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Comparative study of chemical and physical methods for distinguishing between passive and metabolically active mechanisms of water contaminant removal by biofilms

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ABSTRACT

In this study, physical and chemical approaches were employed to distinguish between passive and active mechanisms in biofilms removing contaminants in waste waters and their relative merits were assessed. Respiration, post-exposure recovery and scanning electron microscopic analysis demonstrated that both ultraviolet (UV) treatment (300 mJ/cm^2) and sodium azide (10 mM) completely inhibited metabolic activity at 5 and 24 h exposure, respectively, whilst not damaging the integrity of the biofilms. Amongst the commonly used chemical inhibitors, only sodium azide showed complete inhibition after 24 h incubation with only about 10% (±4%) of biofilm carbon released into the bulk solution, compared to 33–41% (±8%) when exposed to 5 mM and 10 mM 2,4-dinitrophenol (DNP) and 69–80% (±5%) when exposed to 2% and 5% w/v formalin, respectively. Biofilm inhibition with UV and sodium azide was found to be equally effective at inhibiting biofilms for treatment of triethanolamine (TEA) and benzotriazole (BTA): the results confirming that the dominant removal mechanism was biodegradation. However, the rates of glucose removal by sodium azide-inhibited biofilms were similar to controls, suggesting that chemical inhibitors were not effective for distinguishing the removal mechanisms of simple sugars. Statistically similar amounts of metal were removed by biofilms treated with UV and sodium azide in zinc, copper and cadmium single-systems: the results indicated that the removal mechanism is predominantly a passive biosorption process.

1. Introduction

Microorganisms remove organic pollutants from water through biosorption and active biodegradation while simultaneously accumulating heavy metals intracellularly or immobilising them on the cell surface (Malik, 2004; Aksu, 2005; Vijayaraghavan and Yun, 2008). Researchers have compared removal of pollutants by live and metabolically inactivated biomass, with a view to differentiate between mechanisms of removal and to quantify the extent each contributes to the total pollutant removal from water (Doshi et al., 2007; Johnson et al., 2007; Klein and Kennedy, 1997).

To date, thermal treatments through techniques such as autoclaving have been the most common means of inactivating biomass for physicochemical sorption measurements (Tsezos and Bell, 1989; Wang and Grady, 1994; Das and Guha, 2009). Wang and Grady (1994) demonstrated that the sorption of both viable and metabolically inactivated biomass were identical, after taking into consideration the loss of biomass during autoclaving. Das and Guha (2009) reported greater chromium (VI) removal in live compared to thermally-inactivated biomass and attributed the difference to the partial loss of binding sites, and/or the intracellular accumulation of chromium by the viable cells. However, the results were difficult to interpret since exposure to high temperatures (>40^{O}C) affected cell adsorption capacity and morphology. In contrast, Boyer et al. (1998) investigated the metabolic removal of copper by *Thiobacillus ferrooxidans* at 5^{O}C. The lower temperature decreased biological metabolism, whilst not disrupting the integral architecture of the biofilms. However, reducing the temperature lowers the total kinetic energy of the system and alters the thermodynamic parameters, which affect the physicochemical removal rates and provides little indication of their potential in real-world applications (Delle Site, 2001).

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Chemical inhibitors are an alternative approach which have been widely used for distinguishing pollutant removal mechanisms (Klein and Kennedy, 1997; Johnson et al., 2007; Das and Guha, 2009). They quench the metabolic activity of microbes whilst avoiding cell damage or disruption of the biofilm structure. Formalin and sodium azide are among the most commonly employed. Formalin is known to dehydrate cells by inactivation of proteins, forming covalent crosslinks with several functional groups. Hence, employing formalin to inactivate bacteria alters the cell structure which can lead to misleading results. Sodium azide prevents electron transfer by inhibiting oxidative phosphorylation by binding to the terminal cytochromes of active bacteria (Johnson et al., 2007; Das and Guha, 2009). 2,4-dinitrophenol (DNP) and N,N′-dicyclohexylcarbodiimide (DCCD) are less commonly used as un-couplers of oxidative phosphorylation preventing ATP synthesis by inactivating ATP synthetase function (Incharoensakdi and Kitjaharn, 2002). Surprisingly, no study to date has investigated the effects of these metabolic inhibitors on the cell structure and the biomass functional groups, which play a key role in physico-chemical sorption of contaminants.

In this study, we compared physical ultraviolet (UV) inhibition with chemical (sodium azide) methods to distinguish and quantify the relative contributions of biosorption and metabolic removal of chemical contaminants in water. UV treatment is a proven, environmentally friendly technology used for effluent disinfection in wastewater systems (Azimi et al., 2014). With this, UV light is absorbed by the nucleic acids (DNA and RNA) of exposed microbial cells, altering DNA structure and thus preventing metabolic activity and any further cell division. Both UV and sodium azide methods were applied to assess the removal of organics (triethanolamine and benzotriazole) and metals (zinc, copper and cadmium) respectively to: 1) compare UV-treatment and chemical inhibitors method for biofilm inhibition and 2) determine the relative contributions of the different mechanisms (biosorption vs. metabolic uptake) responsible for the individual removal of organics and metals.

2. Materials and methods

2.1. Description of bioreactor system

Suspensions of a mixed consortium bacteria were freeze-dried to ensure uniform inoculum was employed for each experiment. This consortium was obtained from an aged oil-based metal-working fluid (MWF) bioreactor adapted to treating Castrol CoolEdge BI. MWF was selected as a model effluent since 95% of it is water, with the remainder of the composition being organics and metals. There is growing need to develop sustainable methods for its end of life treatment (Van Der Gast et al., 2004). Before freeze-drying, the culture was centrifuged and the resulting cell pellet was resuspended in sterile *Mist dessicans*, which is 1:3 (v/v) solution of Luria-Bertani (LB) broth, with 10 g/L D-glucose anhydrous (Fisher Scientific), in horse serum. Two mL of this was then dispensed into sterile freeze-drying vial, frozen at −80°C overnight, and freeze-dried for 24 h at −55°C.

Two mL of phosphate buffered saline (PBS) solution was used to resuspend a freeze-dried inoculum before it was used. The biofilms were grown using two vials of the freeze-dried stocks in a 4 L bioreactor with 800 mL autoclaved M9 minimal medium and 1% v/v fresh MWF (CoolEdge BI concentrate provided by Castrol) at 25°C with continuous air flow over 3 weeks. The bioreactor was decanted and refilled every 3 days with fresh MWF. Typically, 4 stainless steel sheets, employed as holding matrix for biofilm formation, of 200 cm^2 (10 cm × 20 cm) were placed in the bioreactor. After biofilm formation, each was cut into 10 small strips (20 cm^2 each) for studies in 250 mL Erlenmeyer flasks.

2.2. Measurement of metabolic inhibition

Respiration activity was measured using a micro-plate based respiration system, known as MicroResp™, which measures carbon dioxide (CO_2) evolved. This colorimetric method is based on the colour change of a pH indicator dye (cresol red) caused by the presence of CO_2 (Campbell et al., 2003). Post exposure recovery (PER) tests, using the plate count method (Miles et al., 1938), were performed to enumerate colonies after exposure to the test compounds.

The following chemicals, commonly reported to be used as metabolic inhibitors, were tested against UV: formalin (0.1%, 0.5%, 1%, 2% and 5%), sodium azide (1 mM, 5 mM and 10 mM), DNP (0.5 mM, 1 mM, 5 mM and 10 mM) and DCCD (0.1 mM, 0.2 mM and 0.4 mM). All four were supplied from Sigma-Aldrich. The degree of inhibition was assessed by plotting colony-forming units (CFU/mL expressed as % of control) from post exposure recovery method versus respiration activity (CO_2% of control). This method was also used to determine the minimum UV-exposure time required for complete biofilm inhibition and to assess the toxicity effects of individual organics and metals. Biofilms in our study required a UV dose of approximately 300 mJ/cm^2 for complete inhibition.

2.3. Analytical methods

All chemicals were analytical reagent grade supplied from Sigma-Aldrich. Stock solutions of 10% v/v triethanolamine (TEA) and 2% w/v benzotriazole (BTA) were prepared by suspending triethanolamine (GC) (≥99.0%) and 1-H Benzotriazole (≥98.0%) in deionised (DI) water. Zn (II), Cu (II) and Cd (II) stock solutions (each at 1 g/L concentration) were prepared by suspending $ZnSO_4 \cdot 7H_2O$ (≥99.0%), $CuSO_4 \cdot 5H_2O$ (≥98.0%) and $CdSO_4 \cdot 8/3H_2O$ (≥99.0%) in DI water. These are ubiquitous constituents of MWFs, but were further diluted to obtain concentrations for practical use. Samples collected in all experiments were centrifuged at 12,000 rpm for 15 min to minimise background biofilm content. The total organic carbon (TOC) concentration of the supernatant was measured by a Shimadzu TOC V-CPH. The system was calibrated with standards prepared from potassium hydrogen phthalate in the range of 0–1000 mg/L. Atomic absorption spectroscopy (Agilent 240FS AA) was used to measure metal concentrations, calibrated in the linear ranges of 0.2–1.2 mg/L, 0.5–4 mg/L and 0.4–1.8 mg/L for zinc, copper and cadmium, respectively.

2.4. Comparing UV and chemical inhibitor for pollutant removal

The organic/metal removal Qe was calculated as follows (Gabr et al., 2008):

$$Q_e = V\left(\frac{C_o - C_e}{M}\right) \quad (1)$$

where Q_e is the organic/metal removal (mg TOC/g biofilm); C_O and C_e are the initial and equilibrium organic/metal concentrations in solution (mg/L) respectively; V is the solution volume (L); and M is the biofilm dry weight (g).

To determine dry-weight, biofilms grown on stainless steel strips were first rinsed with DI water, and then were dislodged into a 50 mL test tube by vigorous shaking. The dry weight of the biofilm was calculated as the sum of the dry weight of the centrifuged biofilm cells collected at the bottom of the test tube and the dry weight of the biofilm cake floating on the top of the solution after centrifugation. The dry weight of the biofilms cells was measured after drying them at 70°C for 1 h to constant weight. The dry weight of the biofilm cake was measured after vacuum filtering and then