

Preliminary comparison of *Vibrio fischeri* and native *Vibrio* sp. Persian1 for ZnSO₄-induced bioluminescence inhibition in aqueous media

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Abstract Measurement of environmental contamination in water using bioluminescence systems is known as bioluminescence inhibition assay (BIA). Bioluminescence in these bacteria is regulated by quorum sensing, and BIA is a rapid toxicity assay based on the reduction of bacterial light emission after exposure to toxic compounds. This study was conducted to introduce a native strain with the aim of using bioluminescence for rapid and simple water contamination detection. For this purpose, *Vibrio fischeri* (standard strain) and a native Iranian species (*Vibrio* sp. Persian1) were cultured in different media (ASW, PGSW and CSW) under optimized conditions and used by BIA in order to monitor water contamination. The results of this study showed that a culture medium of artificial seawater (ASW) was suitable for growth and emission of light by these bacteria after 4 hours. The light emission of each bacterial strain was measured at 0 and 5 minutes, with three independent replicates. For this, ZnSO₄ was used as a model toxicant contaminant that may be present in water, with concentrations ranging from 0.25 to 1 M. This bioluminescence-based assay is currently used in various countries for monitoring and assessment of water pollutants. As a result, *Vibrio* sp. Persian1, which is native to our climate, can be used for rapid, simple, and cost-effective detection of environmental pollution in water under optimal conditions, which reduces energy consumption. Studies have indicated that in the presence of 1M zinc sulfate, *Vibrio fischeri* and *Vibrio* sp. Persian1 showed a 41% and 34% reduction, respectively.

Keywords: Bioluminescence Inhibition Assay; *Vibrio*, Zinc Sulfate; Water Toxicity; Bacterial Biosensor.

1.Introduction

Environmental contamination and its effects on drinking water is a major global problem and the assessment of contamination levels is vital for the health of the community. The most important water contaminants are harmful toxic chemicals, metal and biological compounds which come either from natural sources or agricultural or industrial waste [1,2]. The most significant part of this assessment is deciding whether the involvement of unintentional contamination of drinking water (agricultural pesticides) and deliberate (malicious purposes). Traditional and common methods such as gas chromatography-mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC), are used in most laboratories and water quality control centers. Although these methods are very accurate, they have some disadvantages that include being time-consuming, expensive, needing skilled personnel and expert and laboratory facilities [3,4]. The United States Environmental Protection Agency (US EPA) has approved nearly 500 innovative biological methods for measuring water pollution from 1995 to 2013. The sample exposure time for these techniques

ranges from 5 minutes to 2 hours. One such biological approach is the use of bioluminescent bacteria as biosensor, which dates back to the 1950s [1]. These bacteria are able to communicate with each other through small molecules released into the environment. Bacteria measure its density of their surroundings by producing and recognizing autoinducers. Over the past years, non-animal-based pollution measurement systems (mainly cell culture) have been introduced as complementary and in some cases alternative tests. Pollution measurement systems using lower organisms (bacterial tests, such as *Vibrio fischeri*) have been proposed as a rapid, environmentally friendly, low-cost, reasonable and easy method and this measurement test is valuable in water pollution control [5]. Common methods for measuring pollution include, monitoring changes in environmental carbon, sulfur, and nitrogen; determining microbial activity by methods such as ATP assays; measuring enzymes such as triphosphatase, glucose-6-phosphatase, alkaline phosphatase, and other enzymes; measuring growth, mortality, or photosynthesis; determining glucose uptake activity using radioisotopes; measuring oxygen consumption using dissolved oxygen electrodes; and measuring bioluminescence emission [6-8]. For this, scientists are looking to design simple, rapid, and low-cost detection methods. Luminous bacteria, including *Vibrio fischeri*, have a self-assembled mechanism named quorum sensing and could be used for water and Pollution monitoring. Quorum sensing is a bacterial self-induced response against the biotic and abiotic environment which is regulated by the genes coordinated expression and dependent on cell density [9-11]. Threshold assays are performed with the aim of determining the minimum stimulus or stimulus required to produce a measurable light response and are based on the induction or regulation of light in a bioluminescent system. In contrast, the bioluminescence inhibition assay (BIA) is a toxicity response assay in which the intensity of light emitted from the luminescent cells is measured after exposure to toxic compounds. Therefore, in BIA, the main goal is to determine the inhibitory or toxic effect of a substance on metabolic activities and light output, not to determine the threshold for bioluminescence induction. Accordingly, this method is also interpreted in the present study in toxicity assessment and monitoring of pollutants. *V. fischeri* is a distinct nonpathogenic member of the *Vibrionaceae* and an ideal candidate for monitoring pollution rather than pathogenic *Vibrio*. Using this bacterium in the bioluminescence inhibition assay (BIA) design a very fast, and sensitive method that has been taken into consideration by researchers [5,12,13]. The Bioluminescent bacteria

test protocol is usually easy, and this method is especially used for pragmatic to aqueous samples or extracts. Today, various commercial kits, including Mutatox, Microtox, BioTox and Toxalert 500 have been produced which are using these bacteria [14]. Although these bacterial bioluminescence-based methods cannot identify the exact nature of contamination, they provide a simple, rapid, practical, and sensitive approach for determining the presence or absence of contaminants in environmental samples. One of the problems with this method, despite its simplicity and speed, is the lack of reproducibility and sensitivity of this bacterium to temperature. Scientists are trying to identify new strains for more accurate and practical measurements so that commercial kits can be designed and manufactured based on them for measuring contamination. In the present study, we aimed to introduce a highly sensitive native bacterial strain as a potential addition to the existing bacterial systems used for contamination warning. Therefore, optimization and improvement of the performance of these bacteria are of great importance. For this, we investigated *V. fischeri* and *Vibrio* sp. Persian1 as the standard and native Iranian strain, respectively, under optimized culture conditions for the monitoring and warning of environmental agents and biological toxicity through changes in luminescence.

2. Experimental

2.1. Bacteria and chemicals

The chemical and biological materials were obtained from the following sources: *Vibrio fischeri*, prepared by the center for scientific and industrial research organization of Iran collection (industrial microorganisms identification code: PTCC1693) and *Vibrio* sp. Persian1, with access number KC505639 in the NCBI database, from Saman Hosseinkhani labs at Tarbiat Modares University [15].

2.2. Complete Artificial Seawater (ASW) Culture Medium

To prepare 1 liter of ASW culture medium 3 g of yeast extract, 5 g of peptone, 3 mL of glycerol, 24.7 g NaCl, 4.66 g MgCl₂, 1.36 g CaCl₂, 0.67 g KCl, 6.29 g MgSO₄, were purchased from Merck (USA) and HiMedia (India). All chemical materials were of the highest grades available.

2.3. Persian Gulf seawater (PGSW) Culture Medium

To prepare 1 liter of PGSW culture medium, 3 g of yeast extract, 3 mL of glycerol, and 5 g of peptone were weighed and made up to volume with 750 mL of Persian Gulf (Asalouye port and Genave port) seawater and 250 mL of double-distilled water, and autoclaved. It is worth noting that PGSW is one of these culture media [12]. Both bacterial strains were incubated, cultured, and examined in both culture media using samples collected from two separate regions of the Persian Gulf.

2.4. Caspian seawater culture medium (CSW)

To prepare 1 liter of CSW culture medium, 3 g of yeast extract, 3 mL of glycerol, and 5 g of peptone were weighed and made up to volume with 750 mL of Caspian seawater and 250 mL of double-distilled water, and autoclaved. It is worth noting that CSW is one of these culture media. The best culture medium was selected for bacterial growth and continued steps to investigate contamination.

2.5. Bioluminescence Inhibition Assay (BIA)

Vibrio fischeri (standard strain) and the native isolate *Vibrio* sp. Persian1 were initially cultured on solid ASW medium (1–2% agar) and incubated overnight at room temperature. Single luminous

colonies were selected and inoculated into optimized ASW liquid culture medium. The cultures were incubated at 28 °C in an orbital shaker at 200 rpm. Based on preliminary optimization experiments, a 4 h incubation period after inoculation provided suitable physiological conditions for contamination assessment using the *Vibrio fischeri* bioluminescence inhibition assay (BIA). To correlate optical density (OD₆₀₀) with viable cell counts (CFU/mL), a tenfold serial dilution method was employed. Briefly, bacterial cultures (*V. fischeri* and *Vibrio* sp. Persian1) were grown to the target optical densities. Aliquots of 100 µL from the serial dilutions (10⁻¹ to 10⁻⁷) were spread-plated onto ASW Agar plates in triplicates. After incubation at 28°C for 24 h, plates containing 30 to 300 colonies were counted. The colony-forming units per milliliter (CFU/mL) were calculated using the formula:

$$\text{CFU/mL} = \frac{\text{Number of colonies} \times \text{Dilution factor}}{\text{Volume plated (mL)}}$$

A calibration curve was established to convert OD₆₀₀ values to CFU/mL. Consequently, OD₆₀₀ = 0.32 corresponded to approximately 1.38 × 10⁸ CFU/mL for, and OD₆₀₀ = 0.28 *V. fischeri* corresponded to approximately 1.05 × 10⁸ CFU/mL for *Vibrio* sp. Persian1.

At this time point, the bacterial densities were as follows: *V. fischeri* reached an OD₆₀₀ = 0.32, corresponding to 1.38 × 10⁸ CFU, and *Vibrio* sp. Persian1 reached OD₆₀₀ = 0.28, corresponding to 1.05 × 10⁸ CFU. These cultures were used for subsequent toxicity measurements. For the bioluminescence inhibition assay, 200 µL of bacterial suspension was transferred into each well of a sterile 96-well microplate. Zinc sulfate (ZnSO₄) was used as a model toxic compound at concentrations ranging from 0 M + 0.25 M + 0.5 M + 0.75 M + and 1 M. The plates were incubated under controlled conditions and the emitted bioluminescence was measured immediately (0 min) and after 5 min of exposure.

Bioluminescence intensity was recorded using a luminometer (Berthold Detection System, Germany). The results were expressed as relative luminescence units (RLU), and the inhibition of luminescence was calculated by comparing treated samples with untreated controls. All experiments were performed in biological and technical replicates, and the obtained data were analyzed based on changes in luminescence intensity relative to the control samples. It should be noted that the number of times the experiment was repeated was three. Background luminescence was corrected by subtracting the signal obtained from a culture medium without bacteria. Wells containing only the ASW culture medium were used as blanks, and their luminescence values were subtracted from all measured samples before data analysis. The corrected luminescence values were then used to calculate the percentage of bioluminescence inhibition. The percentage of bioluminescence inhibition was calculated using the following equation:

$$\text{Bioluminescence inhibition (\%)} = \frac{(\text{RLU}_{\text{control}} - \text{RLU}_{\text{treated}})}{\text{RLU}_{\text{control}}} \times 100$$

where RLU_{control} represents the luminescence intensity of the control sample (without toxic compound) and RLU_{treated} represents the luminescence intensity of the sample exposed to the toxic compound.

3. Results

Analysis of white and brilliant colonies of *V. fischeri* and *Vibrio* sp. Persian1 was on agar medium in dark places in the laboratory,

individually (Figure 1). The monitoring of bacterial morphology and physiological characteristics were also confirmed relating to gram stain. Both bacterial strains were incubated in both culture media; the artificial seawater medium proved suitable for both strains, and was consequently selected for the continuation of the study.

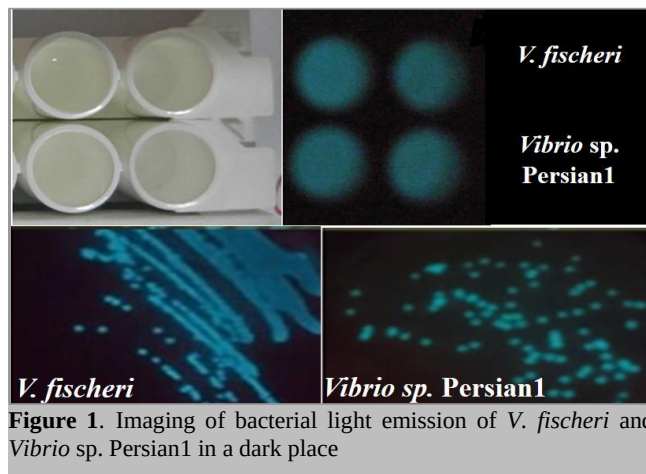


Figure 1. Imaging of bacterial light emission of *V. fischeri* and *Vibrio* sp. Persian1 in a dark place

In the next step, the growth rate and light emission of both strains were analyzed using nutrient liquid cultures for two hours and their growth curve was drawn. Considering the importance of biochemical compounds in ASW, both strains were evaluated and repeated in distinct regions of Iran (Figure 2).

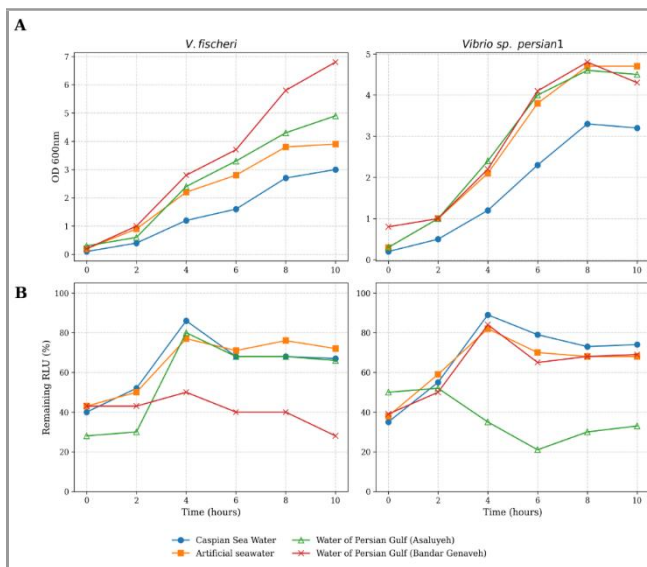


Figure 2. Comparison of growth and bioluminescence profiles for *V. fischeri* and *Vibrio* sp. Persian1 in four different media. **(A)** Growth kinetics (OD_{600}) showing maximum growth in Persian Gulf water (Bandar Genaveh) and minimum in the Caspian Sea water. **(B)** Bioluminescence intensity (Remaining RLU, %) indicating peak emission at 4 h for all conditions, which was selected as the optimal time for the VFBIA. Results show a distinct trade-off where Caspian Sea water supported the highest luminescence despite lower growth. Data are presented as mean $\pm$ SD ($n = 3$).

For toxicity experiments, two strains of *Vibrio* were cultured. different concentrations of zinc sulfate ($ZnSO_4$, 0 to 1 M) were added

and the amount of bioluminescence emission was also measured in the same way using a luminometer. According to figure 3, a significant reduction in the light emission of bacteria is demonstrated in the presence of 1 M $ZnSO_4$.

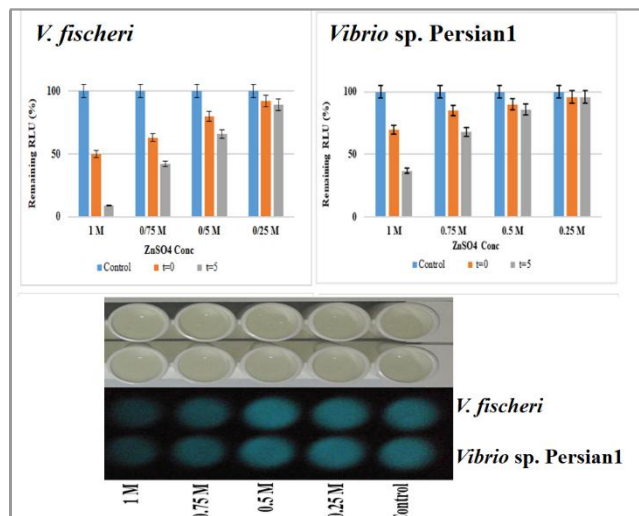


Figure 3. The inhibitory effect of different concentrations of $ZnSO_4$ on the bioluminescence emission of *V. fischeri* and *Vibrio* sp. Persian1

Overall, the strongest inhibition of luminescence was observed in *V. fischeri* exposed to 1 M $ZnSO_4$ at 5 min, where the remaining luminescence decreased to approximately 9% of the control, corresponding to about 41% inhibition. In contrast, the weakest effect was recorded for *Vibrio* sp. Persian1 at 0.25 M $ZnSO_4$ and 0 min, where luminescence remained at approximately 96% of the control, indicating only about 4% inhibition. These results show that *V. fischeri* was more sensitive to $ZnSO_4$ exposure than *Vibrio* sp. Persian1, particularly at higher concentration and longer exposure time.

Table 1. Luminescence inhibition percentage of *V. fischeri* and *Vibrio* sp. Persian1 exposed to different concentrations of $ZnSO_4$ at 0 and 5 min of exposure. Data are presented for two exposure times (0 and 5 min) and normalized to the control group.

$ZnSO_4$ (M)	Control V. <i>fischeri</i>	Control Vibrio sp. Persian1	t=0 min V. <i>fischeri</i>	t=5 min V. <i>fischeri</i>	t=0 min Vibrio sp. Persian1	t=5 min Vibrio sp. Persian1
1	100%	100%	~50%	~9%	~70%	~36%
0.75	100%	100%	~63%	~42%	~85%	~68%
0.5	100%	100%	~80%	~65%	~89%	~84%
0.25	100%	100%	~92%	~88%	~96%	~95%

4. Discussion

Luminescence assays have been used in recent years as one of the most common methods for detecting liquid pollution, especially water pollution. The use of luminescent organisms as a new approach in biological assessments has attracted the attention of researchers [16–19]. The Microtox system, which is based on *V. fischeri* luminescence inhibition, is currently one of the most widely

used laboratory methods for toxicity assessment. However, issues related to reproducibility have been reported. Variations in cell suspension and preparation conditions can lead to differences in measured concentrations and toxicity responses. Nevertheless, the system remains a reliable analytical method, particularly when control samples are included in each measurement [20,21]. In the current research, we used artificial seawater (ASW) as a suitable medium for growth and emission of light by *V. fischeri* and *Vibrio sp. Persian1*, and after 4 hours of cell culture. BIA, a 5-minute incubation with ZnSO₄, a reduction ranging from 4% to 40% was observed in the *V. fischeri*, and from 1% to 34% in the *Vibrio sp. Persian1*.

The bioassay results demonstrated a clear dose-response relationship for both strains exposed to ZnSO₄. *V. fischeri* exhibited the highest sensitivity, with the maximum luminescence inhibition reaching 91% (only 9% remaining RLU) at 1 M concentration after 5 min of exposure. The minimum inhibitory effect for this strain was 8%, observed at 0.25 M at t=0. Conversely, *Vibrio sp. Persian1* showed higher tolerance to the zinc stress. The maximum inhibition for this native strain was recorded at 64% (36% remaining RLU) under the same peak conditions (1 M, 5 min). The minimum response was a negligible 4% inhibition at 0.25 M at t=0. These findings indicate that while *V. fischeri* is a more sensitive biological indicator for rapid toxicity screening, the native *Vibrio sp. Persian1* possesses a more robust bioluminescence system under heavy metal stress, which could be attributed to its adaptation to the specific environmental conditions of its origin. The chemical composition of seawater varies across different seasons and locations in the Persian Gulf; consequently, seawater samples were collected from two distinct regions in southern Iran. The precise composition of the seawater from either region is neither constant nor fully characterized. Given that the indigenous strain had developed within that specific climate, the growth of both strains particularly the indigenous one—was evaluated; however, the results indicated superior bacterial growth for both strains in an artificial seawater culture medium.

The accuracy and applicability of Pollution assessment method using bioluminescent bacteria have been fully established and confirmed and are currently used in rapid water pollution assessment. Although this assessment does not identify the type of potential contaminant, it is considered and used as an easy and accurate final assessment of whether the water is healthy or unhealthy. What we investigated in this study is the introduction of a native strain that has the ability to assess water pollution with better accuracy. practical advantage observed in this study was the ability of the native strain to grow and emit detectable luminescence at laboratory ambient temperature, which is approximately the climatic temperature of the strain for bacterial growth and proliferation. Unlike *V. fischeri*, which has an optimal growth temperature that is lower than laboratory temperature and often requires cooling systems during assessments, *Vibrio sp. Persian1* maintained stable growth and light emission without reducing the ambient temperature. Also, temperature changes themselves disrupt the assessment and test reading time, so the presence of a strain adapted to the Iranian climate is very important. This feature reduces both energy consumption and operational costs in routine monitoring experiments. In this [experiment](#), we tested both bacterial strains at the same temperature, but the optimal temperature is not for *Vibrio fischeri*. Therefore, the bioluminescence inhibition assay (BIA) in optimal conditions could be used for detection of environmental pollution in water and environmental samples.

A comparative evaluation of the two *Vibrio* strains highlights several functional differences relevant to their application in

bioluminescence-based toxicity screening. Under identical culture conditions, *V. fischeri* exhibited slightly higher biomass accumulation, whereas the native *Vibrio sp. Persian1* maintained a more stable and sustained luminescence output over time. When exposed to ZnSO₄, *V. fischeri* showed a pronounced reduction in light emission, while *Vibrio sp. Persian1* demonstrated a noticeably lower degree of inhibition. This pattern suggests that the native strain possesses greater tolerance to metal-induced stress, potentially reflecting its ecological adaptation to the high-salinity and metal-variable marine environments of southern Iran. Taken together, these observations indicate that *Vibrio sp. Persian1* may serve as a complementary or alternative biosensor strain for rapid water toxicity assessment, particularly in settings where luminescence stability and environmental resilience are critical performance attributes. On the other hand, zinc is one of the metals that becomes highly toxic to bacteria when present at high levels.

Due to the toxicity of zinc to bacteria and eukaryotic organisms, including humans, this heavy metal was selected from among the existing contaminants for water. Studies have shown that although its toxic effects have been widely reported, the molecular mechanisms underlying Zn(II)-mediated protection or toxicity are not yet fully understood. In bacterial systems, extracellular Zn(II) can interfere with the uptake of other essential metals, particularly manganese (Mn II), by competing for binding to the solute-binding protein PsaA. Studies have shown that the Zn(II)–PsaA complex exhibits greater thermal stability compared to the Mn(II)–PsaA complex, suggesting that the binding of Zn(II) to this protein may be more persistent and less reversible. In vitro growth experiments have also demonstrated that the presence of extracellular Zn(II) significantly inhibits intracellular Mn(II) accumulation while having minimal impact on intracellular Zn(II) levels. The increase in intracellular zinc concentration $\backslash(\text{Zn}^{2+})$ is likely associated with the partial release and dissociation of zinc from metalloproteins. This process can be mediated by reactive oxygen species (ROS) and free radicals such as HOCl, H₂O₂, and O₂^{•−}. In addition, certain amino acids including L-glutamate, L-aspartate, and L-cysteic acid, as well as disulfide compounds, may contribute to this mobilization. However, it is still unclear whether the elevation of intracellular zinc levels represents a primary trigger of apoptosis or occurs as a secondary consequence for the apoptotic process [22–24].

5. Conclusion

This study represents a preliminary investigation into the sensitivity of a native bioluminescent bacterial strain in comparison with the standard strain *Vibrio fischeri* for environmental contamination detection. The results demonstrated that the native strain can be effectively prepared and used for the initial assessment of pollution, while showing comparable performance to the standard method. An important advantage of the native strain is its ability to grow and produce luminescence at ambient temperature, eliminating the need for cooling systems required for *V. fischeri*, which typically requires temperatures. This characteristic can significantly reduce energy consumption and operational costs in routine monitoring. In addition, the culture conditions and medium required for the native bacterium are compatible with the local environmental conditions, making it a practical candidate for regional applications. Although this study focused on a limited set of experimental conditions, the findings indicate that the native strain has promising potential as a biological tool for pollution monitoring. Further studies are necessary to evaluate the response of this bacterium to a broader range of common water pollutants and to determine its precise sensitivity across different contaminant concentrations.

Nevertheless, the results obtained in this research provide encouraging evidence for the development of a native bioluminescent bacterial system for environmental toxicity assessment.

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Conflict of Interest

The authors declare no conflict of interest.

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