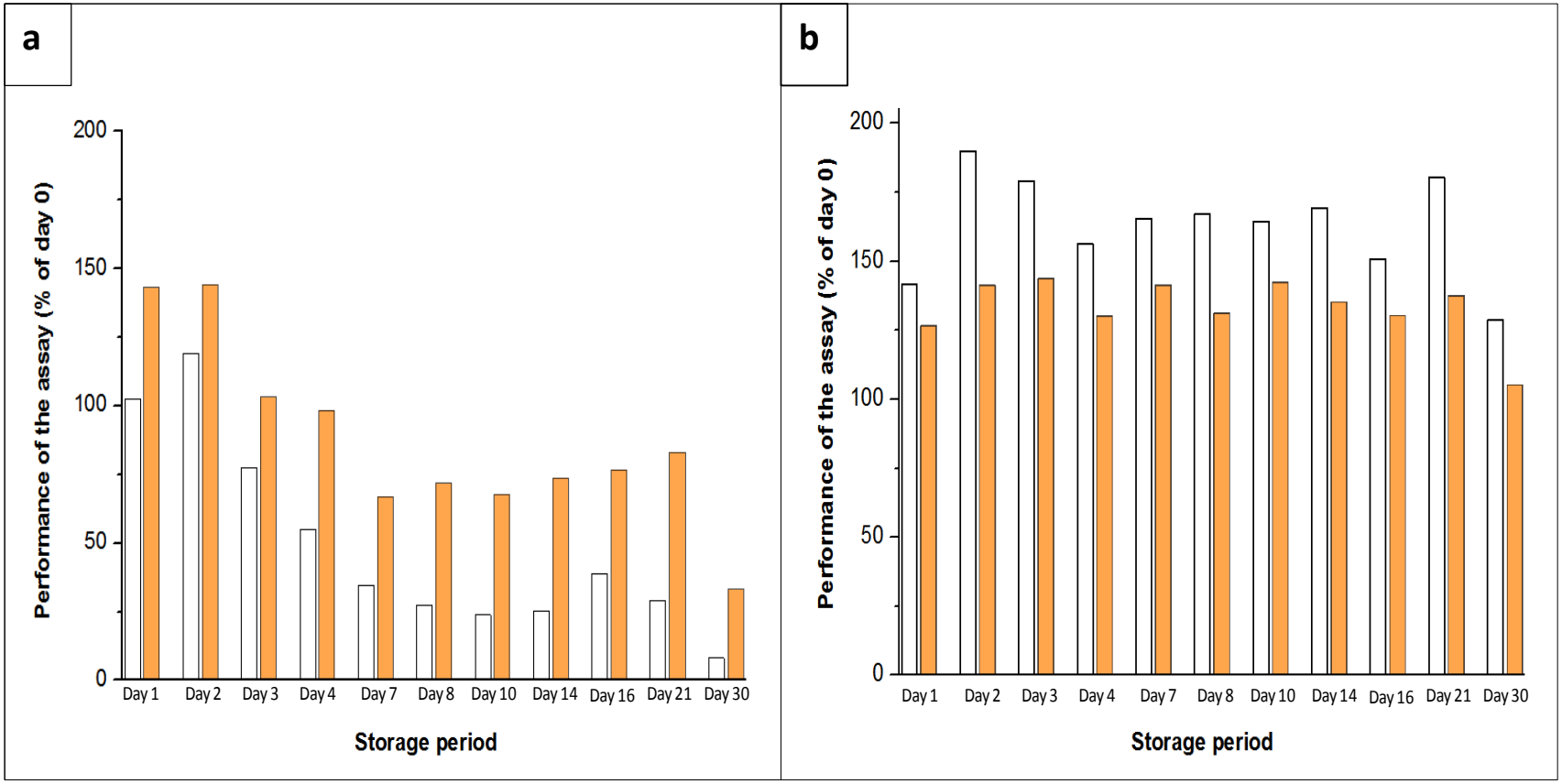
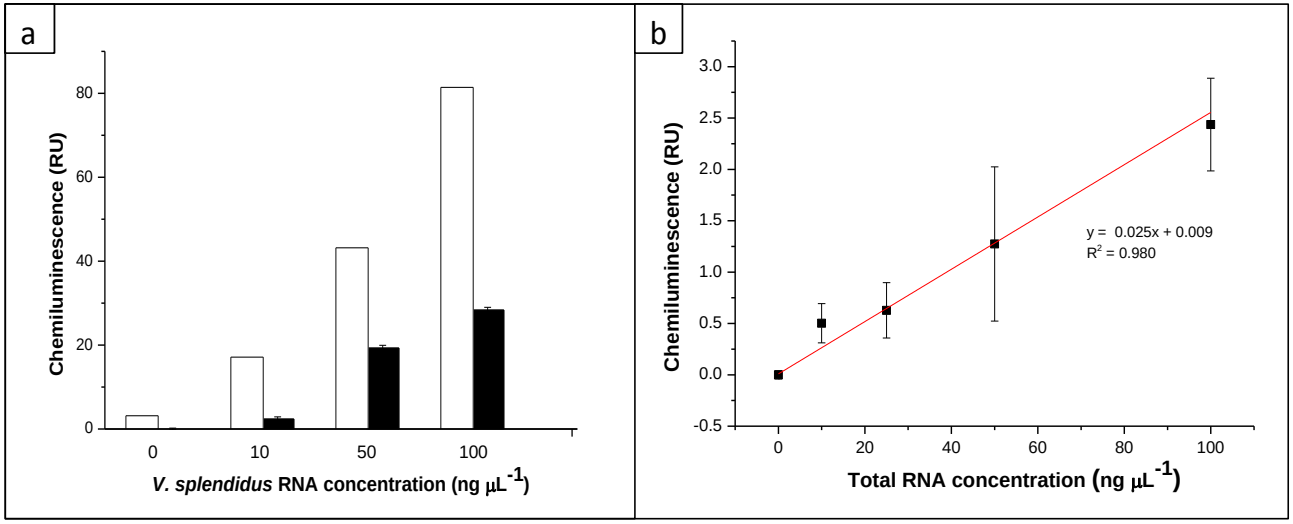

a
Performance of the assay (% of day 0)
200
150
100
50
0
Day 1
Day 2
Day 3
Day 4
Day 7
Day 8
Day 10
Day 14
Day 16
Day 21
Day 30
Storage period
b
Performance of the assay (% of day 0)
200
150
100
50
0
Day 1
Day 2
Day 3
Day 4
Day 7
Day 8
Day 10
Day 14
Day 16
Day 21
Day 30
Storage period

Figure6


a
Chemiluminescence (RU)
0
20
40
60
80
0
10
50
100
V. splendidus RNA concentration (ng μL-1)
b
Chemiluminescence (RU)
-0.5
0.0
0.5
1.0
1.5
2.0
2.5
3.0
0
20
40
60
80
100
y = 0.025x + 0.009
R2 = 0.980
Total RNA concentration (ng μL-1)