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Rapid fluorescence-based measurement of toxicity in anaerobic digestion

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ABSTRACT

A rapid fluorescence measurement based on resazurin reduction was developed and applied for the detection of toxicants/inhibitors to anaerobic digestion metabolism. By initially using a pure facultative anaerobic strain, *Enterococcus faecalis* as a model organism, this technique proved to be fast and sensitive when detecting the model toxicant, pentachlorophenol (PCP). The technique revealed significant metabolic changes in *Enterococcus faecalis* with a PCP spike ranging from 0.05 to 100 mg/L, and could detect PCP's toxicity to *E. faecalis* at a concentration of only 0.05 mg/L in 8 min. Furthermore, by extending this technique to a mixed anaerobic sludge, not only could the effect of 0.05–100 mg/L PCP be determined on anaerobic digestion metabolism within 10 min, but also its rate of biogas production. These results suggest that a resazurin-based fluorescence measurement can potentially be incorporated into a microfluidic system to develop a biosensor for the real-time monitoring, control and early warning of toxicant/inhibitor loads in the influent to an anaerobic digestion system.

1. Introduction

In recent years increasing attention is being focused on anaerobic wastewater treatment due to concerns about energy use, solids production and disposal, carbon footprint, and resource recovery. Anaerobic digestion utilizes a microbial consortium which breaks down biodegradable organic materials in the absence of oxygen, and can treat sewage as well as hospital and industrial effluent to recycle water and produce renewable energy (Rezaee et al., 2005; Show et al., 2010; Torres et al., 2013; Dereli et al., 2014). However, anaerobic digestion is sensitive to a wide range of toxicants, and when they are present in inhibitory concentrations in wastewater, digester upset or failure can occur (Chen et al., 2008). Off-line monitoring of toxicants using GC and HPLC has been widely used, however, measurement delays result in slow responses to these shocks. Such delayed feedback responses can lead to

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difficulties in preventing anaerobic digestion system failure. Real-time monitoring using RANTOX (rapid anaerobic load and toxicity tester) (Rozzi et al., 1999) or MFCs (microbial fuel cells) (Liu et al., 2011; Kaur et al., 2013) show some potential in detecting toxic loads, or reflecting real time microbial activity in anaerobic digestion. However, long response times, or their focus on measuring specific compounds, make these methods limited in scope.

Sensors which give advanced warning when shock/toxic loads are present in the feed are urgently needed in order to treat industrial wastewaters and prevent irreversible damage to the bioprocess. Optical methods are promising as they allow direct and rapid metabolic measurements of the culture activity. The resazurin reduction (RR) assay, previously commercialized under the trade names of Alamar Blue or CellTiter-Blue, utilize resazurin and resorufin as oxidation-reduction indicators that yield colorimetric changes and fluorescent signals in response to intercellular metabolic activity (Fig. 1) (Pratten et al., 2012). The RR assay has been shown to be a simple, fast and cheap way to continuously monitor living cell proliferation, and the viability of bacterial and eukaryotic cells due to its high water solubility, stability in culture medium, non-toxicity and permeability through cell membranes (O'Brien et al., 2000). As an indicator with fluorescent end-points, Alamar Blue was used to develop a rapid method to examine the antimicrobial efficacy of oral care formulations (Sreenivasan et al., 2003), and a rapid resazurin bioassay also proved valuable for screening chemical libraries for fungicides and as a biomarker for detecting the effects of fungicides on non-target fungi (Fai and Grant, 2009). In addition, many tetrazolium salts have become some of the most widely used tools in cell biology for measuring the metabolic activity of cells such as in the MTT assay (Berridge et al., 2005). However, despite yielding sensitive chromophores or fluorophores in response to intercellular metabolic activity, most of these assays with tetrazolium-based dyes were carried out under aerobic conditions.

To the best of our knowledge, the use of resazurin as a biotoxicity indicator for anaerobic digestion has not been rigorously investigated. The properties of resazurin may allow for the development of a rapid assessment sensor which measures the real time metabolic activity change in anaerobic cultures exposed to toxicants. In this work we present a rapid and flexible assay based on optical measurements of resazurin reduction by a pure strain of facultative anaerobes (*Enterococcus faecalis*) and mixed anaerobic sludge with and without a spiked toxicant, pentachlorophenol (PCP). We suggest that the resazurin reduction assay has considerable potential to be developed into a biotoxicity sensor to rapidly assess the inhibition of anaerobic digestion sludge for biogas production.

2. Material and methods

2.1. Chemicals

Resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide), brain heart infusion (BHI) broth and pentachlorophenol (PCP) were of high purity and were from Sigma–Aldrich, Singapore. Other reagents and chemicals used were the highest available purity, and were obtained from Sigma–Aldrich, Singapore.

2.2. Bacterial strain and anaerobic sludge

Enterococcus faecalis (E. faecalis) OG1RF (ATCC® 47077™) stored at −80 °C in BHI broth/25% glycerol was gently thawed and 100 μL injected anaerobically into 10 mL of N_2 bubbled BHI sterile medium. The culture was incubated overnight and 100 μL was inoculated into another 10 mL of BHI medium and subcultured until the middle log phase (~3 h). A 2 L anaerobic reactor was operated at ~30 °C as the seed for the toxicity and activity test. The reactor was fed weekly for 4 circles with a synthetic medium (Table 1) and acetic acid to give a final COD (chemical oxygen demand) around 2 g/L. The methane production was monitored daily by the displacement of a water column after trapping CO_2 with 0.5 M NaOH.

2.3. E. faecalis spiked with PCP: fluorescence and absorbance measurements

Subcultured *Enterococcus faecalis* in their middle log growth phase described above were mixed with autoclaved BHI broth in a 96-well microplate to get a final OD_{600nm} of 0.1 in a 300 μL/well. Resazurin was added to each cell to a final concentration of 25 mg/L, and PCP was spiked to yield final concentrations of 0 (control), 0.05, 0.5, 5, 10 and 100 mg/L. The 96-well microplate was sealed immediately under anaerobic conditions by a sterile film. Triplicate experiments for each test were carried out at 37 °C, and fluorescence was read once every 2 min over 30 min by an Infinite M200 Pro microplate reader (TECAN, Singapore) with TECAN i-control software at $\lambda_{ex} = 530$ nm and $\lambda_{em} = 590$ nm with a gain of 30. For the reaction described in Fig. 1, many researchers, e.g. Zhou et al. (1997) and Perrot et al. (2003), found a maximum emission at 590 nm for resorufin but a negligible fluorescence for resazurin. Although 570 nm was once proposed as the optimal excitation wavelength for resorufin (Perrot et al., 2003), 530 nm is more commonly used (Nakayama et al., 1997; Springer et al., 1998; Evans et al., 2001), while resorufin can be metabolized to dihydroresorufin, a colorless and non-fluorescent metabolite (Natto et al., 2012).

Another aliquot of subcultured bacteria was mixed with autoclaved BHI broth in a 96-well microplate to a final volume of 300 μL, and PCP was spiked into the wells to result in concentrations of: 0 (control), 0.05, 0.5, 2.5, 5, 10, 20, 50 and 100 mg/L. The 96-well microplate was sealed and cultured in a 37 °C incubator, and triplicate experiments were carried out at each PCP concentration. OD_{600nm} was measured at intervals of 6 h by an Infinite M200 Pro microplate reader (TECAN, Singapore) with TECAN i-control software.

2.4. Anaerobic sludge spiked with PCP: fluorescence measurements and methane production

Five mL of fresh anaerobic sludge was collected from the anaerobic reactor one hour after feeding and transferred into a 15 mL test tube in triplicate. PCP was spiked into the tubes, yielding final concentrations of 0 (control), 0.05, 0.5, 5, 10 and 100 mg/L 150 μL PCP spiked anaerobic sludge was collected immediately from these tubes, transferred into a 96-well

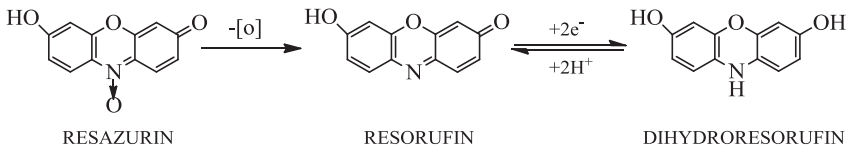

Fig. 1 – Conversion of resazurin to resorufin and dihydroresorufin. At a λ_{ex} of 530 nm and λ_{em} of 590 nm, resazurin is blue and weakly fluorescent; resorufin is pink and strongly fluorescent; dihydroresorufin is colorless and non-fluorescent.

microplate, and mixed with autoclaved DI water to a final volume of 300 μL. Resazurin was added to each well to result in a concentration of 25 mg/L. The microplate was sealed by sterile film, and triplicate experiments were carried out under the same conditions as in the previous section. After shaking under anaerobic conditions at 37 °C for one week, the gas volume of each tube was measured and the concentration of methane determined by GC-TCD (Shimadzu GC-2014 & TCD-2014) equipped with an Agilent J&W Select for Permanent Gases/CO_2 GC column (length of 60 m, film thick of 30 μm and ID of 0.53 mm).

Besides the control set-up described above, the same volume sample was collected daily in the following 5 days from the 2 L anaerobic reactor as well. The RR assay was used to measure the activity of the anaerobic sludge following the same procedure but without PCP addition in the 96-well microplate. These results will be correlated to daily methane production by the anaerobic reactor.

2.5. *Statistical analysis*

The differences in fluorescent signals between the control and each PCP spiked *E. faecalis* or anaerobic sludge sample at the same reading time were determined using a two-sample *t*-test. Accordingly, a Pearson correlation was used to study the relationship between the daily gas production rate, and the kinetics of resazurin reduction.

3. Results

Before studying the complex and mixed microbial anaerobic sludge, *E. faecalis*, a Gram-positive, catalase-negative, non-spore-forming, facultative anaerobic bacterial strain (Fisher and Phillips, 2009), was chosen as a simple model anaerobe to study the effect of toxicants and compare the two toxicity measurements, e.g. the RR assay and the traditional absorbance measurements of bacterial growth. It is known that the second largest group of bacteria in an anaerobic digestion community is the class of Bacilli in the Bacteria domain, of which the most abundant species found in digesters was *E. faecalis*, which participates in the hydrolysis step of anaerobic digestion and possesses hydrogenase activity in its formate dehydrogenase complex (Wirth et al., 2012). Although *E. faecalis* is a facultative anaerobe, all experiments with this strain in the present study were carried out under anaerobic conditions making *E. faecalis* switch metabolism to fermentation or anaerobic respiration. With the novel aim of the present design to correlate fluorescence signal with respect to time, our future plans are to develop an integrated stand-alone biosensor based on the RR assay to give timely advanced warning of toxicants/inhibitors for anaerobic digestion based on changes in the resazurin reduction dynamics. Hence the effect of the model toxicant, PCP, on anaerobic sludge was investigated using the RR assay, and this was applied to measure the activity of anaerobic sludge using biogas production to determine whether both parameters were closely correlated. As a broad-spectrum pesticide, due to its higher number of chlorine atoms, hydrophobicity and acidity, PCP has been considered a priority pollutant since it has a very long half-life in the environment, is harmful at very low concentrations, and has been designated as a priority pollutant and is a probable human carcinogen (Piringer and Bhattacharya, 1999; de Morais et al., 2014). Various residual concentrations of PCP (up to 70 mg L^{-1}) have been found in distribution in the environment (Frick et al., 1988), and IC_{50} values of 0.9–76 mg L^{-1} have been reported to show notable anaerobic inhibition (Meyer and Edwards, 2014).

Table 1 – Synthetic medium used for sludge.

Chemicals	Concentration
Peptone	0.2 g/L
Beef extract	0.14 g/L
Urea	0.01 g/L
$CaCl_2 \cdot 2H_2O$	0.004 g/L
$MgSO_4 \cdot 7H_2O$	0.002 g/L
K_2HPO4	0.011 g/L
NaCl	0.007 g/L
$NaHCO_3$	0.3 g/L

3.1. *Toxicity of PCP to E. faecalis: resazurin reduction and bacterial growth*

Fig. 2 shows the results of resazurin reduction after 30 min by pure *E. faecalis* at OD_{600nm} 0.1 with PCP addition from 0.05 to 100 mg/L. Fluorescence kept on increasing in all the experiments, indicating that the kinetics of resazurin reduction to resorufin was faster than the reduction of resorufin to dihydroresorufin, suggesting that resorufin was accumulating (Fig. 1). In addition, PCP addition clearly slowed down resazurin reduction by the cells, especially for 100 mg/L PCP, which was much higher than 10.2 mg/L, the IC_{50} for *E. faecalis* obtained later. A two-sample *t*-test was used to analyze the differences between PCP-spiked samples and the control over time, and these results are summarized in Table 2. Compared to the control with $P < 0.05$ as the threshold for "statistical significance", it can be concluded that 100 mg/L PCP showed a significant effect after two minutes; while for the 5 and 10 mg/L PCP-spiked samples, it was only after the 4th minute that they began to show a significant effect on resazurin reduction.

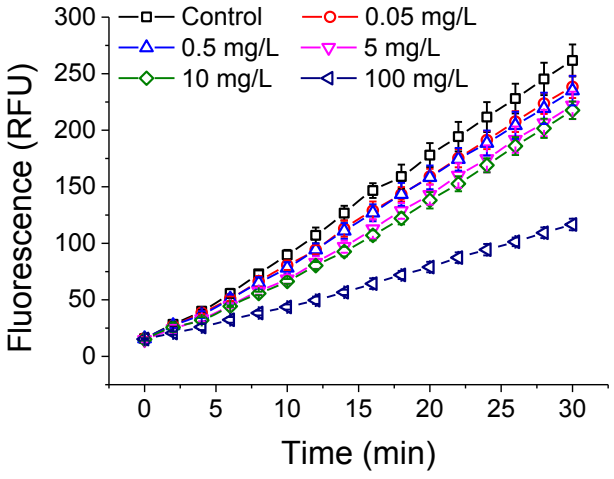

Fig. 2 – Resazurin (25 mg/L) reduction by *E. faecalis* at OD_{600nm}0.1 with different PCP concentrations (□: control (without PCP spiked); ○: 0.05 mg/L; △: 0.5 mg/L; ▽: 5 mg/L; ◇: 10 mg/L; ◁: 100 mg/L) under anaerobic conditions.

However, the 0.05 and 0.5 mg/L PCP-spiked experiments did not show any significant effect until the 8th minute. Hence it is clear that the sensitivity of the present assay to PCP toxicity for *E. faecalis* is time-dependent.

However, absorbance measurements on the effect of PCP addition on *E. faecalis* growth (Fig. 3a) showed that the lower PCP concentrations, e.g., 0.05–5 mg/L, had no effect on the growth of *E. faecalis*. The same maximum OD_{600nm} values (around 0.7), and similar times to reach the same middle-log growth phase (around 2.5 h) were observed for the control experiment. However, PCP at 10 and 20 mg/L showed a significant effect on the growth of *E. faecalis*, reducing the maximum OD_{600nm} to 0.6 and 0.4, respectively, and delaying the middle-log phase to 3.5 and 4.5 h, respectively. For even higher concentrations, such as 50 and 100 mg/L, no statistical growth of *E. faecalis* was observed. The OD_{600nm} of each sample at its middle-log phase was plotted against PCP concentration (Fig. 3b), with an IC_{50} of 10.2 mg/L.

3.2. *Toxicity of PCP to anaerobic sludge: resazurin reduction and methane production*

The fresh anaerobic sludge collected from the seed reactor one hour after feeding with synthetic medium showed a high activity in reducing resazurin to resorufin and eventually to dihydroresorufin (Fig. 4). With the exception of 100 mg/L PCP, the control and other PCP samples increased their fluorescence initially, and after reaching a maximum, decreased to a background level, suggesting that the resorufin had been completely reduced to dihydroresorufin. In contrast, 100 mg/L PCP significantly inhibited sludge activity compared to the other concentrations. The two-sample *t*-test was used to analyze the differences between PCP-spiked samples and the control over time, and the results are summarized in Table 3. Compared to the control, using $P < 0.05$ as the statistically significant threshold, PCP at 100 mg/L showed a significant effect on resazurin reduction after 4 min; it was only after 6 min that 5 and 10 mg/L PCP began to influence resazurin reduction; while after 10 min both 0.05 and 0.5 mg/L significantly influenced resazurin reduction. Fig. 5 shows methane production by anaerobic sludge at different concentrations of spiked PCP. It can be seen that when the PCP concentration was greater than 5 mg/L, there is nearly no methane produced; while 0.56 ± 0.02 and 0.54 ± 0.02 mL of methane was produced in the 0.05 and 0.5 mg/L PCP spiked systems, respectively (less than the 0.6 ± 0.1 mL methane produced in the control). By graphically fitting the methane production with PCP concentration, an IC_{50} of 2.12 mg/L was obtained. This value is lower than the IC_{50} of PCP (10.2 mg/L) with *E. faecalis* we obtained above, suggesting that PCP has a higher toxicity to mixed anaerobic sludge than to a single facultative anaerobic stain. These findings demonstrate the potential applicability of the resazurin reduction assay for identifying toxicants to the anaerobic digestion process. In the present study, the response time of the resazurin reduction assay for 0.5–10 mg/L, which is within the proposed IC_{50} of PCP for acidogenic and methanogenic populations (Chen et al., 2008), was 6–10 min.

3.3. *Activity of anaerobic sludge and biogas production*

Using the resazurin assay, the fastest reduction time was obtained using the anaerobic sludge one hour after feeding, in which the fluorescent signal quickly increased and reached a maximum and then decreased to a steady value (symbol □ in Fig. 6a), suggesting the sludge was active enough to continuously reduce resorufin to dihydroresorufin. After 24 h (day 2-symbol ○ in Fig. 6a), the speed of resazurin reduction was slower than the one hour experiment, suggesting that the activity of anaerobic sludge in day 2 was lower than that one hour after feeding. These observations correlate well with

Table 2 – Results of two-sample *t*-test comparing the fluorescent signal from a PCP spiked *enterococcus faecalis* system to the control system at the same reading time during resazurin reduction.

Signal reading time (min)	P value of comparing different PCP spiked *E. faecalis* to control system				
	0.05 mg/L	0.5 mg/L	5 mg/L	10 mg/L	100 mg/L
0	0.5000	0.3216	0.2338	0.1870	0.2593
2	0.2695	0.1870	0.0825	0.1726	**0.0018***
4	0.0878	0.1026	**0.0028***	**0.0006***	0.0005*
6	0.1021	0.0738	0.0035*	0.0043*	0.0005*
8	**0.0381***	**0.0323***	0.0017*	0.0004*	0.0004*

*: indicates the current result is significantly different to the control according to a *t*-test at $P < 0.05$. The significance of bold suggests that at this moment, the spiked PCP began to show a significant effect on resazurin reduction by *E. faecalis* comparing to control system.

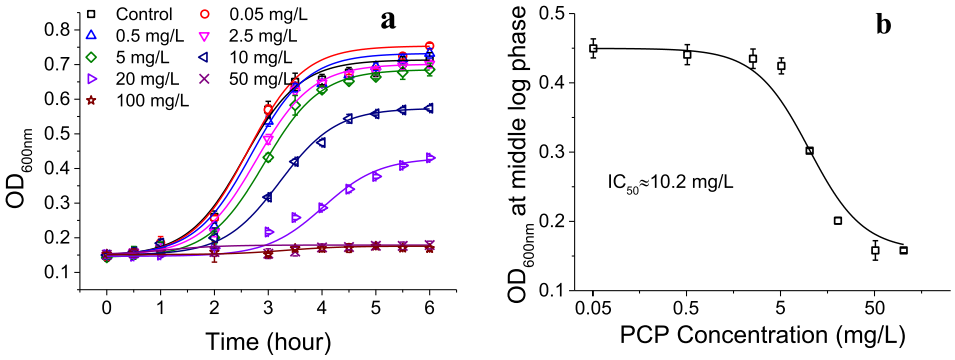

Fig. 3 – Effect of PCP concentration on the growth of *E. faecalis*. a: the growth curve of *E. faecalis* with different PCP additions (□: control (without PCP spiked); ○: 0.05 mg/L; △: 0.5 mg/L; ▽: 2.5 mg/L; ◇: 5 mg/L; ◁: 10 mg/L; ▷: 20 mg/L; ×: 50 mg/L; ☆: 100 mg/L); b: the IC_{50} of PCP to *E. faecalis* was obtained by plotting the OD_{600nm} of each PCP concentration at its middle-log growth phase against PCP concentration.

biogas production (Fig. 6b), i.e. the gas produced in day one after feeding was much more than day two. The assay showed sludge activity in the following 4 days (symbol △, ▽, ⋄ and ◁ in Fig. 6a), however, compared to the first two days their reducing rates were considerably slower, and this was also reinforced by the biogas data. In Fig. 6c, the daily gas production rate, reflecting the activity of anaerobic sludge, was plotted again the gradient obtained by linearly fitting data over 0–10 min in Fig. 6a. A Pearson correlation coefficient at 0.95 was obtained, which is above 0.8, suggesting there is strong correlation between the kinetics of resazurin reduction and the activity of anaerobic sludge.

4. Discussion

Anaerobic digestion is sensitive to a wide range of toxicants (Chen et al., 2014). Thus, an early warning system for the real-time monitoring of feeds to anaerobic digestion processes is essential in order to enhance the chances of avoiding possible instability due to disturbances by toxicants (Orton et al., 2009). However, although conventional anaerobic toxicity assays have been widely used for monitoring anaerobic toxicity over the past few decades, they are time-consuming and this limits their real-time application for the advanced warning of toxicants to anaerobic digestion (Owen et al., 1979; Rozzi and Remigi, 2004). The RANTOX technique has been proven to be effective in the process control of an anaerobic digester based on feed-forward strategies (Rozzi et al., 1999), and was utilized to assess the inhibitory effect of salts and chloroform on anaerobic sludge. However, its slow response time limited its widespread application, e.g., it took 3 h to determine the effect of a step-load of 15 g/L NaCl and 1.07 mg/L chloroform on anaerobic sludge (Pollice et al., 2000, 2001). As a recent and popular real-time sensor for anaerobic digestion most MFCs were designed based on volatile fatty acids (VFAs) sensing, and their accumulation results in a decrease in pH and can ultimately lead to a failure in anaerobic digesters (Siegert and Banks, 2005). However, VFAs in anaerobic digestion only form a bridge between acidogenesis and acetogenesis, and cannot reveal the activity of hydrolysis and methanogenesis, which are generally considered as the rate-limiting step and the key in anaerobic digestion process, respectively (Appels et al., 2008). Although MFCs can determine the concentration of VFAs, and it was reported that the response time to toxicants was around 2 min in an anaerobic reactor (Kaur et al., 2013), the results do not tell us if these compounds are toxic to methanogenesis, the most important step in anaerobic digestion, and offer no metabolic activity assessment of anaerobic biomass. In a study on the toxicity of PCP to other organisms, an LC_{50} of 0.205 mg/L was determined for Fathead Minnow using a static bioassay at 20 °C in 96 h, an EC_{50} at 0.519 mg/L for *Phytobacterium phosphoreum* by the Microtox test in 30 min, and an EC_{50} at 0.09 mg/L for green algae.[1] Besides, many methods have been developed such as the (anti-) yeast estrogen and (anti-) yeast androgen assays, which were reported to have a high sensitivity for PCP of 0.015 μM (~4 μg/L);

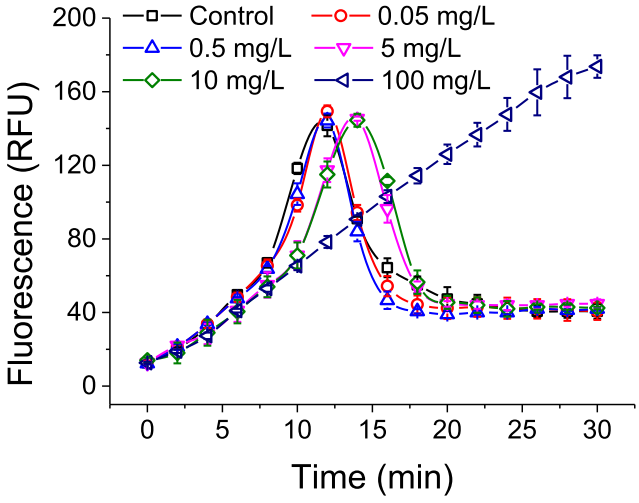

Fig. 4 – Resazurin (25 mg/L) reduction by anaerobic sludge (1:1 dilution in DI water) with different PCP concentrations (□: control (without PCP spiked); ○: 0.05 mg/L; △: 0.5 mg/L; ▽: 5 mg/L; ◇: 10 mg/L; ◁: 100 mg/L) under anaerobic conditions.

[1] https://www.fishersci.ca/viewmsds.do?catNo=AC161120010.

however, the incubation time of 3–6 days limits their application to real-time toxicity monitoring (Orton et al., 2009).

In this present study, we propose a rapid assessment (<10 min) technique of metabolic activity changes in anaerobic cultures exposed to a toxic load based on the biochemical properties of resazurin. Resazurin reduction by cells is postulated to occur via the following steps; i) attachment of resazurin to the bacteria, ii) diffusion through the cell wall and membrane, and iii) reaction with enzymes in the cell (O'Brien et al., 2000). With the oxidation-reduction potential of E_0 = 380 mV, resazurin can be reduced inside the cell by NADPH (E_0 = 320 mV), FADH (E_0 = 220 mV), FMNH (E_0 = 210 mV), NADH (E_0 = 320 mV), as well as the cytochromes (E_0 = 290 mV–80 mV) (Rampersad, 2012). As a refractory organic compound, the model toxicant used in the present study, PCP, is known to be the most toxic to anaerobic digestion amongst the chlorophenols (Chen et al., 2008). When PCP disrupts the proton gradient in the cell membrane, it interferes with cellular energy transduction and competitively inhibits lactate dehydrogenase (LDH) (Young et al., 1999). As a result, the metabolites produced during cellular energy transduction, such as NADH, can be inhibited, and their concentration will decrease due to the reduction of resazurin. For hydrophobic solutes such as PCP, the greater the concentration in bulk solution, the more it will accumulate in the lipid bilayer with concomitant increasing toxicity (Sikkema, 1995). Based on this mechanism, as long as PCP affects the metabolism of living cells, the resazurin assay can highlight this effect through the reduction kinetics changing. These changes in kinetics can be seen in both cultures of *E. faecalis* and anaerobic sludge (Figs. 2 and 4).

Comparing the RR assay to the absorbance measurements for the toxicity of PCP to *E. faecalis*, it can be seen that the RR assay has at least two advantages: it is both quick and sensitive. For the same concentration of PCP, e.g. at the IC_{50} of PCP on *E. faecalis* (around 10 mg/L), the RR assay could tell there was a significant effect of PCP on *E. faecalis* in 4 min, while absorbance measurements offered no such insights in the first 2 h. In addition, in 8 min the RR assay could sense PCP's toxicity to *E. faecalis* at a concentration of only 0.05 mg/L, which is more sensitive than the traditional absorbance measurements which can only detect the toxicity of PCP to *E. faecalis* above 5 mg/L. It can be concluded that the current measurement is flexible since its sensitivity can be increased or decreased by trading off time, e.g. it can be a fast but less sensitive assay to 10 mg/L PCP with a 4 min response, while it can be a very sensitive assay down to 0.05 mg/L PCP with an 8 min response, which allows for it to be tuned towards various anaerobic applications.

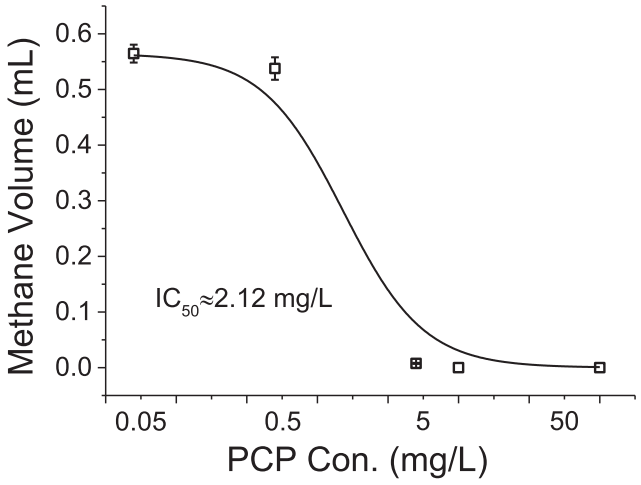

Fig. 5 – The IC_{50} of PCP to anaerobic sludge was obtained by plotting the methane production of each PCP spiked anaerobic sludge against PCP concentration.

Traditionally, biogas (methane) production is another preferred parameter for assessing microbial activity and inhibition under anaerobic conditions. Volumetric methods such as anaerobic toxicity assay (Owen et al., 1979; Rozzi and Remigi, 2004) and specific methanogenic activity test (Jawed and Tare, 1999) are usually used for assessing microbial activity of anaerobic sludge under different conditions. However, these methods are time consuming (hours or even days) with multiple parameters to measure, resulting in a delayed feedback response. In the present study using the RR assay to examine the effect of PCP on anaerobic sludge, it responded rapidly (<10 min) and hence has considerable potential for evaluating the instantaneous activity of anaerobic sludge for biogas production (Fig. 6). The RR assay assesses the entire metabolic pathway of anaerobic digestion, and not a specific step (hydrolysis, acidogenesis/acetogenesis, or methanogenesis). This makes the assay a "gross"

Table 3 – Results of two-sample *t*-test comparing the fluorescent signal from a PCP spiked *anaerobic sludge* system to the control system at the same reading time during resazurin reduction.

Signal reading time (min)	P value of comparing different PCP spiked *sludge* to control system				
	0.05 mg/L	0.5 mg/L	5 mg/L	10 mg/L	100 mg/L
0	0.1151	0.2593	0.5000	0.1096	0.5000
2	0.1254	0.1743	0.1254	0.2473	0.0581
4	0.2536	0.1254	0.1471	0.3048	**0.0010***
6	0.2383	0.0506	**0.0374***	**0.0406***	0.0006*
8	0.2737	0.0659	0.0243*	0.0179*	0.0007*
10	**0.0009***	**0.0107***	0.0003*	0.0008*	<0.0001*

*: indicates the current result is significantly different to the control according to a *t*-test at $P < 0.05$. The significance of bold suggests that at this moment, the spiked PCP began to show a significant effect on resazurin reduction by anaerobic sludge comparing to control system.

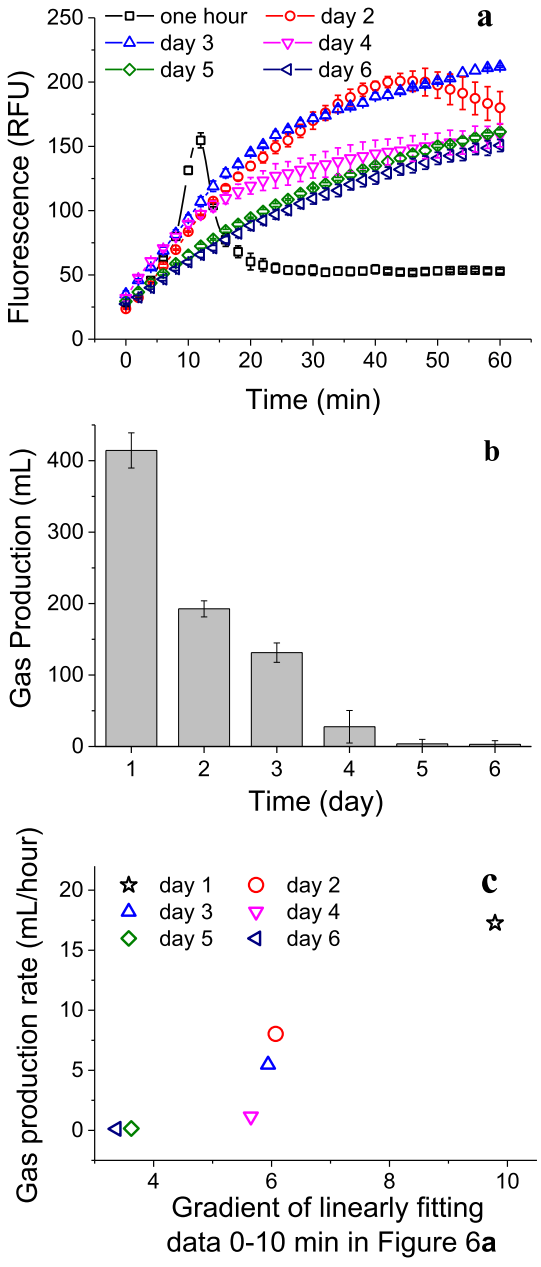

Fig. 6 – Resazurin reduction assay to study the activity of anaerobic sludge (without PCP addition). a: daily kinetic study of resazurin reduction by anaerobic sludge over 6 days after feeding (□: one hour; ○: day 2; △: day 3; ▽: day 4; ◇: day5; ◁: day 6) with synthetic nutrient medium; b: daily monitoring of methane production over 6 days after the synthetic nutrient medium was fed to anaerobic sludge (mean and standard deviation values of 4 circles are shown); c: the daily gas produce rate again the gradient of linearly fitting resazurin reduction date from 0 to 10 min in a.

optical measurement for toxicity which can not only quickly examine toxicants and give advanced warning about anaerobic digester upsets, but also provide information about biogas production rates.

5. Conclusions

- Compared to the traditional toxicity measurements of bacterial growth for a single bacterial strain, and by methane production for anaerobic sludge, the optical measurement based on resazurin reduction proved to be much faster and more sensitive when detecting the model toxicant, pentachlorophenol.
- In addition to toxicity detection, the optical measurement discussed here could also provide the biogas production potential of an anaerobic reactor.
- Future work involves introducing the present optical measurement into a microfluidic system to fabricate a biosensor for the real-time monitoring of anaerobic digestion. The turbulent flow of a droplet in a microfluidic device allows for an increase in the reduction kinetics of resazurin by anaerobic sludge, increasing the toxicity response and increasing the time available for remediation by a variety of means before irreversible damage is done to the anaerobic digestion system. Some work in this area has already been done in our laboratories, and will be submitted in our next paper.

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