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BIODEGRADATION OF DIESEL FUEL BY AN *AZOLLA*-DERIVED BACTERIAL CONSORTIUM

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ABSTRACT

The widely distributed water fern *Azolla* was investigated for use as an amendment in the bioremediation of fuel-contaminated environments. In a field experiment *Azolla pinnata* as well as *Pistia stratiotes* and *Salvinia molesta* were applied to plots containing soil that had been surface-contaminated with diesel fuel ($2.4\,L\,m^{-2}$) and flooded with water. The plants quickly died and bacterial flocs developed around the dead *A. pinnata* fronds. After 16 weeks, diesel concentrations (as determined by levels of gas chromatography-detectable hydrocarbons) in the plant-added plots were less than half that of the control plot, and concentrations of xylenes and ethylbenzene were 50–100 times lower. In laboratory experiments, a consortium composed of *A. pinnata*-derived bacteria displayed dense growth in a 4% diesel-containing mineral salts medium and was found to lower the fluorescence from aromatic compounds by approximately 50% after 19 d. It is concluded that the observed

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enhancement of diesel degradation in the plant-added plots was due to the release of bacteria (bioaugmentation) and physiochemical improvement of the plot conditions (biostimulation).

Key Words: Diesel-contaminated soil; Bioremediation; BTEX; *Azolla* plants

INTRODUCTION

Due to their widespread use, diesel fuel and other petroleum distillates are among the most common environmental pollutants.[1,2] We are presently investigating bioremediation methods to stimulate microbial degradation of diesel hydrocarbons for the treatment of diesel-contaminated soil and water from United States Marine Corps bases on Okinawa, Japan.

The biodegradation rate of diesel compounds varies depending on many factors including their accessibility to microbes, the capacity of the microbial community for hydrocarbon utilization, and the inorganic nutrient composition of the environment. Higher rates of diesel degradation have been reported following seeding with hydrocarbon-degrading bacteria[3] and following the incorporation of dead plant material (hay) into soil.[4] Added plant material increases soil permeability and the amount of surface area available for microbial colonization.[4] Plants synthesize a variety of hydrocarbons and, thus, carry several kinds microbes "preadapted" to degrade the petroleum aliphatic and aromatic hydrocarbons as sources of carbon (C) and energy.[5] The aromatics in diesel, the bulk of which are the two-ring alkyl-naphthalenes and one-ring aromatics, including benzene, toluene, ethylbenzene and *o*-, *m*-, and *p*-xylene (collectively termed BTEX),[6,7] are of particular concern due to their toxicity.[8] Some microbial degradative enzymes induced by plant compounds can co-oxidize compounds that might not otherwise be as readily broken down.[9,10]

Microbial populations in diesel-contaminated environments are likely to be under nitrogen (N) limitation since diesel contains only 0.02% total N.[7,11] Supplementation with inorganic nutrients can greatly increase the rate of diesel degradation in soil.[3] In general, addition of plant material does little to alleviate soil N-deficiency. However, application of the floating freshwater *Azolla* fern ("green manure") can substantially increase soil N content.[12] *Azolla* species are distributed world-wide across the tropics, subtropics and temperate regions.[12] The presence of N_2-fixing symbiotic cyanobacteria[13] and bacteria[14] in specialized leaf cavities permits *Azolla* to accumulate stores of combined-N even in N-deficient environments. Upon death of a plant, N and other nutrients, including phosphate and microelements, become available for use by neighboring plants and microbes.[12,15,16]

Here we describe a field experiment that demonstrates a stimulatory effect of applying *Azolla* and other aquatic plants on the biodegradation of hydrocarbons, including BTEX, in a diesel-contaminated flooded environment. Laboratory experiments characterized the growth kinetics and degradation of aromatic compounds by an *Azolla*-derived bacterial consortium in media having diesel as the sole C-source.

MATERIALS AND METHODS

Design of Field Experiment

A diesel fuel spill simulation was set up April 12, 2001, at USMC Camp Hansen on the subtropical island of Okinawa, Japan (26.5°N, 127.8°E). Approximately 1200 kg of stony clay soil (pH 7.5) from a diesel-contaminated roadbed was mixed and divided into three plastic containers with a surface area of 1.69 m^2. To prevent accidental overflow from rainwater entry, the plots were kept in a covered open-air building. Each plot was splashed with 4 L diesel fuel no. 2 and the surface soil sampled for hydrocarbon analysis. Plots were flooded to a depth of 5 cm, resulting in perturbation of the surface soil. Two plots were inoculated with *Azolla pinnata* plants (1620 cm^2 floating surface area). The other plot was left without inoculation as a control. After 15 d, 540 cm^2 (floating surface area) each of *A. pinnata*, *Pistia stratiotes* (water lettuce), and the non-symbiotic water fern *Salvinia molesta* were added to the already inoculated plots. All plants were obtained from ponds at University of the Ryukyus, Okinawa. One of the plant-containing plots was fertilized with 25 mL Hyponex® 0-6-4 (N-P-K) to provide approximately 25 μM phosphate. Until mid-July 2001 evaporative losses of water were replaced by blown-in rain or by hose.

By August 1 the plots had dried and several grams of soil within 1 cm of the surface from five ~400 cm^2 sites in each plot were combined in a plastic bag. The bags were shaken to mix the soil and submitted for hydrocarbon analysis. Following removal of the samples, the plots were flooded with 5 cm of water and inoculated with 180 cm^2 (floating surface area) of each of the three plants mentioned above.

Chemical Analyses

Soil concentrations of BTEX and diesel hydrocarbons (DHC; 16 hydrocarbons characteristic of No. 2 diesel fuel) were measured by gas chromatography at United States Air Force Detachment 3 Analytical Division, Okinawa. DHC levels in samples were calculated based on a standard curve of DHC in a reference diesel.

Laboratory Cultures

To estimate the numbers of heterotrophic bacteria within the plots, serial dilutions of surface water samples were spread in duplicate onto nutrient agar medium (Difco) and colony forming units (cfu) were counted after a 3 d incubation at 26°C. Surface water was sampled by dipping a sterilized 30 mL centrifuge tube no deeper than 1 cm into a central plot area largely free of particulate matter. To estimate the numbers of bacteria tightly adhering to dead *A. pinnata* tissue, plants were washed twice by vigorous vortexing in sterile water, weighed, crushed and a sample was subjected to the dilution plating procedure described above.

For laboratory experiments a mineral salts bacterial culture medium (pH 7.0) was prepared, containing per liter: 2 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4 \cdot 7H_2O$, 3.0 g KH_2PO_4, 7.0 g K_2HPO_4, and 1.0 mL of a microlements solution from the medium of Allen and Arnon.[17] The medium (25–50 mL) was autoclaved in 125 mL Erlenmyer flasks and cooled to room temperature. Filter sterilized (0.22 μm) No. 2 diesel fuel was added prior to inoculation of the media.

Culturing of an *A. pinnata*-derived bacterial consortium (ADC) was begun by introducing 0.1 g washed whole plant into 4% diesel medium. Source cells for *Rhodococcus* strain APG1 cultures had been cultured in nutrient broth. Micro-Blaze® Emergency Liquid Spill Control, a suspension of endospores from several strains of hydrocarbon-degrading *Bacillus*, was purchased from Verde Environmental Inc. To initiate bacterial cultures, cells or endospores were centrifuged at 7000 g, suspended in medium to 0.005 optical density units at 600 nm (OD_{600}) and incubated at 26°C on a rotary shaker (120 rpm). Growth was monitored by periodic removal of samples and determination of OD_{600} values.

Plants subjected to diesel sensitivity assays were incubated in a plant culture medium (~4 cm depth) with varying levels of diesel at 24°C under an 18/6 h light/dark cycle using combination of fluorescent and incandescent light (100 μmol $m^{-2} s^{-1}$). The medium was identical to a previously described dilution of an N-free Allen and Arnon medium[17] except with phosphate added as 0.3 mM Na_2HPO_4 and 0.2 mM KH_2PO_4. *A. pinnata* was incubated for 7 d in medium without added N while *P. stratiotes* and *S. molesta* were incubated for 7 d and 28 d, respectively, in medium supplemented with 3 mM $NaNO_3$. Evaporative losses were replaced by addition of tap water.

Fluorescence Spectroscopy

To monitor utilization of diesel aromatics, bacteria were cultured in sets of 12 × 100 mm glass screw-capped tubes containing 2 mL 4% diesel medium

and incubated as above. As a control, one set of tubes was left uninoculated. Tubes were periodically removed and placed at 4°C for storage. At the end of the experiment, the cultures were poured into microfuge tubes. The culture tubes were rinsed with distilled water and the rinse added to the microfuge tubes to bring the final volume to 2 mL. Following centrifugation at 18,000 *g* for 10 min the supernatant was returned to the original culture tube and combined with methanolic 0.3% Tween-20. The solution was vortexed and poured into a cuvette. Relative aromatic concentrations were determined by measuring the fluorescence emission spectra of the solutions excited at 254 nm using a fluorescence spectrophotometer (RF-5300PC, Shimadzu, Kyoto, Japan).

RESULTS

Following flooding with water, a nonaqueous phase (~2.5 mm thick) formed on the surface of the three plots containing diesel-contaminated soil. *A. pinnata* plants were applied to two of the plots on day 1 and on day 15. The plants died following both applications due to immediate immersion beneath the oil phase. The fuel also killed *P. stratiotes* and *S. molesta* plants that were applied on day 15. Surface water sampled on day 22 had 8.3×10^6 cfu mL^{-1} in the control plot without added plants, 1.6×10^7 cfu mL^{-1} in the plant-added plot, and 1.7×10^7 cfu mL^{-1} in the fertilized plant-added plot. Bacterial flocculent, covering approximately 5% of the surface area of both plant-added plots, was loosely associated with the dead (still floating) *A. pinnata* plants. None was observed in the control plot. A sample of flocculent taken from the fertilized plant-added plot was found to have 2.8×10^8 cfu mL^{-1}. Bacteria tightly adhering to the dead *A. pinnata* numbered 1.5×10^9 cfu g plant tissue^{-1}.

The higher numbers of bacteria in the plant-added plots positively correlated with greater decreases of DHC and BTEX in soil sampled at week 16 (Fig. 1). Final DHC concentrations in the surface soil of the plant-added plots with and without fertilizer were 44% and 40%, respectively, relative to that of the control plot (Fig. 1). Substantial declines were also observed in BTEX concentrations of the plant-added plots compared to the control plot (Fig. 1).

Changes in the BTEX/ DHC ratios from the start of the experiment to week 16 were calculated. In the control plot, utilization of all BTEX compounds except toluene paralleled that of DHC; toluene was degraded faster than DHC (Table 1). In the plant-added plots, ethylbenzene and the xylenes were degraded at a rate significantly faster than were DHC (Table 1). Fertilizer supplementation appeared to further increased the relative degradation rate of BTEX (Table 1).

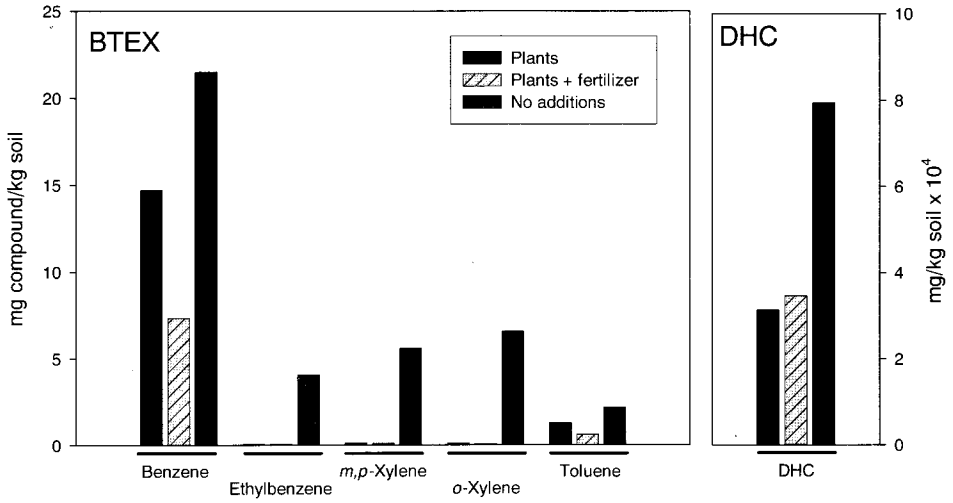

Figure 1. Comparison of total diesel hydrocarbon (DHC) and BTEX concentrations in soil after 16 weeks.

Table 1. Change in Proportions of BTEX Compounds Relative to DHC

	Proportion of BTEX to DHC (10^{-5})			
	Initial ($t = 0$)	Final ($t = 16$ wk)[a]		
Compound	Mean ± S.D. ($n = 3$)	+Plants	+Plants + Fertilizer	Control
Benzene	40.4 ± 21.9	46.8 (0.1)	21.1 (−0.9)	27.1 (−0.5)
Toluene	10.4 ± 7.0	4.1 (−1.5)	1.8* (−4.9)	2.7* (−2.8)
Ethylbenzene	2.7 ± 1.1	0.17** (−14.6)	0.16** (−16.2)	5.1 (0.5)
m,p-Xylene	10.0 ± 3.6	0.38** (−25.5)	0.27** (−36.4)	7.0 (−0.4)
o-Xylene	7.7 ± 1.4	0.28** (−26.7)	0.19** (−40.5)	8.3 (0.1)

[a]Values in parenthesis were calculated according to the equation [1 − (Initial BTEX/DHC) ÷ (Final BTEX/DHC)]; a value of zero indicates no change in the BTEX/DHC proportion.
*Value more than 1 S.D. below the initial mean.
**Value more than 2 S.D. below the initial mean.

Following removal of the soil samples on week 16 the plots were flooded and inoculated with the three plant species. The plants showed differential survival that depended on the species and plot condition. In the control plot all plants were dead within 7 d. In the plant-added plots, death of *A. pinnata* occurred by 7 d, and *S. molesta* by 25 d. *P. stratiotes*, however, was still alive, though slightly withered after 25 d.

Minimal concentrations of diesel required for killing of the three plant species were determined under laboratory conditions. Though in

Table 2. Effects of Diesel on Laboratory-Cultured *A. pinnata*, *P. stratiotes*, and *S. molesta*[a]

% Diesel (v/v)	*A. pinnata*[b]	*P. stratiotes*[c]	*S. molesta*[c]
0	+	+	+
0.005	+		
0.010	−		
0.015	−		
0.020	−		
0.025	−		
0.050	−	+	+
0.10	−	−	+
0.25	−	−	−
0.5	−	−	−
1.0	−	−	−

[a]Abbreviations: −, no growth; +, net growth.
[b]Growth measured by change in surface area.
[c]Growth measured by change in fresh weight.

the field *S. molesta* plants were more sensitive than *P. stratiotes* plants to diesel, they displayed slightly greater tolerance under laboratory conditions (Table 2). *A. pinnata* plants were killed by diesel concentrations 10- and 20-fold lower than those that killed *P. stratiotes* and *S. molesta*, respectively (Table 2).

Laboratory culture experiments were conducted to assess the capacity of the bacterial associates of *A. pinnata* to utilize diesel as a C-source. *A. pinnata* introduced into flasks with 4% diesel medium gave rise to a dense bacterial community predominantly comprised of four distinct colony forming types (Table 3). The density of this *A. pinnata*-derived consortium (ADC) in 4% diesel medium[19] without plant far surpassed that of a commercially available mixture of oil-degrading *Bacillus* species which gave barely detectible growth over the course of 17 d (Fig. 2). Inoculation of *A. pinnata* into an *N*-free plant culture medium containing 4% diesel resulted in the development of flocs around the dead *A. pinnata* tissue.

Fluorescence intensity readings can be used to monitor changes in aromatic concentrations of petroleum-contaminated water.[20] As evidenced by a decline in fluorescence, test tube batch cultures of the ADC had degraded approximately 50% of the aromatic compounds in the 4% diesel medium after a 19 d incubation (Fig. 3). Sterile controls showed a less than 10% decline in fluorescence due to volatilization of aromatics.

Rhodococcus sp. strain APG1, an endophyte previously isolated from surface-sterilized *A. pinnata* frond tissue,[21] grew in 4% diesel medium but achieved an approximately 10-fold lower density than the ADC (Fig. 2) and did not cause any reduction aromatic fluorescence relative to

Table 3. Partial Characterization of Bacterial Strains Comprising the *A. pinnata*-Derived Consortium[a]

Strain	Gram Stain	Cell Dimensions (μm)		Motility	Colony Characteristics[b]		Gelatin Hydrolysis	Growth at:		Nitrate Broth[c]
		Width	Length		Size (mm)	Color		4°C	41°C	NO_2^- Formation
AB1	–	0.7	1.1–2.3	–	> 2.0	Cream	–	–	+	+, d
AB2	–	0.8	2.4–7.4	+	> 2.0	Beige	+	–	–	+, d
AB3	–	0.7	0.7–3.9	+	> 2.0	Beige, bf, cc	+	+	–	+, d
AB4	+	1.0	1.7–6.0	–	~1.0	White	–	–	–	–, sd

[a]All strains displayed cytochrome oxidase and catalase activity, lacked amylase activity and did not form endospores.
[b]Appearence of colonies on nutrient agar after a 3 d incubation at 26°C. All colonies had a smooth margin, convex elevation and shiny surface. Examined for fluorescence under UV light (365 nm). Abbreviations: bf, blue fluorescence; cc, concentric rings.
[c]Cells were cultured in nutrient broth medium with 5 g L^{-1} KNO_3; none of the strains produced gas. Abbreviations for growth pattern in broth: d, diffuse; sd, settled-diffuse.

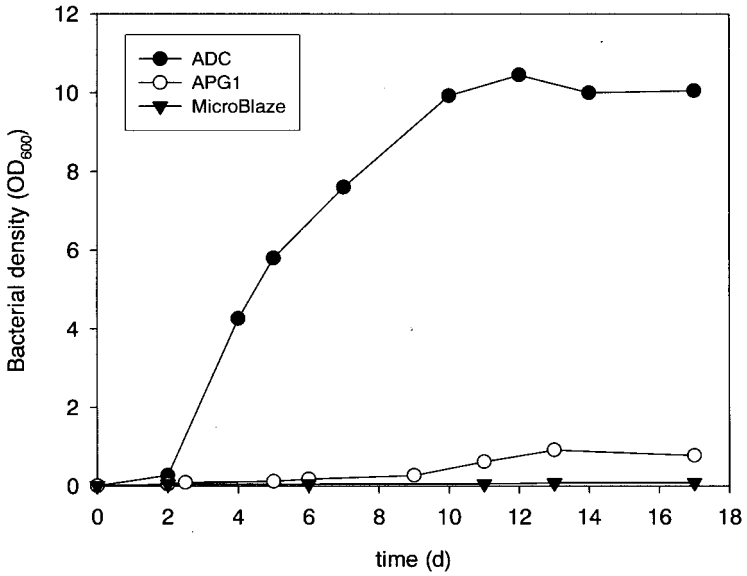

Figure 2. Growth curves of the *A. pinnata*-derived consortium (ADC), *Rhodococcus* sp. strain APG1, and a commercially available mixture of *Bacillus* species (Micro-Blaze®) incubated in 4% diesel medium. 1.0 OD_{600} unit of the ADC culture represents 4×10^8 colony forming units per mL.

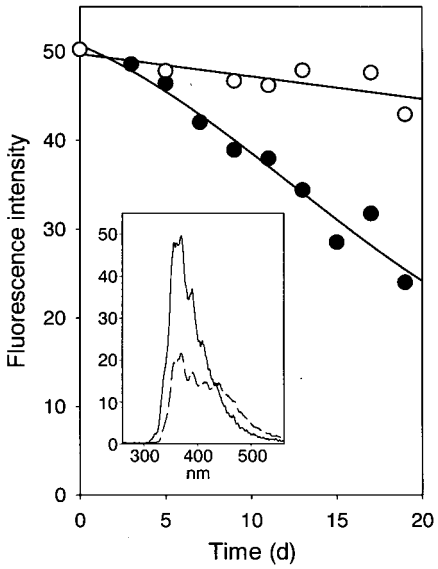

Figure 3. Change in fluorescence of 4% diesel medium in tubes with and without ADC inoculation. Data points of the ADC cultures are the mean average from two tubes inoculated with the same source culture. The inset shows a comparison of fluorescence profiles taken at $t = 0$ from a control tube (solid line) and at $t = 19$ d from an ADC-inoculated tube (dashed line).

sterile controls (data not shown). This bacterium was not found as part of the ADC.

DISCUSSION

A combination of several factors is probably responsible for the higher rate of DHC and BTEX degradation in the plant-added plots. Application of *Azolla* is known to improve the fertility of its surrounding environment.[16] *Azolla* plants are also rich in a variety of phenolic compounds,[22,23] some of which may act as co-metabolites to stimulate degradation of the structurally similar aromatic compounds found in fuel. In this report, we have shown that *Azolla* plants release a consortium of bacteria capable of significantly lowering levels of diesel fuel pollution.

Azolla as a Fertilizer, Bacterial Inoculum, and Colonization Surface

The ADC bacteria, all of which formed visible colonies after overnight incubation on nutrient agar, are most likely epiphytes since culturable endophytes from *Azolla* take approximately 5 days to form visible colonies.[21,24] The inability the *Rhodococcus* APG1 endophyte to establish itself in the diesel-degrading consortium may be due to competitive exclusion by the faster growing epiphytes. Alternatively, dying plants may not release viable endophytes.

Leaching of nutrients from decaying plants should allow for higher growth yields of the ADC bacteria as well as certain endogenous soil bacteria. The gradual release of nutrients from *Azolla* plants is preferable to the application of chemical fertilizer, which can temporarily reduce microbial activity at bioremediation sites.[25,26] Furthermore, residual nitrate or nitrite in fertilized soil returned to the environment can lead to ground water poisoning and surface water eutrophication.[12]

Phosphate supplementation appeared to stimulate degradation of BTEX (Fig. 1, Table 1). Phosphate is often a limiting nutrient for aquatic plants, especially *Azolla*,[12] but, when present in sufficient amounts, can be stored by *Azolla* plants as "luxury" phosphate.[16] The ponds from which the source plants were taken had not been fertilized and contained only 30 μM phosphate. Had the source plants been fertilized to 1.5 to 2.0 mM phosphate, the recommended level for *Azolla*,[12] the apparent phosphate limitation in the treated plots may have been relieved.

Aquatic plants increase the surface area of the water/nonaqueous phase interface, making more of the petroleum hydrocarbons susceptible to attack by microbes. Upon flooding of contaminated soil, the majority of the lighter aromatic hydrocarbons including BTEX desorb from soil particles whereas

about half of the more insoluble saturated components remain bound to the soil.[7,27,28] In the plant-added plots, increased exposure to the aromatic-degrading plant-associated ADC bacteria was likely to have been at least part of the reason for the higher rate of BTEX degradation relative to that of DHC.

Sun Exposure

Though not specifically investigated for this report, the higher relative BTEX degradation rate may also have partially resulted from the stimulation of photodegradation by the dead plant material itself. When plant organics undergo photolysis they generate free radicals, such as the alkylperoxyl radical, that can oxidize aromatic rings and side chain structures of petroleum hydrocarbons.[29] The photodegradative products may be volatile or more soluble, and thus, more susceptible to biodegradation.[30] In regions such as Okinawa that receive a high solar flux, degradation by photochemical processes can approach that resulting from microbial activity.[30] However, in our experiment, plant-enhanced photodegradation was probably minimal since the plots received sun exposure only in the late afternoon hours.

An added benefit of greater radiant energy absorption would be increasing the site temperature to hasten the evaporation by volatile hydrocarbons. Also, at higher temperatures the solubility of non-volatile hydrocarbons increases, making them more available to microbes.[7] Faster evaporation of water would facilitate the cycling of flooded conditions with relatively dry (tilled) conditions. Varying the environmental redox potential in this manner may be a useful strategy for encouraging breakdown of recalcitrant compounds.[9] One study found that mineralization of methoxychlor, an aromatic insecticide typically resistant to biodegradation, could be enhanced by periodic flooding of a contaminated site.[31]

Preparations for *Azolla*-Based Bioremediation and Monitoring

Many of the same characteristics that make *Azolla* a good biofertilizer for farming also bode well for its use in bioremediation. In agricultural settings, *Azolla* is often incorporated into the soil as a carpet-like mat and then applied as a mulch several times during a growing season.[12] For our experiment, plants had to be brought by vehicle, limiting coverage of the plots to less than 20% of the total surface area. Obviously, an operational bioremediation facility should have an area for cultivating the aquatic plants.

The differential susceptibility of *A. pinnata*, *S. molesta* and *P. stratiotes* to diesel could be used to monitor cleanup efforts. The maximum sublethal

diesel concentration we have observed for *A. pinnata* (Table 2) is below that typically documented for other organisms.[32,33] The spraying of diesel has even been used to selectively eliminate nuisance populations of *Azolla*.[15] Survival of *A. pinnata* at a treatment site could serve as a prerequisite to the collection of samples for hydrocarbon analysis and the eventual return of soil to the environment.

Ideally, a full-scale bioremediation treatment facility for our needs would be able to simultaneously accommodate both diesel-contaminated soil and water. Based on our results we recommend that the design and operation of such a facility include the following: direct exposure of the treated areas to the sun, phosphate fertilization of the source plants, variations in physical conditions over time (including dry periods for tilling), and application of relatively greater amounts of plants to both the soil and water.

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