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EXPERIMENTAL ARTICLES

Assessment of Toxicity of Volatile Fatty Acids to *Photobacterium phosphoreum*[1]

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Abstract—The toxicity of four volatile fatty acids (VFAs) as anaerobic digestion (AD) intermediates was investigated at pH 7. *Photobacterium phosphoreum* T3 was used as an indicator organism. Binary, ternary and mixtures of AD intermediates were designated by letters A (acetic acid + propionic acid), B (acetic acid + butyric acid), C (acetic acid + ethanol), D (propionic acid + butyric acid), E (propionic acid + ethanol), F (butyric acid + ethanol), G (acetic acid + propionic acid + butyric acid), H (acetic acid + propionic acid + ethanol), I (acetic acid + butyric acid+ ethanol), J (propionic acid + butyric acid + ethanol) and K (acetic acid + propionic acid + butyric acid + ethanol) to assess the toxicity through equitoxic mixing ratio method. The IC_{50} values of acetic acid, propionic acid, butyric acid and ethanol were 9.812, 7.76, 6.717 and 17.33 g/L respectively, displaying toxicity order of: butyric acid > propionic acid > acetic acid > ethanol being additive in nature. The toxic effects of four VFAs could be designated as synergistic and one additive in nature.

Keywords: anaerobic digestion, toxicity, anaerobic intermediates, *Photobacterium phosphoreum* T3, industrial wastewater

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A number of technologies are being used for wastewater treatment; whereby anaerobic digestion (AD) is relatively cost-effective technology [1–3]. AD is a complex process with several advantages over the conventional aerobic technologies, such as no requirement for aeration, high productivity of biogas and low yield of sludge. AD has been widely applied in environmental engineering practices [4]. AD is accomplished by the concerted action of many bacterial and archaeal communities [5, 6]. The biochemical reactions take place in four steps i.e. hydrolysis, acidogenesis, acetogenesis and methanogenesis which are catalyzed by various microorganisms [7–11]. Complex interaction networks and delicate balance exist among these microbial groups, reactants and their products [12]. A delicate homeostatic balance in the physicochemical conditions and various intermediates is pivotal for the optimal operation of AD. An imbalance in the microbial activities occurs when the concentration of the particular intermediate becomes higher which subsequently disturbs the homeostatic equilibrium of AD causing disturbances and inhibition of the bacterial catalysis [13].

It is well established that the conversion of volatile fatty acids (VFA) into methane is the limiting step during AD of non-particulate substrates or non-excessively complex organic matter [14]. However, VFA are intermediates which may often accumulate aggravating a decrease of reactor pH and the overall operation failure. About 64% of the methane produced during AD comes from acetate, while the remaining 36% originates from hydrogen [15]. Propionate is an important precursor of acetate and hydrogen—approximately 30% of the electron flow directly related to methane production goes through propionate [16]. In addition, propionate, acetate and hydrogen are more sensitive to process upsets than biogas production, methane content, or pH.

Imbalance between the microbial activities might result in the accumulation of the intermediates like ethanol and VFAs, hereafter they are designated as endo-toxicants, and their accumulation may seriously inhibit anaerobic digestion [12, 17]. Since VFAs are important intermediates of AD and their concentrations affect the efficiency of the process treating the industrial wastewater. As process performance indicator, VFA concentration is probably the most sensitive parameter to be monitored. The toxicity of the VFA mixtures to anaerobic digestion has not yet been reported. Currently, Luminescent bacteria are widely used for the assessment of pollutant toxicity [6, 17–20]. So, the luminescent bacterium can be

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employed to assess the single and joint toxicities of VFAs during anaerobic digestion process. In the present study, the *Photobacterium phosphoreum* T3 was used as a indicator organism. The main objectives of the study were: (1) To determine half inhibition concentration of individual VFA (acetic acid, propionic acid, butyric acid and ethanol), and (2) To determine the binary, ternary and joint toxicity of the VFA mixtures.

MATERIALS AND METHODS

Materials and chemicals. The freeze-dried powder of luminescent bacterium *Photobacterium phosphoreum* T3 [21] was supplied by Nanjing Institute of Soil Science, Chinese Academy of Sciences. Anhydrous alcohol, acetic acid, propionic acid, butyric acid were purchased from Sinopharm Chemical Reagent Co., Ltd., China (analytical reagent).

Toxicity assays. The toxicity tests were performed using the Microtox Toxicity Analyzer (DXY-2, manufactured by the Nanjing Institute of Soil Science, The Chinese Academy of Science). Fully mixed 0.5 g freeze-dried luminescent bacteria in 1–1.5 mL 2.5% cold NaCl solutions through 1 mL injector were used to revive luminescent bacteria. Luminescent bacterial solution (10 μL) was placed in a test tube containing 2 mL of 3% NaCl solution and was fully mixed. Test tubes were kept in Microtox Toxicity Analyzer (DXY-2). The scale of initial luminosity was in the range of 600 to 1900 mV. Freshly freeze-dried powder of luminescent bacteria was used for the purpose.

Individual VFA toxicity assay. The single acute toxicity assay for AD intermediates was carried out by using the standard toxicity measurement protocol [22]. For each VFA, eight concentrations were prepared. All samples were diluted by 3% NaCl solution at 20°C. The pH of solutions was adjusted to 7.00 ± 0.05 by 0.1 M NaOH or HCl. There were three replicates for each sample and a control. For the assay, the luminescent bacterium was stored in the ice water bath, 10 μL of activated bacterium was put into the tubes containing 2 mL of VFA solution or 3% NaCl solution (control). The individual toxicities of four VFAs (acetic acid, propionic acid, butyric acid and ethanol) to *P. phosphoreum* were determined after 15 min by using the Microtox Toxicity Analyzer (DXY-2). *Microtox* is a standardized toxicity test system, which employs bacterium as the test organism. The bacteria are exposed to a range of concentrations of the material being tested. The reduction in intensity of light emitted from the bacteria is measured along with standard solutions and control samples. The change in light output and concentration of the toxicant produce a dose/response relationship. The results are normalized and the IC_{50} is calculated.

Equation (1) was used to obtain the relative luminescence units (RLU), which was calculated by the mean luminescence unit (LU) in the samples and the mean luminescence unit (LU0) in the controls (without toxicant).

Equation (1)

$$\mathrm{RLU} = \frac{\mathrm{LU}}{\mathrm{LU}_0}\%. \quad (1)$$

The concentration causing 50% relative luminescence unit was obtained by the regression equation and used as the 15-min half inhibitory concentration (15 min–IC_{50}).

Binary VFAs mixture toxicity assay. According to the 15 min-IC_{50} of single anaerobic intermediate VFAs, six groups of binary mixtures were prepared according to equitoxic ratio mixing method. Letters were assigned to different VFA groups [A (acetic acid + propionic acid); B (acetic acid + butyric acid); C (acetic acid + ethanol); D (propionic acid + butyric acid); E (propionic acid + ethanol); F (butyric acid + ethanol). For each binary component mixture seven concentrations were prepared with three replicates at each concentration and one control was performed.

Ternary and joint VFAs mixtures toxicity assay. Three groups of ternary mixtures and one group of joint all four AD intermediates were prepared according to the 15 min–IC_{50} of single chemical, as per equitoxic ratio mixing method: G (Acetic acid + propionic acid + butyric acid); H (Acetic acid + propionic acid + ethanol); I (propionic acid + butyric acid + ethanol). Seven concentrations were made in triplicates and one control.

Assessment of the binary, ternary and joint toxicities. There are four main types of joint toxicities INdependent Effect (INE), Additive Effect (ADE), SYnergistic Effect (SYE) and ANtagonistic Effect (ANE) [23]. The concentration addition model (CA) is the main assessment method for joint toxicities. Joint toxicities can be determined by the following formula:

$$\frac{1}{\mathrm{IC}_{50(E)}} = \Sigma\frac{x_i}{\mathrm{IC}_{50(i)}}(i = 1, 2, \ldots, n). \quad (2)$$

Where Xi is the mass concentration ratio of a toxicant $a, b, \ldots, n$ in the

$$\sum xi = 1{:}\mathrm{IC}_{50(i)}.$$

Mixture, IC_{50} (i) is the single IC_{50} of a toxicant $a, b, \ldots n$ and $IC_{50(E)}$ is the expected IC_{50} of the multi component mixture.

In this study, according to equitoxic mixing ratio i.e. toxicants were mixed according to IC_{50} of the single mixture for taking joint effects on diverse mixture, so it is:

$$X_a = \frac{\mathrm{IC}_{50(a)}}{\mathrm{IC}_{50(a)} + \mathrm{IC}_{50(b)} + \ldots \mathrm{IC}_{50(n)}}. \quad (3)$$

According to the single anaerobic digestion intermediate IC_{50} and the formula (3), binary mixture IC_{50}, and then R value can be calculated the R as the criterion for determination of joint effect of anaerobic digestion intermediates.

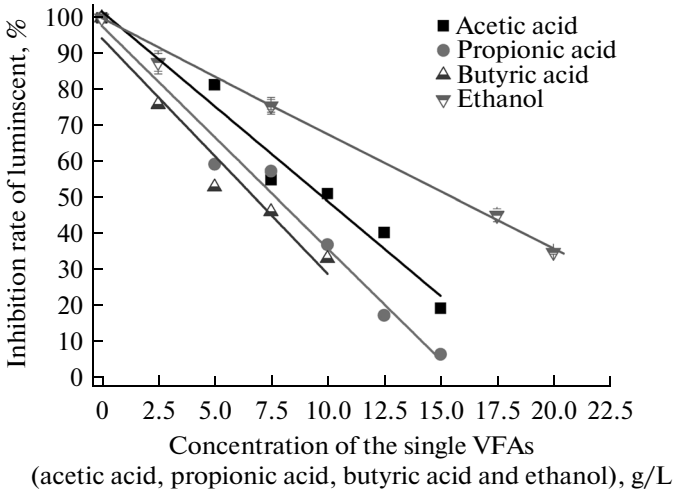

Fig. 1. Relationship of individual VFA concentration with IC_{50} of *P. phosphoreum* T_3.

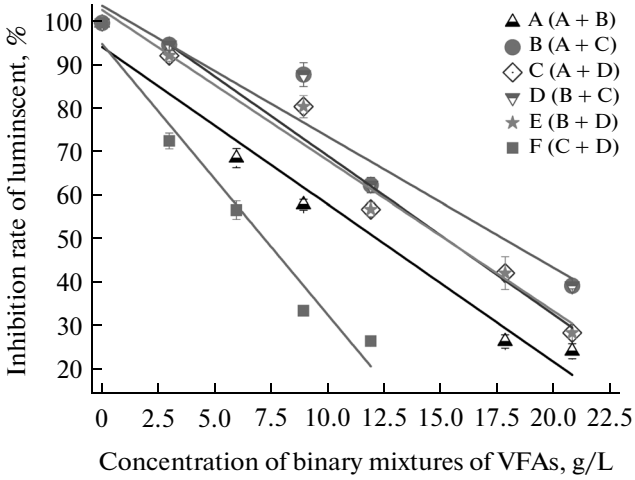

Fig. 2. Relationship between the binary mixtures of VFAs concentration IC_{50} of *P. phosphoreum* T_3.

$$R = \frac{IC_{50(E)}}{IC_{50}}. \quad (4)$$

According to the R value type of joint effect may be judge. Where, if $R < 0.4$, its means the effect is antagonistic, if $R > 2.5$ the effect is synergistic and if $R < 0.4R < 2.5$ the effect is additive.

Data processing and analysis method. Digital data processing and the statistical data analysis of variance, linear fitting, correlation coefficient (R) and confidence interval in the experiments were performed using Origin pro 8 software and SPSS™v.18 statistical software.

RESULTS

Toxicity of individual VFA. The results of toxicity of individual VFA to *P. phophoreum* are shown in Fig. 1. The regression equations, correlation coefficients and the 15 min-IC_{50} are shown in the Table 1.

According to results a positive linear correlation was found between the RLU and the different VFA concentrations. The 15 min IC_{50} of acetic acid, propionic acid, butyric acid and ethanol calculated, based on the regression equation were 9.81, 7.76, 6.78 and 17.33 g/L, respectively. According to 15 min IC_{50}, the order of individual toxicity of intermediates was butyric acid C (6.72) > propionic acid B (7.76) > acetic acid A (9.81) > and ethanol D (17.33), so smaller is the IC_{50} value, greater is the toxicity to luminescent bacterium.

Toxicity of Binary VFA mixtures. The joint toxicity to luminescent bacterium was tested in order to know the joint effect of the anaerobic intermediates. The dose-response relationships for the joint toxicities of binary mixtures were depicted in Fig. 2. Based on Fig. 2, RLU were found positively correlated to the concentrations of different binary mixtures. The linear regression equations, Rand the IC_{50} values of binary mixtures were listed in Table 2. According to the linear regression equations, the IC_{50} values of binary mixtures A + B, A + C, A + D, B + C, B + D, and C + D were calculated as 12.416, 17.449, 15.375, 7.229, 12.317 and 10.538 g/L, respectively. The acute toxicities of binary mixtures descended in the order of B + C, C + D, B + D, A + B, A + D and A + C.

Ternary and joint mixture toxicities of VFAs. In order to acquire the ternary and joint effect of the anaerobic digestion intermediates, the joint toxicity to luminescent bacterium was tested by mixing the three anaerobic digestion intermediates and all four anaerobic digestion intermediates together by equivalent concentration mixing method. Relationship between

Table 1. Toxicities of individual anaerobic intermediates to *P. phosphoreum* T3

Anaerobic intermediates	Linear regression equation	Correlation coefficient, r^2	IC_{50}
A (Acetic acid)	$y = -18.463x + 19.043$	0.96	9.81
B (Propionic acid)	$y = -15.911x + 15.719$	0.98	7.76
C (Butyric acid)	$y = -14.627x + 14.031$	0.95	6.72
D (Ethanol)	$y = -28.815x + 31.746$	0.95	17.33

Y represents the relative luminescence unit. X represents the concentration of the individual toxicant, g/L.

Table 2. Binary mixtures toxicities of anaerobic intermediates to *P. phophoreum* T3

Binary mixtures	Linear regression equation	R^2	Actual IC_{50} (g/L)	Theoretical IC_{50} (g/L)	R	Joint toxicity
A (A + B)	$y = -0.273x + 26.07$	0.97	12.42	8.78	0.70	+
B (A + C)	$y = -0.3122x + 33.06$	0.94	17.45	8.26	0.47	+
C (A + D)	$y = -0.2849x + 29.62$	0.97	15.38	13.57	0.88	+
D (B + C)	$y = -0.1597x + 15.21$	0.97	7.23	7.24	1.00	+
E(B + D)	$y = -0.2566x + 25.15$	0.97	12.32	12.54	1.01	+
F (C + D)	$y = -0.2078x + 20.93$	0.98	10.54	12.02	1.14	+

Table 3. Ternary and joint mixture toxicities of anaerobic intermediates to *Photobacterium phosphoreum* T3

Binary mixtures	Linear regression equation	r^2	Actual IC_{50} (g/L)	Theoretical IC_{50} (g/L)	R	Joint Toxicity
G (A + B + C)	$y = -0.19101x + 18.919$	0.90	9.113	8.09	0.88	+
H (A + B + D)	$y = -0.2959x + 28.293$	0.97	13.746	11.63	0.85	+
I (A + C + D)	$y = -0.2816x + 27.276$	0.93	13.534	11.28	0.83	+
J (B + C + D)	$y = -0.2494x + 23.383$	0.93	11.433	0.72	0.063	Syn
K (A + B + C + D)	$y = -0.2791x + 28.265$	0.92	13.90	13.87	0.99	+

+ Additive effect. Syn: Synergistic.

the joint mixtures of anaerobic digestion intermediates IC_{50} of *P. phosphoreum* T_3 is shown in Fig. 3.

The result of joint toxicity assay for anaerobic digestion intermediates were depicted in the Table 3. It presented a positive correlation between concentrations of the multi-component mixtures and IC_{50} values or the RLU. It was shown that joint effect of ternary groups G, H and I was additive but group J was synergistic in effect. So it means that the effect of group J ternary mixture is greater than the sum of their separate effect. The joint effect of all the anaerobic digestion intermediates group K (A + B + C + D) was additive and the reason may be the similar target organ and the same action mechanism (Fig. 4).

A positive correlation between Microtox and anaerobic activity was developed [24] and it was found feasible to use the standard toxicity measurement protocol for acute toxicity assessment to anaerobic digestion methanogens. Usually, more than one anaerobic digestion intermediates produced during anaerobic process, so the joint toxicities of binary mixtures are usually different from the toxicities of single anaerobic digestion intermediate. The joint toxicities include four main types: the joint toxicities included indepen-

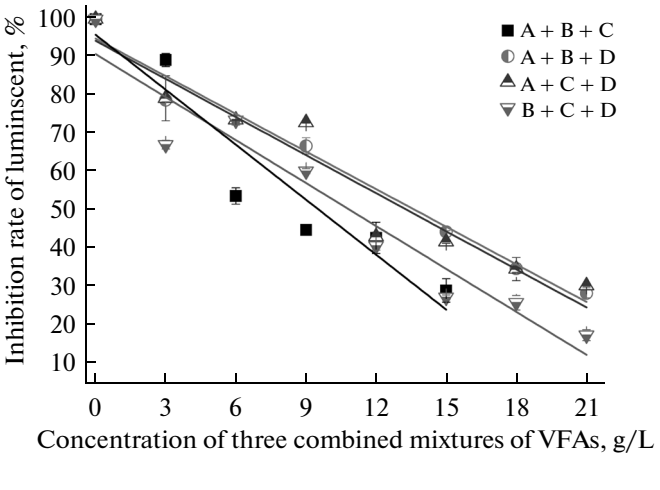

Fig. 3. Relationship between the three combined mixtures of VFAs concentration IC_{50} of *P. phosphoreum* T_3.

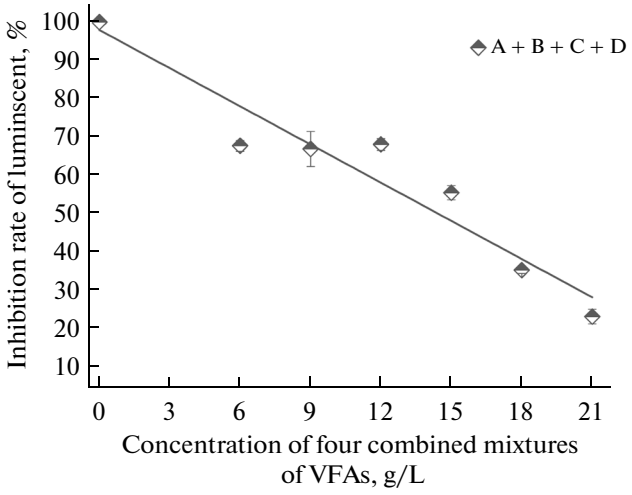

Fig. 4. Relationship between the four combined mixtures of VFA concentration IC_{50} of *P. phosphoreum* T_3.

dent effect, additive effect, synergistic effect and antagonistic effect [23].

DISCUSSION

A good correlation between Microtox and anaerobic bacteria activity was reported by [24]. So, it was practical to assess the chemical toxicities to ADMs through standard toxicity measurement protocol. The IC_{50} values of single VFAs from the results showed that they are toxic to *Photobacterium phosphoreum*. VFA can permeate cell membrane in un-dissociated form; collapse the proton gradient and block ATP synthesis [25–27]. On one hand, the un-dissociated part of these soluble metabolites can permeate the cell membrane of hydrogen-producing bacteria and then dissociate in the cell, which can disrupt the physiological balance in the cell. Thus, some maintenance energy should be used to restore the physiological balance in the cell, which can reduce the energy used for bacterial growth and then inhibit their growth in a sense; on the other hand, if the dissociated part of these soluble metabolites is present in the fermentative hydrogen production system at a high concentration, the ionic strength will increase, which may result in the cell lysis of hydrogen-producing bacteria. As a result, at a high concentration, these soluble metabolites can inhibit hydrogen-producing bacteria growth and then inhibit the fermentative hydrogen production accordingly [28]. Butyrate was found to cause more inhibition compared to acetate for *C. tyrobutyricum* and also for *C. acetobutylicum, C. populeti*, and *C. thermocellum* [29]. Ethanol can denature proteins, and dissolve into the membrane interior, which makes the carbon chains of the lipids more fluid and causes the cell membrane more permeable, and it can also inhibit the activity of intracellular enzymes and hinder the metabolism [30].

In general, the inhibitory effects of the byproducts (e.g., acetate, butanol, ethanol, and acetone) appear in a concentration range that is above the concentrations usually reached during butyric acid fermentations. The inhibition of bacterial activity by VFA may be expected when their concentrations are increased to extremes. High volatile fatty acid (VFA) concentrations in the system cause the inhibition of methanogenesis. Under conditions of overloading and in the presence of inhibitors, methanogenic activity cannot remove hydrogen and volatile organic acids as quickly as they are produced. The accumulation of acids and the depression of pH to levels that also inhibit the hydrolysis/acidogenesis phase. The accumulation of VFAs may contribute to inhibition at extremely high levels (>10 g/L) [31, 32]. As the single IC_{50} of acetic, propionic, butyric and ethanol were 9.81, 7.76, 6.78 and 17.33 g/L taken together, these findings indicate the activity of the *Photobacterium phosphoreum* decreased up to 50%. As *Photobacterium phosphoreum* was used as an indicator organism to predict the toxicity of the VFAs to ADMs. So we can assume these concentrations of the acetic, propionic, butyric and ethanol repressed the activity of the ADMs and pH of the reactor decreased rapidly and results in cessation of the reactor operation in terms of Cod removal efficiency and methane yield.

The joint toxicity effect may be independent effect when toxicants use different paths to transport, use different modes or act on different targets. When the toxicants are similar in their structures and characteristics, target site and mechanism are the same, their joint biological effects may be additive in nature. Additive effect means the effect of the toxicants mixture is equal to the sum of the individual effect. When one toxicant acts as agonistic to other toxicants' absorption and accumulation or propel cells to assimilate and accumulate other toxicants or hunks their degradation and excretion; their joint biological effect results in synergistic effects. It means that in synergistic effect, the effect of the chemical mixture is greater than the sum of their separate effect. When one toxicant can accelerate other toxicants' degradation and excretion or decrease their absorption and accumulation, their joint biological effect may end as antagonistic effects. In the light of the above discussion all six groups [(A + B), B (A + C), C (A + D), D (B + C), E (B + D), F (C + D)] of VFAs binary mixtures effect was additive. This additive effect was due to the allied structure, characteristics and similar mode of action. It was shown that joint effect of ternary groups G, H and I was additive but group J was synergistic in effect. So it means that the effect of group $ ternary mixture is greater than the sum of their separate effect. The joint effect of all the anaerobic digestion intermediates group K (A + B + C + D) was additive and the reason may be the similar target organ and the same action mechanism.

CONCLUSION

The anaerobic digestion intermediates showed the acute toxicity to *Photobacterium phosphoreum* T_3, the invidual toxicity of selected anaerobic digestion intermediates was found in the order of butyric acid > propionic acid > acetic acid > ethanol. All the six binary mixtures and a ternary mixture out of four showed the synergistic effect; while joint mixture of four VFA showed the additive toxic effect. The luminescent bacterium assay can be used to assess the toxicity of VFA to anaerobic digestion treatment.

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