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Characterization and biostimulation of benzene biodegradation in the potting-mix of indoor plants

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Abstract

Over 900 volatile organic compounds (VOCs) have been detected in indoor air, where they cause acute and chronic health problems to building occupants. Potted-plants can significantly reduce VOC levels in indoor air, the root-zone bacteria of the potting mix effecting most of the VOC biodegradation. In this study, a baseline community level physiological profile (CLPP) was established for the potting mix bacteria of the indoor plant species, *Spathiphyllum wallisii* 'Petite', using Biolog EcoPlates, to provide information on the functional abilities of this community. Changes in the CLPP resulting from benzene exposure were then determined and following the identification of the carbon sources associated with changes in the CLPP, biostimulant solutions were formulated and applied to fresh potted-plant specimens. Biostimulation of benzene removal was observed, with increases in removal rates of about 15%, providing proof-of-concept for the biostimulation of this process. The findings further elucidate the mechanisms of bacterial activity associated with removal of indoor airborne benzene, and could be applied to increase VOC biodegradation rates, augmenting the uses of indoor plants in improving building environmental quality.

Key words: VOC, microorganisms, indoor air quality, indoor plants, Biolog EcoPlate, biostimulation

Abbreviations: AWCD: Average well colour development; CLPP: Community level physiological profile; IAQ: Indoor air quality; MANOVA: Multivariate analysis of variance; OD: Optical density; PCA: Principle components analysis; ppmv: parts per million by volume; TVOC: Total volatile organic compounds; UAP: Urban air pollution; VOC: Volatile organic compound

Introduction

A growing proportion of the world is becoming urbanised, so indoor air quality (IAQ) has become a significant international health issue (Mendell *et al.*, 2002; Environment Australia, 2003). Urban air pollution (UAP), of which about 90% is derived from fossil fuel emissions (US EPA, 2011a) is a major international health concern — the World Health Organisation (WHO, 2011) estimates that about 2 million deaths p.a. are directly attributable to indoor air pollution. UAP is usually higher indoors than outdoors, because, as outdoor air enters indoor spaces, it is augmented with indoor-sourced pollutants (Environment Australia, 2003; US EPA, 2011a). One of the main classes of indoor air pollutants are volatile organic compounds (VOCs) outgassing from synthetic furnishings, finishes, solvents etc. (Rehwagen *et al.*, 2003; Seppänen *et al.*, 2006). Even at imperceptible levels, mixtures of VOCs can cause symptoms of sick building syndrome, such as loss of concentration, headache or nausea (Harada *et al.*, 2010; Yu and Kim, 2010; US EPA, 2011b).

It is well established that 'indoor' plants can bring about reductions in many components of indoor air pollution (Wood *et al.*, 2002, 2006; Orwell *et al.*, 2004, 2006; Yoo *et al.*, 2006; Yang *et al.*, 2009; Wang and Zhang, 2011). In this laboratory, research has been focused on the ability of indoor plants to reduce VOCs. Our field studies, conducted in several university buildings, showed that three potted-plants per office were sufficient to reduce total VOCs (TVOCs) by more than 75% (Orwell *et al.*, 2006; Wood *et al.*, 2006), to levels below all international TVOC standards (Environment Australia, 2011).

Test-chamber studies in our laboratory have demonstrated that it is the microorganisms of the potting mix that are the primary VOC removal agents of the indoor plant microcosm (Wood *et al.*, 2002; Orwell *et al.*, 2004, 2006; Burchett *et al.*, 2008; Burchett *et al.*, 2010). Plants appear to contribute to the microbial VOC removal process via the selection and nourishment of the root-zone microbial community (Bais *et al.*, 2006; Kang and Freeman, 2007). The primary purpose of the current study was to characterize further the nature and responses of the microbial community primarily responsible for this biodegradation.

One method of characterising microbial communities is by investigating their carbon source utilization profiles (community level physiological profiling– CLPP), through the use of Biolog MicroPlates, which are designed for ecological applications. The method has previously been applied to distinguish among soil microbial communities (Grayston and Prescott, 2005; Niklińska *et al.*, 2006; Gelsomino *et al.*, 2006) and habitats (Garland and Mills, 1991). Whilst Biolog community profiling can detect only culturable microorganisms, studies have shown that the component of the potting mix microbial community that is involved with benzene biodegradation is an aerobic, culturable bacterial consortium (Wood *et al.*, 2002), with *Pseudomonas* species being predominant.

A demonstration of biostimulation would directly confirm the role

of the bacterial consortium in the removal of the VOC, and in addition, could lead to the development of a means of increasing VOC biodegradation rates in indoor potted-plants, and/or reducing the induction period for the response. Both could be of significant practical benefit in improving the performance of the process. The practice of using bacterial biostimulation for remediation of soils and groundwater contaminated by organic pollutants is well developed (Olaniran *et al.*, 2006; Sarkar *et al.*, 2005). Most biostimulants augment microbial growth *per se,* rather than the enhancement of specific components of the microbial community. The current study was one of the few projects undertaken with the aim of stimulating a specific consortium of soil bacteria for a particular role. We aimed specifically to provide proof-of-concept that biostimulation could be achieved, thus we did not compare our biostimulant with any alternative mixtures.

The experimental objectives of this study were to: (a) establish a baseline CLPP for the potting mix bacteria associated with the indoor plant species *Spathiphyllum wallisii* 'Petite', to provide information on the functional abilities of the indigenous microbial community; (b) investigate the CLPP changes resulting from benzene biodegradation; (c) identify any changes in carbon source usage following benzene exposure; and (d) use the CLPP data to construct a carbon-source supplement that might stimulate specifically the benzene-degrading consortium of the bacterial community, to provide proof-of-concept that biostimulation could be achieved for benzene biodegradation.

Materials and methods

Plant materials: Twelve month old, clonally cultivated potted specimens of *Spathiphyllum* 'Petite' were provided by Ambius (from Dalwood Wholesale Nursery, Dalwood, NSW, Australia). The plants were 0.3-0.4 m in height, in 130 mm diameter plastic pots. Plants did not grow substantially over the course of the experiment, and were tested within 72 h of the addition of the biostimulant, precluding substantive plant growth as a result of the addition of organic matter. Average foliage area was 0.90 ± 0.01 m^2 (Li-Cor LI-3000-A leaf-area meter, Li-Cor Nebraska). The plants were in a standard potting mix of 5% coco peat: 80% composted pine bark: 15% basalt crusher dust; and small amounts of aglime, dolomite and superphosphate (M. Keen, Dalwood Nursery, pers. comm.). The potting mix contained 41.6±4.27% organic content (mean ± SE dry weight basis, as loss at 400°C). Plants had received foliar nitrogen-based soluble fertilizer, Mancozeb fungicide and Crown insecticide, and were top-dressed with controlled-release pelleted fertilizer (Osmocote; Scotts Australia Pty Ltd) at 8-9 months after planting. Several weeks prior to experimentation, plants were fertilized with granular Osmocote Plus fertilizer at approximately 10 g per pot. No plant was used more than once in these trials.

Biodegradation test chambers: Seven replicate 0.216 m^3 Perspex chambers were used. The chambers had removable perspex lids held in place with sprung metal clips. Although sealed, 2-10% per day vapour loss occurred through the chamber seals. All daily removal rates were corrected by subtracting the amount of leakage from the overall removal rate. Each chamber was fitted with a silicone rubber septum through which the dose of VOC was injected and gas samples withdrawn for analysis; a 0.5 m copper tubing coil (i.d. 4 mm) with water circulating from a water-bath (Julabo, Seelbach, Germany) set to 23 ± 0.1 ºC; a thermometer; a 2.4 W fan recirculating chamber air to accelerate atmosphere equilibration; above-tank lighting box with five adjustable-intensity 18 W photosynthesis-specific fluorescent tubes (Wotan L 18/11 Maxilux Daylight, Osram, Germany), with an air gap of 50 mm between box and chamber. Light intensity was adjusted to 120 ± 10 µmol m^{-2} sec^{-1} (Li-Cor LI-185 B photometer). Before each trial, the internal surfaces of the chambers were cleaned with 70% ethanol and left for 24 h with the fans on to remove traces of ethanol and any residual benzene from previous trials.

Test procedures: Plants were watered to field capacity and allowed to drain for 30 min before use. Just prior to placement in the chambers, a pre-benzene treatment sample of potting mix was taken from each pot for EcoPlate inoculation. After plants were sealed in the chambers, a 25 ppmv (80 mg m^{-3} at 1 atm, 23ºC) dose of benzene (AR grade, Sigma) was injected into each chamber and left for 1 h to evaporate. This concentration of benzene vapour is five times the recommended maximum Australian 8-h averaged exposure standard (SafeWork Australia, 2009) and was used to present a strong quantitative challenge to the potted-plant microcosm. After equilibration, a 1.00 mL sample of chamber air was taken for analysis using gas chromatography (Shimadzu GC17A, Sydney, Australia). Subsequent samples were taken at 24 h intervals over the period of testing (up to 14 d). Two successive top-up doses of 25 ppmv were injected into each chamber after 95% of the previous dose had been removed, to observe reduction rates over the full induction period. A post-VOC-exposure potting mix sample for CLPP analysis was taken from each of the pots as they were removed from the chambers.

EcoPlate preparation: The method followed the general approach of Grove *et al.* (2004). Pieces of organic material >3 mm and any residual Osmocote granules were removed from the samples prior to weighing. Samples of 1.0 g were weighed out, and to each was added 10 mL of phosphate buffer (composition: 1.236 g Na_2HPO_4 [BDH], 0.18g NaH_2PO_4 [Sigma], 8.5g NaCl [Caledon] /L deionized water; sterilized by autoclave at 120ºC for 15 min). The phosphate buffer was used in preference to water to reduce bacterial cytolysis. Samples were shaken for 1 h at 300 rpm. The resulting suspensions were filtered through sterile tissue paper to obtain 2.5 mL of suspension, which was diluted 10-fold and used to inoculate the Biolog EcoPlates at 150 µL per well under laminar flow. For each pot, one EcoPlate was prepared before, and one after, benzene exposure. Eleven potted-plants were tested in the initial experimentation. A procedural control was also performed to determine whether the observed changes in the CLPP of the potting mix bacteria were related to benzene exposure or to some other effect related to enclosure in the chambers. For this test, four replicate plants were placed in chambers for 14 d, but with no benzene doses. EcoPlates were prepared from these pots before and after that period.

Plate readings and analysis: The optical density (OD) of the EcoPlate wells were read at 590 nm (Zak *et al.*, 1994) after 4 d incubation (Glimm *et al.*, 1997) at 23 ± 1 ºC, using a Bio-Tek PowerWave HT microplate spectrophotometer. Between readings, EcoPlates were incubated in the dark at 23 ± 1 ºC. Mean ODs for each of the 32 well types were calculated from the three replicate wells on each plate. The ODs for the 31 carbon-source wells were then corrected for any colour development in the blank water

wells. Following the approach of Glimm *et al.* (1997), wells were standardized by dividing the average OD for each well by the average well colour development (AWCD) in the 31 carbon source-containing wells to reduce the effect of differing inoculum densities among EcoPlates (Garland and Mills, 1991).

Testing possible biostimulation: Based on the nutrient utilization profiles obtained from the above procedures, a two-part putative biostimulant solution was made up: (a) a sugar solution containing 75g/L D-cellobiose (AR grade, Sigma) plus 75g/L α-D-lactose (AR grade, BDH); and (b) a 33g/L solution of L-asparagine (AR grade, BDH). The solutions were made separately and mixed after sterilization to avoid any possibility of the Maillard reaction (Finot, 2005). The concentrations used were formulated by determining the concentrations of similar nutrients in various commonly-used bacterial growth media (Oxoid, 1982; Difco, 1984). A total of 3.5 g of D-cellobiose, 3.5 g of α-D-lactose, and 2.45 g of L-asparagine, were added per pot (average wet weight of potting mix: ~0.7 kg). The doses were administered in equal sub-volumes over 4 d, to allow the potting mix bacteria to adapt gradually to the nutrient conditions before exposing them to benzene. The putatively biostimulated potted-plants were then placed in the test chambers and exposed to three doses of 25 ppmv benzene over 14 d. EcoPlates were again prepared from potting mix samples before and after benzene exposure.

Data analysis: Data are displayed as means ± the standard error of the mean. The relationship between the multivariate CLPP data before and after benzene exposure was examined using principal components analysis (PCA) (Lewicki and Hill, 2005). To compare CLPPs, we used MANOVA with Wilk's λ test statistic. All analyses were carried out using Minitab Ver. 14 (Minitab Inc. 2003).

Results

Benzene removal rates before and after application of biostimulant formulation: As anticipated, when the potted-plants were exposed to three doses of 25 ppmv benzene (Fig. 1), removal rates increased with successive doses, after an initial, slower first exposure response ('induction' dose). Fig. 1 also provides a comparison of VOC removal responses before and after the application of the biostimulant. The patterns of response are consistent with those obtained in our previous experimentation with this VOC (Wood *et al.,* 2002; Orwell *et al.,* 2004). We have previously shown that the overwhelming majority of this benzene removal in our system is performed by the potting mix microorganisms (Orwell *et al.,* 2004): whilst the plants themselves may play a small role in benzene sorption (Yoo *et al.*, 2006), the primary mechanism for benzene biodeterioration is microbial. The rate of benzene removal was calculated as the time taken to reach the minimum benzene concentration achieved in the unstimulated treatment group. This calculation method was necessary to allow for the different minimum benzene concentrations achieved in the treatments. Removal rates with the biostimulant solutions were higher than those in the non-biostimulated treatment (Fig. 1). With biostimulation, the removal rates also reached asymptotic levels, *i.e.* full induction, earlier than in the control treatment. With the third dose (*i.e.* at full induction), while the rate in the non-stimulated treatment was approximately 1.5 times faster than the initial dose, in the biostimulated treatment the final rate was over twice as fast, the final time taken to reach the equivalent minimum concentration achieved in the unstimulated treatment being 47.5% faster than in the non-stimulated treatment.

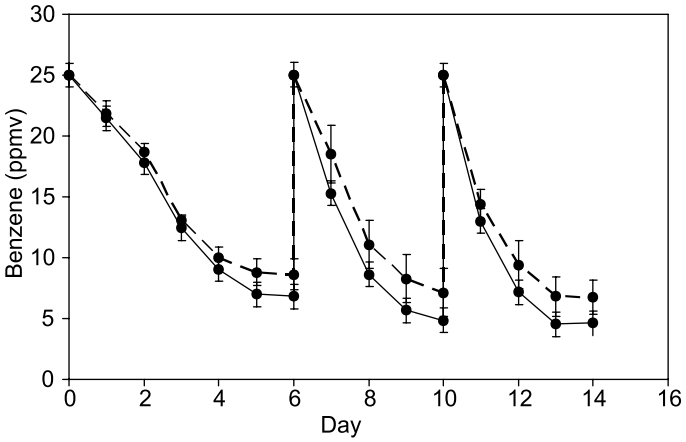

Fig. 1. Rates of benzene removal for non-biostimulated and biostimulated potted plants. A 25 ppm benzene dose was applied to the experimental chambers at days 0, 6 and 10. Solid line = biostimulated plants; dashed line = non-biostimulated plants. (Means ± SEM. n=11 non-biostimulated plants; n=4 biostimulated plants.)

Whilst, it is not possible to identify specifically the bacterial species involved in the benzene biodegradation consortium from their ability to use the three biostimulant components as sole carbon sources, it is known that various *Pseudomonas* spp. are capable of using these compounds (Her *et al.*, 1999; Bultreys and Gheysen, 2000), and this genus was predominant in our previous isolation of a benzene degrading bacterial consortium (Wood *et al.*, 2002).

Comparison of CLPPs before and after benzene exposure, without biostimulation: Results of the PCA comparison of pre- and post-benzene exposure of non-biostimulated plants are shown in Fig. 2. The pre-benzene exposure data points are clearly separated along the primary axis from the post-benzene exposure data points. This result indicates that overall carbon source usage by the bacterial community changed as a result of benzene exposure, a clear effect of the VOC exposure on the potting mix bacterial community. Further analysis showed that the post-benzene samples had an increased usage of D-cellobiose, α-D-lactose and L-asparagine, which were thus utilized to formulate the biostimulant solution.

CLPPs before benzene exposure, in non-biostimulated and biostimulated pots: The PCA for comparison of the pre-benzene exposure in non-biostimulated and biostimulated potting mix treatments (Fig. 3), showed considerable differences between the two treatments along the primary axis. The non-biostimulated treatment showed considerably more variation than the biostimulated group, no doubt, because the biostimulant had augmented the growth of a specific component of the potting mix bacterial community. However, a Wilk's λ test comparing average well colour development of the pre-benzene exposure samples found no significant difference in carbon source usage between the two groups (P=0.910). This result indicates that the bacterial species favoured by the biostimulant treatment were already significant components of the microbial community present in the non-biostimulated pots.

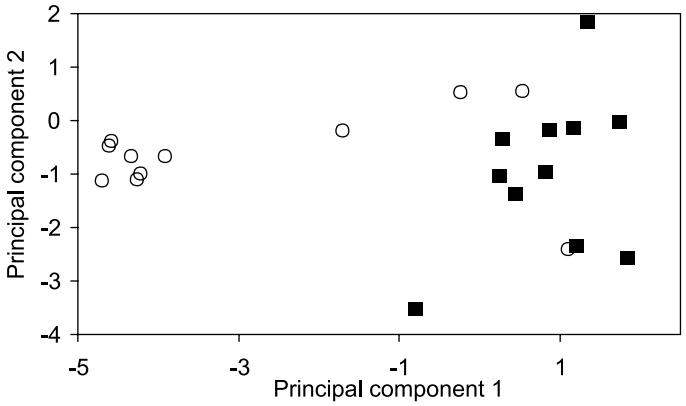

Fig. 2. Ordination for the first two principal components from the PCA of CLPP data, pre- and post-benzene exposure in non-biostimulated potted plants. ■ = pre-benzene; ○ = post-benzene.

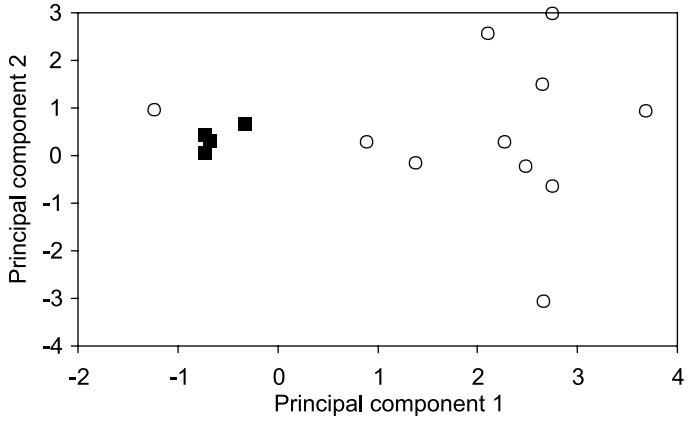

Fig. 3. Ordination for the first two principal components from the PCA of CLPP data, pre-benzene exposure, in non-biostimulated and biostimulated potted plants. ■ = biostimulated; ○ = non-biostimulated

CLPPs after benzene exposure, in non-biostimulated and biostimulated pots: The PCA plot of biostimulated and non-biostimulated CLPP data post-benzene exposure (Fig. 4), indicated clear multivariate differences between the two groups, the stimulated group again showing much less dispersion than the non-stimulated treatment. However, a Wilk's λ test again found no significant difference in carbon source usage between them (P=0.978). This result was not surprising, as both groups had been induced by exposure to benzene — the biostimulant simply increased the rate of induction and subsequent benzene removal rates, but evidently did not have any effect on the species composition of the bacterial consortium responsible for benzene removal.

CLPP of procedural controls: The PCA of CLPP data from pre- and post- (no-benzene) chamber controls (Fig. 5) also showed a difference in distribution between the two data sets on both primary and secondary axes. The pre-chamber data points were more broadly dispersed along both axes, while the post-chamber points were relatively clustered. This test confirmed that 14 d in the test chambers itself leads to changes in the microbial community. A Wilk's λ test comparing AWCD of the pre- and post-chamber control samples found a significant difference in carbon source usage between the two treatments (P=0.0001), and Tukey's *post hoc* tests revealed that utilization of D-galacturonic acid, D-malic acid and L-asparagine increased. L-asparagine was one of the carbon sources with increased uptake after exposure

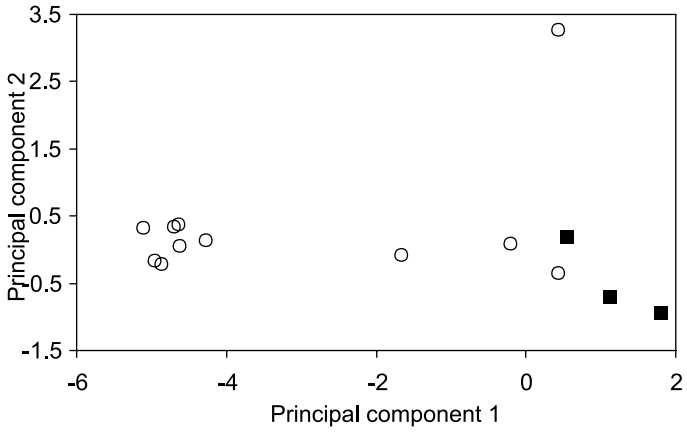

Fig. 4. Ordination for the first two principal components from the PCA of CLPP data, post-benzene exposure, in non-biostimulated and biostimulated potted plants. ■ = biostimulated; ○ = non-biostimulated

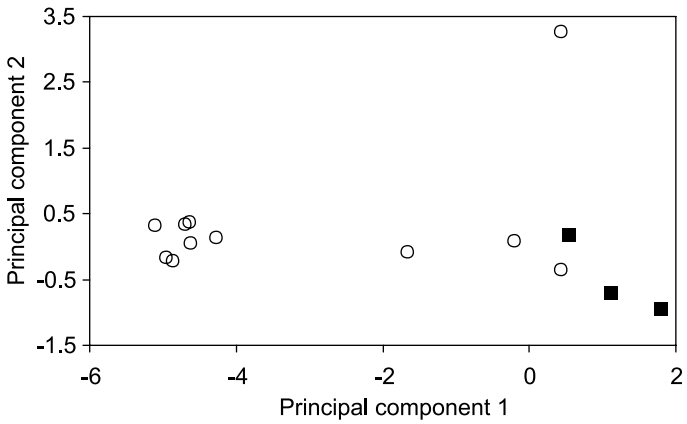

Fig. 5. Ordination for the first two principal components from the PCA of CLPP data, for pre- and post-chamber control potted plants. ■ = pre-chamber; ○ = post-chamber

to benzene in the biostimulated plates. For this and other reasons outlined below, further research is required into the efficacy of biostimulation of airborne VOC removal response in indoor potted-plants.

Discussion

Overall, the application of the biostimulant solutions increased rates of benzene degradation by about 27%. That a stimulated response was observed with the first dose indicates that the biostimulant had a direct effect on the bacterial consortium associated with benzene biodegradation, and is thus fairly specific. The increased rates of removal resulting from the biostimulation could be advantageous in an indoor environment with high levels of airborne VOCs. This study has been the first systematic investigation conducted on the physiology of the normal bacterial community in the potting mix of an indoor plant. The study also throws light on the nature of the responses of the CLPP of this type of bacterial community to exposure to one of the most common urban air pollutants worldwide, benzene, a Class A carcinogen (US EPA, 2006). This compound and other VOCs are often found in higher concentrations in indoor air than outdoors, since they are used in many furnishing and finishing materials, and it is known that these VOCs are degraded by indoor plants *in situ* in the indoor environment. In this study increased usage of three specific carbon sources was found to be associated with the microbial changes brought about by benzene exposure, and when

these compounds were made up into biostimulant solutions and applied to fresh plant pots, they significantly stimulated benzene removal, measurably increasing the performance of the potted plants to remove this pollutant from the surrounding atmosphere, leading to the potential for further performance enhancement with development of the process. The increased rates of removal resulting from the biostimulation could be advantageous in an indoor environment with high levels of airborne VOCs.

The results throw further light on the primary role of the potting mix bacteria in the removal of airborne VOCs by indoor potted-plants, and provide proof-of-concept for specifically enhancing the growth of the benzene-degrading components of the bacterial community by selective biostimulation. The results have the potential to lead to the development of practical ways to augment the VOC biodegradation capacity of indoor plants. More research is needed on the characterisation of potting mix bacteria associated with airborne VOC removal, including investigations on changes in CLPPs that may occur with a range of indoor plant species, and to other VOCs. Testing the capacity of the biostimulant formulation to influence the biodegradation of other hydrocarbons is also of importance, as are matters of determining optimum concentrations of components of the biostimulant solution for particular VOCs, mixtures thereof, and with different plant species. We did not compare the performance of the biostimulant formulation with alternatives (*e.g.* inorganic fertilizers, general bacterial growth media), but rather, have provided novel proof-of-concept that the process has substantial potential to improve the performance of indoor plants as bioremediators of indoor air pollutants.

Various biofilters utilizing VOC degrading bacteria have been developed for a number of engineering applications, but only very rarely are any gaseous contaminants removed via air conditioning systems in city buildings. Cleaner air is healthier air, and leads to improved work performance and reductions in adverse health symptoms– indoor plants are low-cost, flexible, portable air cleaners that have also been shown to provide other benefits to building occupants (*e.g.* Lohr *et al.*, 1996; Bringslimark *et al.*, 2007; Dijkstra *et al.*, 2008; Dravigne *et al.*, 2008), and clearly have underutilized potential for improving the health of many people.

The findings elucidate the mechanisms of bacterial activity associated with removal of indoor airborne benzene, and could be applied to increase VOC biodegradation rates, augmenting the uses of indoor plants in improving building environmental quality.

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