Chiang Mai J. Sci. 2021; 48(1) : 74-89
http://epg.science.cmu.ac.th/ejournal/
Contributed Paper

Effects of Sub-lethal Concentrations of Isothiazolone Biocide on the Performance of Rotating Biological Contactors

Prapaparn Sirikhansaeng [a], Niphaphat Phukoetphim [b], Pattana Laopaiboon [b,c,d] and Lakkana Laopaiboon*[b,d]

[a] Faculty of Science, Buriram Rajabhat University, Buriram 31000, Thailand.
[b] Department of Biotechnology, Faculty of Technology, Khon Kaen University, Khon Kaen 40002, Thailand.
[c] Center for Alternative Energy Research and Development, Khon Kaen University, Khon Kaen 40002, Thailand.
[d] Fermentation Research Center for Value Added Agricultural Products, Khon Kaen University, Khon Kaen 40002, Thailand.

*Author for correspondence; e-mail: lakcha@kku.ac.th

Received: 16 January 2020
Revised: 22 April 2020
Accepted: 27 May 2020

ABSTRACT

The aim of this study was to investigate the effects of operating conditions on chemical oxygen demand (COD) removal and biocide degradation in a synthetic wastewater by rotating biological contactors (RBCs). The RBC operating parameters included the organic loading rate (OLR), hydraulic loading rate (HLR) and hydraulic retention time (HRT). The presence of another carbon source (lab-Lemco broth) in the synthetic wastewater contaminated with isothiazolone (IT) biocide at sub-lethal concentrations was also examined. Biofilms were established on RBC discs and then exposed to the wastewater containing 6 ppm of IT under various operating conditions. After an acclimatization period with an HRT of 36 min, the COD removal was 16.49 ± 1.55% and biocide removal was negligible. IT degradation increased with the OLR of a growth substrate and/or HRT. Acclimatized biofilms exhibited IT degradation through co-metabolism when Lab-Lemco broth was included in the medium. At an OLR (using Lab-Lemco broth) of 17 to 20 g COD/m^2.d, the acclimatized biofilms degraded 73.11 ± 5.01 to 77.38 ± 6.66% and 45.76 ± 5.01% of the 6 ppm of IT at HRT values of 72 and 36 min, respectively. When the OLR of the growth substrate was doubled, IT degradation increased to 59.33 ± 5.58% at an HRT of 36 min. The IT resistant bacteria were tentatively identified as predominantly of the species *Burkholderia cepacia*. Biofilms could be developed in the presence of 3 ppm of IT after an acclimatization period, and the degree of COD removal and biocide degradation depended on the HLR and HRT values. Biochemical oxygen demand (BOD) measurements were not appropriate for investigating treatment of wastewater contaminated with IT due to severe bio-oxidation inhibition. This was true even when using adapted seed and/or increased incubation time.

Keywords: biocide, biofilm, biodegradation, isothiazolone compounds, RBC, wastewater treatment

1. INTRODUCTION

Isothiazolone compounds (5-chloro-2-methly-4-isothiazolin-3-one, CMIT and 2-methly-4-isothiazolin-3-one, MIT) react with thiol containing enzymes to achieve biocidal activity

[1]. They are of used as antimicrobial agents in a variety of commercial activities, such as preventing microbial growth in cooling water, as well as in paper, cosmetics and textile manufacturing [2-3]. It is established that the sessile bacteria in a biofilm are less susceptible to biocides than are those not attached to solid surfaces [4-6]. In water systems, a biofilm can increase resistance to heat transfer, plug filters and pipes as well as promote metal corrosion [7-11]. Over-use of biocides to prevent the formation of biofilms will lead to environmental, ecological and toxicological problems when the water of such systems is discharged into natural bodies of water or is sent to municipal treatment plants. The impacts of biocides on secondary wastewater treatment plants is of growing concern. Environmental regulations in this area are becoming more restrictive. Soon, it may be necessary to treat wastewater containing biocides before discharge. It was reported that the adsorption of isothiazolones by powdered activated carbon was an effective method for the treatment of reverse osmosis (RO) effluents containing isothiazolone biocides [12]. However, the major drawbacks of this method or any membrane technology are severe membrane scaling and fouling [13].

Use of rotating biological contactors (RBCs) are one of the standard methods of secondary (biological) wastewater treatment in which degradation and bio-oxidation are mainly achieved through a biofilm attached to the rotating discs [14-15]. The advantages of RBCs are their high biomass resulting in greater efficiency for degrading organic compounds [16], their simplicity of maintenance and operation, low energy consumption and good sludge settling properties [17]. Additionally, sessile cells in biofilms have higher resistance to a toxic substance than their planktonic counterparts [4-6]. Therefore, laboratory-scale RBCs can be employed to predict the impacts of biocides on full-scale RBCs. The ability of full-scale RBCs to degrade biocides by biofilms and the ability of biocides to control or even eliminate biofilms in continuous systems can be studied.

The quantity of biocide applied is generally determined from laboratory minimum inhibitory concentration (MIC) / minimum lethal concentration (MLC) [18]. In practice, the concentration of active biocides is reduced after addition due to many factors. Some biocides chemically react with other system constituents, e.g., proteins and polysaccharides of the biofilm to bond to metal surfaces or to cells. Consequently, the available concentration of a biocide is reduced in the system. Therefore, microorganisms may be exposed to subinhibitory concentrations of biocides [19].

Our previous study [20] showed that MLC of isothiazolone (IT) on bacterial cells in recycled sludge from a wastewater treatment system was 10-12 ppm. Additionally, it was found that IT at 6 ppm could not be removed by a single-stage RBC unit receiving synthetic wastewater at an organic loading rate (OLR) of 24 g COD/m^2.d and a hydraulic retention time (HRT) of 36 min. The percentage of COD removal under these conditions was far less than that of the untreated control sample. Thus, various operating conditions were examined to establish whether these removal rates can be increased.

The aim of this study was to investigate the effects of operating conditions on COD removal by the RBCs, biodegradation of IT and the microbial population of a biofilm at sub-lethal concentrations of biocide. The parameters examined include HRT and OLR and the presence of another carbon source (Lab-Lemco broth) in synthetic wastewater containing 6 ppm of IT. Changes in microbial susceptibility to IT of non-acclimatized and acclimatized cells as indicated by MLC determination were observed. The use of the BOD value to express the efficiency of wastewater treatment of samples contaminated with IT was also studied.

2. MATERIALS AND METHODS

2.1 Single-stage (Three Discs) Laboratory-scale RBC Units

Single-stage (three disc) laboratory-scale

RBC units were constructed in a group of three with 40% disc submergence [21]. The three units were driven by the same motor and drive shaft. The three discs (267 mm in diameter) with an inter-disc spacing of 15 mm were rotated at a speed of 16 rpm. Each unit had an influent chamber (0.65 L), a disc stage (1.56 L) and a settling tank (1.55 L). They were separated by fiber glass walls, each fabricated with an 18-20 mm diameter transfer hole. This allowed liquor and suspended biomass to pass through the chambers. In each unit, the solids of treated liquid from the disc stage were allowed to settle before discharge through an overflow tube. The settled biomass was removed through a gate valve. The operating parameters of this single-stage laboratory-scale RBC are summarized in Table 1.

2.2 Synthetic Wastewater

The synthetic wastewater used in the current study was made from Lab-Lemco broth, 90 mg/L which was comprised of 33.75 mg/L of Lab-Lemco powder (Oxoid) and 56.25 mg/L bacteriological peptone (Oxoid); NH_4Cl, 54 mg/L; K_2HPO_4, 28 mg/L; NaCl, 7 mg/L; $CaCl_2.2H_2O$, 4 mg/L and $MgSO_4.7H_2O$, 2 mg/L [21].

2.3 Biocide

An aqueous substituted isothiazolinone solution was provided by Ondeo Nalco, Thailand. It was composed of 1.15% (by wt) of 5-chloro-2-methly-4-isothiazolin-3-one and 0.35% of 2-methly-4-isothiazolin-3-one.

2.4 Biofilm Establishment and Biocide Treatments

A volume of 350 mL of recycled sludge from the wastewater treatment system of a local paper manufacturer (Phoenix Pulp & Paper Company, Ltd., Khon Kaen, Thailand) was added to the disc stage of RBC units treating synthetic wastewater. It was operated as a batch system for 24 hours. Then, the synthetic wastewater was fed to the units for two weeks at a flow rate of 2.5 L/h to provide a medium for microbial growth. After one week, the surfaces of the RBC discs were covered by a thin homogeneous biofilm. When the system reached steady state (no change in COD removal), a diluted commercial IT solution was fed separately to give a final concentration of 6 ppm when it was mixed with the synthetic wastewater. A control experiment was run with no IT in the synthetic wastewater.

2.5 Experiments

2.5.1 Impacts of operating conditions on efficiency and IT biodegradation by the RBCs

Seven experimental runs, Runs 1 to 7, were performed. Each run lasted for about one week of stable operation as indicated by constant COD and IT concentrations in the effluent. The details of each run are summarized in Table 1. Untreated synthetic wastewater samples containing no IT were tested under the same conditions in Runs 1, 2 and 4. Under the various operating conditions, influent and effluent samples from the RBC units were collected for analysis as described below.

To study the decreased susceptibility to IT of acclimatized cells, the minimum lethal concentrations (MLC) were determined for biocide on non-acclimatized planktonic cells from the control RBC and on acclimatized planktonic cells from the RBC receiving 6 ppm in Run 6.

At the end of the experiment, bacterial cells in the acclimatized biofilms were isolated on tryptone soya agar (TSA, Oxoid) using a spread plate technique, and tentatively identified using an API 20 NE strip test (BioMérieux SA, France).

Table 1

2.5.2 Capability in biofilm formation in the presence of IT

The planktonic microorganisms (150 mL) of the control RBC and the recycled sludge (200 mL) from the wastewater treatment system of the local paper manufacturer were added to the disc stage of two clean RBCs filled with the synthetic wastewater containing 3 ppm of IT. After 24 hours

Table 1. The experimental runs with the synthetic wastewater containing 0 and 6 ppm of IT.

Run	Composition of influent wastewater			Flow rate of the wastewater (L/h)
	Lab-Lemco broth (mg/L)	IT (ppm)	Mineral salts (mg/L)	
1	90	0, 6	Normal (see Section 2.2)	2.5
2	180	0, 6	Normal × 2	1.25
3	-	6	Normal × 2	1.25
4	45	0, 6	Normal / 2	1.25
5	180	6	Normal × 2	1.25
6	90	6	Normal	2.5
7	180	6	Normal × 2	2.5

operation as a batch system, a flow of synthetic wastewater was introduced to the units at rates of 1.25 and 2.5 L/h. When a biofilm became established, COD and IT concentrations in the influents and effluents were determined. The viable population of biofilms and planktonic cells of the two RBCs were also determined.

2.5.3 BOD_{20}

A synthetic wastewater containing 180 mg/L of Lab-Lemco broth and 6 ppm of IT was mixed with dilution water containing reagents recommended by APHA AWWA and WPCE [22]. Seed cultures consisted of acclimatized planktonic cells from the RBC of Run 4 at levels of 0.5% and 3.5% of the total volume, along with a nitrification inhibitor (2.0 mg of allylthiourea/L) were added to the diluted samples. BOD was measured every 5 days for 20 days or until the BOD values became constant. The BOD values of the synthetic wastewater without the biocide were also determined. Dissolved oxygen (DO) concentrations in BOD determination were determined by the azide modification method [22].

2.6 Sampling and Analysis

2.6.1 Chemical oxygen demand and IT concentration

Influent and effluent samples were passed through a cellulose acetate membrane filter with 0.2-μm pore size (Whatman, Maidstone, Kent, UK). Their COD values were then measured using a modified closed reflux, titrimetric method [22]. Standard ferrous ammonium sulfate (FAS) at 0.025 mol/L was used as a titrant. IT concentrations were measured using HPLC (LC10 AD Shimadzu, Kyoto, Japan). A Spherisorb 5 μm ODS column (46 mm × 300 mm) (Water, Ireland) was used with 65:35 ratio of 0.4% acetic acid:methanol as a mobile phase at 1 mL/min. A UV detector (SPD-10A Shimadzu, Kyoto, Japan) was used at 280 nm [23].

2.6.2 Enumeration of viable populations of biofilm and planktonic bacteria

A single one-cm^2 disc was removed from a biofilm using a sterile cork borer (1.13-cm diameter). It was immersed in 9 mL of a 25% strength Ringer's solution. Each sample was sonicated three times for five min. They were vortex mixed for ten seconds between sonication processes [24]. Then, a total viable count (TVC) on TSA was determined by incubation at 37 °C for 48 h. The liquid in the disc chamber was taken to measure the TVC of the planktonic organisms. The number of colonies was counted on TSA plates that had between 10 and 300 colonies and the TVC was calculated.

2.6.3 Minimum lethal concentration (MLC) of IT

One mL of the planktonic phase from the control RBC unit or the treated RBC in Run 6 was serially inoculated into sterile vials containing 9 mL of synthetic wastewater and the desired IT concentration. The vials were incubated at 37 °C for 48 h. The MLC was determined by adding 1 mL of the aforementioned 48-h incubated samples into sterile vials containing 5 mL of double strength tryptone soya broth (TSB, Oxoid) along with 4 mL of sterile distilled water. Incubation was done at 37°C for 48 h. The lowest concentration of IT showing no growth (turbidity) was determined to be the MLC.

3. RESULTS AND DISCUSSION

3.1 Effects of Operating Conditions on Treatment Efficiency of RBCs Receiving 6 ppm of IT and Susceptibility Changes of Acclimatized Cells to Biocide

3.1.1 COD removal and biodegradation of IT in RBCs receiving 6 ppm of IT under various operating conditions

1) Run 1: Cell acclimation in normal synthetic wastewater (Lab-Lemco broth at 90 mg/L) containing 6 ppm of IT at a flow rate of 2.5 L/h

COD removal and the characteristics of the control RBC (without biocide) as well as the treated RBC under various operating conditions are shown in Figure 1, Tables 2 and 3. From Figure 1 and Table 3, the average influent COD of the treated RBC in Run 1 was 131.33 ± 4.68 mg/L. The RBC in Run 1 achieved steady state at day 8-10. COD removal was 16.49 ± 1.55% corresponding to 14.50 ± 6.74 mg/L of actual COD. Rarely IT was removed by the unit (Figure 2). The average effluent IT concentration was 6.43 ± 0.49 ppm and only 0.06 ± 0.11 ppm of IT was removed by this unit (Table 3).

When COD removal of the control unit and the treatment unit were compared, the untreated control unit showed significantly higher COD removal (69.64 ± 5.92%) than the treated RBC unit (Tables 2 and 3). Since the biocide could not be removed by the unit, COD removal occurred in Run 1 (14.50 ± 6.74 mg/L) was completely

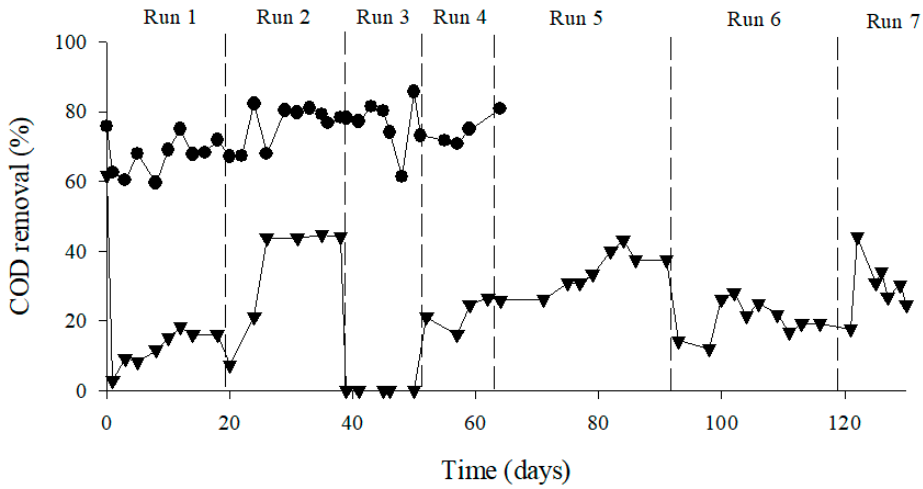

Figure 1. Effect of IT on COD removal in the RBCs: control (●) and 6 ppm (▼). (The operating conditions in Run 3 and Run 4 of the control RBC were identical).

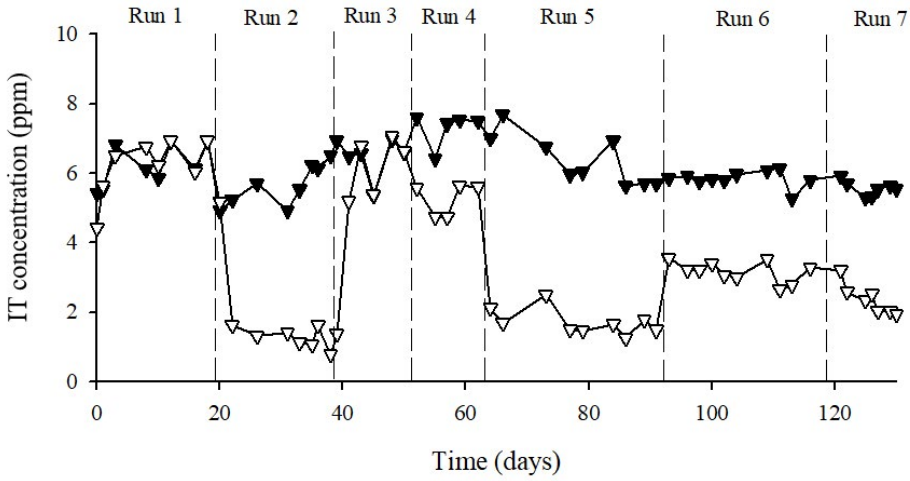

Figure 2. IT removal in the RBCs**:** influent (▼), effluent (∇).

Table 2. Characteristics of the untreated control RBC under various operating conditions.

Run	Flow rate (L/h)	HRT (min)	Lab-Lemco broth (mg/L)	Influent COD (mg/L)	OLR (g COD/ m^2.d)	COD removal	
						%	mg/L
1	2.5	36	90	114.60 ± 11.66	20.63	69.64 ± 5.92	79.11 ± 10.54
2	1.25	72	180	185.50 ± 11.50	16.70	78.27 ± 3.72	143.20 ± 13.90
3	-	-	-	-	-	-	-
4	1.25	72	45	111.00 ± 6.83	10.00	74.10 ± 7.18	74.43 ± 10.67

Table 3. Characteristics of the RBC receiving 6 ppm of IT under various operating conditions.

Run	Flow rate (L/h)	HRT (min)	OLR (g COD/m^2.d)	Influent COD (mg/L)	COD removal		IT removal	
					%	mg/L	%	ppm
1	2.5	36	23.64	131.33 ± 4.68	16.49 ± 1.55	14.50 ± 6.74	0.90 ± 1.62	0.06 ± 0.11
2	1.25	72	17.37	193.00 ± 5.03	44.05 ± 0.44	85.00 ± 2.00	77.38 ± 6.66	4.47 ± 0.79
3	1.25	72	2.74	30.40 ± 8.29	0	0	0	0
4	1.25	72	10.94	121.50 ± 11.93	22.12 ± 4.57	27.00 ± 7.02	27.99 ± 4.74	2.04 ± 0.39
5	1.25	72	17.64	196.00 ± 5.66	38.29 ± 3.61	75.20 ± 9.12	73.11 ± 5.01	4.66 ± 0.64
6	2.5	36	20.78	115.43 ± 11.31	23.02 ± 3.49	26.86 ± 5.98	45.76 ± 5.01	2.67 ± 0.34
7	2.5	36	39.88	221.60 ± 6.07	29.31 ± 3.68	64.80 ± 6.57	59.33 ± 5.58	3.27 ± 0.36

Table 4. COD removal derived from Lab-Lemco broth and IT under various operating conditions.

Run	COD removal in untreated control RBC (mg/L)	COD removal in the RBC treated with 6 ppm of IT (mg/L)		
		derived from Lab-Lemco broth	derived from[a] IT	Total removal
1	79.11 ± 10.54	14.50	0	14.50 ± 6.74
2	143.20 ± 13.90	63.45	21.55	85.00 ± 2.00
3	-	-	-	-
4	74.43 ± 10.67	17.17	9.83	27.00 ± 7.02
5	143.20 ± 13.90	52.74	22.46	75.20 ± 9.12
6	79.11 ± 10.54	13.96	12.90	26.86 ± 5.98
7	-	49.04	15.76	64.80 ± 6.57

*The values were calculated based on IT removal in Table 3 and assuming mineralization of IT occurred.

[a]The value were calculated based on the following equation: 1 ppm of IT = 4.82 mg/L COD.

derived from the Lab-Lemco broth (i.e., the carbon sources in normal synthetic wastewater). The presence of 6 ppm of IT inhibited the RBC's capacity to remove this COD by 87% or ~100 mg/L (114.60 – 14.50 = 100.10 mg/L) (Table 4).

2) Run 2: Cell acclimatization in synthetic wastewater (Lab-Lemco broth at 180 mg/L) containing 6 ppm of IT at a flow rate of 1.25 L/h / Effects of hydraulic retention time

In Run 2, the flow rate of the influent wastewater was decreased to 1.25 L/h, but the OLR derived from the Lab-Lemco broth was kept constant. The average COD of the influent synthetic wastewater containing 6 ppm of IT in Run 2 was 193.00 ± 5.03 mg/L. After switching to the lower flow rate, the system was in steady state in 6 days with an average total COD removal of 44.05 ± 0.44%. The average effluent IT concentration at steady state was 1.27 ± 0.31 ppm corresponding to a 77.38 ± 6.66% IT removal (Figure 2 and Table 3) or 4.47 ± 0.79 ppm of IT was removed by the unit. As 1 ppm of IT is equivalent to 4.82 mg/L COD, the IT removal corresponded to 21.55 mg/L COD. Therefore, COD removal derived from the Lab-Lemco broth and IT was 63.45 and 21.55 mg/L, respectively (Table 4).

The untreated control for Run 2 showed a COD removal of 78.27 ± 3.72%, corresponding to 143.20 ± 13.90 mg/L of actual COD (Table 2). Therefore in Run 2, COD removal derived from the Lab-Lemco broth decreased by 56% (143.20 – 63.45 = 79.75 mg/L) when the RBC was treated with 6 ppm of IT, compared with the untreated control (Table 4). However, the biodegradability of IT was increased from zero (in Run 1) to 77% (Table 3).

Increasing hydraulic retention time (HRT) in the RBC unit showed that 4.47 ± 0.79 ppm of IT could be removed by the unit, corresponding to a 77.38% removal (Table 3). The increase in IT removal compared to that of Run 1 implied that the IT molecule, a more complex carbon source than Lab-Lemco broth, was only slightly broken down in 36 min (retention time at 2.5 L/h). Increasing the HRT to 72 min resulted in an increase in the mass transfer of the biocide into the biofilm, resulting in a decreased IT level in the effluent. The presence of IT did, however, reduce the COD removal derived from Lab-Lemco broth, compared to the control (Table 4).

3) Run 3: IT as a sole carbon source

In Run 3, the flow rate of the synthetic wastewater was kept constant at 1.25 L/h, but the

Lab-Lemco broth was withdrawn from the influent wastewater. The organic loading rate was reduced from 17.37 to 2.74 g COD/m^2.d (in Table 3). The average COD of the influent wastewater containing 6 ppm of IT was 30.40 ± 8.29 mg/L. Figures 1 and 2 show that the percentage of COD removal was 0% and IT concentration in the effluent was markedly increased to constant level after 5 days of the operation. Average effluent IT concentration at steady state was the same as that of the influent. This means that IT was not removed by the unit containing no LabLemco broth in the influent.

4) Run 4: Cell acclimatization in synthetic wastewater (Lab-Lemco broth at 45 mg/L) containing 6 ppm of IT at a flow rate of 1.25 L/h / Effects of OLR derived from Lab-Lemco broth

In Run 3, no COD or IT removal occurred when IT was used as a sole carbon source in the RBC unit. To confirm the relationship between the presence of Lab-Lemco broth and biodegradation of IT, Lab-Lemco broth was re-introduced to the unit at a concentration of 45 mg/L. In Run 4, the organic loading rate was increased to 10.94 g COD/m^2.d, while the flow rate of the synthetic wastewater was kept constant at 1.25 L/h. The average influent wastewater COD increased from 30.40 ± 8.29 mg/L (Run 3) to 121.50 ± 11.93 mg/L (Run 4). After Lab-Lemco broth was reintroduced to the RBC, COD removal increased immediately along with a reduction in IT in the effluent (Figures 1 and 2). The percentage of COD and IT removal at steady state was 22.12 ± 4.57% (27.00 ± 7.02 mg/L) and 27.99 ± 4.74% (2.04 ± 0.39 mg/L), respectively (Table 3). These results suggested that biodegradation of IT occurred only when Lab-Lemco broth was available in the system.

The effects of the organic loading rate on COD and biocide removal were investigated. In Run 4, the flow rate of the synthetic wastewater was kept constant at 1.25 L/h, but the organic loading derived from Lab-Lemco broth was reduced by twofold compared to that of Run 2, thus limiting the availability of easily degradable carbon. The results showed that when the organic loading of the Lab-Lemco broth was decreased, both COD and biocide removals were significantly decreased (Run 2 & Run 4). This result was unexpected as IT removal in Run 4 did not change when the concentration of Lab-Lemco broth was decreased. IT removal decreased from 77.38% (in Run 2) to 27.99% (in Run 4). This decrease in IT removal suggested that the concentration of LabLemco broth played an important role in biocide degradation.

5) Run 5: Cell acclimatization in normal synthetic wastewater (Lab-Lemco broth at 90 mg/L) containing 6 ppm of IT at a flow rate of 1.25 L/h (i.e., as in Run 2) / Effects of acclimatization period and Lab-Lemco broth concentration on IT degradation

In Run 5, the flow rate of the synthetic wastewater was kept constant at 1.25 L/h, but the organic loading rate derived from Lab-Lemco broth was increased by twofold. The average COD of the influent wastewater containing 6 ppm of IT was increased from 121.50 ± 11.93 (Run 4) to 196.00 ± 5.66 mg/L (Run 5). COD and biocide removal in Run 5 increased by 16% and 46%, respectively, compared to those of Run 4 (Figures 1 and 2 and Table 3). This increase in IT removal suggested that the concentration of Lab-Lemco broth played an important role in biocide degradation.

The operating conditions in Run 5 were identical to those of Run 2. This was done to determine whether COD and IT removal would change with longer acclimatization. Even though cells had now been acclimatized to 6 ppm of IT for over 2 months (Runs 1 to 4), COD and IT removal of Run 5 were similar with those of Run 2 (Figures 1 and 2 and Table 3). This implied that further acclimatization of the cells beyond the initial 25 day period at the same biocide concentration

did not lead to an increased biodegradation of IT.

6) Run 6: Cell acclimatization in normal synthetic wastewater (Lab-Lemco broth at 90 mg/L) containing 6 ppm of IT at a flow rate of 2.5 L/h (i.e., as in Run 1) / Effects of acclimatization period on IT degradation

In Run 6, the conditions were identical to those of Run 1 with the average COD of the influent wastewater being 115.43 ± 11.31 mg/L. After the normal synthetic wastewater containing 6 ppm of IT was re-introduced to the RBC at a flow rate of 2.5 L/h, the system reached steady state in a few days (Figures 1 and 2). Compared to Run 1, the total COD removal of Run 6 increased from 16.49 ± 1.55 to 23.02 ± 3.49% and biocide removal increased from zero to 45.76 ± 5.01%. This dramatic increase in both the total COD and IT removal under the same operating conditions suggested that a period of cell acclimatization under biocide stress together with suitable operating conditions are very important to increase bio-oxidation and biodegradation of IT.

7) Run 7: Cell acclimatization in synthetic wastewater (Lab-Lemco broth at 180 mg/L) containing 6 ppm of IT at a flow rate of 2.5 L/h / Effects of Lab-Lemco broth concentration on IT degradation

In Run 7, the flow rate of the synthetic wastewater was kept constant at 2.5 L/h, but the concentration of Lab-Lemco broth was increased by twofold. Organic loading was increased from 20.78 to be 39.88 g COD/m^2.d. The average COD of the influent wastewater containing 6 ppm of IT increased to 221.60 ± 6.07 mg/L.

Figures 1 and 2 show that COD removal increased to a constant level of 29.31 ± 3.68%, corresponding to 64.80 ± 6.57 mg/L of COD. IT removal also increased to 59.33 ± 5.58%. The IT removal corresponded to 15.76 mg/L of COD. Therefore, COD removal derived from Lab-Lemco broth and IT was 64.80 – 15.76 = 49.04 and 15.76 mg/L, respectively (Table 4). These increases in COD and IT removal indicated that the amount of readily available carbon significantly affected treatment efficiency of the RBC in terms of COD removal and biocide degradation.

The results from Runs 1 to 7 showed that acclimatized cells cannot utilize IT as a sole carbon or energy source. IT was degraded biologically through cometabolism. Metabolism of this compound (as a cosubstrate) occurs in the presence of a second organic compound (growth substrate) that is used as the primary energy source or carbon source. In order to degrade a cosubstrate, the microorganisms need enzymes and reducing equivalents or energy sources which are provided by the primary substrate (growth substrate) [25-26]. In this study, in order to degrade IT, the cells needed an energy source that is generated during the transformation of the Lab-Lemco broth (primary or growth substrate) and enzymes that are induced by Lab-Lemco broth.

When the OLR of the growth substrate was constant (Runs 1 & 2), increasing the HRT resulted in an increased growth substrate removal. Consequently, more enzymes and reducing equivalents or energy sources were generated during the degradation of the growth substrate. As a result, greater degradation of IT was observed (in Run 2). When Lab-Lemco broth was removed from the influent wastewater (Run 3), the enzymes and reducing power or storage compounds that were still present in the system were used to degrade IT until a new steady state was reached after 5 days.

When the hydraulic loading rate (HLR) was constant (Runs 2 & 4 and Runs 6 & 7), IT degradation increased with increasing primary substrate loading rate. This probably arose from Lab-Lemco broth supplementation. The metabolic enzyme system of the cells was increasingly induced for IT degradation. The dramatic increase in treatment efficiency in terms of both total COD and IT removal under the same operating conditions (Runs 1 & 6) implied that a period of cell acclimatization under biocide

stress together with suitable operating conditions were very important to increase bio-oxidation and biodegradation of IT.

3.1.2 Viable microbial population of RBCs receiving 6 ppm of IT under various operating conditions

Figures 3a and 3b show a viable population of microorganisms in the RBCs receiving 6 ppm of IT under various operating conditions. In the control treatment, TVCs of the biofilms and planktonic cells were approximately 10^7 to 10^8 cfu/cm^2 and 10^6 to 10^7 cfu/mL, respectively, throughout the experiment. In Run 1, IT at 6 ppm caused approximately 10^3-fold and 10^2-fold reductions of the numbers of colony-forming units in the biofilms and planktonic phase, respectively, when compared to those of the control unit. In Run 2, when the HRT was increased twofold, TVCs of the biofilms and planktonic cells were markedly increased to the control levels in three days, indicating that the growth rate of biofilm was increased and more biofilm was sloughed-off into the planktonic phase. In Run 3, when only IT was introduced to the unit, TVCs of biofilm and planktonic cells were decreased after 10 days due to the lack of usable carbon or growth substrate. However, even though no growth substrate was

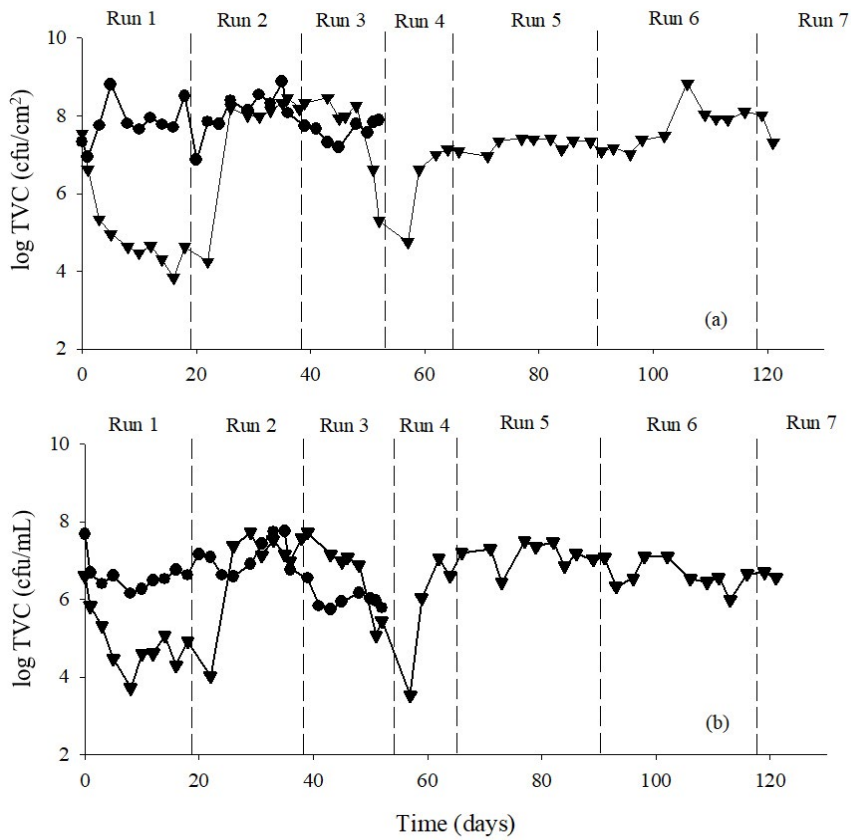

Figure 3. Effect of IT at 6 ppm on the viability of (a) biofilms and (b) apparent planktonic cells in the RBC during acclimatization: control (●) and 6 ppm (▼). (The operating conditions in Run 3 of the control RBC were identical to those in Run 4 of the treated RBC).

available, the viable cell count of the biofilms and planktonic cells remained at approximately log 5 cfu/cm^2 and log 5 cfu/mL at the end of the run. In Run 4, when Lab-Lemco broth was added to the synthetic wastewater, TVC of biofilms and planktonic cells recovered to the control levels in about a week. In Runs 4 to 7, TVC of biofilms and planktonic cells were similar, suggesting that after an acclimatization period, introducing synthetic wastewater containing 6 ppm of IT to the RBC at various operating conditions did not affect the viability of bacterial cells in the biofilm, as long as the growth substrate was present in the wastewater.

3.1.3 Decreased susceptibility of acclimatized cells to biocide

Changes in the susceptibility of cells to the bactericidal activity of IT can be assessed by determination of their MLC. A higher MLC indicates lower susceptibility. The MLC of the non-acclimatized planktonic cells from the control RBC and the acclimatized planktonic cells from the treated RBC in Run 6 are shown in Table 5. The results showed that susceptibility of the nonacclimatized and acclimatized cells to IT in terms of MLC was similar, 10-12 ppm. They also imply that biodegradability of IT may not be totally related to the susceptibility of cells to the biocide. IT removal by the non-acclimatized biofilms in Run 1 and the acclimatized biofilms in Run 6 was significantly different with values of 0.90 ± 1.62% and 45.76 ± 5.01%, respectively (Table 3), even though the MLC of IT to the planktonic cells detached from those biofilms was similar, 10-12 ppm.

3.1.4 Tentative identification of acclimatized cells

There were many distinct colonies from the non-acclimatized biofilm of the control RBC. The isolates were tentatively identified as *Burkholderia cepacia*, *Sphingomonas paucimobilis*, *Chryseobacterium indologenes, Aeromonas hydrophila* and *Aeromonas salmonicida*. Less bacterial diversity was seen in RBCs receiving wastewater containing IT. At the end of the experiments in Run 2, bacterial cells from the biofilms were isolated. All isolates were oxidase positive Gram-negative rods. The bacterium most resistant to IT was tentatively identified as *Burkhol. cepacia*. This microorganism is in a group of catalase-producing, non-lactose fermenting Gram-negative bacteria. Similar results were reported by Bae and Rittmann [26] who observed that the most prevalent species in a continuous-flow reactor containing mixed phenolic compounds were *Burkhol. cepacia* and *Pseudomonas testosteroni*. Chavan and Mukherji [27] also reported that microorganisms in RBC biofilms for treatment of hydrocarbon-rich wastewater were *Burkhol. cepacia* and cyanobacteria (*Phormidium*, *Oscillatoria* and *Chroococcus*).

3.2 Capability in Biofilm Formation in the Presence of 3 ppm of IT

Biofilms on the RBC discs in all previous experiments were established by feeding normal synthetic wastewater to the units, which were then

Table 5. MLC of IT on the non-acclimatized planktonic cells and acclimatized planktonic cells.

Inoculum	MLC [a] (ppm)	Tested cell concentration [a] (cfu/mL)
non-acclimatized planktonic cells-1	12	3.90×10^6
non-acclimatized planktonic cells-2	10	1.96×10^6
acclimatized planktonic cells	10	5.40×10^5

[a] The values are expressed as mean ± SD of triplicate experiments.

treated with biocide. The aim of this experiment was to investigate the capability of microorganisms (non-acclimatized planktonic cells) under a sublethal IT concentration (3 ppm) to form a biofilm on the discs. This was done to simulate biofilm formation under sub-lethal concentrations of IT. It also enabled investigation of treatment efficiency of the units and the biocide efficacy against viable populations of biofilm and planktonic cells under biocide stress. The effects of HRT on all measured parameters were also observed.

3.2.1 Visual observation of biofilm formation

Biofilm formation was observed after two days in the RBCs receiving wastewater containing 3 ppm of IT at 1.25 and 2.5 L/h, which was similar to the control unit receiving no biocide.

3.2.2 COD and biocide removal by the RBCs

Synthetic wastewater containing 3 ppm of IT was applied to the RBC units at flow rates of 1.25 and 2.5 L/h (corresponding to the HRTs of 72 and 36 min, respectively). COD and IT concentrations in the influent and effluent wastewater were determined beginning on the second day of the experiment. Figures 4 and 5 show that the degree of COD removal and biocide degradation depended on the acclimation period. COD removal in the unit receiving 3 ppm of IT under both HRTs was initially quite low, approximately 20% removal. With four days of acclimatization, COD removal had significantly increased to 50.42 ± 0.84% (59.00 ± 1.15 mg/L) after ten days in the RBCs receiving biocide at a low a HRT. At high HRTs, COD removal reached steady state on day 15 with the values of 77.27 ± 0% (136.00 ± 0 mg/L), comparable to the control (Figure 6).

Figures 5a and 5b show biocide removal at high and low HRTs, respectively. The biocide removal after two days was slight, but then it sharply increased. The average effluent IT concentration at flow rates of 1.25 and 2.5 L/h was 0.89 ± 0.07 and 0.69 ± 0.03 ppm, respectively. The degree of IT removal at flow rates of 1.25 and 2.5 L/h was similar, approximately 73.69 ± 2.25 and 76.39 ± 1.84%, respectively.

The degree of COD removal at the longer HRTs was higher than that at the low HRTs, while the degree of IT removal at high and low HRTs was similar. A possible reason for this is that the biocide was not completely decomposed to CO_2 at low HRTs. Rather, it was converted to intermediates undetectable by IT determination, but it was detected as COD.

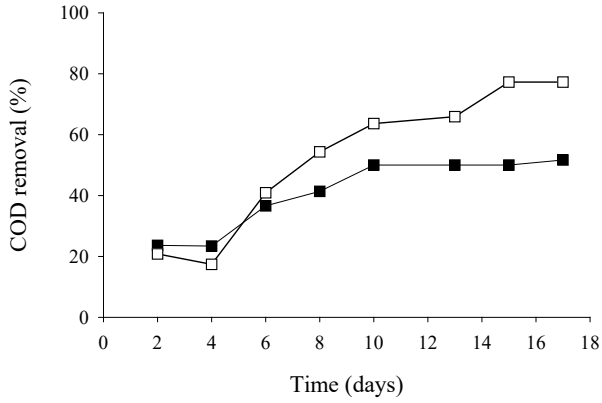

Figure 4. Effect of IT on COD removal in the RBCs: 3 ppm of IT at a flow rate of 1.25 L/h (□) and 3 ppm of IT at a flow rate of 2.5 L/h (■).

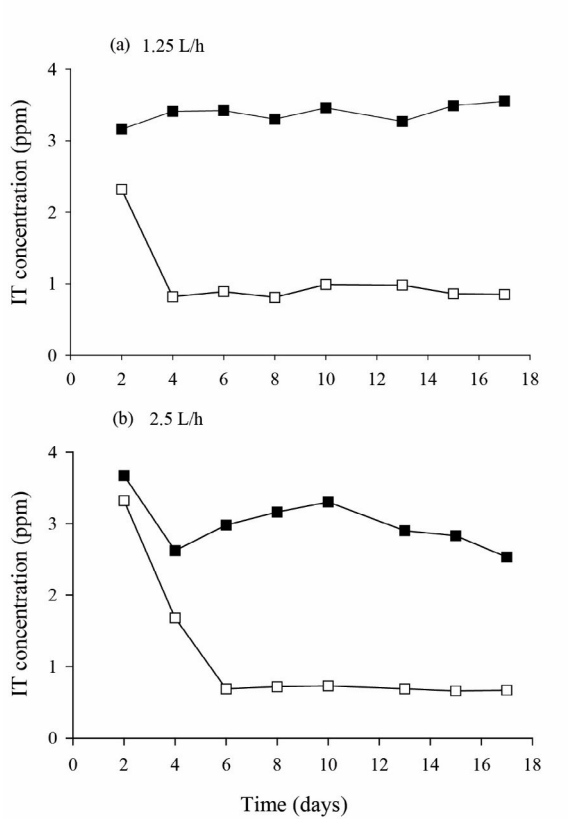

Figure 5. IT removal in the RBCs receiving the synthetic wastewater containing 3 ppm of IT at flow rates of 1.25 L/h (a) and 2.5 L/h (b): influent (■) and effluent (□).

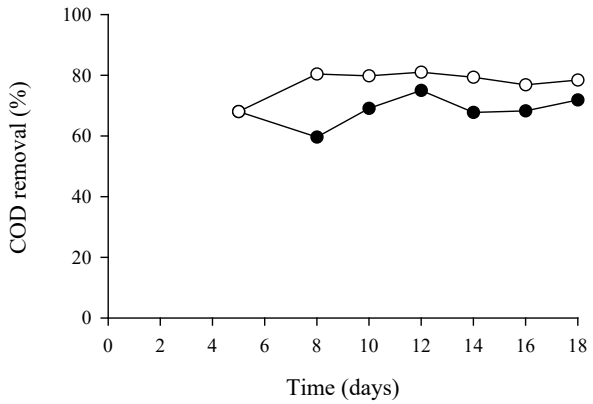

Figure 6. COD removal in the control RBCs receiving the normal synthetic wastewater at flow rates of 1.25 (○) and 2.5 L/h (●).

3.2.3 Viable populations

The viable microorganisms in the RBC receiving the wastewater containing 3 ppm of IT at 1.25 and 2.5 L/h were determined after five days. The average viable populations are shown in Table 6. The results indicate that IT had no adverse effects on the viability of the biofilms and apparent planktonic bacteria at IT concentrations of up to 3 ppm after a period of acclimation. The TVCs of the biofilms on the discs and the planktonic cells of the treated RBCs were similar to those of the control RBC, approximately 10^8 cfu/cm^2 and 10^7 to 10^8 cfu/mL, respectively.

3.3 BOD_{20}

Use of the BOD_5 test is not appropriate in this work. When the acclimatized cells at 0.5% were used as seed, the BOD_5 of the synthetic wastewater without IT was 107 mg/L, while the BOD_5 of the wastewater containing 6 ppm of IT was only 30 mg/L (the IT concentration in the BOD bottle was 0.3 ppm). Longer periods of incubation before DO measurements, as well as increased seed volume might lead to reduced bio-oxidation inhibition.

When acclimatized cells were used as a seed at 0.5 and 3.5% for BOD measurement of the synthetic wastewater without biocide, an incubation time at five days was sufficient to reach the maximum BOD values (Figure 7). Longer incubation caused slightly lower BODs values. The BOD values obtained using 3.5% acclimatized cells were lower than those using 0.5% acclimatized cells. This might have been due to the effects of high biocide concentrations in the acclimatized cells. As the acclimatized cell suspension contained approximately 3 ppm of IT, the IT concentration in the BOD bottles using 0.5 and 3.5% seed would be 0.015 and 0.105 ppm, respectively. The results also imply that IT at 0.015 ppm did not cause any adverse effect on biooxidation during the BOD test, while 0.105 ppm caused approximately a 20% reduction in the BOD value of the synthetic wastewater.

When the acclimatized cells were used as a seed at 0.5 and 3.5% levels for BOD tests of the synthetic wastewater containing 6 ppm of IT, BOD values increased with incubation time (Figure 7). The results indicated that a higher volume of seed (3.5%) resulted in lower BOD_5 and BOD_{10} values, as previously discussed. BOD_{20} using 3.5% acclimatized cells was higher than that using 0.5% acclimatized cells, indicating that seed volume and incubation time markedly affected the BOD value of the wastewater contaminated with the biocide. As the sample was diluted by 20-fold prior to the BOD test, the concentration of IT in the BOD bottle was only 0.3 ppm. Nevertheless this was sufficient to decrease the metabolic activity of the planktonic bacteria present in the BOD_{20} test by 63 and 44%, when acclimatized cells at 0.5 and 3.5% levels were used, respectively (compared to the BOD_5 of the control sample).

Table 6. Viability of biofilms and planktonic cells in the control RBC and the RBCs receiving the wastewater containing 3 ppm of IT at 1.25 and 2.5 L/h.

Viability	Control RBC		Treated RBC	
	1.25 L/h	2.5 L/h	1.25 L/h	2.5 L/h
TVC of biofilms (log cfu/cm^2)	7.82 ± 0.53	8.10 ± 0.57	8.50 ± 0.22	7.96 ± 0.47
TVC of planktonic cells (log cfu/mL)	7.53 ± 0.60	7.12 ± 0.44	7.21 ± 0.44	7.83 ± 0.10

: The results were expressed as mean and ± SD during steady state for at least 4 values of the TVCs.

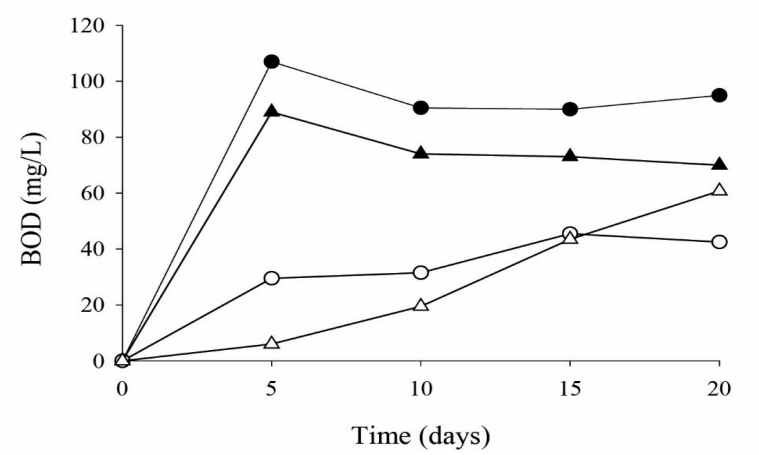

Figure 7. Effect of IT on BOD_{20}: influent seed 0.5% control (●), influent seed 0.5% control + 6 ppm of IT (○), influent seed 3.5% control (▲) and influent seed 3.5% control + 6 ppm of IT (Δ).

The results showed that acclimatized cells or adapted seed and longer incubation time led to increased BOD values or reduce the inhibitory effect on bio-oxidation to some extent. The results implied that IT severely affected biooxidation. BOD tests were not useful for investigating the efficiency of wastewater treatment units contaminated with IT, even using adapted seed and/or a prolonged incubation time.

4. Conclusions

RBC units were used to simulate wastewater treatment and predict the level of COD removal and IT degradation. Degradation of IT under aerobic conditions occurred via co-metabolism using Lab-Lemco broth as a growth substrate. Acclimatization under biocide stress, the presence of growth substrates and operating conditions (OLR, HLR and HRT) were important parameters that promoted treatment efficiency of the system. After acclimatization with an OLR, from Lab-Lemco broth, of 17 to 20 g $COD/m^2.d$, ~73 to 77% and 46% of the 6 ppm of IT was removed at HRTs of 72 and 36 min, respectively. Higher biocide removal was obtained when the OLR of the growth substrate was increased. As biofilms could be established under sub-lethal IT concentrations after an acclimatization period, industrial application of biocides can be considered.

Acknowledgements

This research was funded by the National Research Council of Thailand (NRTC), the Post-doctoral Program from Research Affairs and Graduate School, Khon Kaen University (KKU) (Grant No. 59153), Thailand. The authors wish to thank Phoenix Pulp & Paper Public Company Ltd., Khon Kaen, Thailand for providing the recycled sludge and Ondeo Nalco (Thailand) for providing the biocide.

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