

## **Effect of concentrating and exposing the bioluminescent bacteria to the non-luminescent allo-bacterial extra cellular products on their luminescence**

Ravindran J<sup>1</sup>, Geetha Priya G<sup>2</sup> and \*Kannapiran E<sup>3</sup>

<sup>1</sup> National Institute of Oceanography, Regional Centre, Council of Scientific and Industrial Research (CSIR),  
Dr. Salim Ali Road, PB. No. 1913, Kochi – 682 018, India

<sup>2</sup> Department of Biotechnology, Bishop Heber College, Tiruchirappali – 620017, Tamil Nadu, India

<sup>3</sup> Alagappa University, Directorate of Distance Education, Karaikudi – 630 003, Tamil Nadu, India

Correspondance to Dr. E. Kannapiran, Directorate of Distance Education, Alagappa University,  
Karaikudi – 630 003, Tamil Nadu, India

### **Abstract:**

Bioluminescence is a biochemical process occurring in many organisms. Bacterial bioluminescence has been investigated extensively that lead to many application of such knowledge. Quorum sensing in the bioluminescent bacteria is a chemical signal process to recognize the strength of its own population to start luminescence in harmony. There is a mechanism in these bacteria to recognize inter-species strength also. When there is higher number of these bacteria, the possibility and frequency of cell-cell physical contact will be high. In this study, the physical proximity was artificially enhanced between cells and the effect on luminescence in the concentrated cells in the normal culture medium and in presence of other non-bacterial cell free supernatant were investigated. The role of such physical contact in the quorum sensing in the bioluminescence is not known. Increase in the luminescence of *V. fischeri* when concentrated shows the presence of physical proximity facilitates the quorum sensing for their bioluminescence.

**Key words:** *V. harveyi*, *V. fisheri*, *V. parahaemolyticus*, Luminescence, Quorum sensing

## Introduction

Bioluminescence is the production and emission of visible light as a result of a chemical reaction. During this process chemical energy of an exergonic reaction is converted to light energy which may or may not be visible. The mechanism of light emission is an enzyme mediated intracellular process in which the luciferase enzyme catalyses the oxidation of the long chain aldehydes and FMNH<sub>2</sub> into organic acids and FMN respectively (1). Light emission is the by product of this reaction.

All bacteria communicate by a process called quorum sensing in which the bacteria secrete small molecules and the other bacteria with the help of selective receptors sense the same (2). The low molecular weight molecules secreted are called autoinducer that act as “Molecular Messengers” (3). The auto inducers increase with increase in bacterial population and are able to involve in quorum sensing cascade only after reaching a threshold concentration (4). Quorum sensing is ineffective in case of single bacterial activity and is beneficial when a large number of bacteria act as multicellular organisms.

There are two types of auto inducers, AI-1 and AI-2 secreted by bacteria. The biochemical nature of AI-1 is Acyl home serine lactone (AHL) and the AI-2 is furanosyl-borate-diester (5). Intra species quorum sensing in gram negative bacteria is carried out by AHL molecules and whereas the interspecies communication is mediated by autoinducer-2 (AI-2) (6). This type of signaling between cells can lead to the co -ordination of group bacterial activities. AI-2 is reported to be an universal communication molecule and the AI-2 from other organisms could trigger luminescence in the *V. harveyi* (5). Another autoinducer namely CAI-1 (for *cholerae* autoinducer 1) was reported by Miller et al. (7) and its structure has not been determined. This autoinducer is found to be novel as this is not to be similar to Lux M or any other synthases reported previously. AI-1, CAI-1 and AI-2 are suggested to mediate intraspecies, intra-genera and inter-species cell–cell communication respectively (8).

There were 40 different bacterial species have been shown to possess quorum sensing system based on the AHL as autoinducers (9, 10). It is not known if there is any role for the physical contact of the bacterial cells in the quorum sensing. In this study the role of the physical proximity while artificially concentrating the bioluminescence bacterial cultures and their luminescence in presence of non-luminescent bacterial extra-cellular product were investigated.

## **Materials and Methods**

### **Isolation of Bacteria**

The water sample was collected from a shrimp culture farm located at south east coast of Tamil Nadu, India. The collected water samples were transported to the field laboratory in ice within two hours. The water sample was serially diluted and spread plated in Luminous Agar medium (Nutrient broth 8.0g; NaCl 30g; Glycerol 3g; CaCO<sub>3</sub> 5g; Agar 15g; Seawater 1000ml;pH 7.2.). The plates were then incubated at 37°C for 24 hours under dark. Luminescent colonies were picked by observation in dark. Morphologically distinct and brightly glowing colonies were purified further in the fresh Luminous Agar plates. A common non-luminescent bacteria screened by the morphological characters were isolated in the Luminous Agar medium and purified. The isolated bacteria were identified based on the 16S rDNA sequences using universal primers.

### **Concentrating bacteria in the water sample**

The water sample was taken in the three 50ml centrifuge tubes and spun at 20,000 RPM for 20 min in the centrifuge (REMI India; Model R-24) to pelletise the contents. The pellets were suspended in the 1.5 ml, 1 ml and 0.5 ml of filter-sterilized seawater to make it 20 times, 50 times and 100 times concentrated respectively. All the preparations were in triplicates. Similarly, after filtering the water sample through 5µm mesh size plankton net to eliminate planktonic organisms, 20, 50 and 100 times concentrates were prepared. Initial luminescence reading was measured in the freshly collected sample and after passing through the plankton net using a Luminometer (Turner, USA; Model 20/20n). Luminescence expressed in relative light units (RLU). Luminescences of the concentrated samples were measured immediately after preparation as 0 hr and measured upto 8 hrs with 1 hr interval.

### **Preparation of Cell Free supernatant**

The purified non-luminescent bacterium was inoculated in the luminous broth (Nutrient broth 8.0g; NaCl 30g; Glycerol 3g; CaCO<sub>3</sub> 5g; Seawater 1000ml; pH 7.2). The luminous broth tubes were incubated at 37°C for 24 hours. The cell free extract was prepared by removing the cells from the growth medium by centrifugation at 20,000rpm for 20 minutes. The cell free extract was further filter sterilized using sterile Whatman 0.2µ-nuclepore-membrane filter.

## Concentration of Bacterial Strain

The two distinct luminescent strains isolated were inoculated in the luminous broth and incubated at 37°C for 24 hours. Then they were concentrated into 20 times, 50 times and 100 times following the procedure as mentioned above using the sterile luminescent broth.

Another set of the culture broth containing the luminous bacterial strains was pelleted and concentration of 20 times, 50 times and 100 times were prepared using the cell-free supernatant of the non-luminescent bacterium as per the procedure followed earlier. The tubes were incubated at 37°C. Luminescence of the concentrated samples were measured immediately after preparation as 0 hr and measured upto 8 hrs with 1 hr interval.

## Colony forming units (CFU) in the samples concentrated in the non-luminescent culture supernatant

The cell density was measured using the same aliquots used to measure the luminescence was plated in the Luminous medium. The plates were then incubated overnight at 37°C and the resulting colonies were counted next day.

## Statistical Analysis

All the data were processed and analyzed for the significance of variance using Microsoft excel package.

## Results

The distinct luminescent bacteria isolated from the shrimp farm were *Vibrio harveyi* and *Vibrio fischeri*. They are commonly occurring luminous bacterial species in the aquaculture system. *V.harveyi* is cosmopolitan found as free living in sea and in the guts of marine animals. *V.fischeri* occurs as symbiotic form in the guts of marine animals and in light organs of squid and fishes. The non-bioluminescent bacterium isolated was identified to be *Vibrio parahaemolyticus*. RLU in the concentrated water sample initially increased and the subsequently decreased. RLU of the water sample increased initially followed by a decrease and the mixed trend followed till the end of the observation time. The RLU was not proportional to the concentration factor. The RLUs of the concentrated samples of with and without plankton did not vary significantly when concentrated upto 20 times and 50 times ( $P<0.01$  &  $P<0.05$  respectively) (Table 1; Fig. 1a,b,c& d). The concentration at 100 times yielded significant variation between the sample with plankton and without them ( $P>0.05$ ) (Table

1; Fig. 1 e & f). The concentrated sample with plankton showed nearly three folds higher luminescence than the value of sample before concentration where as the concentrated sample without plankton showed 1.5 folds of the initial value. It is also observed that the P value shows increasing trend with concentration factor. That means the variation would be higher with higher concentrations.

Luminescence in the *V. harveyi* was not increased and in *V. fischeri* it decreased when concentrating compared to the luminescence without concentration (Table 1; Fig. 2 a & b, 3 a & b). The significance was  $P < 0.05$ ,  $P < 0.05$  and  $P < 0.01$  respectively for 20, 50 and 100 times concentrations (Table 1; Fig. 4 a, b & c). In the case of *V. fischeri* there was an increase in the luminescence when concentrated ( $P > 0.05$ ) in all the concentrations of 20, 50 and 100 times (Table 1; Fig. 5 a, b & c). The increase is not proportional to the concentration factor.

Luminescence of the 20 and 100 times concentrated cells of *V. harveyi* in the *V. paraheamolyticus* supernatant was increased ( $P > 0.05$ ) (Table 1) compared to the similarly concentrated cells in the luminous broth. The 50 times concentration did not show any variation in luminescence ( $P < 0.05$ ) (Table 1). It had a weak positive correlation between CFU and RLU in 20 times concentration ( $r = 0.17$ ) and negative correlation in 50 times and 100 concentrations ( $r = -0.65$  and  $-0.34$  respectively). Luminescence of the concentrated cells of *V. fischeri* showed an increase in the luminescence both in the luminous broth and in the *V. paraheamolyticus* supernatant with out significant variation between them (20 times =  $P < 0.05$ ; 50 and 100 times =  $P < 0.01$ ) (Table 1). Positive correlation between CFU and RLU in the cultures grown in the *V. paraheamolyticus* supernatant at the concentrations of 100 and 20 times ( $r = 0.61$  and  $0.28$  respectively). At 50 times concentrations there was a negative correlation ( $r = -0.48$ ). CFU in the *V. paraheamolyticus* supernatant of both *V. harveyi* and *V. fischeri* shows that there is an increase in the cell numbers from the initial numbers in all concentrations. After a mixed trend in the CFU, the final value after eight hrs observations was not less than the initial value in *V. fischeri* (Fig. 6b) but the RLU was not higher than the initial value (Fig. 5c). In the case of *V. harveyi* the final CFUs were less than the initial value (Fig. 6a) but the RLU was higher than the initial value (Fig. 4c).

## Discussion

Water sample concentration results indicate that the physical proximity of the bioluminescent bacteria during high cell density conditions might not have any role in the bioluminescence process. Increase in the luminescence of *V. fischeri* when concentrated shows the presence of physical quorum sensing for their bioluminescence in addition to autoinducer based quorum sensing. The luminescence

of the sample concentrated with planktonic organism is the combined luminescence of both luminescent plankton and bacteria. It further shows that there could be an inhibition on the bacterial bioluminescence by the planktonic organisms and other bacterial communities when concentrated. The inhibition appears to be high by the concentrated planktonic organisms and less by the bacterial communities. The luminescence from the planktonic organisms appears to be unaffected.

Increase in the luminescence non-proportionally with the concentration factor shows that the luminescent bacterial numbers and their luminescence are not in the stoichiometry of 1:1. It is evident from all the experiments that the 0 hr reading did not reflect the concentration factor. That means, the light intensity will not actually reflect the actual number of cells. It is also possible that the increased cell density may cause shadow that could lead to an error in the measurement of luminescence and/or the accumulation of toxic metabolites and competition for nutrients also may reduce the luminescence during high cell density. In all the concentration experiments, though the luminescence decreases initially, it fluctuates subsequently giving two or more peaks, for which the reason is not known. In *Vibrio harveyi* accumulation of auto inducers will have the influence on the timing of gene expression in their mixed- or mono-species environments (11). If this presumption is applied for the current experiment, it is not known what effect the possible excess autoinducers accumulated as in the case of concentrated samples will have on the timing of gene expression. Further studies will lead to the answer for the fluctuation in the luminescence while concentrating the samples.

*V. paraheamolyticus* supernatant enhanced the luminescence in both *V. harveyi* and *V. fischeri*. The non-luminescent organism also can secrete AI-2 and trigger luminescence in the *V. harveyi* (12,13). It is also shown that the *E. coli* transcribes AI-2 (14, 15).

The inverse relationship between CFU and RLU at 50 and 100 times concentrations found in the *V. harveyi* when cultured in the *V. paraheamolyticus* supernatant could be because of the quality of the medium. Since, *V. paraheamolyticus* cultured for 24 hrs initially might have depleted the dissolved oxygen (DO) considerably. The depleted DO is insufficient for the bioluminescence as the luminescence shares about 20% of the total oxygen consumption of the cells (16). This does not explain the positive correlation found in the *V. fischeri* in the same concentration. *V. fischeri* has a dual quorum sensing system (17). One is AinS enzyme, a LuxM type N-acyl homoserine lactone synthase which synthesizes N-octanoyl homoserine lactone signal and the other is Lux system (18). The quorum sensing function *V. fischeri*, which well known for its symbiotic relationship acts in two steps. In the initial proliferation and colonization in the animal host, the AinS system ensures that the flagellar movement is activated. The *V. fischeri* LuxS signal AI-2 was shown to be involved in the regulation of

luminescence expression and colonization competence in addition to that of *ain* and *lux* quorum sensing but quantitatively the impact of *ain* and *lux* quorum sensing is comparatively higher than the impact of AI-2. (19). So, *V. fischeri* grown in the culture filtrate of the *V. parahaemolyticus* is mostly dependent on the Ain and Lux quorum sensing that had to be secreted by its own. However, the bioluminescence in *V. harveyi* could be triggered by its own AI-2 molecules together with the AI-2 secreted by the *V. parahaemolyticus* in the culture filtrate as the AI-2 from other organisms could also trigger luminescence in the *V. harveyi* (5) and so the negative correlation exists between CFU and RLU. It can be interpreted that the negative correlation between CFU and RLU in *V. harveyi* is the outcome of the enhanced availability AI-2 for its bioluminescence whereas in the *V. fischeri* bioluminescence was solely dependent on its own quorum sensing molecules. Moreover, another autoinducer CAI-1 which acts for sensing inter genera quorum, may have a role for the inverse relationship between RLU and CFU (8).

In aquaculture farms, occurrence of bioluminescent bacteria causes significant economic loss. It cannot be predicted when they will appear. The present solution is frequent exchange of water to dilute the risk. It is not known the kind response of the luminescent bacteria in presence of various non-bioluminescent bacteria. But, it can be emphasized here that monitoring the total number of non-luminescent bacteria or elevated number of any specific type of bacteria will help in anticipating or further understanding outbreak of an episode of bioluminescent bacterial bloom in the aquaculture system.

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Table1 Summary of water sample concentration and the related RLU

Concentration factor	Mean RLU of the sample concentrated with plankton	Initial sample RLU before concentration	Mean RLU of the sample concentrated without plankton	Initial sample RLU before concentration	P -value
20 times	787	606	1126	873	<0.01
50 times	1108		2123		<0.05
100 times	1716 (2.8 folds)		1338 (1.5 folds)		>0.05

Fig.1. Water sample concentration and the related RLUs

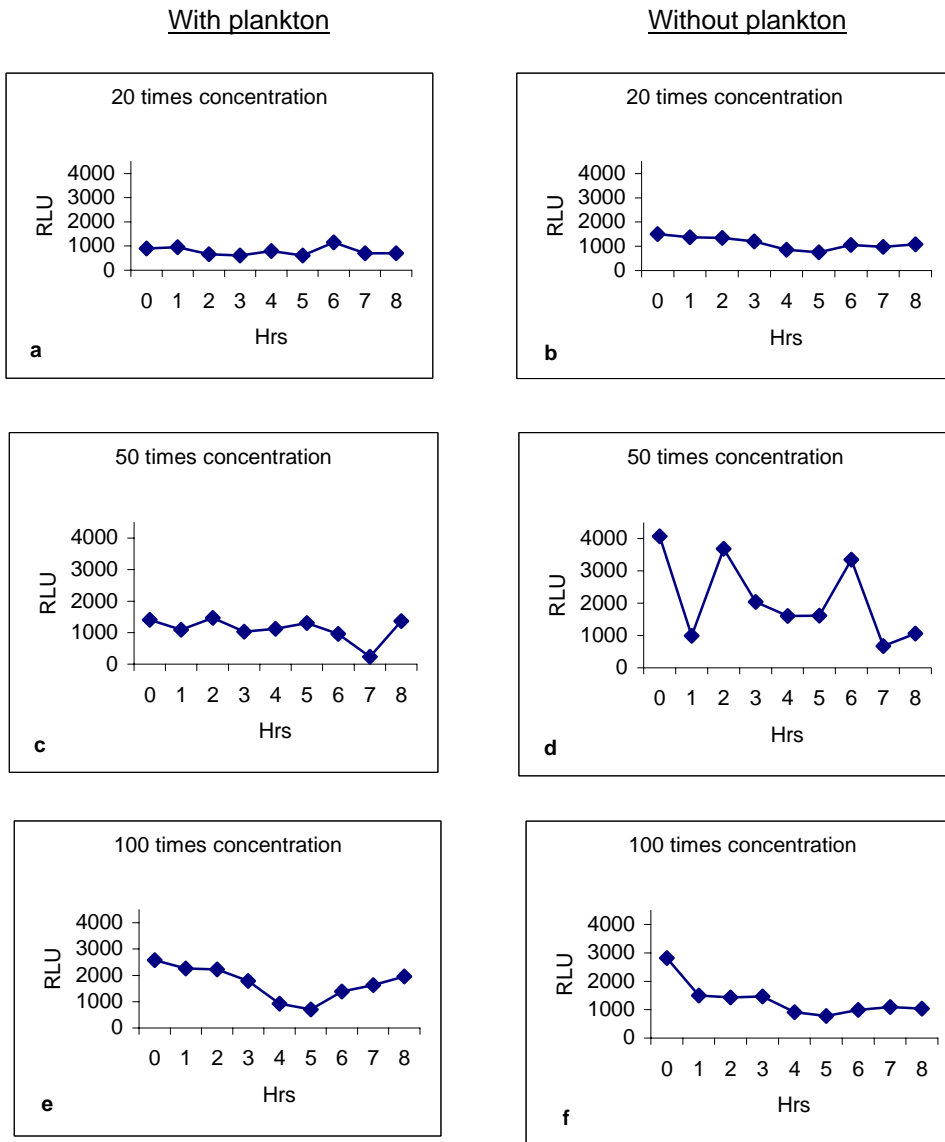


Fig 2. Luminescence of *V. harveyi* in the luminous broth and in the *V. paraheamolyticus* cultured cell free supernatant

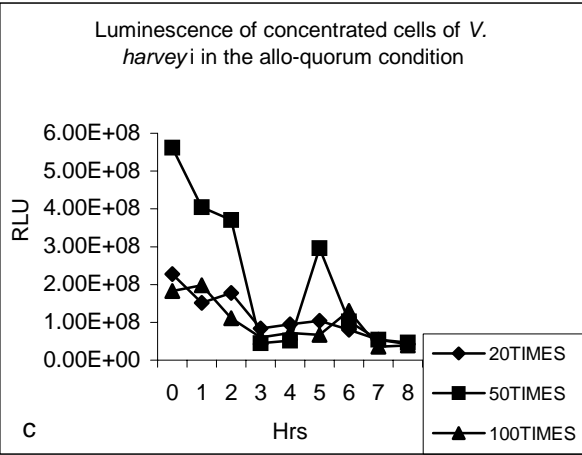
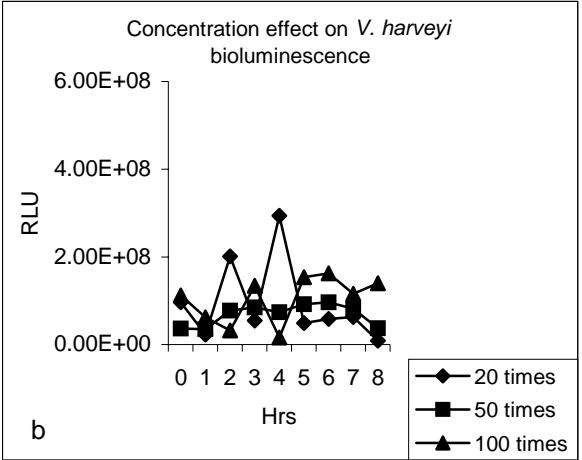
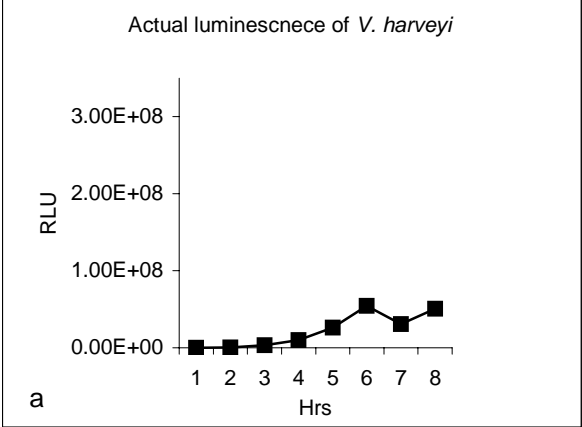


Fig 3. Luminescence of *V. fischeri* in the luminous broth and in the *V. paraheamolyticus* cultured cell free supernatant

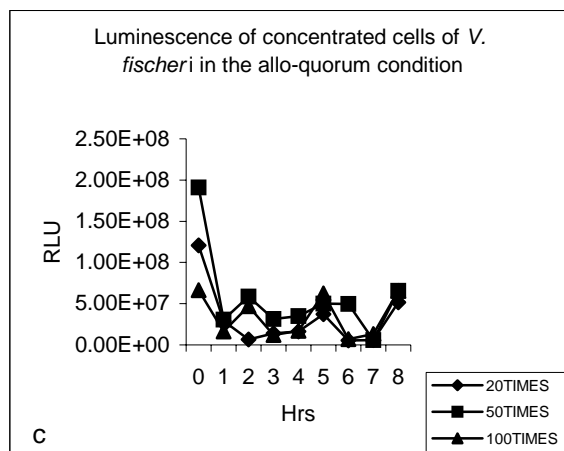
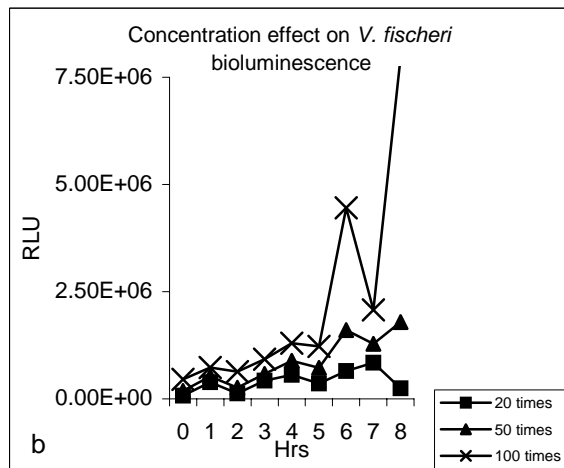
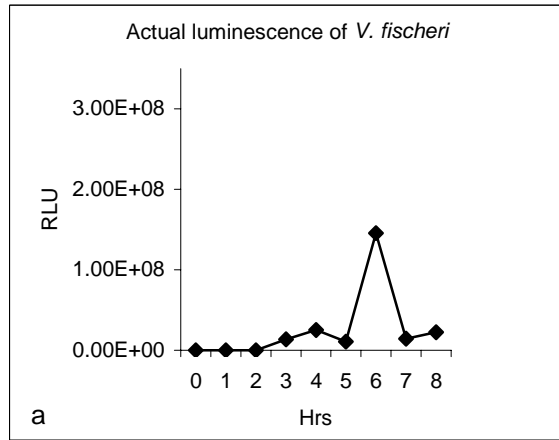


Fig. 4. Luminescence of concentrated cells of *V. harveyi* in the luminous broth and in the *V. parahaemolyticus* cultured cell free supernatant

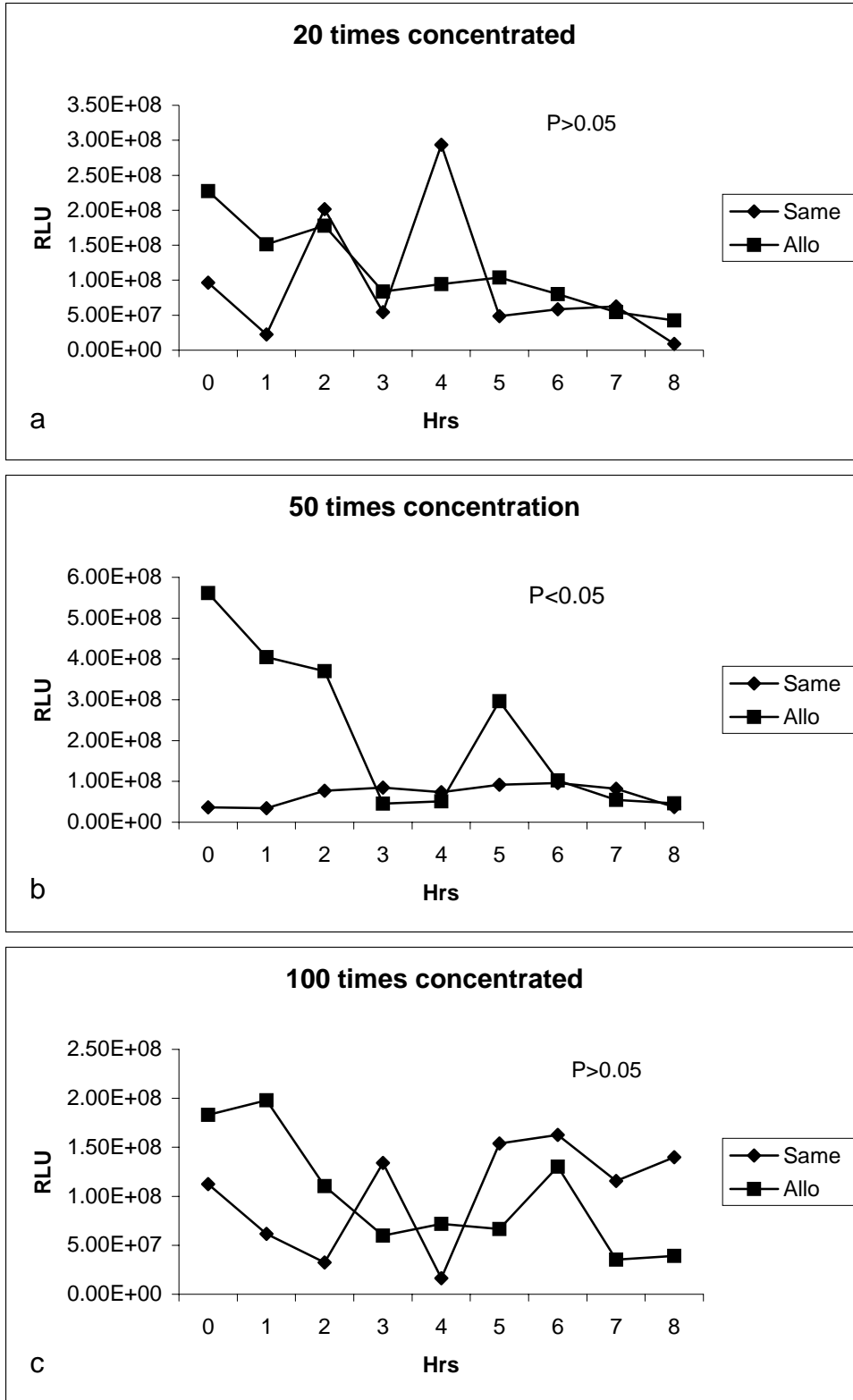


Fig. 5. Luminescence of concentrated cells of *V. fischeri* in the luminous broth and in the *V. parahaemolyticus* cultured cell free supernatant

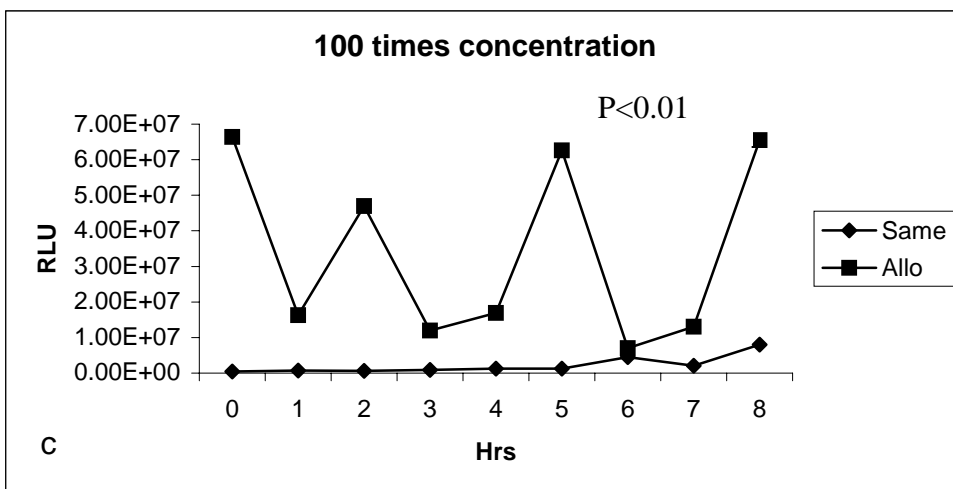
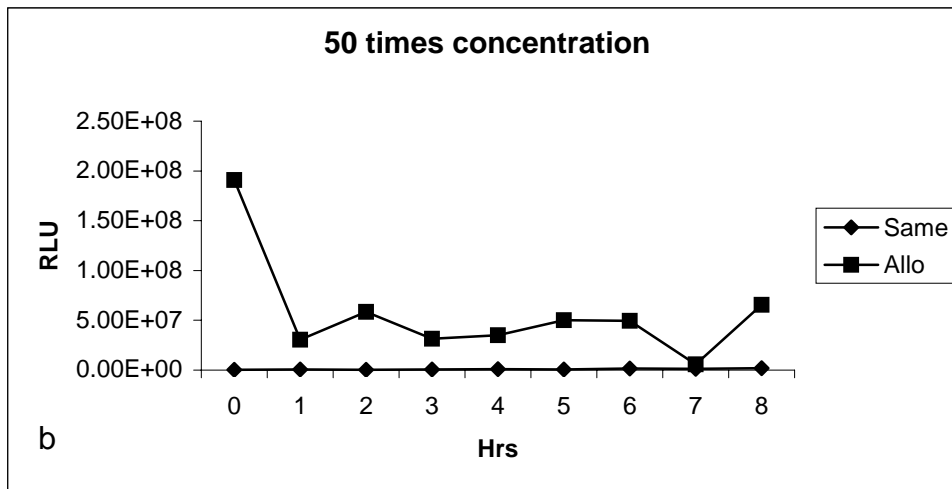
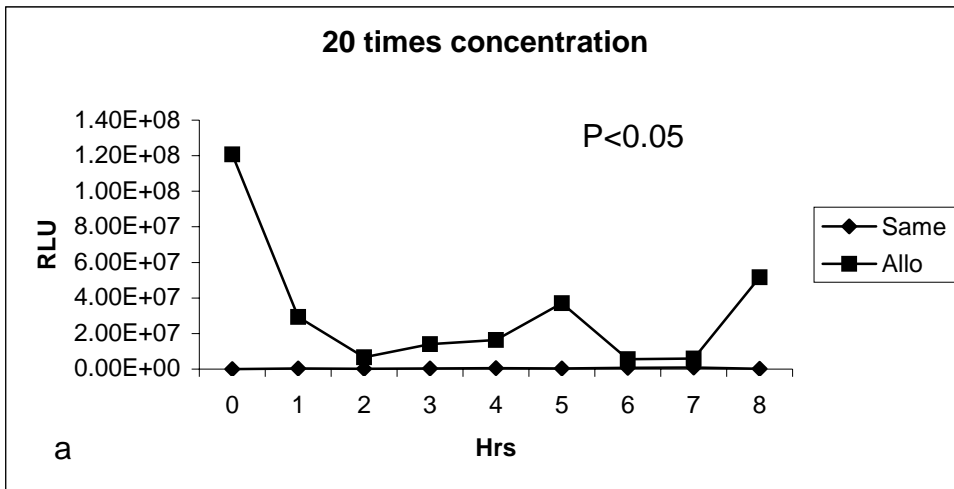


Fig. 6. CFU of luminescent bacteria in the *V. parahaemolyticus* cultured cell free supernatant at various concentrations

