Research Article

Response of mangrove propagules to the presence of oil- and hydrocarbon-degrading bacteria during an experimental oil spill

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ABSTRACT. The aim of this work was to investigate the effect of marine diesel oil on the development and survival of three different species of mangrove propagules with or without a hydrocarbon-degrading bacterial consortium and the possible use of propagules for the recovery of mangroves impacted by oil. The study was conducted in a greenhouse, near a mangrove from which we collected samples of sediments and propagules of *Laguncularia racemosa*, *Avicennia schaueriana* and *Rhizophora mangle*. The bacterial consortium comprised *Bacillus* spp., *Rhizobium* spp., *Pseudomonas* spp., *Ochrobactrum* spp. and *Brevundimonas* spp. After six months, *L. racemosa* and *A. schaueriana* only survived in control treatments and *R. mangle* showed the highest survival rates of the three species, indicating that different mangrove species do not respond uniformly to oil spills. Propagules of *R. mangle* are much more resistant and the hydrocarbon-degrading bacterial consortium we tested can be applied in the phytoremediation of pollutants.

Keywords: *Laguncularia racemosa*, *Avicennia schaueriana*, *Rhizophora mangle*, esterase enzyme, dehydrogenase enzyme, pollution, bioremediation.

INTRODUCTION

Mangroves are coastal wetlands that provide a range of environmental services including: protection from coastal erosion, buffering of pollutants, efficient mobilization of carbon and energy, and nutrient recycling (Othman, 1994; McKee & Faulkner, 2000; Chmura, 2003; Moberg & Rönnbäck, 2003; Valiela *et al.*, 2004). This ecosystem serves as a feeding and reproductive area and provides protection for a wide range of organisms, many of which are of special commercial importance to humans (Lee & Shang-Shu, 2004). Despite their great ecological and economic importance, mangroves are one of the principle habitats threatened by human actions (Burns *et al.*, 1993; Li *et al.*, 2009). Anthropogenic practices such as industrial

processing, oil spills and incomplete combustion of fossil fuels have caused an accumulation of polycyclic aromatic hydrocarbons (PAHs) in the environment (Chang *et al.,* 2008) and, because mangroves are coastal ecosystems, they are among the primary locations where oil spills are concentrated.

The impact of oil on mangroves, as well as on other ecosystems, is related to the type of pollutant, amount spilled, toxicity, deposition pattern, retention time and the prevailing climatic conditions and tides. When petroleum and its derivatives reach mangroves, their physical and toxicological effects may be acute (*e.g.*, defoliation or death of fauna) and/or chronic (*e.g.*, reduced plant reproduction, seed survival, or faunal population size) (Burns & Codi, 1998; NOAA, 2002). Pollutants that reduce the survival or growth of seedlings

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may impact the regeneration rate or species composition of disturbed mangroves (Proffitt *et al.,* 1995).

Microbial degradation is believed to be one of the major processes remediating PAH-contaminated mangrove sediments (Hughes *et al.,* 1997). Previous studies have reported that indigenous microbial communities can exhibit considerable potential to assist oil-contaminated sediment recovery (Ramsay *et al.,* 2000). The complexity of the metabolic processes needed to degrade PAHs suggests that no single species of microorganism can completely degrade petroleum. Instead, it is likely that petroleum degradation occurs more efficiently when carried out by complex microbial consortia (Komukai-Nakamura *et al.*, 1996; Sugiura *et al.*, 1997; Alexander, 1999; Crapez *et al.*, 2002; Brito *et al.*, 2006). In nature, biodegradation of oil typically involves the activities of a succession of species with broad enzymatic capabilities within the microbial consortium (Komukai-Nakamura *et al.,* 1996; Foght *et al.,* 1999). Bioremediation of polluted sediments using oil-degrading and plant growth-promoting bacteria (phytoremediation) is considered to be a less invasive environmental cleanup approach compared to chemical solutions (Korda *et al.,* 1997; Crapez *et al.,* 2002).

Phytoremediation is an inexpensive strategy, especially compared to the removal and relocation of contaminants. The benefits of phytoremediation include healthier soil, and promotion and preservation of the indigenous microbial communities that are essential to long-term soil bioremediation (Pilon-Smits, 2005; Mendez & Maier, 2008; Wang *et al.*, 2008). Many studies in the literature have reported the application of phytoremediation technologies for the recovery of environments impacted by oil (Atlas, 1981, 1995; Balba *et al.*, 1998; Duke *et al.*, 2000; Ramsay *et al.*, 2000; Moreira *et al.*, 2011, 2013). However, studies focused on the recovery of mangroves impacted by oil are scarce (Burns *et al.*, 1999; Ramsay *et al.*, 2000; Ke *et al.*, 2003; Brito *et al.*, 2009), especially studies using mangrove propagules (Proffitt *et al.*, 1995; Proffitt & Devlin, 1998; Ye & Tam, 2007; Zhang *et al.*, 2007). Here, in a greenhouse experiment, we investigated the effect of marine diesel oil on the development and survival of three different species of mangrove propagules, with or without hydrocarbon-degrading bacterial consortia, and assessed the potential use of these propagules for the recovery of mangroves impacted by oil.

MATERIALS AND METHODS

Experimental design: bioassays on mangrove propagules

All bioassays were conducted in a greenhouse, near to the mangrove from which sediment samples were collected (Suruí Mangrove, Guanabara Bay, RJ, Brazil; 22º40'S, 43º06'W). We used a distillate of marine diesel, marine diesel oil (MDO), in our bioassays that has a lower cetane index and a higher density than marine diesel. MDO has a sulfur content of between approximately 0.3 and 2.0 m/m % (EPA, 1999). Propagules of *Laguncularia racemosa*, *Avicennia schaueriana* and *Rhizophora mangle* were harvested from mangrove swamps in Rio de Janeiro, Brazil and planted in perforated plastic bags (30×18 cm) filled with 0.8 kg half sandy and half muddy fresh sediments for cultivation in a greenhouse. Propagules were subjected to three different treatments: 1) Control, 2) MDO, and 3) MDO and a hydrocarbon-degrading bacterial consortium (MDO & HDB). For the control, mangrove propagules did not receive any treatments. For the MDO treatment, propagule rhizospheres received 3% MDO. For the MDO & HDB treatment, propagule rhizospheres received 3% MDO and an inoculation of 10^7 cells of the hydrocarbon-degrading bacterial consortium (see below). Bioassays were conducted over the course of six months and parameters were quantified at the beginning of the bioassay (T0) and every two months thereafter (T2, T4, T6). A total of 450 propagules in the perforated plastic bags was set up for each treatment, comprising 150 propagules of each of the three mangrove species. Propagule growth was evaluated by measuring their height, diameter and number of leaves of all propagules. The height of propagules was measured from where the stem emerged to the bottom of the most distally-opened pair of leaves. Diameter was determined at the midpoint of the lowest inter-node using a digital caliper. The number of emerged leaves was counted manually.

Temperature, pH and oxyreduction potential (Eh) were obtained *in situ* using specific electrodes (YSI 556 MPS, Multi Probe System) and they were measured at the same time on each sampling day. Sediment samples were collected at random from three locations in the rhizosphere of each propagule. These sediment samples were collected using a stainless steel spoons and were immediately placed in a separate clean glass pot. These samples were conditioned on ice and taken to the laboratory for analysis.

Isolation and taxonomic identification of the hydrocarbon-degrading bacterial consortium (HDB) and bacterial analysis

We collected sediment samples from Suruí Mangrove; an environment impacted by MDO (Fontana *et al.,* 2010). These samples were stored for 2 h in sealed polythene bags, conditioned on ice, and taken to the laboratory for analysis. Sediment samples (1 g) were

distributed in Erlenmeyer bottles containing 9 mL of filtered seawater (0.45 μm pore), urea (5 g L^{-1}), starch (10 g L^{-1}), and MDO (2 mL L^{-1}). Samples were incubated for 15 days at room temperature. Aliquots of the liquid medium were then plated onto solid medium with red Congo agar (0.08%) for 15 days (Krepsky *et al.*, 2007), after which the reddish-pink colonies were selected and reinoculated into liquid medium to attain a bacterial biomass of 10^7 cells. This medium was added with a graduated cylinder to propagule rhizospheres once a month for six months. Species within the bacterial consortium were identified once bioassays were completed (T6).

DNA extraction of 30 mL HDB cultures was performed using a PureLink^{TM} Genomic DNA Mini Kit (Invitrogen) according to the manufacturer's instructions. Extracted DNA was analyzed in 1% agarose gels and quantified using a Nanodrop^{TM} spectrophotometer (Thermo Fisher Scientific Inc.). Small subunit (16S) rRNA genes were amplified by PCR using the primer pair 27F-1401R (Lane, 1991) and a PCR Master mix (Promega) was prepared according to the manufacturer's instructions. PCR conditions were: 95°C for 5 min; 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 5 min. All amplicons were analyzed in a 1.5% sybr-safe-stained agarose gel and were cloned with a TOPO TA cloning kit (Invitrogen Ltd.) according to the manufacturer's instructions. Cloning products were re-amplified by PCR with the primer pair M13F-1401r. PCR-amplified vector inserts of the correct size were purified with a PureLink™ PCR Purification Kit (Invitrogen). A total of 50 cloning products of each sample were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) by the company Genomic Engenharia Molecular (Brazil), using the universal primer T7. DNA sequences were assembled with the Bio-Edit Sequence Alignment Editor and all trimming, clustering and classifications were performed in Mothur (Schloss *et al.,* 2009). Sequences were compared using the BLASTX algorithm against the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) database with the Ribosomal Database Project (RDP) (Wang *et al*., 2007; Cole *et al*., 2009).

Bacterial cells was enumerated by epifluorescent microscopy (Axiosp 1, Zeiss, triple filter Texas Red-DAPI-fluorescein isothiocyanate, 1000x magnification) and using fluorochrome fluorescein diacetate and UV-radiation (Kepner & Pratt, 1994). Esterase activity (EST) was analyzed according to Stubberfield & Shaw (1990). Dehydrogenase activity (DHA) was analyzed using the method described by Houri-Davignon & Relexans (1989). Kruskal-Wallis analysis was used to test the significance of differences among treatments and times for each species of mangrove propagule. All microbial analysis was done in triplicate and data computation employed the Statistic 10.0 software. The significance level was $P \leq 0.05$ and the results are shown in Table 1.

RESULTS

Our analysis at the end of the experiment revealed that the bacterial consortium was formed by five bacterial species: *Bacillus* spp., *Rhizobium* spp., *Pseudomonas* spp., *Ochrobactrum* spp. and *Brevundimonas* spp. This bacterial consortium can utilize oil as a source of carbon and energy.

Temperature was not significantly different among the three treatments ($P > 0.05$) (Table 1), but temperature varied over time ($P \leq 0.05$), ranging from 25.37 ± 0.27°C (T0 - Control) to 27.00 ± 0.06°C (T2 - MDO & HDB) for the treatment of *R. mangle*; from 25.37 ± 0.27°C (T0 - Control) to 27.10 ± 0.21°C (T4 - MDO & HDB) for *L. racemosa* and from 25.63 ± 0.15°C (T0 - MDO & HDB) to 26.90 ± 0.32°C (T6 - MDO & HDB) for *A. schaueriana* (Fig. 1a). The pH of the rhizosphere of propagules also did not differ significantly between treatments and times ($P > 0.05$) (Fig. 1b). Eh was positive (Fig. 1c), and results were significantly different over time for all mangrove propagules ($P \leq 0.05$, Table 1), with highest mean found in T4 (MDO) for the treatment of *R. mangle*, in T6 (MDO & HDB) for *L. racemosa* and in T4 (MDO & HDB) for *A. schaueriana*, and the means were 258 ± 1 mV, 247 ± 0.3 mV and 264 ± 2 mV, respectively.

After two months (T2), *A. schaueriana* exhibited the highest mortality in all three treatments (86, 80 and 90% in control, MDO and MDO & HDB treatments, respectively). After six months (T6), *L. racemosa* and *A. schaueriana* only survived in the control treatment (50 and 10%, respectively). At T6, *R. mangle* had the highest survival rates of the three species; 66% in the control treatment, 48% in the MDO treatment, and 59% in the MDO & HDB treatment.

At T6, heights of *L. racemosa* were the lowest in control (3.18 ± 0.20 cm) (Fig. 1d). *R. mangle* was tallest at T6, with mean values of 25.32 ± 0.80 cm for the control treatment, 25.40 ± 0.83 cm for the MDO treatment, and 27.17 ± 0.76 cm for the MDO & HDB treatment. At T6, *A. schaueriana* survived only in the control, with a mean height of 34.40 ± 2.99 cm. These differences in heights among species were significant over time and between treatments (both $P \leq 0.05$, Table 1). Propagule diameters (Fig. 1e) were significantly different over time and between treatments for the three species ($P \leq 0.05$, Table 1).

Table 1. Kruskal-Wallis summary (*P*-values) of parameters analyzed during bioassays of mangrove propagules impacted by oil. Significant effects are indicated in bold. EST: Esterase activity, DHA: Dehydrogenase activity.

Species	Factors	Temperature	Eh	pH	Height	Diameter	Number of leaves	EST	DHA	Bacterial density
R. mangle	Times	**0.0002**	≤ 0.05	0.6567	≤ 0.05	≤ 0.05	≤ 0.05	**0.0348**	≤ 0.05	≤ 0.05
	Treatments	0.9825	0.4317	0.0641	**0.0175**	≤ 0.05	**0.0183**	**0.0116**	0.7577	0.7734
L. racemosa	Times	**0.0003**	**0.0053**	0.4251	≤ 0.05	≤ 0.05	≤ 0.05	**0.0001**	≤ 0.05	≤ 0.05
	Treatments	0.9014	0.1212	0.1457	≤ 0.05	≤ 0.05	≤ 0.05	0.2450	0.4373	0.6353
A. schaueriana	Times	**0.0006**	≤ 0.05	0.8157	≤ 0.05	≤ 0.05	≤ 0.05	**0.0087**	**0.0001**	≤ 0.05
	Treatments	0.8197	0.3359	0.6491	**0.0406**	**0.0032**	**0.0462**	0.1309	0.6593	0.5801

By the end of the first two months (T2), all of the species had produced leaves under each of the three treatments (Fig. 1f). Thereafter, production of leaves was significantly different over time and between treatments for the three species ($P \leq 0.05$, Table 1). In the control treatment, only for *A. schaueriana* did leaf production increase after two months; for the other two species, leaf production was constant from T2 until the end of the study. Leaf production was mostly similar for all species under MDO and MDO & HDB treatments.

Esterase activity in the rhizosphere of the propagules was similar for the treatments of *L. racemosa* and *A. schaueriana*, but there were significant differences among the three treatments for *R. mangle* ($P = 0.012$, Table 1). In general, the control and MDO & HDB treatments showed higher esterase activity compared with the MDO treatment. For the MDO & HDB treatment, highest esterase activities were recorded at T2 for all species (Fig. 1g). Mean values of esterase activity ranged from 0.07 ± 0.03 µg fluorescein g^{-1} h^{-1} at T4 for the MDO & HDB treatment of *A. schaueriana* to 2.31 ± 0.12 µg fluorescein g^{-1} h^{-1} at T2 for the MDO & HDB treatment of *R. mangle*. Dehydrogenase activities were highest at the beginning of the experiment (T0), but decreased thereafter (Fig. 1h). Mean values of dehydrogenase activity ranged from 0.001 ± 0.001 mg INT-F g^{-1} (*A. schaueriana* - MDO at T6) to 0.041 ± 0.002 mg INT-F g^{-1} (*L. racemosa* - control at T0), with mean values only differing significantly over time ($P \leq 0.05$, Table 1). Bacterial density in all propagule rhizospheres increased up to the end of bioassays (Fig. 1i). The results ranged from $2.76 \times 10^1 \pm 3.17 \times 10$ cells cm^{-3} (all mangrove species - MDO & HDB at T0) to $7.90 \times 10^9 \pm 4.12 \times 10^8$ cells cm^{-3} µC cm^{-3} (*R. mangle* - MDO & HDB at T6), with significant differences over time ($P \leq 0.05$, Table 1).

DISCUSSION

Our results reveal that mangrove species respond differently to simulated oil spills. We found that *R. mangle* coped much better under an oil spill scenario than the other two species, and this finding is in agreement with a report showing that propagules of *R. mangle* are resistant to dispersed and undispersed oil (Proffitt *et al.*, 1995). Oil coating propagules probably forms a blockage, resulting in oxygen deficiency and suffocation (Getter *et al.*, 1985; Snedaker *et al.*, 1996). The resistance of *R. mangle* to the effect of oil is possibly due to their ability to prevent oil uptake by their roots (Getter *et al.*, 1985), in a similar way to how this species excludes salt (Tomlinson, 1986; Touchette *et al.*, 1992). The high survival of *R. mangle* in our bioassay indicates that propagules of this species are well adapted to the conditions found in contaminated sediments.

L. racemosa and *A. schaueriana* survived and grew only under our control conditions, but *R. mangle* exhibited steady growth under all three treatments until the end of the study. The effects of oil on survival and growth rates of *L. racemosa* were also tested by Nardes *et al.* (2013), who found that height and diameter of propagules were significantly higher for the control specimens compared to oil-treated specimens. This same pattern was observed by Proffitt *et al.* (1995) in an experiment on the development of *R. mangle* impacted by oil. Other studies on the genus *Avicennia* have shown that oil-treated individuals were shorter than controls (Proffitt *et al.*, 1995; Ye & Tam, 2007; Naidoo *et al.*, 2010).

Toxicity may also differ among mangrove species. For example, along the coast of São Paulo (Brazil), an oil spill caused defoliation of 25.9% in *R. mangle*, 43.4% in *L. racemosa*, and 64.5% in *A. schaueriana* (Lamparelli *et al.*, 1997). Nardes *et al.* (2013) found for *L. racemosa* that, in the first seven weeks of bioassay, defoliation and mortality was significantly higher in plants subjected to all the different oil treatments compared to the control group.

Esterase hydrolyzes organic matter and produces monomers and oligomers (Weiss *et al.*, 1991), which
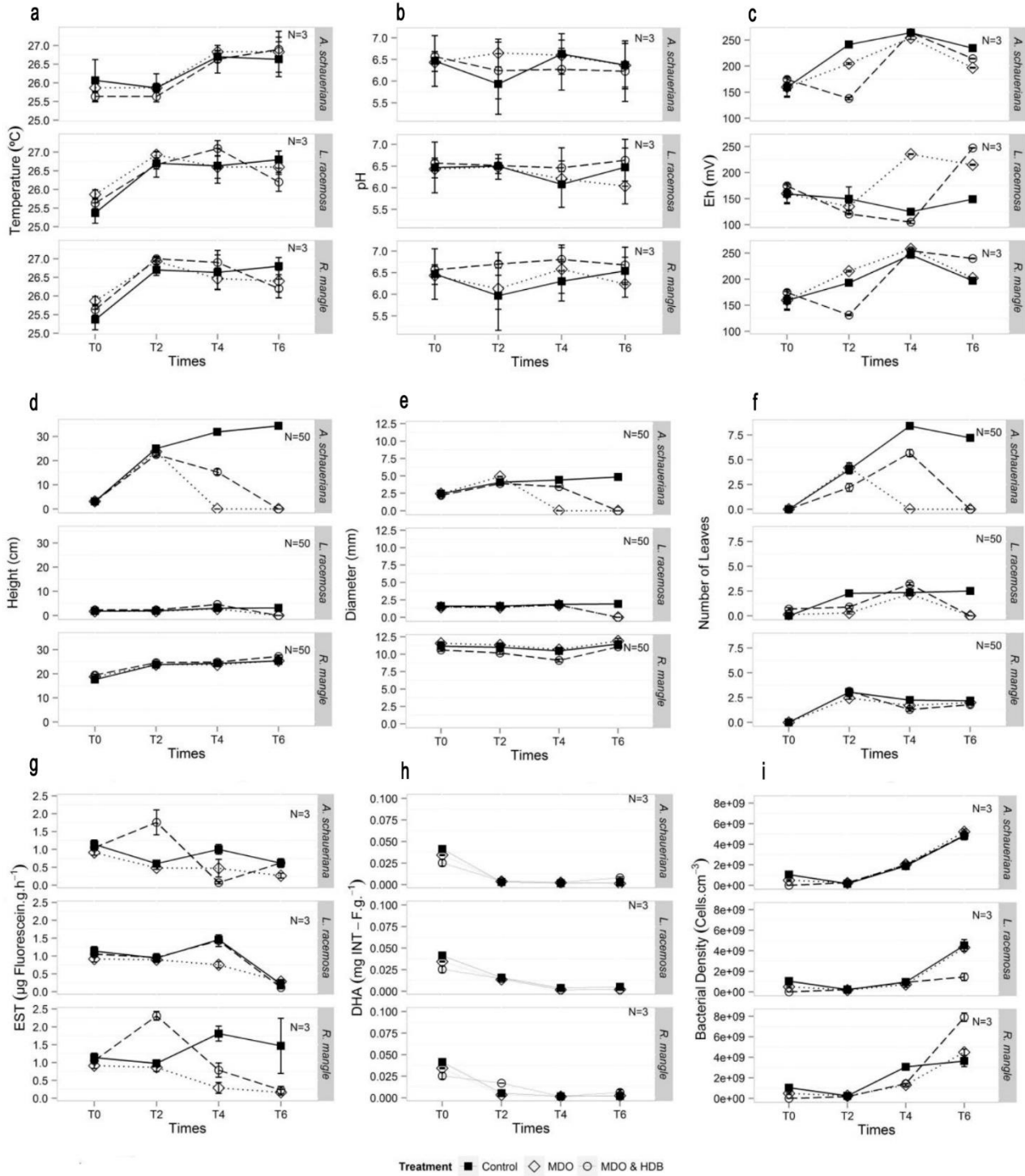
Figure 1. a) Temperature, b) pH, c) Eh, oxyreduction potential, d) height, e) diameter, f) number of leaves, g) esterase activity (EST), h) dehydrogenase activity (DHA) and i) bacterial density of propagules of *A. schaueriana, L. racemosa* and *R. mangle* impacted by marine diesel oil (MDO) with or without a hydrocarbon-degrading bacterial consortium (HDB).

enter bacterial cells to be oxidized by dehydrogenases, thereby providing the energy necessary to proliferate (Meyer-Reil & Koster, 2000; Fenchel *et al.*, 2012). However, in the absence of a mature root, interactions between bacteria and propagules are reduced (Bais *et al.,* 2004), and in our study the bacterial community was not able to promote the growth of propagules, with consequent high mortality under the MDO and MDO &

HDB treatments. Most of our dead propagules exhibited almost no epicotyl or root system expansion, as observed by Scherrer (1988) during a simulated oil spill experiment. Thus, inhibition of root development by oil compounds may induce mortality in propagules planted on polluted sediments (Scherrer & Blasco, 1989).

Our data suggest that MDO significantly affects the development and survival of propagules of *L. racemosa* and *A. schaueriana*. On the basis of our results, neither of these species should be planted on MDO-contaminated sediments because the success of phytoremediation depends on the ability of the plants and microbes to tolerate and survive in the sediment. In contrast, the high survival of *R. mangle* propagules is linked to the physiology of this species, which has a stronger capacity to develop and survive in oil-contaminated sediments. Thus, propagules of *R. mangle* could be used for phytoremediation of mangroves impacted by oil. However, further studies are needed to investigate the role of roots and associated hydrocarbon-degrading bacteria.

ACKNOWLEDGEMENTS

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