

## **Long term response of bacterial bioluminescence to reverse osmosis treated effluents from dye industries**

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### **ABSTRACT**

Bacterial bioluminescence assay is one of the novel means for toxicity detection. The bioluminescence response of two marine bioluminescent bacteria was tested upon their long term exposure against nine different Reverse Osmosis (RO) rejects with varying chemical composition sampled from various dye industries. Bioluminescent bacteria were cultured in the RO reject samples itself, at different concentrations and their growth rate and luminescence was measured for 24 hrs. The RO reject samples caused sub-lethal effects upon exposure and retarded the growth of bacteria, confirming their toxic nature. Further, continuation of the exposure showed that the initial luminescence though reduced, it recovered and increased beyond the control cultures irrespective of the cell density and finally decreased once again. The present study emphasis the need of evolving a long term exposure assay and suitability of the method followed in this study to evaluate the toxicants that exert delayed toxicity with lower concentrations of toxicants and also to use for colored samples.

**Key words:** Bioluminescence, RO reject, Dye Industry, Sub lethal effect, Delayed toxicity

## INTRODUCTION

The effect of wastes from the dye industries on the biological component of an environment in which they are released is minimized by pre-treating it. The textile dyeing industries employ various separation techniques including the Reverse Osmosis (RO) to treat the effluents. Though, the RO process is proved to be efficient in treating the effluents, the RO reject water obtained from this process is characterized by high amounts of Total dissolved solids (TDS), nutrients and other trace metals. TDS are the sum of inorganic, organic and other dissolved materials in water (US EPA 1986). High amounts of TDS can cause changes in the ionic composition of water and salinity, which in turn could cause toxic effects to the organisms (Webber-Scanell & Duffy 2007). Assessing the toxicity of such RO rejects before their discharge in to the environment is very essential and important in view of environmental pollution. Analysis of these effluents using physical and chemical methods is insufficient due to their complex nature and they may not reflect the biological effects (Coombe et al. 1999). *In-vivo* tests using macro organisms have their own drawbacks and so their use to assess the toxic nature of the compound are limited (De Marini 1991). The bioluminescence inhibition assay proved to be capable of assessing the toxic nature of a substance (ISO 1998) and its usage therefore increased in recent years.

Bioluminescence is the production and emission of visible light by living organisms. The bioluminescence continued to interest the researchers for long and this led to its wide spread applications including coastal pollution surveillance (Ramaiah & Chandramohan 1993). Bioluminescence inhibition assay is one of the rapid methods to assess the toxicity of a substance. In this assay, the toxicity of a substance is related to the reduction in the intensity of luminescence (Ulitzur 1997). This assay employs a gram negative marine bacterium *Vibrio fischeri* to assess the acute toxicity of the substance upon short term exposure. It yields a measure of the sub lethal response through reduction in the intensity of luminescence, which corresponds to the metabolic activity of the bacterial cell in response to the toxic substance. Various bioluminescence inhibition assays commercialized in the name of Microtox, LUMISTox and Tox Alert use a specific strain NRRL B-1117 to assess the short term toxicity of a substance (Farre & Barcelo 2003). This assay was used by various researchers to assess the toxicity of substances like organic pollutants (Parvez et al. 2006, Ren & Frymier 2005), pesticides (Farre et al. 2002, Ruiz et al. 1997), antibiotics (Backhaus & Grimme 1999) and genotoxic potential of ground waters (Dizer et al. 2002) etc.

The short term toxicity test underestimate the toxic effects of certain chemicals (Backhaus et al. 1997), whereas the more reliable estimate was given by the long term bio assay test. In the long term bioassay test, the bioluminescent bacteria are exposed to the toxic substance for 24 hours in

contrast to the 15 or 30 minutes in short term toxicity tests. Certain substances may exert delayed toxic effects at low concentration and this may not be revealed in the standardized short term bioluminescence inhibition assays. The short term bioluminescence inhibition assay can misinterpret certain chemicals as non toxic despite the fact that those chemicals would exert delayed toxicity during long term exposure (Froehner et al. 2000).

This study focuses on the bioluminescence pattern of two marine bioluminescent bacteria in response to their long term exposure to the RO reject samples. The luminescence pattern was observed every 3 hours for 24 hrs to get the first hand information about the toxic nature of the RO reject samples. The RO reject samples were characterized by a complex mixture of high amount of TDS, Total Organic Carbon (TOC) and Nutrients. Besides this, the chemical analysis of the RO reject samples was done to emphasize their role in the inhibition of bioluminescence.

## **MATERIALS AND METHODS**

Seawater was collected from a depth of 2 meters from Palk Bay, South east coast of India in clean, sterilized 50ml poly propylene containers by skin diving. The collected water samples were stored in ice and transported to the lab within 3 hrs. The water samples were serially diluted in a filter sterilized, autoclaved sea water and then spread plated in a modified Luria bertani (LB) agar (casein enzymic hydrolysate- 10g; yeast extract-5g; sodium chloride – 10g; glycerol- 30ml; aged seawater – 970ml) plates prepared with aged sea water and supplemented with glycerol. The plates were incubated at 28°C for 24 hrs. Luminescent colonies were picked up by observation in the dark. Morphologically distinct and brightly glowing colonies were purified further in fresh LB agar plates prepared using aged sea water.

The Bioluminescent bacteria were identified by 16S rRNA sequencing. Overnight bacterial cultures were used for the extraction of genomic DNA. Extraction was done using a modified phenol-chloroform method and the quality of the product was checked in 0.8% agarose gel. The sequencing was done using a forward primer 27F (5'AGAGTTTGATCCTGG CTCAG 3') (Weisberg et al. 1991), and the reverse primer 1492 R (5'GGTTACCTTACGACTT 3') (Reysenbach et al. 1992). The PCR was done in a thermocycler (Applied Biosystems, USA) using the following cyclic program. Initial denaturation at 94°C for 5 minutes followed by 30 cycles of denaturation at 94°C for 1 minute; annealing at 55°C for 1 minute; extension at 72°C for 1 minute and final extension at 72°C for 10 minutes. The PCR products were purified using Poly ethylene glycol precipitation (Liss 1980). The purified PCR products were further extended using the 27 F and 1492 R primers in a thermocycler using the following program. Initial denaturation at 96°C for 1 minute

followed by 30 cycles of denaturation at 96°C for 10 seconds; annealing at 50°C for 5 seconds, extension at 60°C for 4 minutes and final extension at 60°C for 1 minute. The final extension products were cleaned as described by Jayshankar & Ramaiah (2007) and the sequencing was done using ABI sequencer (ABI PRISOM model no. 3700). Forward and reverse sequences obtained were aligned using Sequencher V4.10.1 and identified by BLAST analysis. The sequences were submitted in the GenBank (GenBank accession numbers: JQ361646-JQ361647).

The Bioluminescent bacteria was inoculated in LB broths and incubated in an orbital shaker for 24 hours. 0.1ml of this 24 hr old culture was inoculated in a freshly prepared LB broth containing filter sterilized RO reject samples in three different concentrations i.e. 25% (v/v), 50% (v/v) and 100% (v/v). Bioluminescent bacteria inoculated in LB broth without RO reject serves as the control. The experiment was conducted in triplicates and at stable temperature of 25°C to ensure the reproducibility of the results. Immediately after inoculation, the growth was monitored by measuring the absorbance at 600nm against the uninoculated LB broth as reference using a UV-Vis double beam spectrophotometer (Shimadzu UV 2550, Japan). Uninoculated broths containing 25% (v/v), 50% (v/v) and 100% (v/v) concentration of RO reject samples were used as a reference for the respective test samples inoculated with bioluminescent bacteria. The measurements were taken every 3 hours for a period of 24 hours. The rate of bacterial growth cultured with different concentrations of RO reject was compared with the control cultures using two sample t- test. Cell density at the arrival of stationary phase has been taken as the variable factor. The analysis was performed using Microsoft Excel 2007.

1.5ml of the control and RO reject treated bioluminescent bacterial cultures were transferred to a sterile micro centrifuge tubes and centrifuged at 12000rpm for 15 minutes. The pelletized cells were resuspended in 1.5ml of filter sterilized and autoclaved sea water. This was done to prevent the absorption of luminescence by the culture media and the colored RO reject samples. The luminescence was measured at once using a Luminometer (Turner Biosystems, model 20/20<sup>n</sup> USA) and thereafter every 3 hours for 24 hours. The results are expressed as the percentage of luminescence existing in the RO reject treated cultures, with reference to the control cultures. It is calculated using the following formula.

$$\% \text{ of existing luminescence} = 100 * S_t / C_t$$

where,  $S_t$  = luminescence in relative light units of the RO reject treated bacterial cultures at the given time interval.

$C_t$  = luminescence in relative units of the control bacteria cultures at the given time interval.

The experiments were conducted in triplicates to ensure the reproducibility of the results achieved. The absorbance values obtained from the growth assay and the Relative light units obtained from the luminescence assay were plotted and analyzed using Microsoft excel package 2007.

The RO reject samples were collected in clean, 1000 ml capacity polyethylene sampling bottles from different effluent treatment plants of textile dyeing industries and were brought to the lab for the analysis of nutrients, total organic carbon (TOC) and trace metals. pH was measured using a portable pH meter (Eutech Tutor) with an accuracy of  $\pm 0.1$  pH units. Turbidity of the samples was measured in terms of Nephelometric Turbidity Units (NTU) using a portable turbidity meter (Eutech TBDW - 1000), with an accuracy of  $\pm 0.01$  NTU. Total suspended solids (TSS) were measured by filtration using Filtration pump (Aspirator Vacuum) (Preston et al. 1997). Millipore vacuum filter pump was used for the filtration and the samples were filtered through pre-weighed  $0.45\mu\text{m}$  filter papers. TSS was calculated from the initial and final weights of the filter paper after filtration.

Nutrients such as phosphate, nitrite, nitrate, ammonia, urea and silicate were determined using the spectrophotometric methods (Strickland & Parsons 1979). The heavy metals were analyzed using ICP-AES. Trace metals were analyzed by Ion chromatography (DIONEX ICS-2500), while the Mercury was analyzed by Mercury analyzer (BUCK Scientific 410 Mercury analyzer). The Total Organic Carbon (TOC) analyses were carried out using TOC analyzers (SHIMADZU TOC-VCPH).

## **RESULTS**

The two isolated bioluminescent bacteria were identified as *Vibrio sagamiensis* and *Vibrio azureus*. The luminescence intensity of both *V. sagamiensis* and *V. azureus* cultured in RO reject sample was low compared to the control when measured within 15 minutes of inoculation. With continued incubation, the luminescence intensity of both the species surpasses the control and displayed an unusual response of increase and decrease in the intensity of luminescence regardless of their cell density and the concentration of RO reject. On the other hand, the luminescence intensity of the control cultures were proportional to their cell density at the given time interval.

The luminescence intensity of *V. sagamiensis* cultured with RO reject sample 1 and 7 (Fig 1a and 1b) and *V. azureus* (Fig 1c and 1d) cultured with RO reject sample 1 and 4 increased and decreased all through the incubation period showing no correlation with the concentration of RO reject or with the duration of incubation. However, fluctuation in their luminescence intensity was lower than that of control culture in terms of relative light units during the entire incubation period. The luminescence pattern of *V. sagamiensis* and *V. azureus* cultured with RO reject samples 2 and 5 differs with the concentration of the RO reject in the culture media. While the luminescence pattern

of both the species cultured with 25% (v/v) of RO reject surpass the control, those cultured with 50% (v/v) and 100% (v/v) falls behind the control (Fig 2a-2d). The luminescence pattern of *V. sagamiensis* (Fig 3a - e) and *V. azureus* (Fig 4a – 4e) cultured with rest of the RO reject samples at 25% (v/v) and 50% (v/v) exhibited a mixed style of increase and decrease regardless of the cell density and the duration of incubation. However the luminescence pattern with 100% (v/v) concentration of RO reject samples rarely exceeded the luminescence intensity of control cultures.

The growth of *V. sagamiensis* cultured with 25%, 50% and 100% concentrations of RO reject samples 4, 5, 7 and 9 differed significantly (P value < 0.05) from the control cultures (Table 1). Similarly, difference between the growth of *V. azureus* cultured with three concentrations of RO reject samples 1, 3, 4 and 5 from that of control cultures was significant (Table 2). Both *V. sagamiensis* and *V. azureus* differed in their sensitivity to the RO reject samples in different concentrations (Table 1). The sensitivity of the bioluminescent bacteria to RO reject samples were described by the 50% inhibition of luminescence to that of control culture measured at the given time interval. 50% inhibition of luminescence was obtained within 15 minutes of inoculation in both *V. sagamiensis* and *V. azureus* cultured with RO reject sample 1 and 4 in all the three concentrations. *V. sagamiensis* showed some degree of resistance to the sample 3 compared to *V. azureus* as 50% inhibition of luminescence is observed immediately in the latter and only after 3 hours incubation in the former. *V. sagamiensis* cultured with 25% (v/v) concentration of RO reject samples 2, 6 and 7 showed 50% inhibition of luminescence following incubation between 6 to 12 hours, whereas it is observed within 15 minutes with those cultured with 50% (v/v) and 100% (v/v). *V. azureus* also showed similar response with RO reject samples 2, 6 and 7 except that the 50% inhibition was attained following incubation up to 3 hours at a concentration of 50% (v/v) and 100% (v/v). RO reject sample 5 and 8 at a concentration of 25% (v/v) were not capable of inhibiting 50% of the luminescence in the *V. sagamiensis*. Similar result was observed with the *V. azureus* cultured with samples 6 and 8.

The concentration of nutrients, pH, turbidity, TOC in the RO reject samples are presented in Table 4. In the RO reject samples, the concentration of phosphate ranges from a maximum of 823.27µmol/l to a minimum of 0.97µmol/l. The concentration of nutrients is very high in the RO reject sample 1 and it exceeds the normal acceptable range. The concentration of urea ranges a maximum of 454.75µmol/l to a minimum of 41.34µmol/l. The TDS in the RO reject samples varies between a maximum of 39000mg/l in RO reject sample 8 and a minimum of 1248 mg/l in RO reject sample 5. The concentration of trace metals in the effluent samples are presented in Table 5. Zinc, Iron and Manganese are predominantly present in all the RO reject samples whereas mercury and

cadmium are found to be present only in few samples. The concentration of copper is very high in RO reject samples 1, 3 and 4 exceeding the normal acceptable limit.

The results of this study indicate that the RO reject samples with high organic content, nutrients and TDS are capable of retarding the growth and luminescence of the bioluminescent bacteria. However, the luminescence recovered and rose further following prolonged exposure. The luminescence which reflects metabolic status of the cell showed a diverse picture. The RO reject samples with their unknown mechanisms of action influenced the intensity of the luminescence by increasing and decreasing it. Bioluminescence is a complex process that depends upon the energy status of the cell in addition to the availability of the substrate and the auto inducer molecules (Meighan & Dunlap 1993). The initial reduction in the intensity of luminescence in response to the RO reject reflects the disturbances in the biochemical processes of the cell. Later, the luminescence intensity increased several times higher than the control though the cell density was lower. It is experimentally proved that the cell density do not reproduce the intensity of luminescence (Ravindran et al. 2009).

The increase in the intensity of the luminescence of the bacterial cultures grown in the RO reject samples suggested that some of the constituents of the RO reject sample might enhance the intensity of the luminescence. One of the possible factors could be the availability of excess nutrients especially the readily soluble forms as an energy source due to the metabolic activity of the cells. These excessive nutrients could be available to the bacterial cultures grown in RO reject samples even after their stationary phase and that could be understood by the less cell density compared to the control samples. However, the increase in the luminescence is inconsistent and it fluctuated at regular intervals during the entire incubation period.

The RO reject samples were complex in nature consisting of very high organic content, nutrients and TDS, exceeding the stipulated level for the environmental discharge except that of pH and turbidity, which are in the normal acceptable range according to the standards set by the Central Pollution Control Board of India (CPCB 2010). Depending on the content, natural degradation and degradation due to bacterial activity, it either reduced or enhanced the intensity of the luminescence in bacteria upon their exposure to these samples for a longer duration. It is hypothesized that the bioluminescent bacteria through their metabolic processes could breakdown the complex organic substances present in the RO reject samples into smaller units by their extra cellular enzymes. These metabolic products could be in a mixture of both stimulants and toxicants affecting the bacterial luminescence. Depending on its availability to the cell at a given time and the concentration, these metabolic products may either increase or decrease the intensity of the luminescence.

The RO reject samples consist of urea in different concentrations. Increase in the nutrient concentrations like Nitrate and Urea has been showed to interfere with physiological process such as bioluminescence. However, it remains harmless to the whole luminescent bacterial population upon continuous exposure (Frischer et al. 2005). Thus, the initial reduction in the intensity of luminescence could be due to interference of urea present in the RO reject on the physiology of bioluminescence and by other compounds with similar property. In particular, RO reject sample 1 has very high concentration of urea and the luminescence intensity of the bacterial cultures inoculated with this sample never exceeded the luminescence intensity of control cultures all through the incubation period. However, with other samples in which the concentration of urea is low, the cells were shown to recover with enhanced luminescence during prolonged incubation. This suggested that the short term exposure bioluminescence inhibition assay would misinterpret a substance to be highly toxic if its constituent includes urea. The short term bioluminescence inhibition assay is not reliable to estimate the toxicity of substances that exert delayed toxicity on the experimental organisms (Backhaus et al. 1997). There is every possibility that high concentration of urea in the RO reject samples will increase the level of organic nitrogen compounds upon discharge and thereby increase the biological activity. In addition to urea, high concentration of  $\text{Cu}^{2+}$  is present in the samples 1, 3 and 4. This might have adverse effect over the bacterial growth (Wang et al. 2009).

In the standard bioluminescence inhibition assay, the toxicant at different concentration is added directly to the bacterial cell suspension and the variation in the luminescence intensity is measured within 15 - 30 minutes of incubation period. The  $\text{EC}_{50}$  value tends to change depending up on the concentration of the toxicant and the duration of the exposure. In this study, the bacteria were cultured directly in to the RO reject samples at different concentrations and the 50% inhibition of luminescence relative to the control cultures was measured. The 50% inhibition of luminescence of the bioluminescent bacteria exposed to 50% (v/v) and 100% (v/v) is observed within 15 minutes of incubation. Whereas, in this study, it was observed only after a minimum incubation period of 6 hours in those exposed to 25% (v/v) concentration of RO reject samples. This suggested that the long term exposure approach may be further improved to develop into an assay to determine the  $\text{EC}_{50}$  values especially to the samples that show delayed toxicity or with low concentration of toxicants.

Culturing bacteria with the toxicant itself will directly reflect any detectable changes in the growth depending on the concentration but the growth assay was less sensitive than the bioluminescence inhibition assay for most of the analyzed toxicants (Gellert 2000). There was a risk of absorption of bioluminescence by the toxicant media while culturing the bacterial cells directly in



the toxicant. It will lead to misinterpretation of the response of bacterial luminescence to the toxicant and the nature of the toxicant. Drawback of the colored samples is that it absorbs luminescence while measuring but it was mitigated in this study by separating the bacterial cells from the experimental sample by centrifugation during the luminescence measurement.

In conclusion, this study emphasize that culturing the bacterial cells directly in the toxic substance will facilitate the direct exposure of the organisms to the toxicant without other interference. The approach of long term exposure of the bioluminescent bacteria showed that the RO reject samples exerted their inhibitory effects at different time intervals and retarded the growth of the bacteria. Moreover, the procedure followed in this study has an added advantage that it will be able to mitigate the interference of colored samples especially when their absorption wavelengths are same as that of bioluminescence.

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### **REFERENCES**

1. U.S. Environmental Protection Agency. 1986. Office of Water Quality criteria for water (Gold Book). EPA 440/5-86-001, Washington, D.C.
2. Webber-Scanell, P.K., and Duffy, L.K. 2007. Effects of total dissolved solids on aquatic organism: A review of literature and recommendation for salmonid species. *Am. J Environ. Sci.* 3: 1-6.
3. Coombe, V.T., Moore, K.W., and Hutchings, M.J. 1999. TIE and TRE: An abbreviated guide to dealing with toxicity. *Water sci. and tech.* 39: 91-97.
4. DeMarini, D.M. 1991. Environmental mutagens/complex mixtures. *In Genetic toxicology. Edited by A.P. Li and R.H. Heflich.* CRC press, Boca Raton, pp. 285-302.
5. ISO 11348-1:1998 (E). 1998. Water Quality determination of the Inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescence Bacteria test) - Part-I: Method Using freshly prepared Bacteria, International Organization for Standardization, 1-16.
6. Ramaiah, N., and Chandramohan, D. 1993. Ecological and Laboratory studies on the role of luminous bacteria and their luminescence in coastal pollution surveillance. *Mar. Pollut. Bull.* 26: 190-201.

7. Ulitzur, S. 1997. Established technologies and new approaches in applying luminous bacteria for analytical purposes. *J. Biolumin. Chemilumin.* 12: 179-192.
8. Farre, M., and Barcelo, D. 2003. Toxicity testing of wastewater and sewage sludge by biosensors, bioassays and chemical analysis. *Trends. Anal. Chem.* 22: 299-310.
9. Parvez, S., Venkataraman, C., and Mukherji, S. 2006. A review on advantages of implementing luminescence inhibition test (*Vibrio fischeri*) for acute toxicity prediction of chemicals. *Environ. Int.* 32: 265-268.
10. Ren, S., Frymier, P.D. 2005. Toxicity of metals and organic chemicals evaluated with bioluminescence assays. *Chemosphere* 58: 543-550.
11. Farre, M., Goncalves, C., Lacorte, S., Barcelo, D., and Alpendurada, M. 2002. Pesticide toxicity assessment using an electrochemical biosensor with *Pseudomonas putida* and a bioluminescence inhibition assay with *Vibrio fischeri*. *Anal. Bioanal. Chem.* 373: 696-703.
12. Ruiz, M.J., Lopez-Jaramillo, L., Redondo, M.J., and Font, G. 1997. Toxicity assessment of Pesticides using the Microtox Test: Application to Environmental Samples. *Bull. Environ. Contam. Toxicol.* 59: 619-625.
13. Backhaus, T., and Grimme, L.H. 1999. The toxicity of antibiotic agents to the luminescent bacterium *Vibrio fischeri*. *Chemosphere* 38: 3291-3301.
14. Dizer, H., Wittekindt, B., Fischer, B., and Hansen, P.D. 2002. The cytotoxic and genotoxic potential of surface water and waste water effluents as determined by bioluminescence, *umu* assays and selected biomarkers. *Chemosphere* 46(2): 225-233.
15. Backhaus, T., Froehner, K., Altenburger, R., and Grimme, L.H. 1997. Toxicity testing with *Vibrio fischeri*: A comparison between the long term (24h) and the short term (30min) bioassay. *Chemosphere* 35: 2925-2938.
16. Froehner, K., Backhaus, T., and Grimme L.H. 2000. Bioassays with *Vibrio fischeri* for the assessment of delayed toxicity. *Chemosphere* 40: 821-828.
17. Weisberg, W.G., Barns, S.M., Pelletier, D.A., and Lane, D.J. 1991. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* 173: 697-703.
18. Reysenbach, A.L., Giver, L.J., Wickham, G.S., and Pace, N.R. 1992. Differential amplification of rDNA genes by polymerase chain reaction. *Appl. Environ. Microbiol.* 58: 3417-3418.
19. Liss, T.J. 1980. Fractionation of DNA fragments by Polyethylene glycol Induced Precipitation. *Method. Enzymol* 65: 347-353.
20. Jaysankar. De., and Ramaiah, N. 2007. Characterization of marine bacteria highly resistant to mercury exhibiting multiple resistances to toxic chemicals. *Ecol. Indicators.* 7: 511-520.

21. Preston, N.P., Macleod, I., Rothlisberg, P.C., and Long, B. 1997. Environmentally sustainable aquaculture production—an Australian perspective. *In* Second World Fisheries Congress: Developing and Sustaining World Fisheries Resources: The State of Science and Management Brisbane, Vol 2: Proceedings. *Edited by* D.C. Hancock, A.G.Smith, and J.P. Beumer. CSIRO, Melbourne, pp. 471-477.
22. Strickland, S.C., and Parsons, T.R. 1979. A practical Handbook of Seawater Analyses. Bull Fish Res. Board of Canada, Ottawa.
23. Meighan, E.A., and Dunlap, V.P. 1993. Physiological, biochemical and genetic control of bacterial bioluminescence. *Adv. Microb. Physiol* 34: 2-12.
24. Ravindran, J., Geethapriya, G., and Kannapiran, E. 2009. Effect of concentrating and exposing the bioluminescent bacteria to the non-luminescent allo bacterial extracellular products on their bioluminescence. *Luminescence* 26: 23-28.
25. CPCB (Central Pollution Control Board). 2010. Industry specific standards- Dye and Dye Intermediate Industry, Environment (Protection) Third (Amendment) rules, Part II, Section 3 (i), New Delhi, pp 5-6.
26. Frischer, M.E., Danfort, J.M., Foy, T.F., and Juraske, R. 2005. Bioluminescent bacteria as Indicators of chemical contamination of coastal waters. *J. Env. Qual.* 34: 1328-1336.
27. Wang, W., Lampi, M.A., Huang, X.D., Gerhardt, D., Dixon, G., and Greenberg, B.M. 2009. Assessment of mixture toxicity of copper, cadmium and phenanthrenequinone to the marine bacterium *Vibrio fischeri*. *Env. Tox* 24: 166-177.
28. Gellert, G. 2000. Sensitivity and significance of luminescent bacteria in chronic toxicity testing based on growth and bioluminescence. *Ecotoxicol. Environ. Saf.* 45: 87-91.

**Table 1:** Two sample t-test to compare the growth of *V. sagamiensis* between control and cultures exposed to RO reject samples

RO reject sample	Control vs 25%			Control vs 50%			Control vs 100%		
	t <sub>stat</sub>	t <sub>crit</sub>	P value	t <sub>stat</sub>	t <sub>crit</sub>	P value	t <sub>stat</sub>	t <sub>crit</sub>	P value
1	4	4.302	0.057191	<b>1.27x10<sup>15</sup></b>	<b>4.302</b>	<b>0.61x10<sup>-31</sup></b>	<b>1.27x10<sup>15</sup></b>	<b>4.302</b>	<b>0.61x10<sup>-30</sup></b>
2	1	4.302	0.42265	1	4.302	0.42265	<b>10</b>	<b>4.302</b>	<b>0.009852</b>
3	<b>11</b>	<b>4.302</b>	<b>0.008163</b>	2	4.302	0.183503	<b>11</b>	<b>4.302</b>	<b>0.008163</b>
4	<b>14</b>	<b>4.302</b>	<b>0.005063</b>	<b>8.69</b>	<b>4.302</b>	<b>0.012976</b>	<b>25</b>	<b>4.302</b>	<b>0.001596</b>
5	<b>0.63x10<sup>15</sup></b>	<b>4.302</b>	<b>2.47x10<sup>-30</sup></b>	<b>8</b>	<b>4.302</b>	<b>0.007634</b>	<b>17</b>	<b>4.302</b>	<b>0.001721</b>
6	1.732051	4.302	0.1124	<b>2.54x10<sup>15</sup></b>	<b>4.302</b>	<b>0.07x10<sup>-30</sup></b>	<b>3.18x10<sup>15</sup></b>	<b>4.302</b>	<b>0.04x10<sup>-30</sup></b>
7	<b>0.63x10<sup>15</sup></b>	<b>4.302</b>	<b>1.23x10<sup>-30</sup></b>	<b>0.63x10<sup>15</sup></b>	<b>4.302</b>	<b>1.23x10<sup>-30</sup></b>	<b>5</b>	<b>4.302</b>	<b>0.018875</b>
8	1	4.302	0.211325	<b>4</b>	<b>4.302</b>	<b>0.028595</b>	2.5	4.302	0.064806
9	<b>1.27x10<sup>15</sup></b>	<b>4.302</b>	<b>0.3x10<sup>-30</sup></b>	<b>1.27x10<sup>15</sup></b>	<b>4.302</b>	<b>0.3x10<sup>-30</sup></b>	<b>4</b>	<b>4.302</b>	<b>0.028595</b>

\* bold lettered values indicate significant difference between growth

**Table 2:** Two sample t-test to compare the growth of *V. azureus* between control and cultures exposed to RO reject samples

RO reject sample	Control vs 25%			Control vs 50%			Control vs 100%		
	t <sub>stat</sub>	t <sub>crit</sub>	P value	T <sub>stat</sub>	T <sub>crit</sub>	P value	t <sub>stat</sub>	t <sub>crit</sub>	P value
1	<b>1.27x10<sup>15</sup></b>	<b>4.302</b>	<b>0.3x10<sup>-31</sup></b>	<b>5</b>	<b>4.302</b>	<b>0.018875</b>	<b>2.55x10<sup>15</sup></b>	<b>4.302</b>	<b>0.07x10<sup>-30</sup></b>
2	1	4.302	0.211325	1	4.302	0.211325	<b>12.12</b>	<b>4.302</b>	<b>0.003367</b>
3	<b>8</b>	<b>4.302</b>	<b>0.007634</b>	<b>0.63x10<sup>15</sup></b>	<b>4.302</b>	<b>1.23x10<sup>-30</sup></b>	<b>10</b>	<b>4.302</b>	<b>0.0049262</b>
4	<b>11</b>	<b>4.302</b>	<b>0.0040817</b>	<b>16</b>	<b>4.302</b>	<b>0.0019418</b>	<b>10.19x10<sup>15</sup></b>	<b>4.302</b>	<b>0.004x10<sup>-30</sup></b>
5	<b>0.63x10<sup>15</sup></b>	<b>4.302</b>	<b>1.233E-30</b>	<b>7</b>	<b>4.302</b>	<b>0.0099</b>	<b>16</b>	<b>4.302</b>	<b>0.001942</b>
6	1	4.302	0.211325	1	4.302	0.211325	<b>20</b>	<b>4.302</b>	<b>0.001245</b>
7	1	4.302	0.211325	1	4.302	0.211325	<b>13</b>	<b>4.302</b>	<b>0.002933</b>
8	1	4.302	0.211325	1	4.302	0.211325	1	4.302	0.211325
9	1	4.302	0.211325	1	4.302	0.211325	<b>11</b>	<b>4.302</b>	<b>0.004082</b>

\* bold lettered values indicate significant difference between growth

**Table 3:** Time interval during which 50% inhibition of luminescence is observed with *V. sagamiensis* and *V. azureus* exposed to different concentrations of RO rejects effluents.

RO reject Sample	<i>V. sagamiensis</i>			<i>V. azureus</i>		
	25% (v/v)	50% (v/v)	100% (v/v)	25% (v/v)	50% (v/v)	100% (v/v)
1	0-15 m	0-15 m	0-15 m	0-15 m	0-15 m	0-15 m
2	9-12 h	0-15 m	0-15 m	9-12 h	0-15 m	0-15 m
3	18-21h	9-12 h	3-6 h	0-15 m	0-15 m	0-15 m
4	0-15 m	0-15 m	0-15 m	0-15 m	0-15 m	0-15 m
5	*	0-15 m	0-15 m	6-9 h	3-6 h	3-6 h
6	9-12 h	0-15 m	0-15 m	*	*	3-6 h
7	6-9 h	3-6 h	3-6 h	6-9 h	3-6 h	3-6 h
8	*	*	0-15 m	*	0-15 m	3-6 h
9	18-21h	3-6 h	0-15 m	0-15 m	0-15 m	3-6 h

\* 50% inhibition of luminescence was not observed.

**Table 4:** Concentration of Nutrients in the RO reject samples.

RO reject Sample No.	pH	Turbidity (NTU)	PO <sub>4</sub> <sup>3-</sup> (umol/L)	NO <sub>2</sub> <sup>-</sup> (umol/L)	NO <sub>3</sub> <sup>-</sup> (umol/L)	NH <sub>4</sub> <sup>+</sup> (umol/L)	Si (umol/L)	Urea (umol/L)	TDS (mg/l)
1	8.48	5.91	823.27	230.26	744.31	342.39	832.3332	454.75	38645
2	7.63	7.53	64.99	2.19	50.30	1566.88	161.0161	48.32	23712
3	7.81	4.14	783.53	51.54	512.16	365.94	997.3497	74.86	23252
4	7.94	46.77	875.14	23.03	60.85	1403.52	871.0871	126.54	28200
5	7.88	66.4	198.54	7.68	20.71	3048.94	1934.943	46.93	1248
6	8.24	2.23	421.50	15.90	571.90	73.06	1707.421	45.53	5604
7	6.34	11.4	0.97	0.55	126.49	74.53	858.5859	41.34	3284
8	7.78	1.21	3.18	127.74	931.47	232.01	208.5209	65.08	39000
9	7.62	5.94	153.29	37.83	829.12	284.99	817.3317	79.05	20400

**Table 5:** Concentration of trace metals in the RO reject samples. All trace metal values in ppm (mg/l)

RO reject Sample #	Fe <sup>2+</sup>	Cu <sup>2+</sup>	Ni <sup>2+</sup>	Zn <sup>2+</sup>	Co <sup>2+</sup>	Cd <sup>2+</sup>	Mn <sup>2+</sup>	Hg <sup>2+</sup>	TOC
1	0.4826	0.2305	*	0.5035	*	*	0.0063	*	2065.0
2	0.0559	0.0098	*	0.1380	*	*	0.1071	*	306.05
3	2.0718	1.1966	*	0.4828	*	0.5904	0.1449	0.0018	223.65
4	2.9046	1.1476	*	0.7794	0.0033	*	0.1386	0.0030	419.15
5	0.0082	0.0608	*	0.0662	*	*	0.1134	*	101.90
6	0.5262	0.0804	0.0144	0.1159	0.0013	*	0.0025	0.0004	74.40
7	0.0212	0.0804	0.0112	0.0221	*	*	0.1335	*	21.42
8	0.0059	0.0255	0.0096	0.0276	*	*	*	*	46.70
9	0.8487	0.0098	0.0016	0.2290	*	*	0.4284	*	121.70

\* The concentration of trace metals in the RO reject samples was lower than the detection limit of the measurement technique.

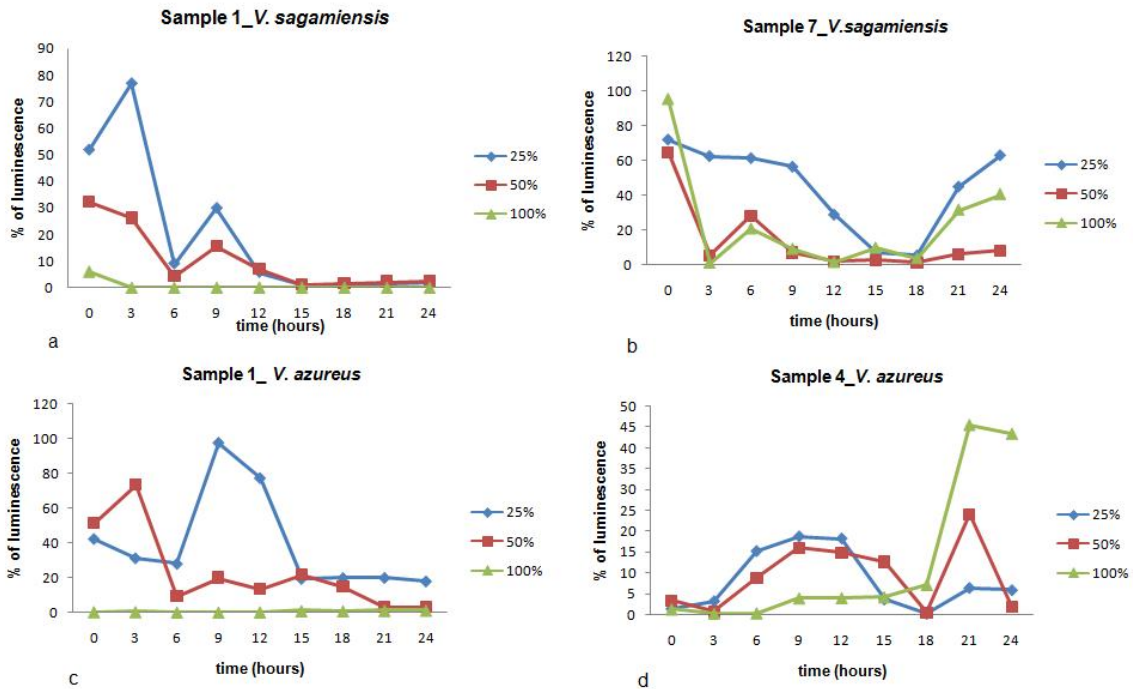


Fig. 1. 1a- Luminescence pattern of *V. sagamiensis* exposed to the RO reject sample 1, 1b – Luminescence pattern of *V. sagamiensis* exposed to the RO reject sample 7, 1c- luminescence pattern of *V. azureus* exposed to the RO reject sample 1, 1d- Luminescence pattern of *V. azureus* exposed to the RO reject sample 4. The mixed trend in the pattern of luminescence intensity of the bacteria exposed to RO reject in all the concentrations is less than the luminescence intensity of the control cultures. The luminescence intensity values are presented as the percentage of existing luminescence with reference to the control.

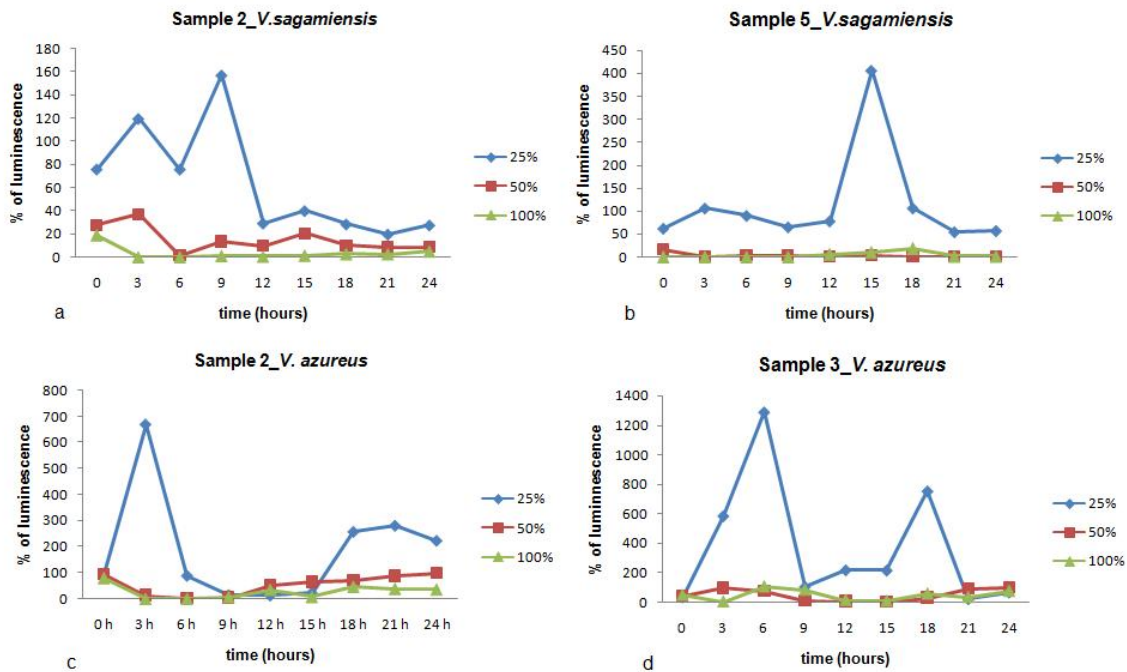


Fig. 2. 2a- Luminescence pattern of *V. sagamiensis* exposed to the RO reject sample 2, 2b- Luminescence pattern of *V. sagamiensis* exposed to the RO reject sample 5, 2c- Luminescence pattern of *V. azureus* exposed to the RO reject sample 2, 2d- Luminescence pattern of *V. azureus* exposed to the RO reject sample 3. The Mixed trend in the luminescence pattern of the bacteria exposed to RO reject at 25% (v/v) concentration is higher than the control culture, whereas those exposed to 50% (v/v) and 100% (v/v) is less than the control culture.



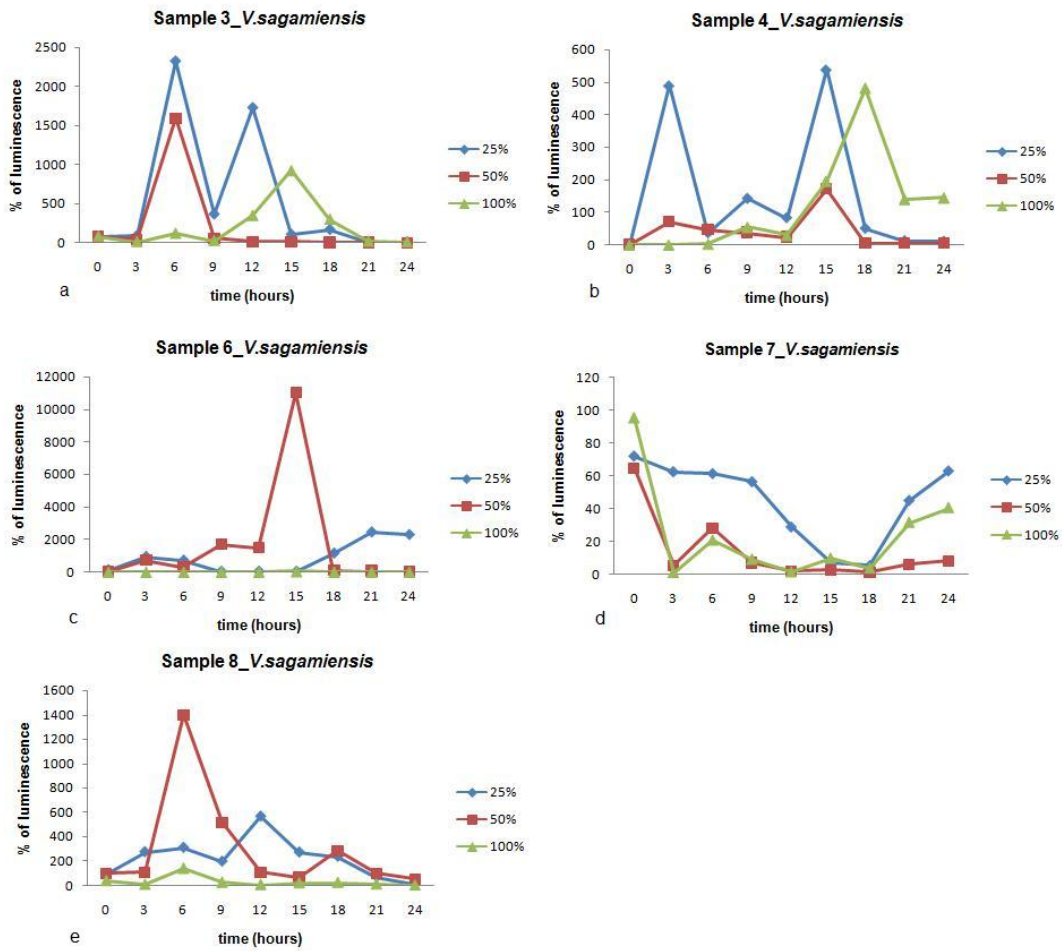


Fig. 3 (a-e). Luminescence pattern of *V. sagamiensis* exposed to the RO reject samples 3, 4, 6, 7 and 8 respectively at different concentrations. The Mixed trend continues for the entire incubation period.

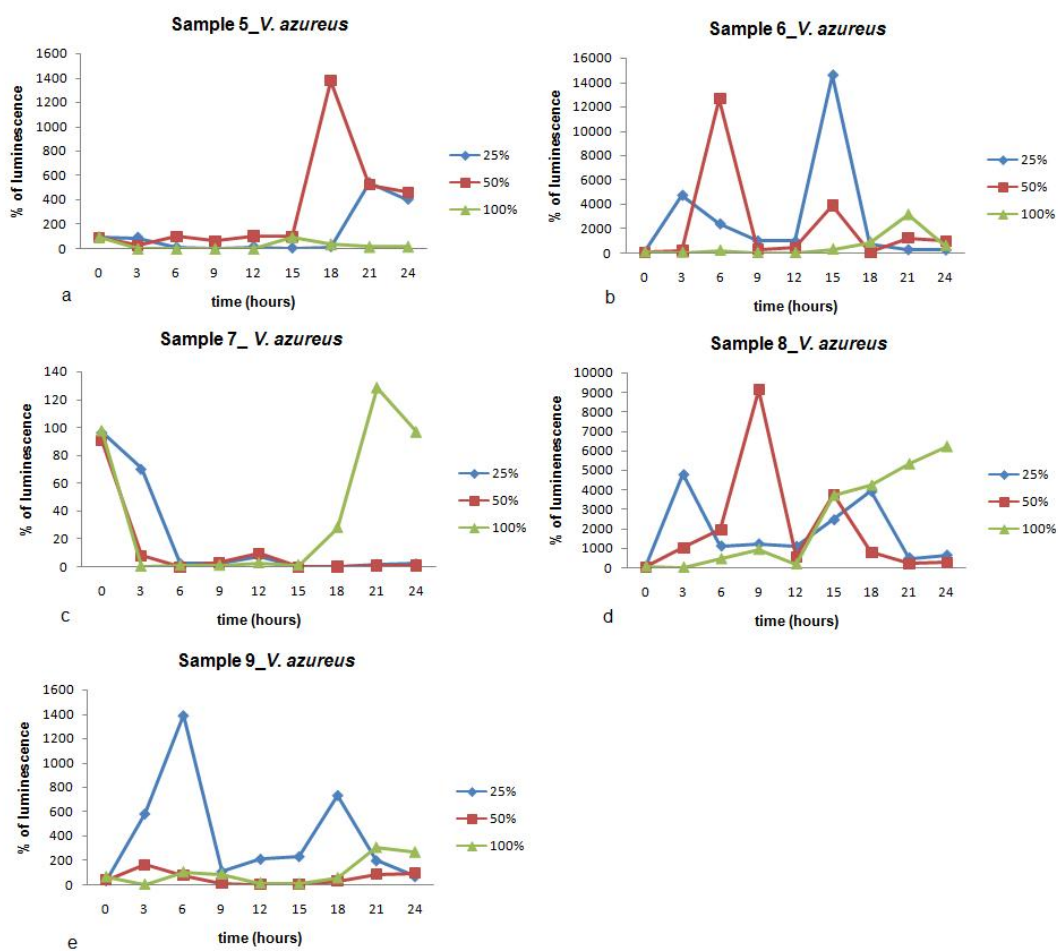


Fig. 4 (a-e). Luminescence pattern of *V. azureus* exposed to the RO reject samples 5, 6, 7, 8 and 9 respectively at different concentrations. The luminescence intensity exhibits a mixed trend of rise and fall during the long term incubation.