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Original Research

Model Anaerobic Microbe *Photobacterium phosphoreum*: A Potential Biosensor for Different Metals and Volatile Fatty Acids Toxicity during Wastewater Treatment

Bibi Saima Zeb[1, 2], Zheng Ping[1], Qiu Lin[1], Mohammed Salim Akhter[3], Allah Ditta[4, 5], Qaisar Mahmood[2, 6]*, Abeer Hashem[7], Turki M. Dawoud[7], Elsayed Fathi Abd Allah[8]

[1]Department of Environmental Engineering, Zhejiang University, Hangzhou 310058, China
[2]Department of Environmental Sciences, COMSATS University Islamabad, Abbottabad Campus 22060, Pakistan
[3]Department of Chemistry, College of Science, University of Bahrain, Sakhir 32038, Bahrain
[4]Department of Environmental Sciences, Shaheed Benazir Bhutto University Sheringal, Dir (Upper), Khyber Pakhtunkhwa 18000, Pakistan
[5]School of Biological Sciences, The University of Western Australia, 35 Stirling Highway, Perth, WA 6009, Australia
[6]Department of Biology, College of Science, University of Bahrain, Sakhir 32038, Bahrain
[7]Botany and Microbiology Department, College of Science, King Saud University, P.O. Box. 2460, Riyadh 11451, Saudi Arabia
[8]Plant Production Department, College of Food and Agricultural Sciences, King Saud University, P.O. Box. 2460, Riyadh 11451, Saudi Arabia

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Abstract

The presence of various heavy metals may influence the transformation of many organics by anaerobes under anaerobic conditions. Sometimes, the coexistence of both heavy metals and volatile fatty acids (VFAs) may cause an inhibitory impact on microbial species involved in such biotransformation. These toxic mixtures create disturbances in the bioreactors by inhibiting the ability of the microorganism to degrade the waste. To keep the anaerobic process horizontal, it is essential to know the concentrations of the heavy metals and VFAs during the wastewater treatment operations, otherwise, it would result in halting various operations. Other methods are time-consuming. However, the Microtox analysis provides quick and reliable information about the toxic concentrations of the mixtures by calculating the IC50 15 min luminosity inhibition through *Photobacterium phosphoreum* T3S. The current research work aimed to assess the impacts of Pb, Cd, Cu, and VFAs on luminescent anaerobic microbial species *P. phosphoreum* under laboratory conditions. The individual and combined

*e-mail: mahmoodzju@gmail.com

acute toxicity assays were conducted following standard protocols. The results revealed that the combined treatments of individual heavy metals along VFAs caused partly additive toxic effects. The effects of two heavy metals along VFAs resulted in the synergistic type of toxic effects. The application of tri-mixtures of metals along VFAs also caused synergistic effects. The current data would be quite handy to conclude *P. phosphoreum* as a potential biosensor for metals and volatile fatty acids toxicity and to optimize various bioreactors against different heavy metals and VFA shocks by taking timely corrective measures.

Keywords: toxic metals, cadmium, copper, lead, toxicity, VFAS

Introduction

Environmental pollutants toxicity has gained serious concern and emerged as a global environmental pollution challenge. Contamination of the aquatic environments by pollutants like heavy metals has become a very serious problem in recent years [1, 2]. In anaerobic wastewater treatment plants, the presence of such toxic pollutants results in decreasing treatment efficiency. Moreover, water bodies are receiving mixtures of toxicants, when effluents are discharged by various industrial units like textile, leather, dying, metal plating facilities, oil, and vegetable ghee mills, and manufacturing units of storage batteries contain heavy metals [3]. The industrial wastewater originating from various unit operations may contain a variety of pollutants and is anticipated to cause serious operational issues to anaerobic digesters in Wastewater Treatment Plants (WWTP). The presence of various heavy metals may cause inhibition of methanogenic microbial populations resulting in the accumulation of VFAs. In these circumstances, heavy metals and VFAs would be anticipated to cause serious inhibitory effects on anaerobic microbial communities. Currently, there is a lack of evidence on how various combinations of metals and VFAs cause combined toxicity to anaerobic digesters. It has been demonstrated that metals affect ion displacement and/or substitution causing alterations in the molecular structure of the enzymes, nucleic acids, and nutrient transport systems [4]. A complex interaction among the number of bacterial groups is responsible for anaerobic wastewater treatment. The inhibition effects of heavy metals on anaerobic wastewater treatment systems depend on wastewater pollution load, pH, metal specie concentration, the solubility of metal ions, and also on the concentration of the volatile fatty acids (VFAs).

Effective utilization of VFAs keeps the anaerobic digesters in a balanced state of functioning. The accumulation of VFA in anaerobic digesters is indicative of the unbalanced conditions [5,6] which are accompanied by the souring of the digesters (decreasing pH) resulting in the failure of the anaerobic digestion [7]. Previously, the toxicity of various VFAs on the *P. phosphoreum* has been reported [8], likewise the toxic effects of various heavy metals from industrial sources on *P. phosphoreum* T3S have also been reported [3]. However joint toxicity data of heavy metals and VFAs has not been reported so far.

The bioluminescence inhibition assay of luminescent bacteria is widely employed in the toxicological assessment of environmental pollutants [1, 9-11], hence the major emphasis of this research work was to shed the light on the joint toxicity of heavy metals and VFAs on a model anaerobic bacterium. The objective of the current work was to use *P. phosphoreum* T3 in a Microtox system to predict the acute toxicity of heavy metals and VFAs, which is not reported in the literature so far.

Materials and Methods

P. phosphoreum and Chemicals

The luminescent bacterium i.e., *P. phosphoreum* (T3 mutation) was procured from the Institute of Soil Science, Chinese Academy of Sciences, Nanjing, PR China for the present study. All reagents were of analytical grade and were purchased from Sinopharm Chemical Reagent Co., Ltd., China.

Samples Preparation

Preparation of Single Toxicity Assay

The individual VFA acute toxicity assay of acetic acid, butyric acid, propionic acid, and ethanol was carried out according to standard toxicity protocol [12]. Eight concentrations were prepared for each VFA. Dilution of all concentrations was done with 3% NaCl and was kept at 20ºC. The pH was maintained by either HCl or 0.1 M NaOH to 7.00±0.05. Each concentration was prepared in triplicate with one control. A water bath was used to keep the Luminescent bacterial strain in the ice. Tubes were filled with 2 mL of each VFA concentration and then 2 µL containing active *P. phosphoreum* T_3 was administered into the tubes. A tube holding 2 mL NaCl solution (3%) served as a control. A Toxicity Analyzer (DXY-2, Institute of Soil Science, Chinese Academy of Sciences, Nanjing, China) analyzed the treated samples for 15- min at 20ºC~25ºC. The initial luminosity scale was adjusted in the range of 600 to 1900 mV. If the initial luminosity did not reach the standard, a newly freeze-dried powder of

luminescent bacteria was used. After the exposure of the model bacterium to selected metals and VFAs, the luminosity was measured and compared with standard solutions and control samples. A dose/response association was calculated by using the light change data and the amount of the toxicant employed. The calculations of the IC_{50} and relative luminescence units (RLU), were based on the mean luminescence unit (LU) of the samples and the mean luminescence unit (LU0) for the controls (without toxicant) [8].

Single Toxicity Assay of Cd, Cu, and Pb

The single metal toxicity assay to determine the IC_{50} value for Cd, Cu and Pb was performed as described in earlier research [13]. The Cd, Cu, and Pb inhibitory effects on luminescent *P. phosphoreum* were discussed in detail in the previous article [3].

Combined Toxicity of Cd, Cu, Pb, and VFAs

For the determination of the joint toxicity of various metals and VFA mixtures, the equitoxic ratios were used to set concentration gradients [14, 12]. The procedure was similar to that used for assessing the individual toxicity assessment tests. The concentration addition procedure was used to prepare the seven batches of metals and VFAs mixtures including Cu, Cd, Pb, acetic acid, butyric acid, propionic acid, and ethanol. Such a procedure involves the actions of a chemical as a dilution of other and its consequence can be achieved by its replacement fully or partially by the equieffective quantity of another chemical. Metals and VFAs were mixed according to the IC_{50} of the individual mixtures to investigate joint effects on diverse mixtures. The joint toxicity was calculated by the following formula:

$$X_a = \frac{IC_{50(a)}}{IC_{50(a)} + IC_{50(b)} + \cdots IC_{50(n)}} \quad (1)$$

For each combination, various treatments were applied in triplicates. The toxicity of the combined treatments was examined similarly as carried out for the individual toxicity tests.

Toxic Unit (TU) and Mixture Toxic Index (MTI) Approaches for Assessment of Cd, Cu, Pb, and VFAs toxicity

In this research work, joint toxicities of the Cd, Cu Pb, and VFAs were assessed by TU and MTI [15, 16].

Toxic Unit (TU)

The toxic unit (TU) was calculated by the following formula:

$$TU_i = \frac{C_i}{IC_{50,i}} \quad (2)$$

Where, ci = concentration of "i" when the mixture is at its IC_{50}
IC_{50}, i = median inhibition concentration
TUi = toxic unit of i.

$$M = \sum_{i=1}^{n} TU_i = \frac{C_1}{IC_{50,1}} + \frac{C_2}{IC_{50,2}} + \cdots + \frac{C_n}{IC_{50,n}} \quad (3)$$

Where M = sum of the toxic units

$$M_0 = \frac{M}{(TU_i)_{max}} \quad (4)$$

Where,
M0 = ratio of M and (TUi) max
(TUi) max = maximum toxic unit of the mixture

Mixture Toxic Index (MTI)

The joint toxicity effect was also determined by another approach known as Mixture Toxic Index (MTI), which can be calculated by the following formula [16].

$$MTI = 1 - \frac{\log M}{\log M_0} \quad (5)$$

Where,
MTI < 0 the mixture effect is antagonistic
MTI = 0 the mixture effect is not additive
MTI > 0 <1 the mixture effect is a partial additive
MTI = 1 the mixture effect is additive
MTI > 1 the mixture effect is supra-additive

Moreover, the joint toxicity effect was also calculated as M and M_0 values.

Data Analysis

Origin pro 8 software and SPSSTM v.18 analyzed the data for its statistical significance.

Results

Toxic Effects of the Mixture of Individual Metal with VFAs

The toxicity of the single metals Cd+ [VFAs], Cu+ [VFAS], and Pb+ [VFAs] to *P. phosphoreum* was presented in Table 1. Data related to experimental and theoretical IC_{50} values were also presented. The respective theoretical IC_{50} values for Cd + VFAs, Cu + VFAs, and Pb + VFAs included 8.334, 8.693, and 8.567 mg L^{-1}. The R^2 values for the mixture of single metals with VFAs were 0.906, 0.885, and 0.954 respectively. The relationship is presented in Fig. 1(a-c).

Toxic Effects of Two Metals Along VFA

Various theoretical IC_{50} values of 7.253, 7.148, and 7.447 mg L^{-1} were noted for Cd + Cu + VFAs, Cd + Pb

Table 1. Experimental IC_{50} of Cd, CU, Pb, and VFAs toxicity.

Mixtures	Experimental IC_{50} (mg L^{-1})	Theoretical IC_{50} (mg L^{-1})
Cd+[VFAs]	15.275	8.334
Cu+[VFAs]	7.379	8.694
Pb+[VFAs]	8.790	8.567
Cd+Cu+[VFAs]	4.247	7.253
Cd+Pb+[VFAs]	12.604	7.148
Cu+Pb+[VFAs]	4.576	7.447
Cd+Cu+Pb+[VFAs]	4.987	6.391

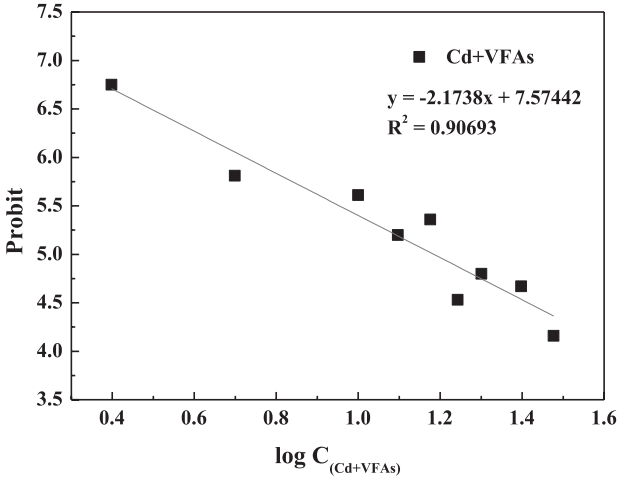

Fig. 1. The concentration mixture of Cd +VFAs (log C) and luminosity (%) Probit relationship.

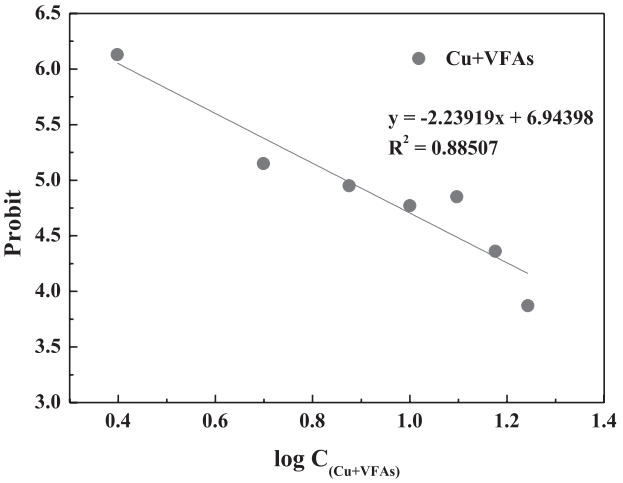

Fig. 2. The concentration mixture of Cu + VFAs (log C) and luminosity (%) Probit relationship.

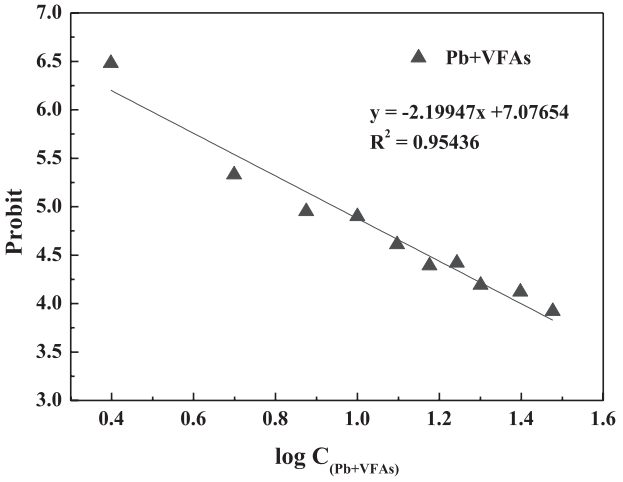

Fig. 3. The concentration mixture of Pb + VFAs (log C) and luminosity (%) Probit relationship.

+ VFAs, and Cu + Pb + VFAs, respectively as presented in Table 1. The respective R2 values (Fig. 2) were 0.919, 0.909, 0.968, and 0.950. A linear relationship existed between the relative luminosity (%), two combined heavy metals, and VFAs concentration in a mixture.

Joint Toxicity of Three Metals Along with VFAs

The notional and experimental IC_{50} values for joint toxic effects of three metals and VFA i.e., [Cd + Cu + Pb + VFAs] were 6.391 mg L^{-1} and 4.987 mg L^{-1}, which were given in Table 1. A direct or linear relation was revealed when *P. phosphoreum* was exposed to mixtures of three metals and VFAs and the results were presented in Fig. 3.

The Interactive Affects of Metal Combinations

To unravel the interactive effects of various combinations of metals along with VFAs, two scientific lines of the study were employed. Following Eq. (3) and Eq. (4), toxic units were assessed for single, two metals + VFA, and three metals with VFAs by using Toxic Unit (TU) approach.

The results were presented in Tables 2 and 3. The results showed that the adverse effects of different combinations had low toxicity (higher IC_{50} or lower TU values) than the sum of the toxic effects of a single component. Such results suggested an antagonism between the single, two-metal combination with VFA and three combined metals with VFAs. The comparison of the two different mathematical approaches was presented in Table 4. According to the MTI approach MTI>0<1 and according to the TU approach, when M0>M>1 the effect was partly additive. The results were 0.623, 1.102, 0.984, and 0.959>0<1 for Cd + [VFAs], Cu + [VFAs], Pb + [VFAs], and Cd + Pb + VFAs as given in Table 4 at 95% confidence interval (CI). While the other combination demonstrated a synergistic effect.

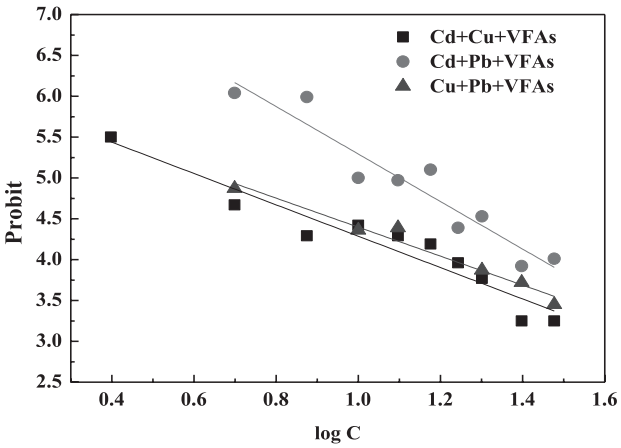

Fig. 4. The concentration (log C) and luminosity (%) Probit relationship of Cd + Cu + VFAs, Cd + Pb + VFAs, and Cu + Pb + VFAs.

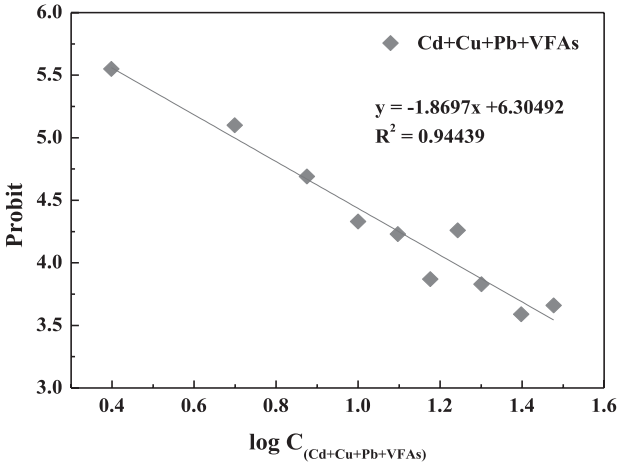

Fig. 5. The concentration (log C) and luminosity (%) Probit relationship of Cd + Cu + Pb + VFAs.

Discussion

A positive direct relation existed among relative luminescence units (RLU) and the volatile fatty acids (VFAs) concentrations tested. The present results are comparable with an earlier study which concluded that toxicity to tested bacterial strain was higher for a lower IC_{50} value [3]. *P. phosphoreum* exhibits luminescent property which is the most abundantly present in the environmental system. VFA toxicity is normally increased at lower pH values. Upon the entry of VFAs in cells where pH values are generally around neutral, VFAs get ionized thereby producing cations that lower pH values inside the cells [17, 18]. The presence of VFAs like acetic acid, propionic acid, and butyric acid may represent a sensitivity indicator for methanogenic systems [19, 20]. Various studies have reported the toxic effects of metals on different stages of anaerobic digestion [3, 21, 22]. It was suggested that Zn and Cu had greater toxicity to acidogens as compared with Pb. Other reports [23] showed inhibition to acidogens caused by Cu and Zn at a concentration of 1-10 mg dm^{-3} and 5-40 mg dm^{-3}, respectively [23, 24]. It was suggested that metals may inhibit the conversion of VFA to methane during anaerobic digestion [25]. The most toxic heavy metal reported so far is mercury [26, 27]. Owing to its lipophilic properties Hg can penetrate biological membranes. Some bacterial species convert the inorganic mercury to methyl mercury (MeHg) and thus Hg may bioaccumulate in many biological tissues in fish and other animal tissues [26, 28]. Many studies reported low sensitivity of the Gram-negative bacteria to Cd^{2+} which might be due to the presence of exopolysaccharides in the bacterial membrane which traps Cd [29-31].

The toxicity effects of mixtures of potentially toxic ingredients on organisms can be additive, synergistic,

Table 2. Toxic Units of the single HMS+VFAs.

Mixtures	Experimental IC_{50}	Theoretical IC_{50}	Toxic Unit of Individual Heavy metals & VFAs							**Sum of TUs**
			TU_{Cd}	**TU_{Cu}**	**TU_{Pb}**	**TU_A**	**TU_B**	**TU_C**	**TU_D**	
Cd + [VFAs]	15.275	8.334	0.366	_	_	0.366	0.366	0.366	0.366	1.832
Cu + [VFAs]	7.379	8.693	_	0.169	_	0.169	0.170	0.169	0.169	0.848
Pb + [VFAs]	8.790	8.567	_	_	0.205	0.205	0.205	0.205	0.205	1.026

Table 3. Toxic Units of two combined and three combined HMs+ VFAs.

Mixtures	Experimental IC_{50}	Theoretical IC_{50}	Toxic Unit of Individual Heavy metals							**Sum of TUs in a mixture**
			TU_{Cd}	**TU_{Cu}**	**TU_{Pb}**	**TU_A**	**TU_B**	**TU_C**	**TU_D**	
Cd+Cu+[VFAs]	4.247	7.253	0.097	0.09	_	0.097	0.097	0.097	0.097	0.585
Cd+Pb+[VFAs]	12.605	7.148	0.294	_	0.294	0.294	0.294	0.294	0.294	1.763
Cu+Pb+[VFAs]	4.576	7.447	_	0.10	0.102	0.102	0.102	0.102	0.102	0.614
Cd+Cu+Pb+[VFAs]	4.988	6.391	0.111	0.11	0.111	0.111	0.111	0.111	0.111	0.780

Table 4. Comparison of the TUs and MTI approach.

Mixtures	(r^2)	IC_{50} (mg L^{-1})	95% CI	M	Interact effect	MTI	Interact effect
Cd + [VFAs]	0.906	15.275	14.168~16.382	1.832 1.700~1.965	Partly add	0.623 0.580~0.670	Partly add
Cu + [VFAs]	0.885	7.379	7.016~7.741	0.848 0.807~0.89	Partly add	1.102 1.072~1.133	Partly add
Pb + [VFAs]	0.954	8.790	8.349~9.231	1.026 0.974~1.077	Partly add	0.984 0.953~1.016	Partly add
Cd + Cu + [VFAs]	0.919	4.2474	4.012~4.482	0.585 0.553~0.617	Syn	1.038 1.034~1.042	Syn
Cd + Pb + [VFAs]	0.909	12.604	11.534~13.677	1.763 1.613~1.913	Partly add	0.958 0.953~0.965	Partly add
Cu + Pb + [VFAs]	0.963	4.576	4.191~4.961	0.614 0.562~0.666	Syn	1.035 1.029~1.041	Syn
Cd + Cu + Pb + [VFAs]	0.950	4.987	4.776~5.199	0.780 0.747~0.813	Syn	1.015 1.012~1.018	Syn

Add = Additive; Syn: Synergistic

or antagonistic [32-33]. In this study, the effect of the single metal and VFAs mixture [Cd + VFAs, Cu + VFAs, and Pb + VFAs] was determined. The experimental IC_{50} was 15.27 mg L^{-1}, 7.37 mg $L^{-1,}$ and 8.79 mg L^{-1}, respectively, for Cd + VFAs, Cu + VFAs, and Pb + VFAs mixtures. According to TU and MTI approach, Cd + VFAs, Cu + VFAs, and Pb + VFAs mixtures interaction effect was partly additive for all three combinations. It implied that the combined effect of the sum of the Cd + VFAs, Cu + VFAs, and Pb + VFAs mixtures were equivalent to the sum of the impact of individual metals.

The extent of pollutant toxicity is not only restricted to the exposed concentration, but the time of exposure is also considered a significant influencing factor. Cd + Cu + VFAs and Cu + Pb + VFAs interaction effect was synergistic, while Cd + Pb + VFAs was partly additive and Cd + Cu + Pb + VFAs was also synergistic as suggested by comparisons based on TU and MTI approaches. This showed the synergistic effect as the luminescence bacterium was exposed to only two or more toxic substances at the same time their effect was greater than the sum of the individual toxic substance alone in a mixture.

In a previous study, Cu^{2+}, $Ag^{+,}$ and Hg^{2+} metals luminescence toxicity tests were conducted, and the individual metals were found very toxic to the luminescence bacterium. However, the joint metals Hg^{2+} + Ag^{+}, Hg^{2+} + Cu^{2+}, Ag^{+} + Cu^{2+}, Hg^{2+} + Ag^{+}, and Cu^{2+} toxicity and their interactive effect were found to be very weak. One of the possibilities is that when these substances are in a mixture, their interaction lowers the toxicity of the most active substance in a mixture, and overall, the joint mixture showed a weaker toxic effect [34]. Authors argued that Hg^{2+}, $Ag^{+,}$ and Cu^{2+} caused high sensitivity and toxicity to bacterium when tested individually but when these metals were tested in the mixture or combination hypothetically, they must target the luciferase; however, it resulted in completion among the metals for the binding sites on the luminescent bacterium which might hinder their interaction [35]. The luminescence phenomenon in *Vibrio fischeri* seemed to be connected with the respirational process and thus serves as a good gauge of metabolism and cytotoxicity caused by mixed compounds [36].

In another study, individual metal toxicity of Cu^{2+} and Cd^{2+} was tested on *P.* sp. The toxicity results showed that the toxicity of Cu^{2+}>Cd^{2+}, while the combined effect was antagonistic as TU shows M>1 and MTI<0. The antagonistic effect means, that in the mixture the low-efficacy toxic metal competes with the high-efficacy toxic metal [34]. Usually, luminescence inhibition studies on the interaction effects of combined metals are conducted for 5 to 30 minutes. A previous study by the authors was conducted on IC_{50} luminescence inhibition for 15 minutes [37]. The effect was additive and synergistic while for antagonistic effect needs more inhibition time i.e., 6 h. In another toxicity study of Cu^{2+} + Zn^{2+}, their additive interaction effect changed to antagonistic in 30 minutes to 6 h. The additive effect of the two combined heavy metals additive effect was due to the same mode of action or mechanism, they only differ in their potencies. Moreover, the same metals mixture Cu^{2+} + Zn^{2+} combined toxicity effect was synergistic to *V. fischeri* at 15 minutes, the EC_{50} values were 0.125 mg L^{-1} and 0.14 mg L^{-1} for Zn^{2+} and Cu^{2+} respectively [38]. When the chronic test was conducted on both the mixtures of zinc and copper, an antagonistic interaction effect was observed after 6 h. In antagonistic interaction after 6 h Cu may enhance the Zinc toxicity, while Zn was found less sensitive to model bacterial species. The reason was that the Cu^{2+} cellular uptake was more as compared to Zn^{2+}, which ultimately affects the luciferase activity. In the present work, the study focused on the combined effects of metals and VFA which produced further insights into combines

metal and VFA toxicity to model microbe and will be useful in understanding the toxicity tolerance management.

Conclusions

These toxic mixtures create disturbances in the bioreactors by inhibiting the ability of the microorganism to degrade the waste. To keep the anaerobic process horizontal, it's essential to know the concentrations of the heavy metals VFAs during the wastewater treatment operations, otherwise, it will be halted. Other methods are time-consuming. However, the Microtox analysis provides quick and reliable information about the toxic concentrations of the mixtures by calculating the IC_{50} 15 min luminosity inhibition through *P. phosphoreum* T3S. This study will contribute to the current knowledge and understanding of how to determine the toxicity of mixtures to microbial species.

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Conflicts of Interest

The authors declare no conflict of interest.

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