

Remediation of the effect of adding cyanides on an algal/bacterial treatment of a mixture of organic pollutants in a continuous photobioreactor

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Abstract The effect of inorganic pollutants on the treatment of organic pollutants using algal/bacterial microcosm was investigated in a continuous photobioreactor. The microcosm was composed of *Chlorella vulgaris* MM1 and *Pseudomonas* MT1 and was able to efficiently treat artificial waste-water contaminated with 6.4 salicylate and 2.2 mM phenol at a hydraulic retention time of 4 days. No negative effect was recorded when the waste-water was supplemented with 1.6 mM thiocyanate; however, the treatment efficiency severely deteriorated when the system was challenged with 0.74 mM cyanide. Addition of 2 g $\text{NaHCO}_3 \text{ l}^{-1}$ did not improve the efficiency of the treatment. Toxicity of the pollutants to the alga was cyanide > thiocyanate > phenol > salicylate. The high toxicity of the waste-water was eliminated either by a 25-fold dilution or by photocatalytic pre-treatment which allowed the subsequent efficient biological treatment.

Keywords Chlorella · Cyanide · Microcosm · Photosynthesis · Photocatalytic pretreatment · Phenol · Pseudomonas · Salicylate · Thiocyanate

Introduction

Algal/bacterial culture biotechnology as a promising strategy for the treatment of waste-waters (Essam 2006; Essam et al. 2013). Under illuminated conditions, algae produce O_2 by photosynthesis, which is required by the bacteria to mineralize organic matter. This strategy helps to avoid the high risk of volatilization of aromatic pollutants, reduce the relative high cost of the mechanical aeration and mitigate the amount of CO_2 released by the bacteria thereby preventing greenhouse gas emission (Muñoz and Guieysse 2006; Essam et al. 2013). Most of the studies, however, focused on the treatment of organic pollutants in waste-waters (Muñoz and Guieysse 2006). Moreover, many of these studies were conducted using organic pollutants on individual basis (Muñoz and Guieysse 2006) and very few were conducted on a mixture of organic pollutants (Essam et al. 2007, 2013). Indeed, industrial discharges usually contain complex mixtures of organic and inorganic pollutants (Essam 2006) where the organic load represents the major problem. The inorganic load, though, may considerably contribute to the waste-water toxicity consequently in the global environmental problem (Essam 2006; Dash et al. 2009).

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In these regards, cyanides represent an interesting model of pollutants as they can be found in a wide variety of organic and inorganic compounds (Dash et al. 2009; Manar et al. 2011). Both forms of cyanides (organic and inorganic) have been detected in waste-waters where the common forms of organic cyanides are the nitriles (e.g. acetonitrile, propionitrile, etc.) (Muñoz et al. 2005) while common inorganic cyanides are cyanide salts and thiocyanate (Dash et al. 2009; Manar et al. 2011). Cyanides may enter surface water or appear as contaminants in waste-waters through various industries including metal cleaning, metal processing, steel tempering, mining, pharmaceuticals, coal coking, ore leaching, plastics, etc. (Luque-Almagro et al. 2005; Dash et al. 2009). However, inorganic forms of cyanides are much more toxic than organic ones and free cyanide is the most toxic form of all cyanides [Dash et al. 2009].

The present study was conducted to investigate and optimize the algal/bacterial biodegradation of artificial waste-waters with increasing load and number of organic and inorganic pollutants in a continuous photobioreactor dynamic system consisting of a phenol degrading bacterial strain (*Pseudomonas* MT1) and a microalgal strain (*Chlorella vulgaris* MM1). The effect of the combined toxicity of the pollutants mixtures was studied and the impact of the addition of two forms of cyanide (thiocyanate and/or free cyanide) was investigated and the most toxic pollutant was identified. Possible solutions to avoid waste-water toxicity fluctuation were investigated and evaluated.

Materials and methods

Unless otherwise specified, all tests were conducted under aseptic conditions and in triplicate.

Microorganisms

Pseudomonas strain MT1 (Genbank accession number JQ178342) and a microalgal strain, morphologically characterized as *Chlorella vulgaris* MM1, were isolated from soil and water samples respectively, collected previously from Cairo, Egypt (ElRakaiby et al. 2012). The bacterial and algal strains were cultivated and maintained in metal salt medium (MSM) according to Essam et al. (2010) and El-Rakaiby et al. (2012).

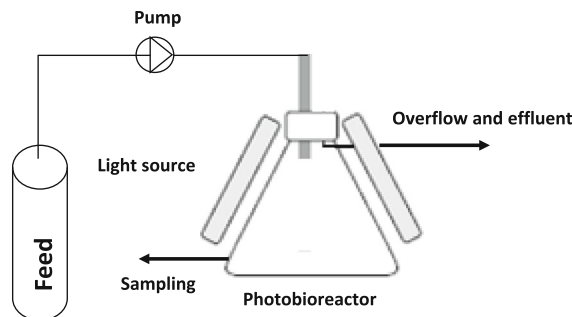


Fig. 1 Schematic set up of the experimental algal—bacterial photobioreactor

Establishment of the photobioreactor

The dynamic system was established using a 1,100 ml conical glass photobioreactor (PBR) in a continuous stirred tank reactor “CSTR” mode according to Essam et al. 2006 and Essam et al. 2013, with minor modifications (Fig. 1). Illumination was provided to the CSTR by three fluorescent lamps (Gelia E27, 36 W) placed in a parallel configuration around the reactor (illumination intensity of 5,000 lux at the reactor surface). The PBR was initially filled with the artificial waste-water (composed of MSM, supplemented with $1,000 \text{ mg salicylate l}^{-1}$) and inoculated with 10 % (v/v) of the algal/bacterial consortium in a ratio of 5:1 according to Guieysse et al. (2002).

Once microbial growth was established, the waste stream was supplied to the reactor in a continuous mode using a peristaltic pump (Watson Marlow, 101U, England) at a HRT of 4 days. The PBR was challenged by increasing the influent load in terms of increasing the number of the pollutants and the introduced chemical oxygen demand (COD) (see Table 1 below) at constant environmental conditions; room temperature ($25 \pm 2 \text{ }^\circ\text{C}$), continuous stirring (200 rpm) and HRT of 4 days. When the toxicity level of the artificial waste-water severely inhibited algal/bacterial growth and the treatment efficiency significantly deteriorated, the artificial waste-water was fed with $2 \text{ g NaHCO}_3 \text{ l}^{-1}$ (Table 1). Samples, 50 ml, were periodically taken from the reactor outlet for analysis of phenol, salicylate, thiocyanate, cyanide, COD, pH, chlorophyll-*a* content, OD_{600} , $\text{CO}_2/\text{CO}_3^{-2}$ and phytotoxicity.

Temperature and dissolved O_2 (DO) were measured using a DO meter coupled with a temperature sensor.

Table 1 A summary of the conducted sets of experiments on the photobioreactor and the corresponding recorded treatment and detoxification efficiencies where the PBR was inoculated with 10 % v/v of algal-bacterial microcosm and operated at room temperature (25 ± 2 °C), continuous illumination (5,000 lux), agitation (200 rpm) and HRT (4 days)

Set	n ^a	Pollutants concentration (mM)			COD (mg l ⁻¹)	COD removal %	Pollutant removal %				Phytotoxicity X2 ^d
		Salicylate	Phenol	SCN ^b			Salicylate	Phenol	SCN ⁻	CN ⁻	
#1	10	6.4 ± 0.11	0 ± 0	0 ± 0	1,460 ± 26	79 ± 2	100 ± 0	NA	NA	NA	0 ± 0
#2	13	6.4 ± 0.13	2.2 ± 0.11	0 ± 0	2,030 ± 31	82 ± 1	100 ± 0	100 ± 0	NA	NA	0 ± 0
#3	12	6.3 ± 0.1	2.2 ± 0.15	1.1 ± 0.1	2,150 ± 29	78 ± 1	100 ± 0	100 ± 0	100 ± 0	NA	0 ± 0
#4	14	6.4 ± 0.15	2.2 ± 0.11	1.6 ± 0.1	2,217 ± 35	73 ± 3	100 ± 0	97 ± 1	95 ± 3	NA	0 ± 0
#5	12	6.6 ± 0.1	2.2 ± 0.14	1.6 ± 0.12	2,306 ± 20	22 ± 2	20 ± 0	12 ± 1	11 ± 1	8 ± 0	100 ± 0
#6 ^c	12	6.2 ± 0.12	2.1 ± 0.1	1.6 ± 0.1	2,309 ± 40	19 ± 1	21 ± 1	11 ± 1	14 ± 0	9 ± 1	100 ± 0
UV-TiO ₂	-	6.4 ± 0.1	2.2 ± 0.1	1.6 ± 0.1	2,378 ± 42	22 ± 1	32 ± 1	34 ± 1	58 ± 1	100 ± 0	78 ± 3
#7 ^g	8	4.4 ± 0.1 ^h	1.4 ± 0.1 ^h	0.7 ± 0.05 ^h	1,865 ± 27	88 ± 2	100 ± 0	100 ± 0	100 ± 0	100 ± 0	0 ± 0

The presented data is the mean ± standard deviation

^a The number of samples used to calculate the average of the recorded parameters

^b Thiocyanate concentration

^c Cyanide concentration

^d The attempted dilution factor

^e The feed of the PBR was supplemented with 2 g NaHCO₃ l⁻¹ under all previously listed conditions

^f The artificial wastewater was pretreated photocatalytically for 36 h with UV irradiation in presence of 1 g TiO₂ subsequently introduced into the PBR l⁻¹

^g The feed of the PBR was photocatalytically pretreated for 36 h

^h The recorded remaining concentrations of the pollutants after the photocatalytic pretreatment

Each time a parameter was changed, the system was tested for a period of at least 3–4 HRT before a new change was made.

Photocatalytic pre-treatment

Photocatalytic pre-treatment was conducted on the simulated waste-water supplemented with the pollutants mixture and 1 g TiO₂ l⁻¹ (anatase, nanotubes 5–15 nm). Wastewater was sonicated for 10 min to obtain a homogenous suspension and then divided into portions of 40 ml. Each portion was transferred into screw-capped glass tubes (25 × 20 cm) that were mechanically agitated and irradiated at 1.8 × 10⁻⁵ Einstein s⁻¹ using three 30 W UV blue-lamps (Sylvania Reptistar, Sylvania, USA, with up to 30 UVA–5 % UVB placed 15 cm away from the tubes). Samples of 1 ml were periodically withdrawn from three randomly selected test-tubes to monitor the concentration of the remaining pollutants and preserved at 4 °C prior to analysis. The liquid fractions from each set of the experiment were collected and mixed after 36 h of irradiation following the removal of TiO₂ by centrifuging the tubes at 1,400×g for 15 min. This experiment was repeated in order to collect a sufficient volume from which samples were withdrawn for COD, phytotoxicity, and algal toxicity analyses. The collected photocatalytically-treated waste-water was then subjected to biological treatment.

Algal toxicity assay

The algal toxicity of all the tested pollutants at different concentrations was estimated using a monoculture of *Chlorella vulgaris* MM1 and according to ElRakaiby et al. (2012). Algal toxicity was calculated as the reduction % in the chlorophyll-*a* content in the test sample (with pollutants) compared to that of blank (without pollutants). The artificial waste-water with the highest recorded toxicity to the dynamic algal/bacterial system was subjected to several folds of dilutions and the algal toxicity of these dilutions was similarly estimated.

Analysis

Analysis of phenol and salicylate was conducted by HPLC using an LC-18 column according to Muñoz

et al. (2004) and ElRakaiby et al. (2012). Cyanide and thiocyanate were measured colorimetrically according to Luque-Almagro et al. (2005) and APHA (2005), respectively. Cell density was estimated from the OD₆₀₀ value. Chlorophyll-*a* content was measured according to Chen et al. (2011) and the COD was measured according to Essam et al. (2007). The remaining CO₂/CO₃²⁻ were measured by acid–base titration using 0.1 M HCl and Methyl Orange as indicator. Phytotoxicity was conducted according to ElRakaiby et al. (2012) using seeds of *Lepidium sativum*. Potential outliers were identified and rejected using the Grubb's test at the 5 % significant level.

Results

Initially, the algal/bacterial microcosm completely removed and detoxified 6.4 mM salicylate at a HRT of 4 days. This was accompanied by a removal of 79 % of the introduced COD (Table 1). The addition of 2.2 mM phenol to the feed did not affect the treatment or the detoxification efficiencies (Table 1). Furthermore, the algal/bacterial microcosm completely detoxified a mixture of three pollutants; two organic compounds (salicylate and phenol) and one inorganic compound (1.1 mM thiocyanate) under the same HRT (4 days). When the concentration of thiocyanate increased to 1.6 mM, the treatment efficiency slightly declined where 100, 97 and 95 % removal of salicylate, phenol and thiocyanate, respectively, were recorded (Table 1). Under these conditions, the COD removal efficiency slightly decreased to 73 %; however, complete effluent detoxification was still observed.

When the feed was supplemented with the fourth pollutant (0.74 mM cyanide), the treatment and detoxification efficiencies of the system severely deteriorated and all pollutants accumulated (Table 1). A COD removal of 22 % was recorded. In addition, the effluent showed complete inhibition to germination of seeds (complete phytotoxicity). Addition to this artificial waste-water of 24 mM NaHCO₃ neither improved the treatment nor the detoxification efficiencies (Table 1).

The treatment and detoxification efficiencies of the photobioreactor were monitored by analyzing several parameters. First, the COD of the effluent was measured and recorded. Increasing the number of the

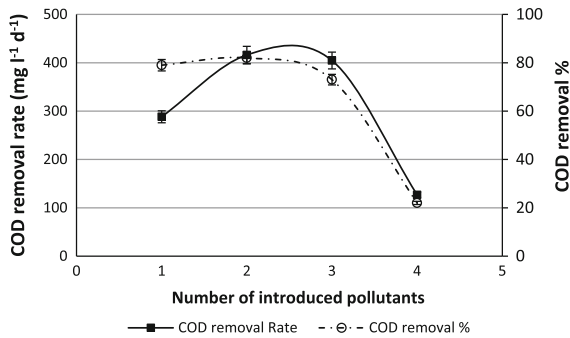


Fig. 2 COD removal % (closed squares) and COD removal rate (open circles) in the photobioreactor inoculated with the algal-bacterial microcosm, supplied with simulated waste-water stream containing increasing number of pollutants and operated at room temperature (25 ± 2 °C), continuous illumination (5,000 lux), agitation (200 rpm) and HRT (4 days)

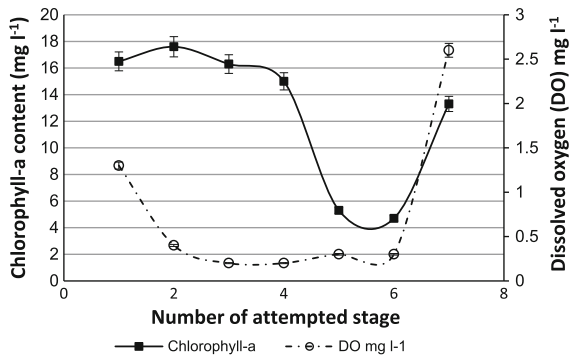


Fig. 3 Chlorophyll-*a* content (closed squares) and dissolved oxygen (open circles) in the PBR, inoculated with the algal-bacterial microcosm through different sets of experiments where the PBR was continually supplied with simulated waste-water stream supplemented with an increasing number of pollutants and COD influent load, operated at room temperature (25 ± 2 °C), continuous illumination (5,000 lux), agitation (200 rpm) and HRT (4 days)

introduced pollutants to two (salicylate and phenol) increased the COD load (Table 1) which was accompanied by an increase in the COD removal rate (Fig. 2). Although the COD removal declined slightly (73 %) when the artificial waste-water was supplemented with three pollutants (salicylate, phenol and thiocyanate), the COD removal rate remained almost the same ($410 \text{ mg l}^{-1} \text{ day}^{-1}$). However, the addition of 0.74 mM cyanide greatly reduced the COD removal and its rate (Fig. 2).

Algal growth and O_2 generation were monitored by measuring the chlorophyll-*a* content and dissolved O_2

concentration (DO), respectively. When the PBR was supplied with the artificial waste-water containing only salicylate, the chlorophyll-*a* content and the DO concentration were around 16 and 1.3 mg l^{-1} , respectively. When the influent was additionally supplied with phenol, the chlorophyll-*a* content slightly increased while the DO concentration was reduced to below 0.5 mg l^{-1} (Fig. 3). The addition of thiocyanate (sets 3 and 4) resulted in a slight and gradual reduction in the chlorophyll-*a* content (Fig. 3). A severe reduction in the chlorophyll-*a* content was observed when cyanide was supplied to the artificial waste-water (set 5; Fig. 3). Addition of 24 mM NaHCO_3 neither improved the chlorophyll-*a* content nor the DO concentration (set 6 and Fig. 3).

The toxicity of the effluent was estimated by the algal toxicity assay. Cyanide was highly toxic to the alga where complete inhibition was recorded at 0.15 mM (Fig. 4a). However, thiocyanate was less toxic since algal growth started to decline at 1.2 mM while complete inhibition was recorded at 3.7 mM (Fig. 4b). Phenol showed a similar toxicity pattern to thiocyanate at lower concentrations up to 2.2 mM (Fig. 4b and c). However, at higher concentrations, phenol showed milder toxicity (Fig. 4c). The lowest toxicity was recorded to salicylate where a relatively high concentration (12.5 mM) was required to completely inhibit algal growth (Fig. 4d).

To overcome the high algal toxicity of the pollutants-loaded waste-water (6.4, 2.2, 1.6 and 0.74 mM salicylate, phenol, thiocyanate and cyanide, respectively), the artificial waste-water was diluted. Dilutions up to 10-fold did not reduce the algal toxicity; however, 15-fold dilution reduced the toxicity to 60 % (Fig. 5). Dilutions ≥ 20 -fold considerably reduced the algal toxicity to less than 20 % and no algal toxicity was recorded when the artificial waste-water was diluted by 50-fold (Fig. 5). When the 20 -old diluted artificial waste-water was introduced as influent, the PBR achieved complete pollutants removal, detoxification and up to 86 % COD removal.

Artificial waste-water was also treated by UV irradiation, where there was no significant difference in the pollutants concentrations after up to 96 h (data not shown). Comparatively, photocatalytic pre-treatment of the artificial waste-water for 24 h using nanotubes of TiO_2 removed 12 % of the COD. However, the algal toxicity assay showed that the pre-treated waste-water still retained significant

Fig. 4 Algal toxicity calculated as reduction % in the chlorophyll-*a* content in 12 ml tubes, supplemented with MSM containing 24 mM NaHCO₃ and increasing concentrations of the tested pollutants: cyanide (a), thiocyanate (b), phenol (c) and salicylate (d) where the tubes were inoculated with 5 % v/v algae and incubated at room temperature (25 ± 2 °C), continuous illumination (5,000 lux) and agitation (200 rpm)

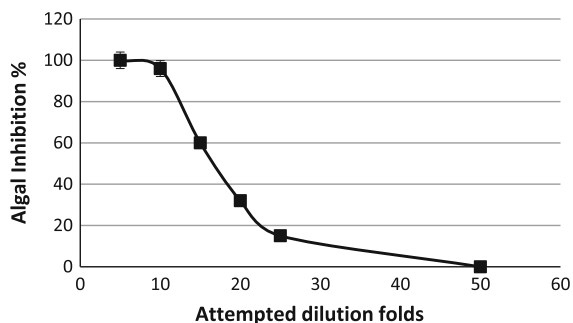
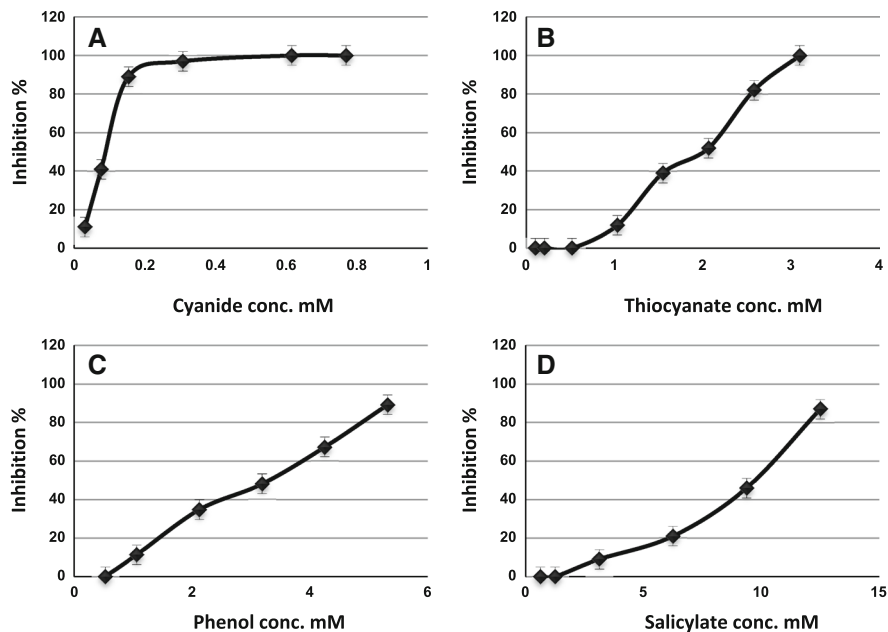


Fig. 5 Algal toxicity calculated as reduction % in the chlorophyll-*a* content in 12 ml tubes, supplemented with different dilution folds of artificial waste-water with MSM and supplemented with 24 mM NaHCO₃ where the tubes were inoculated with 5 % v/v algae and incubated at room temperature (25 ± 2 °C), continuous illumination (5,000 lux) and agitation (200 rpm)

toxicity (data not shown). Photocatalysis for 36 h completely removed cyanide in addition to 58, 34 and 32 % of the thiocyanate, phenol and salicylate loads, respectively (Table 1). This photocatalytically-treated waste-water was then introduced into the PBR under the same conditions of operation. The PBR effluent showed 88 % removal of the introduced COD load with complete removal and detoxification of all the introduced organic and inorganic pollutants (Table 1).

Contrasting the theoretical oxygen demand (ThOD) calculations with the experimentally estimated chemical

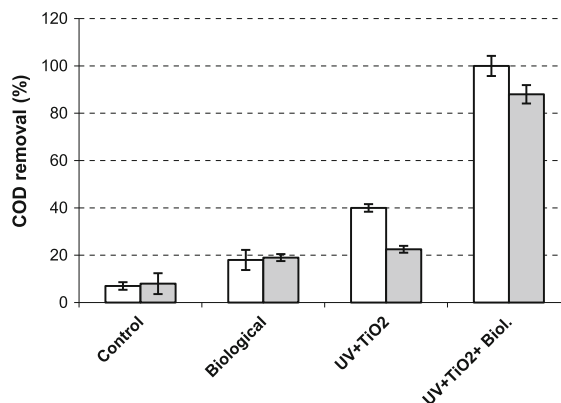


Fig. 6 COD removal % (grey bars) compared to the theoretical COD (ThCOD) removal % (white bars) of artificial waste-water consisting of MSM supplemented with 6.4, 2.2, 1.6 and 0.75 mM of salicylate, phenol, SCN and CN; and enriched with 24 mM NaHCO₃. This artificial waste-water was treated biologically in the PBR, photocatalytically (UV+TiO₂) or sequentially (photocatalytic followed by biological treatment in the PBR). The PBR was operated at room temperature (25 ± 2 °C), continuous illumination (5,000 lux), agitation (200 rpm). ThCOD was calculated from pollutant concentration multiplied by the practically or theoretically assigned equivalent ThCOD (1.46, 2.38, 1.13 and 9.75 for salicylate, phenol, SCN and CN, respectively). The control was the untreated artificial waste-water

oxygen demand (COD), there is no significant difference in the COD removal % values in case of the control and the biological treatment (Fig. 6). On the contrary, in case

of the photocatalytic treatment, the ThOD calculated was 40 % whereas the COD measured was ≈ 22 %. Similarly, in case of the sequential treatment (photocatalytic followed by biological treatment), the experimentally estimated COD was 12 % less than the theoretically calculated oxygen demand (Fig. 6).

Discussion

Initially, the algal/bacterial microcosm was efficiently established on a single organic pollutant (salicylate) within a PBR. When a second pollutant (phenol) was introduced, the microcosm retained its homogenous performance. A similar microcosm was able to efficiently treat and detoxify a simulated waste-water loaded with mixtures of phenol and pyridine up to 4.6 and 4.4 mM, respectively, at a HRT of 2.7 days (Essam et al. 2013).

Although in a previous study (Essam et al. 2013), as well as in the current study, the established microcosm was able to treat and detoxify a mixture of organic pollutants, real waste-waters are usually heterogeneous and contaminated with organic and inorganic substances of various molecular weights (Bitton 2010; Huang et al. 2010). Moreover, the inorganic portion of waste-water has been reported to cause severe problems to the efficiency of the biological treatment (Mara and Horan 2003). Therefore, it was vital that the algal/bacterial based biological treatment of a mixture of organic and inorganic pollutants be attempted and optimized.

Starting with a mixture of two organic pollutants, the PBR efficiently treated and detoxified the influent loaded at a rate of $\approx 500 \text{ mg l}^{-1} \text{ d}^{-1}$ with a COD removal and rate up to 80 % and $400 \text{ mg l}^{-1} \text{ d}^{-1}$, respectively. These results were homogenous and consistent with those reported previously by the same algal/bacterial microcosm for the treatment of an influent of combined organic pollutants of phenol and pyridine (Essam et al. 2013). Although the addition of a third pollutant (inorganic thiocyanate at 1.1 mM) had no significant effect on its PBR, increasing its concentration to 1.6 mM had slight negative impact on the treatment efficiency. Again, the addition of the fourth pollutant (0.75 mM cyanide) caused a severe deterioration of the PBR treatment efficiency. Muñoz (2005), has reported that increasing the concentration of the pollutant(s) or the presence of a highly toxic

pollutant may have a negative effect at a certain threshold beyond which a complete collapse of the system may occur.

Previous studies (Essam et al. 2006, 2013) have reported that fertilization of the PBR with NaHCO_3 was a good tool to restore the biodegradation and detoxification efficiencies. Unfortunately, in the present study, the addition of 24 mM NaHCO_3 had no effect which indicated that a severe inhibition occurred to the microcosm, especially to the algae (ElRakaiby et al. 2012). This conclusion was confirmed in the current study through the monitored DO and the chlorophyll-*a* content.

The algal toxicity assay of the four pollutants on individual basis revealed that the toxicity was in the following order; cyanide > thiocyanate > phenol > salicylate. This order is in agreement with the work of Dash et al. (2009) and Manar et al. (2011), who reported that inorganic cyanide is the most toxic form of cyanides. Yet, cyanide is more toxic than phenol where the cyanide had an EC_{50} –72 h of 0.017 mM on the microalgae (Manar et al. 2011) compared to an EC_{50} –96 h of 4 mM for phenol (Essam et al. 2007). Borde et al. (2003), Essam et al. (2006) and Muñoz et al. (2009) have reported that phenol was much more toxic than salicylate. Hence, the failure of the treatment efficiency was attributed to the high toxicity of the cyanide portion introduced to the influent.

Dilution can be applied to decrease toxicity and allow the bioremediation of highly toxic effluents (Aggelis et al. 2003; Muñoz and Guieysse 2006). In the current study, 20- and 25-fold dilutions with free MSM reduced the effluent toxicity and allowed the algal/bacterial degradation. Interestingly, this is in agreement with the algal toxicity assay where the algae had inhibition cut off of 0.075 mM cyanide. However, the application of such dilution would prolong the treatment time or it could be limited by the unavailability of enough landscape (Muñoz 2005).

As an alternative, photochemical pretreatment was attempted to reduce the influent toxicity. Photolysis neither reduced the pollutants concentration nor the toxicity. Essam et al. (2007) reported that photolysis alone was an insufficient pretreatment to reduce the toxicity of highly contaminated influents. Therefore, UV/ TiO_2 photocatalysis was opted as a pretreatment process. Although this pretreatment completely removed the most toxic portion (cyanide) after only

36 h. it removed only 22 % of the introduced COD. Similarly, Essam et al. (2007), reported that photocatalytic pretreatment remove the most toxic pollutant (pentachlorophenol) in a mixture of four chlorophenols and allowed the subsequent biodegradation after 56 h irradiation. Hence, the use of nano-tubes of TiO₂ shortened the required time for photocatalytic degradation and consequently would reduce the cost (He and Chen 2012).

Interestingly, although photocatalytic pretreatment should theoretically remove 40 % of the COD, only 22 % were removed. This indicated the formation of photoproducts; however, these products were less toxic than the parent compounds (Essam et al. 2007). Again, although, sequential photocatalytic-biological treatment completely detoxified the artificial wastewater, it did not achieve complete removal of the COD. This may be attributed to the produced microbial byproducts such as extracellular enzymes (Mazotto et al. 2011). Thus, the sequential photocatalytic-biological treatment of this organic/inorganic polluted waste-water was proven applicable and cost effective provided it would be further optimized.

Conclusion

Inorganic pollutants, such as cyanides, have a negative impact on the biological treatment of highly contaminated influents. Photocatalytic pretreatment selectively removed cyanide and allowed the subsequent algal/bacterial treatment. Hence, physical or photochemical pre-treatment may provide feasible solutions to reduce the influent toxicity and allow the cost-effective, eco-friendly biological treatment.

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