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Fast Assessment of Quality of Water Containing Inorganic Pollutants Using Laser Biospeckles in Microbioassay

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Abstract: Recently, bioassay techniques have been gaining prominence in assessing water toxicity, offering comprehensive evaluations without identifying the individual chemical component. However, microscopic observation is a crucial component in microbioassays to know the critical features of the targeted microorganisms. However, as the microorganism's size becomes smaller, observation becomes more difficult due to the narrower focal depth of the imaging system. To address this challenge, we propose a novel laser biospeckle non-imaging technique utilizing biospeckle patterns generated by microorganisms, enabling non-imaging assessments of their swimming ability. Paramecium and Euglena were used as microorganisms. Paramecium and Euglena were subjected to varying concentrations of heavy metal pollutants ($Zn(NO_3)_2 \cdot 6H_2O$ and $FeSO_4 \cdot 7H_2O$), and their swimming activity was quantified using a dynamic biospeckle analysis. The results show a concentration-dependent effect of Zn on both species, leading to decreased swimming ability at increased concentration. Conversely, Fe exhibited varying effects on Paramecia and Euglena, with the latter displaying tolerance at lower concentrations but a notable response at higher concentrations. The advantage of the method is that owing to the non-imaging system, an enormous number of microorganisms can be processed. Moreover, the method allows for an immediate and statistically significant estimation of their swimming ability in response to environmental pollution.

Keywords: microbioassay; biospeckle; microorganisms; Euglena; Paramecia; water toxicity; heavy metals; Zn; Fe environmental pollution; swimming ability

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1. Introduction

In recent years, the proliferation of heavy metals in the environment has emerged as a significant environmental concern, with profound implications for ecosystem health and human wellbeing [1]. Among these metals, iron (Fe) and zinc (Zn) play pivotal roles in various industrial processes, agricultural practices, and household applications, leading to their widespread presence in natural ecosystems [2]. While essential in trace amounts for biological functions, elevated concentrations of these metals can harm aquatic organisms, terrestrial flora, and, ultimately, human populations through biomagnification and bioaccumulation processes [3,4]. The emerging heavy metal pollution requires innovative approaches that quantify metal concentrations and assess their biological impacts with precision and sensitivity. Direct toxicity assessment (DTA) methods or bioassays have recently garnered significant attention for their ability to comprehensively evaluate toxicity without specifying individual chemical substances. These bioassays measure the response of organisms to contaminants compared to controls [5,6]. By using microorganisms that are sensitive to environmental change, the combined biological activities of a mixture of substances extracted from a water sample or any other polluted environment can be assessed. It enables unknown compounds to be detected based on their activity and provides toxicological relevance to the broad chemical characterization of this mixture [7].

Bioassays using microorganisms or bacteria are called microbioassays. Microbioassays for toxicity assessments have many advantages over conventional methods; they are inexpensive, have short life cycles, less time-consuming, and are sensitive to different toxic chemicals, such as heavy metals, phenolic compounds, organic and inorganic pollutants, and other effluents from industrial activities [8]. However, in this technique, microscopic observation is necessary to derive the critical features of the targeted microorganisms, such as their state (alive/dead) and swimming ability. As the microorganism's size becomes smaller, observation becomes more difficult due to the narrower focal depth of the imaging system [9].

In our study, to overcome these difficulties, we have proposed a novel technique for the microbioassay utilizing biospeckles in the diffraction field generated from the microorganisms under coherent light illumination [10]. Based on the dynamic properties of the biospeckle generated by the microorganisms under different toxicity conditions, it is easy to determine the organisms' activities or healthiness. Here, we employed two microorganisms, Paramecia and Euglena. When a living biological organism is illuminated by a coherence light, a biospeckle pattern is generated [11]. If a sample that is being observed is a biological object, such as a microorganism that can move, then the intensity of the speckle ceases to be constant, and a dynamic biospeckle pattern is observed [12]. The inhibition of the swimming ability of microorganisms due to toxicity can be evaluated by analyzing the dynamic properties of biospeckles generated from their movement. The biospeckle pattern reflects Paramecia and Euglena's movement and the organelles' internal activity within microorganisms.

The proposed method offers several advantages, including the ability to process many microorganisms simultaneously without the need for imaging systems. Owing to a non-imaging system, an enormous number of microorganisms, such as Paramecia and Euglena, can be processed at a time, and a statistically significant estimation of the swimming ability of microorganisms under different water toxicity concentrations can be derived immediately and efficiently.

2. Materials and Methods

2.1. Organism Breeding

For this study, two different types of microorganisms were employed. The first was Paramecia caudatum, with a size of 200 to 300 μm; the other was Euglena gracilis of size 150 to 200 μm. Both microorganisms were cultured in the laboratory on a clean bench (BH-900UVAX; As One Co., Ltd., Osaka, Japan) at an optimum temperature of 25 °C and were exposed to fluorescent light for 24 h/day with loose fitting covers. The organisms were cultured in 500 mL jars and fed every two weeks to maintain the stable culture density. For Euglena, 1000-fold diluted Hyponex solution along with soy milk was employed as nutrients, and for Paramecia, Chalkley's medium, including wheat grains, was utilized. These culture media were utilized as a control or baseline to investigate their swimming ability in a clean and unpolluted environment. The ideal density for Paramecia and Euglena was attained after 1–2 weeks, and all experiments were conducted 7–10 days after the culture was divided.

2.2. Sample Preparation

2.2.1. Sample Preparation for Rheology Study

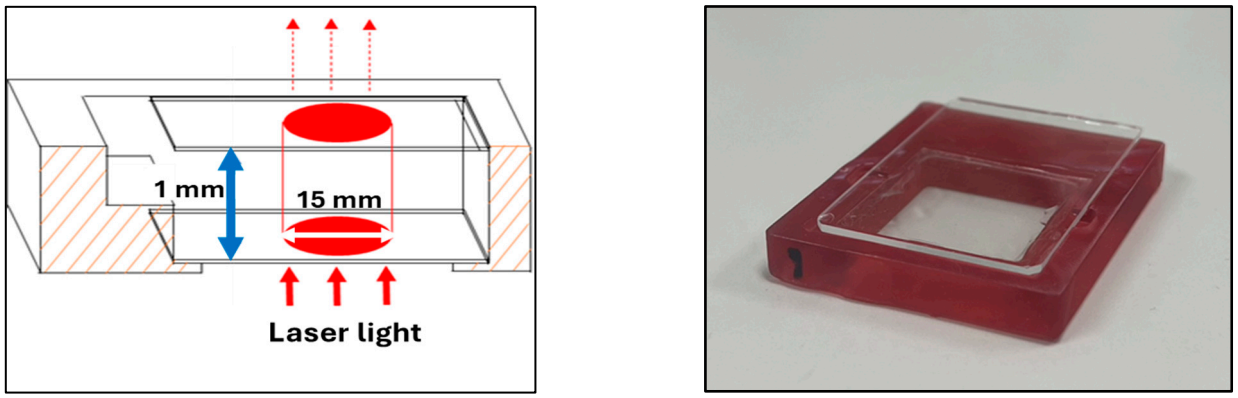
Methylcellulose (MC) 100 was purchased online from Fujifilm Wako Pure Chemical Corporation. Different concentrations of MC solutions, 1, 2, 3.2, and 5 mg/L, corresponding to 2.5, 5, 8, and 12 cP in viscosity, respectively, were prepared and filtered. Then, 1 mL of a concentrated culture of Paramecia (by centrifuging at 300× *g*) was pipetted and incubated with 100 mL of the prepared samples with different viscosities in containers at room temperature for 1.5 h. The culture media viscosity was considered 1 cP and used as a control.

2.2.2. Sample Preparation for Water Toxicity Assessment Study

This study used iron (Fe) and zinc (Zn) as heavy metal water pollution. The concentrations of the pollutant were selected based on their permissible level (PL) in drinking water recommended by the World Health Organization (WHO) [13,14]. Here, we selected two sets of concentrations: high and low. The chosen concentrations for the low sets were 1, 2, 10, and 20 times the concentration of PL in comparison to 0× (the water media control), and the selected concentrations for the high sets were 10, 200, 500, and 1000 times the concentration of PL in comparison to control. In the experiments, zinc nitrate hexahydrate ($Zn(NO_3)_2 \cdot 6H_2O$) and iron (II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), purchased from Fujifilm Wako Pure Chemical Corporation, were used as Zn and Fe sources, respectively. The concentrations used were as follows: (1) zinc at 0 mg/L, 5 mg/L, 10 mg/L, 50 mg/L, and 100 mg/L; (2) iron at low concentration set of 0 mg/L, 0.1 mg/L, 0.2 mg/L, 1 mg/L, and 2 mg/L and at high concentrations of 0 mg/L, 1 mg/L, 20 mg/L, 50 mg/L, and 100 mg/L.

2.3. Sample Cell

A custom sample cell (illustrated in Figure 1) was fabricated using a 3D printer, featuring a window size of 20 × 20 mm. During experiments, an additional glass plate was placed on the top of the cell. Each treatment utilized a sample cell with a 1 mm cell gap. The diameter of the probing volume was 15 mm. For each treatment condition, 8×10^6 Paramecia and 5–6.0 × 10^4 Euglena cells were collected volumetrically. Following mounting the sample cell, a stabilization time of 2 min was given before recording the biospeckles. Furthermore, before recording the movies, the viability of the samples was thoroughly assessed under a microscope (S/NO. 1L0791, Olympus, Japan).



(**a**) (**b**)

Figure 1. A schematic of the sample cell used in the experiments (**a**) and a photograph of the actual cell (**b**). The cell gap was 1 mm, and the diameter of the probing area was 15 mm (red shaded area).

2.4. Experimental Setup

The experimental setup to record the dynamic biospeckle is shown in Figure 2. The system consisted of a laser diode of wavelength 638 nm: two mirrors, M1 and M2, lenses L1 and L2; a polarizer; a spatial filter; and a CCD camera (MCM-303MVC, Gazo Co., Ltd., Tokyo, Japan). The laser light was collimated by lens L1 of focal length 30 mm. The sample cell with a gap of 1 mm was illuminated vertically from the bottom with the light reflected by mirror M1. The illuminated or probe volume on the sample cell was 15 mm in diameter. The light transmitted through the sample cell was focused by lens L2 of focal length 250 mm. A spatial filter was placed at the Fourier plane of the sample to filter out the specular wave components of transmitted light. The dynamic biospeckle reflecting the movement of Paramecia and Euglena under different water toxicities was captured with a CCD camera.

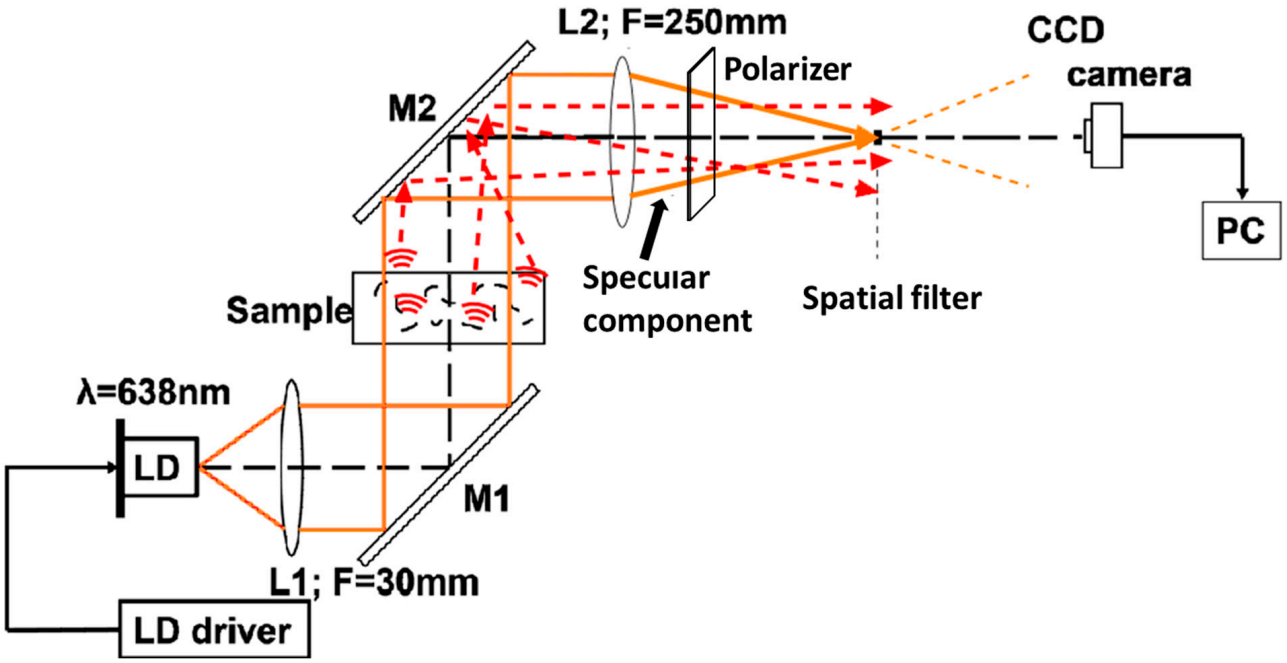


Figure 2. A schematic diagram of the experimental system consisting of M1~M2 mirrors, L1~L2 lenses, a polarizer, and a CCD camera. The parallel beam irradiated a sample cell (cell gap: 1 mm) containing microorganisms.

2.5. Statistical Analysis

The recorded dynamic biospeckle movies were analyzed with a custom-made MATLAB program using the vision and image analysis toolbox of MATLAB (Math work, USA R2016a). All data in this study represent the mean of nine identical experiments performed in triplicate and are presented as the mean ± standard deviation. Significant differences between the treatments and control were determined with the Student's *t*-test. For comparing different concentrations of heavy metals against different time points, two-way ANOVA was used. For either statistical test, the significance threshold was set at $p < 0.05$.

2.6. Biospeckle Cross-Correlation Method

The cross-correlation analysis method was used to evaluate the microorganisms' swimming ability under different concentrations of heavy metal pollution. In this method, the correlation coefficient $\rho(t_i)$ between the initial frame $I(t_o; x, y)$ being fixed as a reference frame and the frame $I(t_i; x, y)$ at the time t_i of the recorded frame of biospeckle video was calculated as given by Equation (1):

$$\rho(t_i) = \frac{\sum_{x,y}\{I(t_i;x,y) - \langle I(x,y)\rangle\}\{I(t_0;x,y) - \langle I(x,y)\rangle\}}{\sqrt{\sum_{x,y}\left\{I(t_i;x,y) - \langle I(x,y)\rangle\right\}^2 \sum_{x,y}\{I(t_0;x,y) - \langle I(x,y)\rangle\}^2}} \quad (1)$$

where $I(t_i;x,y)$ is the intensity at coordinate (x, y) at time t_i and $\langle I(x,y)\rangle$ denoted time average of the intensity given by Equation (2):

$$\langle I(x,y)\rangle = \frac{1}{N}\sum_{i=1}^{N} I(t_i;x,y) \quad (2)$$

where N is the total number of frames.

Next, in this study, the correlation time, denoted as τ_c, served as a quantitative measure of the swimming ability of microorganisms. The concept of correlation time is rooted in the analysis of biospeckle patterns generated by the movement of microorganisms. These speckle patterns fluctuate over time as the microorganisms move, and the rate of these

fluctuations provides insights into their motility. The correlation time τ_c was defined as the time at which the cross-correlation coefficient falls to $\frac{1}{e}$ of its initial value.

The correlation time is inversely related to the speed of movement of the microorganisms. A larger correlation time indicates slower movement of the microorganisms, which could imply that they are experiencing stress, reduced motility, or poor health. Conversely, a smaller correlation time indicates swifter movement of the microorganisms, suggesting that they are healthier and more active. By measuring the correlation time, it is possible to observe the swimming ability of microorganisms or, indirectly, their overall health and physiological state. Finally, the correlation time for both microorganisms was plotted as a function of varying concentrations of Fe and Zn.

3. Results and Discussion

3.1. Indirect Measurement of Viscosity

In this study, we aim to study the deviation in the swimming ability of ubiquitous microorganisms (Paramecia and Euglena) in contaminated water due to the presence of emerging pollutants Fe and Zn. The method is based on a cost-effective implementation that permits the assessment of water quality by evaluating the collective influence of heavy metal pollutants on the swimming ability of aquatic microorganisms. Additionally, we have measured the swimming ability of Paramecia in solutions of different viscosity, η, to see how their swimming behavior changes with increased drag. Since the relationship between an environment's viscosity and the swimming ability of Paramecia has previously been measured numerous times [15,16], we therefore used this study to correlate the proposed/novel biospeckle technique to earlier findings before applying it to the actual water quality assessment.

The advantage of selected organisms is that in addition to being common in nature, their swimming ability is tremendously and significantly influenced by the viscosity of their habitats [17].

The cross-correlation function as a function of time in different viscosity solutions is shown in Figure 3. The graph indicates that the width of the cross-correlation function spreads out as the viscosity of the media increases, indicating the slow movement of plankton under different viscous solutions. In the control group (1 cP methylcellulose), Paramecia exhibited their typical fast and erratic swimming patterns. At 2.5 cP methylcellulose, a slight reduction in speed was noted, but the Paramecia still managed to navigate relatively well. However, at viscosity concentrations of 2.5 cP and above, the Paramecia's swimming speed was markedly reduced, and their movements became more sluggish and less coordinated. At the highest viscosity concentration (8 and 12 cP), Paramecia struggled to move at all, often appearing to be trapped in the viscous medium. The cross-correlation time at 2.5, 5, and 8 cP was observed to be approximately 2, 4, and 4.5 times larger than the control, respectively. As reported in previous studies, the swimming ability of Paramecia is strongly affected by the viscosity in the range of 1–5 cP, showing an average relative drop of 29.2% in the correlation time at 1 cP per increment. However, on a further increase in the viscosity range 5–12.5 cP, the Paramecia movement was completely hindered, and the correlation time was observed to be the same as 5 cP. This reduction in swimming ability at higher methylcellulose concentrations can be attributed to the increased resistance against the ciliary beat, which Paramecia are unable to overcome. The tendency of the presented data with the previous studies regarding the swimming behavior of Paramecia and their dependency on the viscosity of the surrounding media is noteworthy and suggests the suitability of the swimming behavior as a strong indicator for general use.

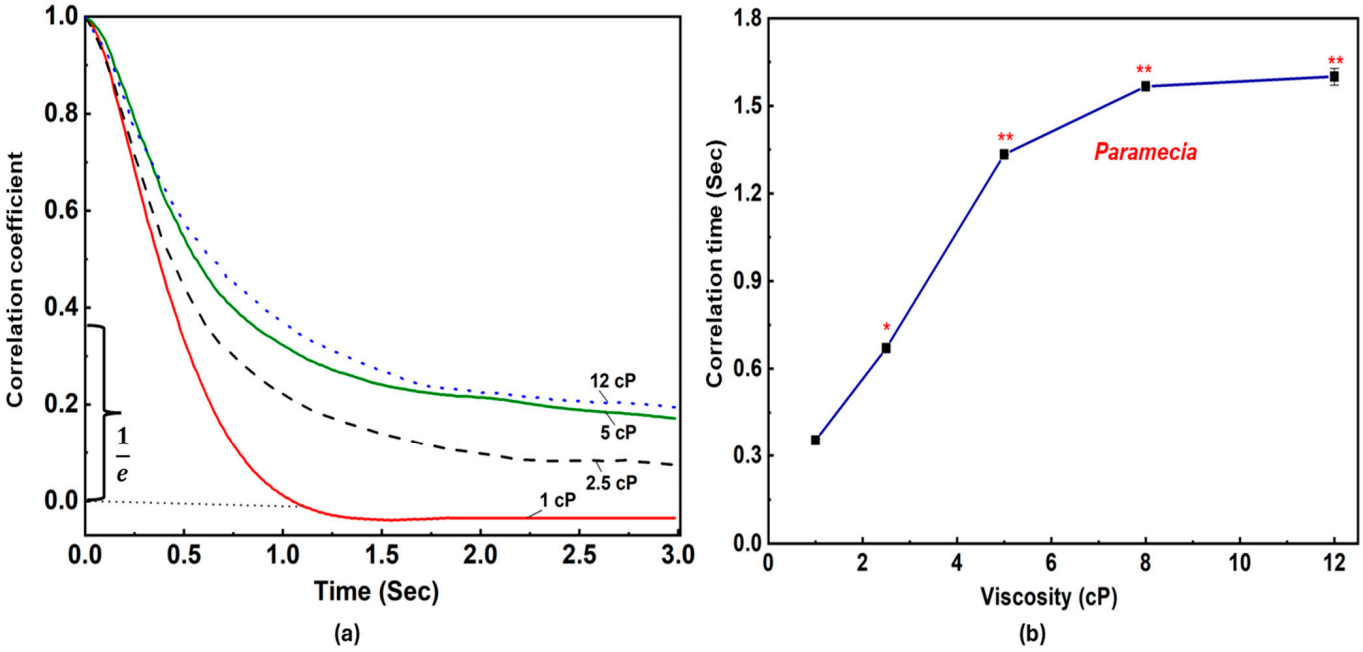


Figure 3. (**a**) The cross-correlation function as a function of time for Paramecia under different viscosity media. (**b**) The correlation time as a function of viscosity for Paramecia. The error bars indicate the standard deviation. *, ** indicated the statistical significance of data $p < 0.05$ and $p < 0.01$. N = 9.

3.2. Detection of Water Toxicity

3.2.1. Fe as a Water Toxicity

Euglena and Paramecia were initially exposed to varying concentrations of an emerging heavy water pollutant, iron (II) sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$). The permissible level (PL) for Fe, as determined by the World Health Organization, is 0.1 mg/L. The microorganisms were exposed to concentrations of 2× (0.2 mg/L), 10× (1 mg/L), and 20× (2 mg/L) the PL of Fe. The exposure time was 1.5 h for each study. The experimental results revealed that on increasing the heavy metal concentration, the cross-correlation function was spread out, indicating the slow movement of the Paramecia, as shown in Figure 4a. The correlation time as a function of Fe is shown in Figure 4b, and a concentration dependency can be seen clearly in the case of Paramecia. On increasing the Fe concentration, especially after two times PL, a significant reduction in the swimming ability of Paramecia was observed compared to the control, indicating the toxic water condition. The correlation time increased 1.5 and 2 times compared to the control for concentrations of 10 and 20× PL.

However, interestingly, at 2× PL, the width of the cross-correlation function or the correlation time was reduced by 8% from the control. This indicates that Paramecia exhibited increased activity or improved health conditions compared to the control group at this concentration. It is reported that Fe at low concentration behaves as a growth promotor for Paramecia caudatum, which is well supported by the smaller correlation time [18]. Additionally, at PL, at a concentration of 0.1 mg/L of Fe, no significant change in their swimming ability was observed. This might be because at this concentration, Paramecia developed resistance/tolerance. However, as the Fe concentration increased from 2× PL to 10× and 20× the PL, the correlation time was observed to be significantly higher, indicating the slower movement of the Paramecia under a toxic water environment. This suggests that concentrations above 2× the PL of Fe were toxic to Paramecia.

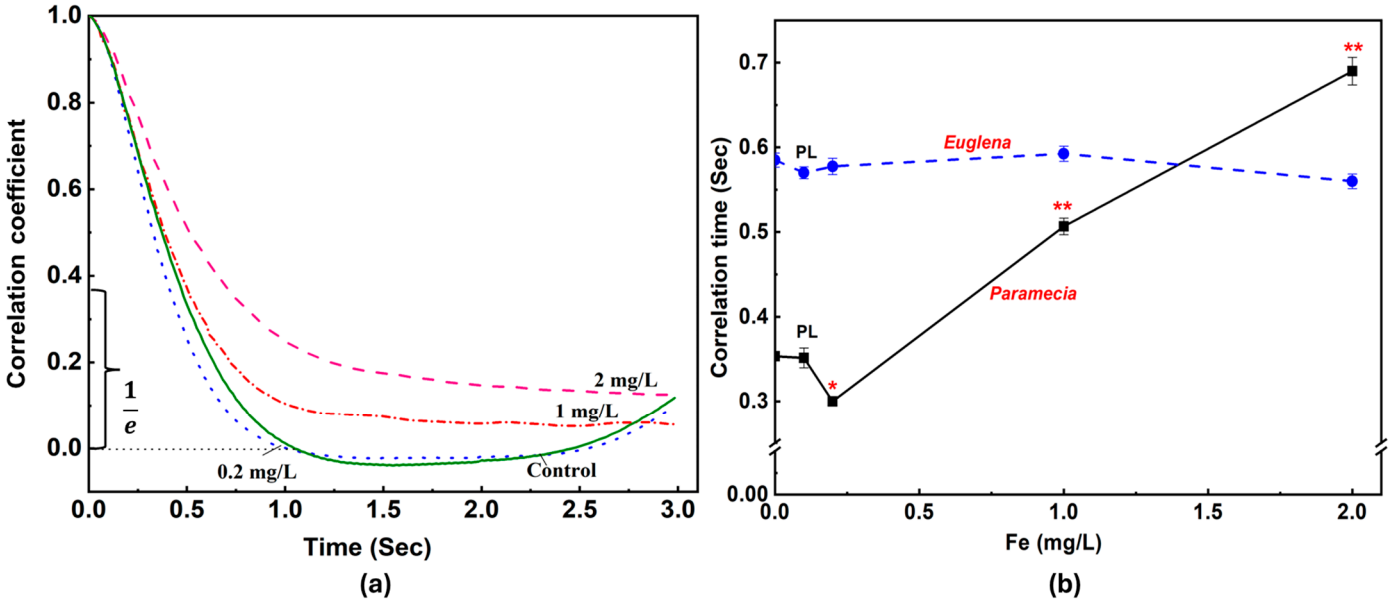


Figure 4. (**a**) Cross-correlation function of Paramecia per unit time under different Fe concentrations and (**b**) the correlation time as a function of varying Fe concentrations (mg/L) for Paramecia and Euglena *, ** indicated the statistical significance of data $p < 0.05$ and $p < 0.01$ (N = 9).

On the other hand, when Euglena was exposed to the same set of concentrations, no significant change was observed in their swimming ability. The correlation time curve remained relatively flat compared to Paramecia at this concentration range, as depicted in Figure 4b. More specifically, the correlation times for concentrations of 2× PL, 10× PL, and 20× PL (0.2, 1, and 2 mg/L) were noted to be nearly equal to that of the control, approximately 0.57 s for Euglena. This suggests that Euglena showed tolerance behavior to this concentration range (0.1–2 mg/L).

However, as Fe concentrations increased further, specifically at 200× PL (20 mg/L), 500× PL (50 mg/L), and 1000× PL (100 mg/L), a significant change in cross-correlation function and the correlation time was observed as compared to the control for Euglena, shown in Figure 5a,b. This increment indicates the slower movement of Euglena as the environmental toxicity increased. A distinct concentration dependency emerged at elevated Fe concentrations. This behaviour suggested a transition from tolerance to responsiveness at elevated/higher Fe concentrations, as shown in Figure 5b. Specifically, the correlation times were noted to be 1.3-, 7-, and 12-fold larger than that of the control at 200× PL, 500× PL, and 1000× PL, respectively.

However, Paramecia retained its sensitivity at elevated concentrations, with the correlation time increasing significantly beyond 2× PL. For Paramecia, the correlation times at 200× PL, 500× PL, and 1000× PL were observed to be 1.6, 5.2, and 7.5 s, respectively, representing 4.5, 14, and 21.4 times larger than the control, depicted in Figure 5b. At elevated Fe concentrations, Euglena and Paramecia exhibited nearly identical behavior, likely because such high doses of Fe water toxicity could prove fatal to them.

These findings indicate that Paramecia was proven to be more sensitive to water toxicity assessments in the case of Fe. Interestingly, unlike Paramecia, no significant change in the correlation time was observed for Euglena when the concentration of Fe increased to 2× PL. This suggests that Euglena has a higher tolerance to the pollutant. However, a clear trend emerged with further increments in the concentration of Fe.

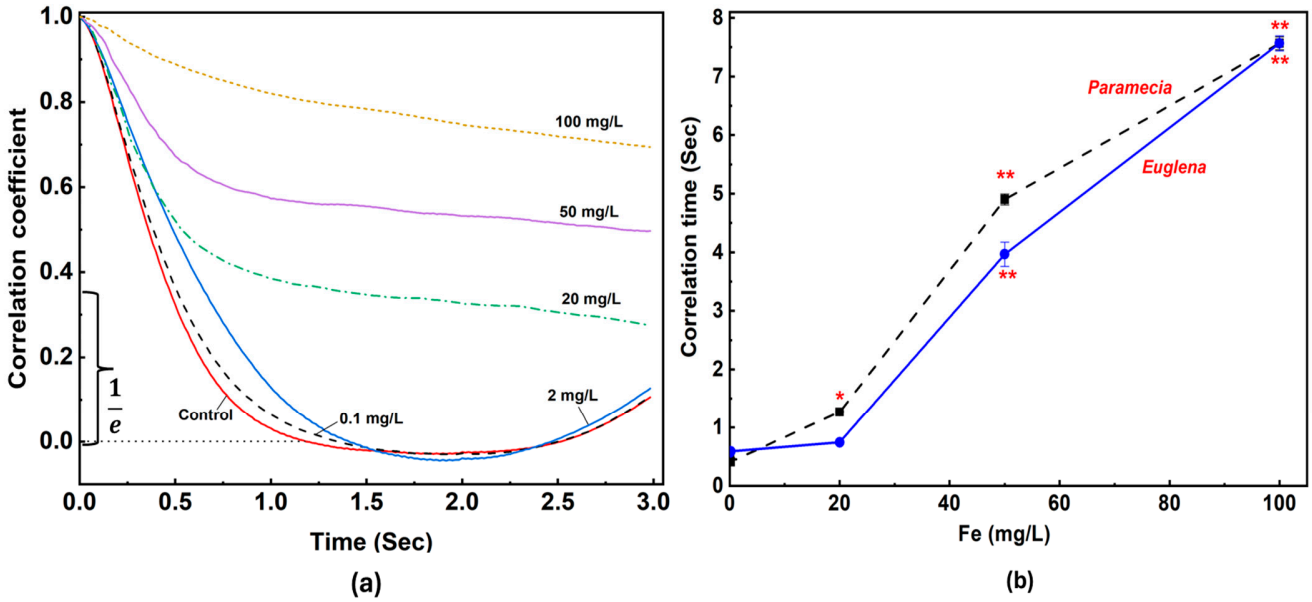


Figure 5. (**a**) The cross-correlation function of Euglena per unit time under different Fe concentrations and (**b**) the correlation time as a function of varying Fe concentrations (mg/L) for Euglena and Paramecia, *, ** indicated the statistical significance of data $p < 0.05$ and $p < 0.01$ (N = 9).

3.2.2. Zn as Water Toxicity

Euglena and Paramecia were exposed to varying concentrations of zinc nitrate hexahydrate ($Zn(NO_3)_2 \cdot 6H_2O$), another heavy metal water pollutant. The permissible level (PL) for Zn, as determined by the World Health Organization (WHO), is 5 mg/L. The Euglena and Paramecia were exposed to concentrations equivalent to 2, 10, and 20 times the PL.

The cross-correlation function as a function of time was employed to assess the swimming ability of Paramecia under different Zn concentrations, as shown in Figure 6a. Additionally, Figure 6b illustrates the correlation time as a function of the concentration of Zn. A clear concentration-dependent response of Paramecia to Zn exposure could be seen. A significantly higher correlation time was observed as the Zn concentration exceeded the PL 10 times. Specifically, at 20 times the PL, the correlation time increased by approximately 30 times compared to that of the control. This significantly higher correlation time indicates excessive water toxicity conditions at 20× PL or 100 mg/L Zn concentration. At this concentration, the swimming ability of Paramecia was completely hindered due to the toxic environment. In other words, no variation in biospeckle patterns was observed, indicating diminished activity.

Surprisingly, contrasting results were observed at concentrations equivalent to the PL (5 mg/L) and 2× PL (10 mg/L), where a significant reduction in correlation time compared to the control was observed in Paramecia. This reduction, approximately 18% less than the control, suggests the heightened activity and rapid movement of Paramecia at these lower concentrations. Similar behavior was observed in the case of Fe for Paramecia. This phenomenon could be attributed to the beneficial role of Zn as a micronutrient at lower concentrations, supporting the health and vitality of the Paramecia. In summary, the dual impact of Zn was seen on Paramecia, with concentrations exceeding 10 times the PL, proving high water toxicity. In contrast, lower concentrations demonstrate a stimulatory effect on microorganism activity, indicating relatively low water toxicity. This behavior might be due to the micronutrient properties of Zn.

Figure 7a shows the cross-correlation function as a function of time, and Figure 7b shows the correlation time as a function of Zn concentrations for Euglena. The experimental results revealed a clear concentration-dependent response of Euglena to Zn exposure. As shown in Figure 7b, a significantly larger correlation time was observed compared to that of the control with increasing Zn concentrations beyond 2× PL. More specifically, the

correlation time becomes approximately 2 to 4 times greater than that for the control. At 10 and 20× PL Zn concentrations, the correlation time was significantly high, indicating the slower swimming ability of Euglena. This significant increase in correlation times suggests that concentrations of 50 and 100 mg/L of Zn were found to be toxic for Euglena, resulting in a complete hindrance of their swimming ability due to heightened water toxicity. Interestingly, at PL and 2× PL, a significant reduction in the correlation time or cross-correlation function was observed compared to the control. This result indicates that within this range (5–10 mg/L) of Zn exposure, Euglena's swimming ability was increased compared to the control. This might be because of the micronutrient behavior of Zn. A similar kind of behavior was also observed for Paramecia in the case of lower Zn concentrations [19].

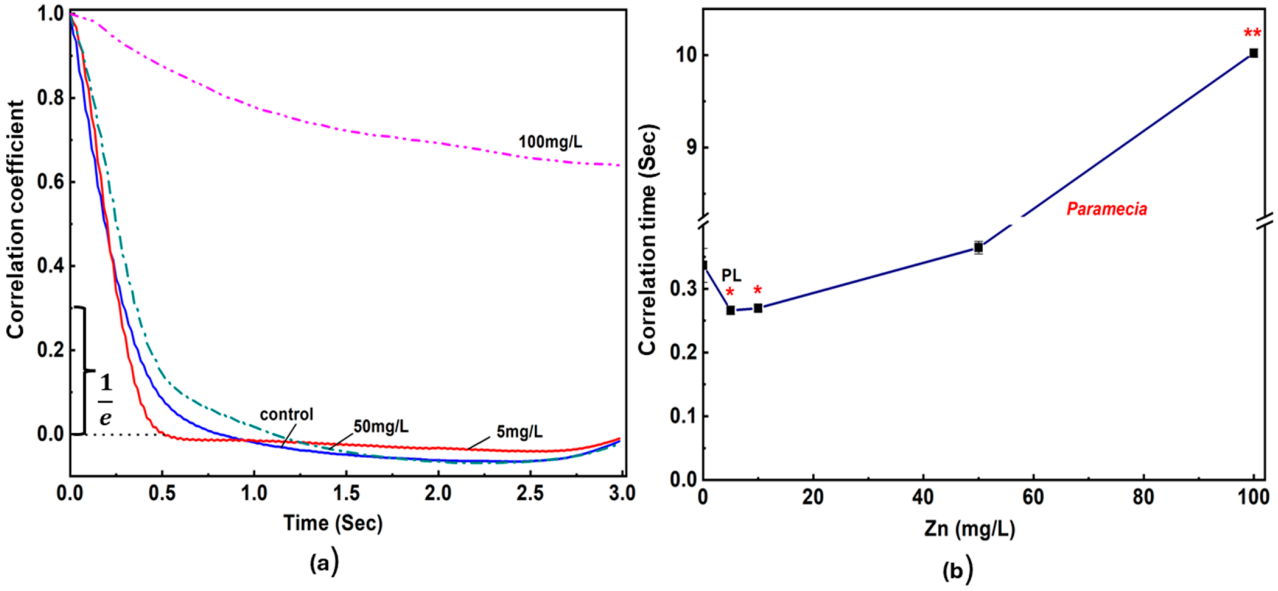


Figure 6. (**a**) The correlation function as a function of time for Paramecia under different Zn concentrations shown, (**b**) the correlation time as a function of Zn concentration (mg/L) for Paramecia. *, ** indicated the statistical significance of data $p < 0.05$ and $p < 0.01$. N = 9.

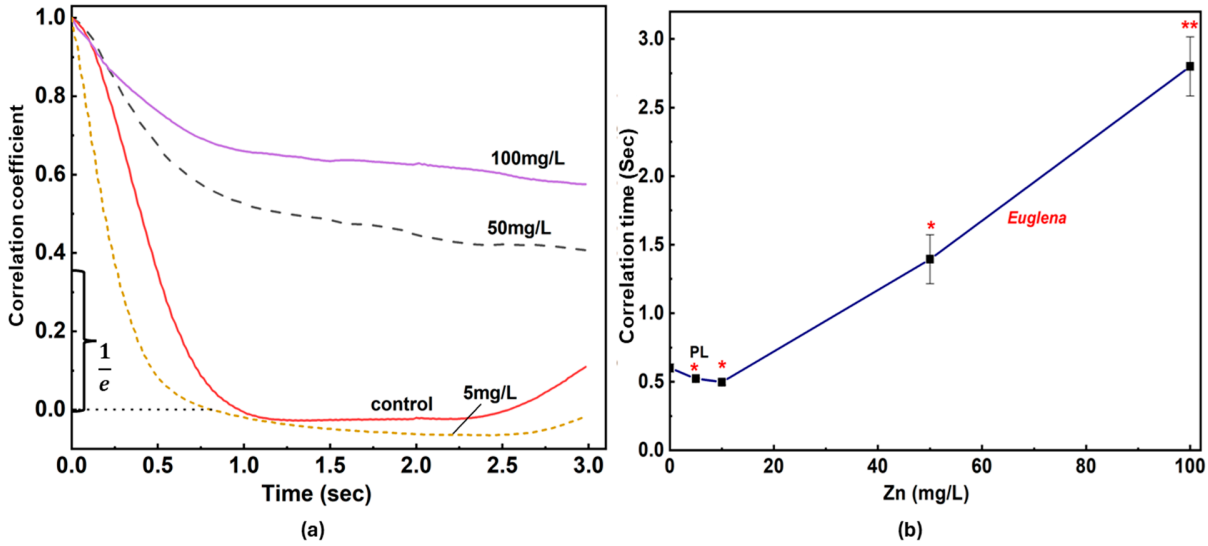


Figure 7. (**a**) The cross-correlation function for Euglena under different Zn concentrations, (**b**) the cross-correlation time as a function of Zn concentration (mg/L) for Euglena. *, ** indicated the statistical significance of data $p < 0.05$ and $p < 0.01$. N = 9.

However, like Paramecia, Euglena did not show a sudden decrease in correlation time, suggesting the absence of Zn's micronutrient behavior for green microalgae Euglena. However, Euglena exhibits such tolerance behavior up to 2× PL for Zn exposure.

3.3. Exposure Dependency Behavior of Microorganisms

After initially assessing the swimming ability of microorganisms at 1.5 h. The comprehensive evaluation of the swimming ability under prolonged exposure conditions, specifically at 24 and 48 h intervals, was also performed. The exposure of Euglena and Paramecia to varying concentrations of Fe and Zn for 24 and 48 h revealed intriguing dynamics in their behavior. For 1.5 h, a clear concentration-dependent response was observed of exposure, exhibiting distinct behavioral changes in response to Fe and Zn metal concentrations. However, as the exposure duration increased, a remarkable shift occurred in the microorganisms' swimming ability. Surprisingly, the concentration dependency was observed initially after just 1.5 h of exposure vanished after longer exposure periods of 24 and 48 h.

Figure 8a,b illustrate the Paramecia and Euglena behavior at varying Fe concentrations for different exposures, 24 and 48 h, along with 1.5 h. A two-way ANOVA was performed to analyze the effect of the concentration and time of exposure dependence on the correlation time. A statistically significant interaction was observed between them under varying Fe conditions (1.5 h of exposure was observed to be statistically significant, as compared to 24 and 48 h, for Euglena and Paramecia, respectively; however, no significance was observed between 24 h and 48 h of exposure in either microorganism). Similarly, under varying Zn conditions, a statistically significant interaction was observed (1.5 h of exposure was observed to be statistically significant, as compared to 24 and 48 h, for Euglena and Paramecia, respectively; however, no significance was observed between 24 h and 48 h of exposure in either microorganism). This disappearance of concentration dependency suggests a dynamic adaptation of microorganisms to their environment over time. A common pattern was observed in the case of both microorganisms under Zn and Fe exposure. There was an immediate and meaningful reduction in their swimming speed after 1.5 h. In addition to that, Paramecia and Euglena achieved a constant swimming state in the first 24 h of exposure to both heavy metal pollutants. This behavior may be due to the adjustment/adaption of the microorganism to the surroundings. It indicates that microorganisms may undergo physiological or behavioral changes in response to prolonged exposure to metals, leading to diminished sensitivity to metal concentrations. The precise mechanisms underlying this phenomenon require further investigation but may involve processes such as metal detoxification, metabolic adjustments, or changes in gene expression. The observed behavior of Euglena and Paramecia raised two possible scenarios for using swimming ability for water quality. First, the dependency of the organisms on the pollutants is rather complicated, especially at a low concentration range. Therefore, it is important to select the microorganism carefully. The disappearance in the concentration dependency or the adaptation behavior of microorganisms suggests the importance of considering the short exposure duration in water toxicity assessments for bioassays.

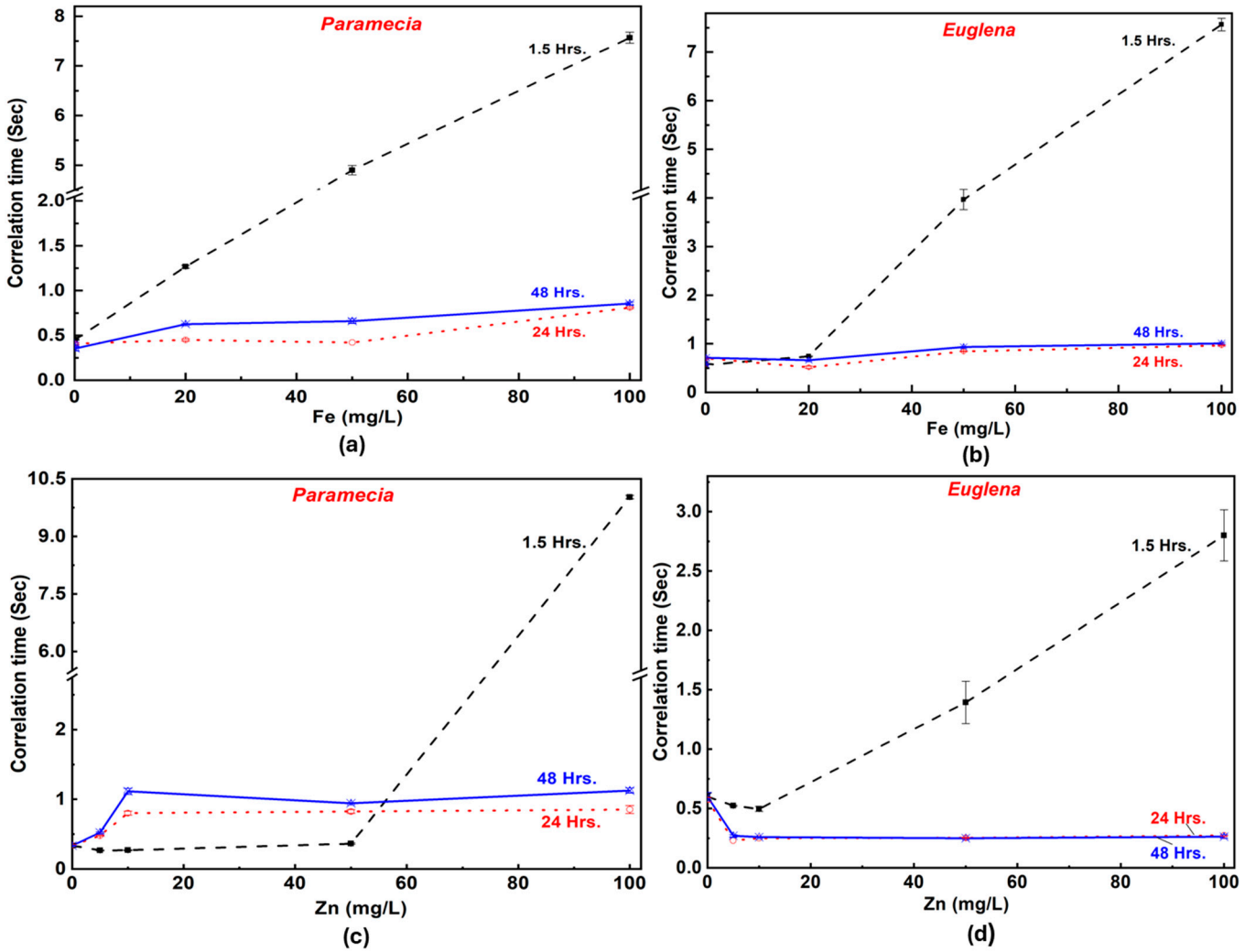


Figure 8. (**a**) The correlation time for Paramecia and (**b**) Euglena as a function of Fe concentrations for 1.5, 24, and 48 h, (**c**) the correlation time for Paramecia and (**d**) Euglena as a function of Zn concentration for 1.5, 24, and 48 h.

4. Discussion

The main objective of this study was to demonstrate the fast, reliable, and simple biospeckle technique for microbioassays using two different types of microorganisms and two heavy metal pollutants, i.e., Zn and Fe. In addition, a rheological study of their swimming speed was conducted using different viscosity media. The result of the proposed bioassay method revealed that significant reductions in the swimming ability of Paramecia and Euglena under different water toxicities were observed just after 1.5 h at higher concentrations of Fe and Zn. This demonstrates the potential of the observation speed of this method.

Several studies suggested that Fe and Zn are essential micronutrients for various organisms, including microorganisms such as Euglena and Paramecium [18,19]. Fe and Zn play critical roles in various cellular processes, such as growth, metabolism, and survival. At low concentrations, Fe and Zn act as cofactors for enzymes involved in various biochemical reactions, including photosynthesis, respiration, and DNA replication [20,21]. However, at elevated concentrations, they can become toxic and adversely affect cellular functions. The obtained results show that Euglena's tolerance to low Fe levels can be attributed to its ability to regulate iron uptake and utilization through sophisticated physiological and molecular mechanisms. At low Fe concentrations, Euglena employs high-affinity Fe transporters and Fe scavenging systems to acquire iron from the environment efficiently [22]. Additionally,

cells, including Euglena, possess iron storage proteins, such as ferritin, which sequester excess iron to prevent oxidative damage and maintain cellular homeostasis [23].

Furthermore, microorganisms can adjust their metabolic pathways and cellular processes to optimize iron utilization under low iron conditions. For example, Euglena may upregulate the expression of iron-dependent enzymes involved in photosynthesis and respiration to maximize energy production. This adaptive response allows for Euglena to thrive in environments with limited iron availability, exhibiting enhanced growth and metabolic activity compared to other microorganisms. It is also confirmed by Choon pin et al. that iron at optimal levels enhances cellular functions and promotes growth in microorganisms [24].

Euglena employs intracellular sequestration to mitigate the toxic effects of heavy metals such as Fe. Metals are compartmentalized within chloroplasts and vacuoles as a protective mechanism against the damaging effects of this metal and also reduces their interaction with vital cellular components [25]. Additionally, Euglena gracilis, a facultative photosynthetic alga, utilizes a cytoplasmic detoxification mechanism involving the synthesis of metal-binding peptides such as phytochelatins and glutathione to inactivate heavy metal ions. This process enables Euglena gracilis to survive in environments contaminated with metals, protecting its cells from the toxic effects of these ions [26]. In addition to that, stress conditions, such as heavy metal stress, exert evolutionary pressures on all organisms, which have developed sophisticated responses to cope and survive. Heavy metal tolerance in Euglena is also mediated through changes in gene expression and the activation of specific signaling pathways. [27]. Stress conditions, including heavy metal exposure, can upregulate genes encoding for heat shock proteins, antioxidants, and metal-binding proteins. These proteins play crucial roles in protecting cellular components from metal-induced damage and maintaining cellular homeostasis. Signaling pathways such as the mitogen-activated protein kinase (MAPK) pathway, this protein kinase appears to mediate oxidative stress responses and are often activated under stress conditions, coordinating a comprehensive stress response that includes the induction of detoxification and repair mechanisms [28].

The obtained results of the current research at low and high concentrations of Zn exposure showing dual behavior corroborate with the study performed by Omar et al., in which the low Zn concentration permitted a gradual increase in the growth rate of two green microalgae, Scenedesmus obliquus and Scenedesmus quadricauda. In contrast, the sudden exposure to high Zn concentrations suppressed the growth rate [29]. It is also observed that higher Zn concentrations behave like the growth inhibition of Spirulina platensis cultures. The surface of microalgae such as Euglena is negatively charged due to functional groups such as carboxyl, hydroxyl, and phosphate groups on the cell membrane [30,31]. These negatively charged sites provide binding sites for metal cations, such as Zn^{2+} and Fe^{2+}. At low concentrations of Fe and Zn, microalgae's negatively charged surface binds with metal ions, reducing their availability in the surrounding environment and mitigating their toxic effects on the cells. However, at high concentrations of Fe and Zn, the excess metal ions saturate the binding sites on the microalgae's surface, leading to the accumulation of free metal ions in the surrounding medium. These free metal ions can then enter the microalgae cells, causing cellular damage and potentially resulting in cell death. In addition to that, metals such as Zn and Fe are crucial for cellular metabolism. These metals are also found in metalloproteins, which are involved in various cellular processes, including electron transport and protection from reactive oxygen species at lower concentrations [30,32].

According to the existing literature, the resistance/adaptation of organisms towards heavy metal is linked with signalling pathways. These signalling pathway transduction systems in bacteria and organisms allow them to sense, react, and adjust to the changes that occur in their environment by responding to signals and stimuli, such as nutrients and cellular redox states [33]. In addition to that, this signaling system reacts to various environmental stresses and regulates functions such as division, metabolism, motility, and stress adaptation [34]. Other studies show that during a short-time exposure of

1 h to cadmium, severe impairment in the motility behavior of flagellated algal species was detected, manifested in movement around the spot, and an abrupt decrease in swimming speed as a response to stress. The swimming speed recovery that was observed after 3 h could indicate a cell adaptation response accompanied by the biosynthesis of proteins [35].

5. Conclusions

The variation in the swimming ability of Paramecia and Euglena is strongly related to water toxicity. It was confirmed that their swimming abilities could be quantitatively evaluated by converting the image of microorganisms into a speckle image with a further analysis using a cross-correlation method. Utilizing two distinct heavy metal water pollutants with two different microorganisms, our results effectively demonstrated the versatility and robustness of the proposed bioassay method. Unlike conventional microbioassays, our method does not require microscope observation to discern the condition of microorganisms, such as viability, which is particularly challenging with smaller microorganisms due to the limited focal depth of imaging systems. Furthermore, our approach offers several advantages for rapid environmental toxicity assessment, including simple data collection based on dynamic biospeckle properties, real-time toxicity insights, and simple data analysis. Evaluating microorganisms collectively enhances the reliability and efficiency compared to conventional individual assessments of microorganism viability under a microscope. Rather than focusing on an individual, swimming ability as an aggregate/swimming inhibition due to environmental toxicity can be evaluated quickly and efficiently. Since the image of any object can be converted into a speckle pattern, the same evaluation method can be applied to any organism, such as fungi and animal cells. In future research, we intend to explore other environmental toxicities, such as inorganic pollutants, especially combinations of these pollutants found in real-life polluted water, with other sensitive organisms and animal cells to further expand the applicability and understanding of our methodology.

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