

Development of a bioreactor system for the remediation of endosulphan

K. Jesitha^a and P. S. Harikumar^{b,*}

^a Sree Sankara College, Kalady, Ernakulam, Kerala, India

^b Centre for Water Resources Development and Management, Kozhikode 673 571, Kerala, India

*Corresponding author. E-mail: hps@cwrdm.org

Abstract

A bioreactor system that consisted of *Pseudomonas fluorescens* cells immobilised in calcium-alginate beads was utilised to remediate endosulphan contaminated water and soil. A packed bed reactor system was designed for the bio-degradation of endosulphan in artificially spiked water samples (initial concentration of endosulphan: 350 µg/L). Reactor studies with cell-immobilised Ca-alginate beads were conducted after checking their efficiency through batch and column degradation studies. The results showed that the concentration of toxic isomers of endosulphan (endosulphan alpha and endosulphan beta) was below the limit in the bioreactor during the 7th day of the experiment. Experiments conducted with contaminated soil samples (initial concentration of endosulphan: 1,000 µg/kg) indicated that the toxic isomers of endosulphan degraded to below the detection limit within 10 days and monitoring of endosulphan residues on the 14th day revealed that almost complete degradation of metabolites of endosulphan had occurred. The bioreactor system designed can be scaled up for remediation of endosulphan in contaminated areas.

Key words: bioreactor system, Ca-alginate beads, endosulphan, *Pseudomonas fluorescens*

INTRODUCTION

The continuous unrestricted use of pesticides may cause harmful and unpredictable health impacts. The persistence of organochlorine pesticides including endosulphan is a matter of concern due to its unexplored environmental impacts. Availability of clean water is of prime importance in limiting several water-borne diseases (Santacruz *et al.* 2005; Harikumar *et al.* 2014; Musthafa *et al.* 2014; Megha *et al.* 2015; Jaseela *et al.* 2016). Many of the physical processes used for the decontamination of hazardous waste have been reported to be uneconomical and unsustainable. Generally, biological methods have been found to be more environmentally friendly, cost effective and socially acceptable (Harikumar *et al.* 2012).

Bioremediation helps to destroy toxic pollutants utilising natural biological activity. Bioreactor systems consist of mechanical vessels in which organisms are cultured in a controlled manner and materials are transformed by specific reactions. The design and conditions to be maintained in bioreactor systems are important to provide a higher degree of control over process upsets and contaminations, since the organisms are more sensitive and less stable than chemicals. Biological organisms can alter the biochemistry of the bioreaction which in turn can lead to the degradation of toxic pollutants. The major challenge in the development of a bioreactor system is minimising undesired activities and maintaining the desired

This is an Open Access article distributed under the terms of the Creative Commons Attribution Licence (CC BY 4.0), which permits copying, adaptation and redistribution, provided the original work is properly cited (<http://creativecommons.org/licenses/by/4.0/>).

biological activity. Organisms are influenced by their morphology and the bioreaction medium and are shear-sensitive to varying degrees. Still there are a number of microorganisms that are relatively tolerant to high-shear environments and they exhibit robustness in high-energy mixing bioreactor vessels. Mixing within the bioreactor system is an important requirement to facilitate efficient heat and mass transfer during the production phase (Williams 2002; Gonzalez *et al.* 2008).

Different types of microbial cultures were introduced for the degradation of organophosphates, PCBs, PAHs endrin, etc. Naturally occurring bacteria capable of destroying some hazardous wastes and chemicals include *Flavobacterium* sp. for organophosphates, *Cunniinghamella elegans* and *Candida tropicalis* for PCBs and PAHs, *Closteridium* for lindane and *Arthrobacter* and *Bacillus* for endrin degradation (Sinha *et al.* 2009). Treatment technology using entrapped microorganisms is a novel biological engineering technology developed in recent years. Immobilised cells have prolonged microbial cell viability and improved capacity to tolerate higher concentrations of pollutants. Hence immobilisation of the pure cultures can help to improve the bioremediation potential (Richins *et al.* 2000; Chen & Georgiou 2002; Meleiro-Porto *et al.* 2011).

In the present study the bacterial strain *Pseudomonas fluorescens* was isolated and tested for its potential to degrade endosulphan. The main objective of the work was to develop a bioreactor system with immobilised *Pseudomonas fluorescens* for the degradation of endosulphan. We have already reported the pathway for microbial degradation of endosulphan (Jesitha *et al.* 2015).

MATERIALS AND METHODS

Preparation of endosulphan standard solution

An endosulphan solution with an initial concentration of 1 mg/L was prepared by diluting 5 mL of a 100 mg/L stock solution to 500 mL with chromatographic grade n-hexane. The desired concentration of the test solution was prepared from the stock solution.

Isolation of *Pseudomonas fluorescens* and inoculum preparation

A pure culture of *Pseudomonas fluorescens* capable of degrading endosulphan was isolated from a talc-based formulation of *Pseudomonas fluorescens*. The isolated bacterial species was identified using biochemical tests, viz. gelatin liquefaction, starch hydrolysis, catalase activity, oxidase activity, IAA production, siderophore production and hydrogen cyanide production.

The media used for the development of the seed culture for biodegradation experiments contained broth minimal medium (pH maintained at 6.8) and endosulphan (100 µg/L) as the sole carbon and energy source. The seed culture medium (500 mL) was placed in an Erlenmeyer flask (1,000 mL) and inoculated with a loop full of the culture, freshly grown on agar slants and incubated for 5 days at 28 °C and 150 rpm. The cells were harvested by centrifugation (8,000 rpm for 10 min) and washed by suspending in sterile deionised water and re-pelleting by centrifugation. The harvested cells were used for the degradation studies as described below (Jesitha *et al.* 2015).

Immobilisation of *Pseudomonas fluorescens* cells

The alginate entrapment for immobilisation of the bacterial cells was performed as per the method described by Kierstan & Coughlan (1985). The cells were harvested in the late exponential growth stage by centrifuging the culture suspension at 8,000 rpm for 10 min. The harvested cells were washed once with sterilised saline solution (0.85% w/v, pH 7.0) and further suspended in a 0.05 mol/L phosphate buffer solution (pH 7.2). A 4% (w/v) sodium alginate solution was autoclaved

and cooled to room temperature before adding to the pre-cultured cells. Bacterial cells were further resuspended in sterilised deionised water, and the optical density of the suspension was controlled in the range of 0.72 OD at 610 nm.

Immobilised cells for the batch degradation studies were prepared by adding 3 mL of the cell suspension having 0.72 OD at 610 nm (cell content in terms of wet weight was equivalent to 0.068 g of cells) to 25 mL of a 4% w/v sterilised sodium alginate solution. For the packed column study, 15 mL of the cell suspension at the same optical density (cell content in terms of wet weight was equivalent to 0.34 g of cells) was mixed with 300 mL of a 4% w/v sterilised Na-alginate solution. The solutions were subsequently stirred for 10 min, and the resulting alginate/cell mixtures were dripped into a beaker containing ice cold sterile 0.3 M CaCl₂ through a needleless 10 mL syringe from a height of 5 cm. The prepared beads were cured in CaCl₂ solution for 12 h at room temperature, and then washed with sterilised saline solution (0.85% w/v, pH 7.0) and stored in CaCl₂ solution at 4 °C for further use (Jesitha *et al.* 2015).

Bioreactor system with immobilised bacterial cells for the degradation and removal of endosulphan from water

Studies on the degradation of endosulphan using a bioreactor with *Pseudomonas* cells immobilised in calcium-alginate beads were conducted. The optimum condition for bioremediation of endosulphan and the feasibility of using a packed bed reactor were evaluated. Reactor studies were conducted with cell-immobilised Ca-alginate beads after checking their efficiency through batch and column degradation studies (Jesitha *et al.* 2015). A disposable polyethylene terephthalate (PET) cylindrical container (12.56 cm² × 18 cm) with a flow regulating system and a lid was used as the reactor. The total reactor working volume was 188.40 cm³. A Whatman (No. 1) paper filter was placed at the bottom to support the bed. The reactor was filled with cell-immobilised Ca-alginate beads up to 15 cm in height. Endosulphan (100 mL of 350 µg/L) was passed through the bioreactor at a flow rate of 0.59 mL/min. The reactor packed with alginate immobilised cells was operated in a semi-continuous flow mode (every 7 hours) during each day of the experiment. An endosulphan solution (350 ± 0.2 µg/L) was added to this reactor and allowed to react with immobilised Ca-alginate beads. A 25 mL aliquot of sample was collected after a fixed time interval (0, 3, 5, 7, 20 days). The collected samples were extracted and analysed to determine the amount of degradation of endosulphan. The bioreactor system for the removal of endosulphan from water is shown in Figures 1 and 2.

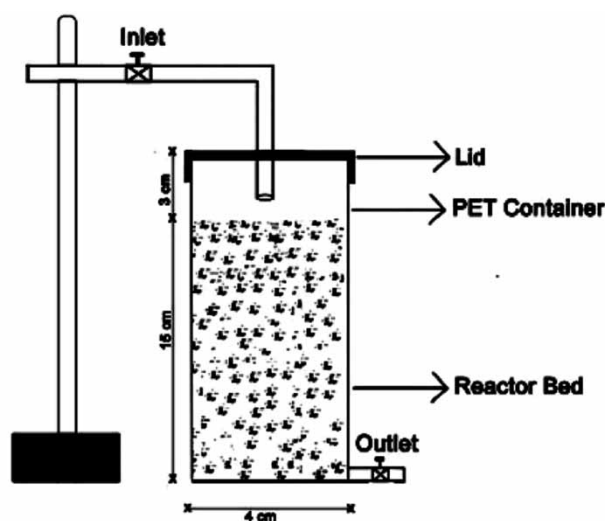


Figure 1 | Schematic diagram of the miniature bioreactor system for the removal of endosulphan from water.



Figure 2 | Immobilised Ca-alginate beads inside the bioreactor system.

Study of the degradation of endosulphan in soil using a bioreactor system

Batch degradation studies for the removal of endosulphan from soil utilising bacterial cell immobilised Ca-alginate beads were conducted to find out the optimum bacterial concentration required for degradation. Sandy soil was taken and the soil employed in all the biotransformation experiments was sterilised by keeping it in a hot air oven at 150 °C for 2 h (Kumar & Philip 2006).

Batch experimental studies were conducted in soil (10 g) with different amounts of Ca-alginate beads. Soil (10 g) artificially spiked with endosulphan (to a concentration of 100 µg/Kg) was placed in three conical flasks and mixed with 2.5, 5 and 10 g of cell-immobilised Ca-alginate beads. The flasks were shaken continuously for 24 hours. After 24 hours the solution was filtered, extracted and analysed.

A bioreactor system utilising *Pseudomonas fluorescens* cell-immobilised calcium-alginate beads was developed for the degradation of endosulphan from soil (Figure 3). The feasibility of using a packed bed reactor for biodegradation of endosulphan was studied under semi-continuous conditions. A PET cylindrical vessel (490.625 cm² × 35 cm) with a flow regulating system, lid and a stirrer was used as the reactor. The total reactor working volume was 9,812.50 cm³. Soil (2 kg) was placed at the bottom of the reactor. *Pseudomonas fluorescens* cell-immobilised Ca-alginate beads (2 g) were mixed with the soil. The soil and the beads were continuously mixed using the stirrer of the reactor system. Sterile broth medium (72 mL, pH 7.0) spiked with 1,000 mL of 1,000 µg/L endosulphan was passed through the bioreactor. The reactor packed with alginate immobilised cells was operated in a semi-continuous mode (every 7 hours) during each day of the experiment. Endosulphan solution was allowed to react with the immobilised Ca-alginate beads. After a fixed time interval (0, 3, 5, 7, 10, 14 days), 25 mL of samples were collected, extracted and analysed.

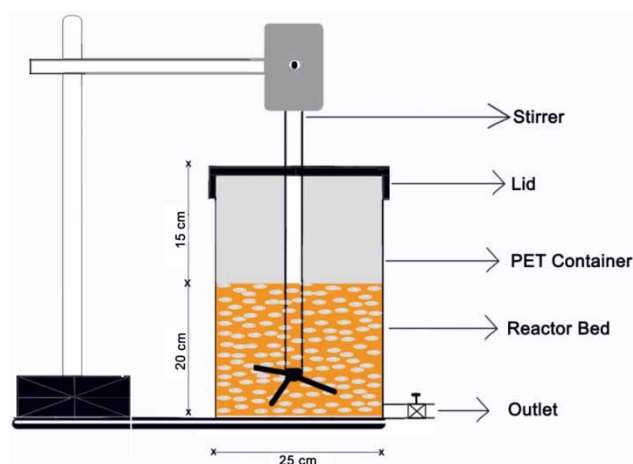


Figure 3 | Schematic diagram of miniature bioreactor system for the removal of endosulphan from soil.

Extraction of endosulphan

The samples were extracted using a mixture of chromatographic grade n-hexane and acetone. Extraction of pesticide residues was carried out following standard methods (USEPA 1996; APHA 2012).

The sample (10 g) was placed in a 250 mL conical flask. Concentrated HCl (0.1 mL) was added to the conical flask. The mixture was shaken well and then transferred into a separating funnel with a capacity of 1 L. A total of 10 mL of a hexane:acetone (80:20) mixture was added to the sample, shaken for about 10 minutes and kept for layer separation. The aqueous layer was collected in a beaker. The n-hexane layer was transferred to a standard flask from the separating funnel. A second volume of n-hexane (5 mL) was added to the aqueous layer and shaken well for 10 minutes, to ensure complete extraction of the pesticide residues. Then the aqueous layer was discarded and the n-hexane layer was transferred from the separating funnel to a conical flask (100 mL). Anhydrous sodium sulphate (3 g) was added to the n-hexane layer for dehydration. The n-hexane extracts were pooled and passed through a chromatography column of anhydrous sodium sulphate (5 g) overlaid with alumina (7 g) with glass wool at the bottom. The extract was concentrated to 10 mL and stored in an amber coloured vial. Quantitative and qualitative analysis of the pesticides was conducted using a gas chromatograph with an electron capture detector and mass spectrometer.

Analysis of endosulphan residues

After processing and clean-up, the samples were analysed for endosulphan residues by gas chromatography using an electron capture detector (GC-ECD). The retention times for endosulphan alpha, endosulphan beta, endosulphan sulphate, endosulphan ether, endosulphan lactone and endosulphan diol were 18.73, 21.94, 24.15, 12.30, 16.9 and 28.8 min, respectively. A Varian CP-3800 gas chromatograph equipped with an Ni electron capture detector was used for the analysis. The experimental conditions used for the estimation of endosulphan were reported in previous publications (Harikumar *et al.* 2013).

RESULTS AND DISCUSSION

Degradation of endosulphan in artificially spiked water using a bioreactor system with immobilised bacterial cells

The results of this study of the degradation of endosulphan, performed by passing an artificially spiked endosulphan solution (initial concentration: 348.21 µg/L) through the bioreactor, are shown in Table 1.

This study shows the feasibility of using Ca-alginate immobilised cells of *Pseudomonas fluorescens* for the degradation of endosulphan in a packed bed bioreactor system. The results showed

Table 1 | Results of the degradation of endosulphan in an artificially spiked water sample using the bioreactor system

Days	Endosulphan alpha (µg/L)	Endosulphan beta (µg/L)	Endosulphan diol (µg/L)	Endosulphan ether (µg/L)	Endosulphan lactone (µg/L)
0	252.53 ± 1.61	95.68 ± 2.48	BDL	BDL	BDL
3	98.96 ± 1.71	32.30 ± 2.59	120.48 ± 2.18	2.18 ± 0.05	6.18 ± 0.46
5	16.81 ± 2.73	2.23 ± 0.95	246.64 ± 1.90	4.59 ± 1.08	9.36 ± 1.31
7	BDL	BDL	252.850 ± 1.82	6.28 ± 0.31	15.65 ± 1.12
20	BDL	BDL	BDL	BDL	BDL

BDL: Below detection limit.

approximately 100% removal of the toxic isomers of endosulphan (endosulphan alpha and endosulphan beta) in the bioreactor with cell-immobilised Ca-alginate beads during the 7th day of experiment (Figure 4). This rapid removal of endosulphan is mainly due to the presence of an increased cell concentration in the Ca-alginate beads, which is derived from the incorporation of 0.34 g wet cells. The metabolites of endosulphan detected during the study included endosulphan diol, endosulphan ether and endosulphan lactone, which are reported to be less toxic than the parent isomers (endosulphan alpha and endosulphan beta). Endosulphan and its metabolites were completely removed within 20 days using the bioreactor system.

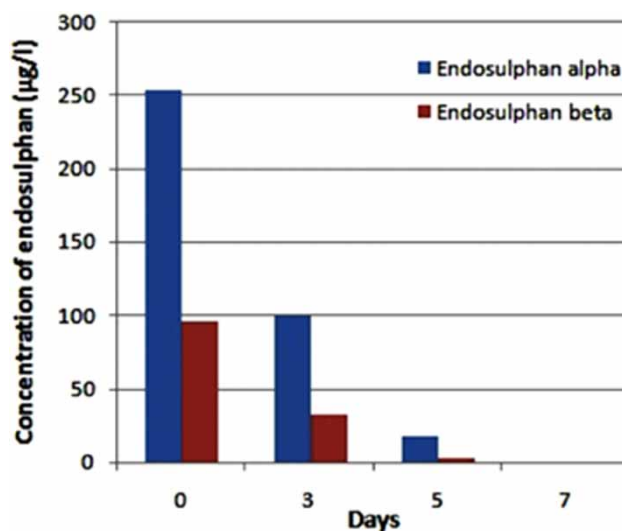


Figure 4 | Results of the degradation of endosulphan (alpha and beta) in the bioreactor system.

The cell immobilised calcium alginate beads were found to remain stable throughout the experiment.

Bioreactor system with immobilised bacterial cells for the degradation of endosulphan in soil

Results of the batch experimental studies to determine the amount of bacterial cell immobilised Ca-alginate beads required to optimise the bioreactor system for removal of endosulphan from soil is shown in Table 2.

The degradation of endosulphan was found to be most efficient when 10 g of soil was treated with 10 g of bacterial cell immobilised Ca-alginate beads. Hence, the soil to beads ratio was selected as 1:1 for the degradation of endosulphan from soil in the bioreactor system. The decrease in the concentration of endosulphan was monitored at fixed time intervals. The results of the degradation of endosulphan in the soil bioreactor system are given in Table 3. The results of the study of degradation of endosulphan (alpha and beta) in the soil bioreactor system are given in Figure 5.

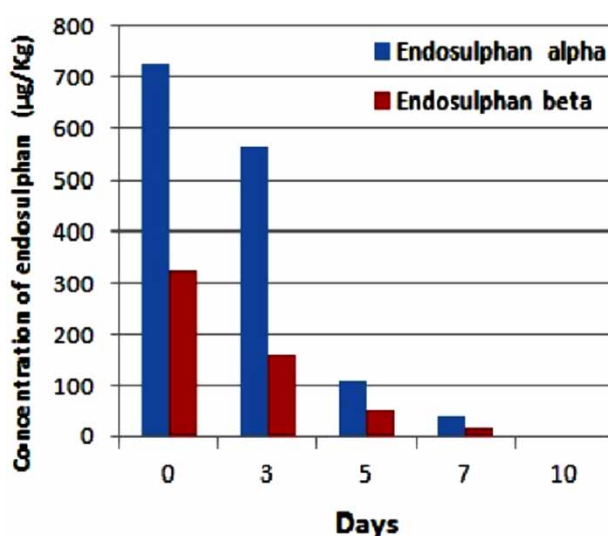
Table 2 | Results of the batch degradation of endosulphan using Ca-alginate immobilised cells in soil

Amount of immobilised beads in 10 g soil (g)	Concentration of endosulphan alpha (µg/kg)	Concentration of endosulphan beta (µg/kg)
2.5	80.23 ± 2.43	15.86 ± 1.16
5	61.55 ± 1.86	11.83 ± 1.60
10	49.24 ± 1.36	10.43 ± 1.06

Table 3 | Results of the degradation of endosulphan in a packed bed soil bioreactor

Days	Endosulphan alpha ($\mu\text{g}/\text{kg}$)	Endosulphan beta ($\mu\text{g}/\text{kg}$)	Endosulphan diol ($\mu\text{g}/\text{kg}$)	Endosulphan ether ($\mu\text{g}/\text{kg}$)	Endosulphan lactone ($\mu\text{g}/\text{kg}$)
0	728.47 \pm 1.92	321.63 \pm 2.64	BDL	BDL	BDL
3	568.23 \pm 1.47	158.29 \pm 1.51	196.19 \pm 2.01	10.85 \pm 1.16	6.68 \pm 0.88
5	110.97 \pm 1.69	52.34 \pm 0.95	350.86 \pm 1.02	56.52 \pm 2.09	29.84 \pm 1.83
7	38.42 \pm 1.43	14.37 \pm 1.49	522.17 \pm 2.31	77.43 \pm 1.63	36.73
10	BDL	BDL	810.58	80.85	40.97
14	BDL	BDL	BDL	BDL	BDL

BDL: Below detection limit.

**Figure 5** | Degradation of endosulphan (alpha and beta) in the soil bioreactor system.

In the bioreactor system experiments, toxic isomers of endosulphan (alpha and beta) were found to be below the detection limit within 10 days. However, the experimental study was continued to determine the time necessary for complete degradation of the metabolites of endosulphan. Monitoring of endosulphan residues on the 14th day revealed that complete degradation of endosulphan had occurred.

Stability of the Ca-alginate beads

The Ca-alginate beads showed no signs of breaking up or disintegrating. It appeared that the Ca-alginate beads maintained their integrity until the end of the experiment. This may be due to the better cell holding capacity and flexibility of the Ca-alginate gel that allowed accommodation of more cells that were generated during cell growth.

CONCLUSIONS

A bioreactor system was developed with enriched cell cultures immobilised in Ca-alginate gel beads. The degradation of endosulphan in the bioreactor system was studied using the isolated bacterial species *Pseudomonas fluorescens*. The biodegradation systems developed were found to be highly efficient for the removal of the toxic pesticide endosulphan from both water and soil matrices. The

presence of intermediate metabolites formed during the study was confirmed, and the metabolites were identified as endosulphan diol, endosulphan ether and endosulphan lactone. The detection of these metabolites suggests a hydrolytic pathway for the biodegradation of endosulphan using *Pseudomonas fluorescens*. The developed design of the bioreactor system can be scaled up for field level applications.

REFERENCES

- APHA 2012 *Standard Methods for the Examination of Water and Wastewater*, 21st edn. American Public Health Association, Washington.
- Chen, W. & Georgiou, G. 2002 Cell-surface display of heterologous proteins: from high throughput screening to environmental applications. *Biotechnol. Bioeng.* **5**, 496–503.
- Gonzalez, I. V. R., Fabio, F. & Varaldo, H. M. P. 2008 A review on slurry bioreactors for bioremediation of soils and sediments. *Microb. Cell Factories* **7** (5), 1–16.
- Harikumar, P. S., Jaseela, C. & Megha, T. 2012 Defluoridation of water using biosorbents. *Nat. Sci.* **4** (4), 245–251.
- Harikumar, P. S., Jesitha, K. & Sreechithra, M. 2013 Remediation of endosulfan by biotic and abiotic methods. *J. Environ. Prot.* **4**, 418–425.
- Harikumar, P. S., Jesitha, K., Megha, T. & Kamalakshan, K. 2014 Persistence of endosulfan in selected areas of Kasaragod district, Kerala. *Curr. Sci.* **106** (10), 1421–1429.
- Jaseela, C., Prabhakar, K. & Harikumar, P. S. 2016 Application of GIS and DRASTIC modelling for evaluation of groundwater vulnerability near a solid waste disposal site. *Int. J. Geosci.* **7** (4), 558–571.
- Jesitha, K., Nimisha, K. M., Manjusha, C. M. & Harikumar, P. S. 2015 Biodegradation of endosulfan by *Pseudomonas fluorescens*. *Environ. Process.* **2**, 225–240.
- Kierstan, M. P. J. & Coughlan, M. P. 1985 Immobilisation of cells and enzymes by gel entrapment. In: *Immobilized Cells and Enzymes, A Practical Approach* (Woodward, J., ed.). IRL Press, Oxford, pp. 39–54.
- Kumar, M. & Philip, L. 2006 Bioremediation of endosulfan contaminated soil and water – optimization of operating conditions in laboratory scale reactors. *J. Hazard. Mater. B* **136**, 354–364.
- Megha, P. U., Kavya, P., Murugan, S. & Harikumar, P. S. 2015 Sanitation mapping of groundwater contamination in a rural village of India. *J. Environ. Protect.* **6** (1), 34–44.
- Meleiro-Porto, A. L., Zelayaran, M. G., Consiglio Kasemod, M. & Nitschke, M. 2011 Biodegradation of pesticides. In: *Pesticides in the Modern World – Pesticides Use and Management* (Stoytcheva, M., ed.). InTech, Croatia, pp. 408–438.
- Musthafa, O. M., Subhankar, K. & Harikumar, P. S. 2014 Assessment and rationalization of water quality monitoring network: a multivariate statistical approach to the Kabbini River (India). *Environ. Sci. Pollut. Res. Int.* **21** (17), 10045–10066.
- Richins, R., Mulchandani, A. & Chen, W. 2000 Expression, immobilization, and enzymatic characterisation of cellulose-binding domain-organophosphorus hydrolase fusion enzymes. *Biotechnol. Bioeng.* **69**, 591–596.
- Santacruz, G., Bandala, E. R. & Torres, L. G. 2005 Chlorinated pesticides (2,4-D and DDT) biodegradation at high concentrations using immobilized *Pseudomonas fluorescens*. *J. Environ. Sci. Health B* **40**, 571–583.
- Sinha, R. K., Valani, D., Sinha, S., Singh, S. & Heart, S. 2009 Bioremediation of contaminated sites: a low-cost nature’s biotechnology for environmental clean up by versatile microbes, plants and earthworms. In: *Solid Waste Management and Environmental Remediation* (Faerber, T. & Herzog, J., eds). Nova Science Publishers, Inc., Commack, pp. 1–72.
- USEPA 1996 *Methods for Organic Chemical Analysis of Municipal and Industrial Wastewater*. United States Environmental Protection Agency, Washington, EPA – 821-B-96-005.
- Williams, J. A. 2002 *Keys to Bioreactor Selections*. [www.cepmagazine](http://www.cepmagazine.com), New York, pp. 34–41.

First received 30 August 2018; accepted in revised form 5 December 2018. Available online 24 January 2019