Suitability of different salt marsh plants for petroleum hydrocarbons remediation

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A B S T R A C T

The suitability of the salt-marsh species Halimione portulacoides, Scirpus maritimus, Juncus maritimus and an association of the last two for remediation of petroleum hydrocarbons (PHC) in soil was investigated. An outdoor laboratory experiment (microcosm-scale) was carried out using contaminated soil collected in a refinery, as a complement of another study carried out in the refinery environment (mesocosm-scale). Soil samples with old contamination (mainly crude oil) and with a mixture of the old and recent (turbine oil) contamination were tested. Studies in both micro- and mesocosm-scale provided results coherent in substance. The presence of S. maritimus caused removal of old contamination which was refractory to natural attenuation (after 7 months of exposure, efficiency was 13% when only old contamination was present and 40% when the soil also contained recent contamination). H. portulacoides (only included in the microcosm-scale study) revealed also potentiality for PHC remediation, although with less efficiency than S. maritimus. Degradation of recent contamination was also faster in the presence of plants (after 7 months: 100% in the presence of S. maritimus vs. 63% in its absence). As these species are common in salt marsh areas in Atlantic coast of Europe, it is probable they will be also useful for recovering coast sediments. In contrast, J. maritimus and association did not reveal capability to remove PHC from soil, the presence of J. maritimus inhibiting the capability of S. maritimus.

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1. Introduction

Spills, discharges and leaks of petroleum hydrocarbons (PHC) represent a humankind problem, since contamination can be easily spread and damage the environment, sometimes with disastrous consequences. Therefore, elimination of contamination is necessary and it should take place as soon as possible after its occurrence and, whenever possible, the remediation technology used should not damage the structure of the soil. Biological remediation (that is, the use of microorganisms and/or plants for decontamination purposes) in addition to be a non-destructive natural option, it is at the same time cost-effective. Contaminant-degrading bacteria can be found, virtually, in all soils (Gerhardt et al., 2009) and start to proliferate when a contamination occurs as they can use PHC as a carbon source. The action of indigenous microorganisms in removing contamination is usually called “natural attenuation”. To deserve a practical application, any induced bioremediation process should demonstrate that the degradation rate is greater than the natural rate of decontamination (Bento et al., 2005).

The use of vegetation to improve removal of PHC has been extensively studied (Phillips et al., 2006; Euliss et al., 2008; Peng et al., 2009; Phillips et al., 2009) because it will have the additional advantage of being a sustainable approach as it is solar driven (Cheng et al., 2008). Vegetated soil generally presents higher numbers of microorganisms when comparing to bulk soil (Macek et al., 2000; Hutchinson et al., 2003; Kaimi et al., 2008; Glick, 2010) as plants can exude specific organic substances (Macek et al., 2000; Banks et al., 2003) that can be used as carbon and energy sources by microbial communities (Chaudhry et al., 2005). A positive effect of plants is the possibility to enhance the chemical extractability and perhaps biological availability of initially unextractable molecules (Liste and Prutz, 2006), especially in aged or weathered soil where PHC bioavailability is significantly reduced (Banks et al., 2003).

Most of the reported studies have demonstrated that the presence of plants could contribute for removal of contamination stimulating soil bioremediation, particularly in soil adjacent to the root (Macek et al., 2000; Gerhardt et al., 2009; Peng et al., 2009; Glick, 2010). However, some tested plant species have shown no potential in terms of enhance PHC remediation (Lalande et al., 2003; Euliss et al., 2008). Information on suitability of salt marsh plants for PHC remediation is hard to find in the literature.

The present work aimed at investigating the potential of different salt-marsh halophytes (or facultative halophytes) to improve degradation of PHC present in soil from a petroleum refinery which had suffered a contamination by crude oil a few years ago (it is unknown the exact date). Salt-marsh plants were chosen for this purpose...
because they tolerate salinity, which will make them potentially suitable for remediation of PHC in coastal environments, where petroleum refineries are often installed and accidental oil spills sometimes occur. Two different situations were tested, in parallel: (i) soil with the old contamination (crude oil) mentioned above which had been contaminated with turbine oil 21 months before the beginning of this work; and (ii) the soil used in (i) but recontaminated with turbine oil just at the beginning of this work. An outdoor pot experiment was conducted to evaluate the effect of each one of the three following species: *Halimione portulacoides* (from Chenopodiaceae family), *Scirpus maritimus* (from Cyperaceae family) and *Juncus maritimus* (from Juncaceae family), as well as an association of the *S. maritimus* and *J. maritimus*. We decided to include this plant association because, despite most of the phytoremediation studies have been based on a single plant species, when plants grow as a multi-species mixture, the interaction between roots of different species may alter root physiology and colonization capacity (Cheema et al., 2010). Therefore, combinations of root types and exudate patterns have been assumed to allow greater infiltration and stimulation of microbial communities (Cheema et al., 2010), which are factors that may favour bioremediation of contaminants.

The different plant species used are very common in salt marsh areas in the Atlantic coast of Europe. The remediation potential of these species has been already tested for metals (Almeida et al., 2006; Reboreda and Caçador, 2007; Almeida et al., 2009) and tributyltin (Carvalho et al., 2010) but for PHC available data are scarce (Couto et al., 2011a).

This work in microcosm-scale was carried out as a complement of another one performed in mesocosm, which was focused on the same soil (with weathered contamination mixed with recent contamination by turbine oil), but took place in the environment of the oil refinery where there was the contaminated soil being studied in this work. Similarities and differences of the results obtained in the two studies are also discussed here.

2. Experimental

2.1. Phytoremediation trials

About 40 kg of soil from a petroleum refinery, which had been subjected to contamination by crude oil a few years ago and contaminated, again, with turbine oil 21 months ago, were collected in March 2009. Soil was divided in two parts. One part was left as it was, that is with 10.4 ± 0.9 mg g⁻¹ of total petroleum hydrocarbons (TPHC) (from now on called as old contamination). The remaining soil was again contaminated with turbine oil, the final concentration (mixture of old and recent contamination) being of 30 ± 2 mg g⁻¹ TPHC. Re-contamination was performed manually with physical homogenization, to prevent the use of solvents (e.g. acetone) that could lead to a reduction of the indigenous microorganisms of the soil (Couto et al., 2010), and soil remained untreated for 2 d (until the absence of perceptible odor).

Twenty-four pots of 1.5 L of capacity were used for remediation trials. Small stones and aluminium foil were pierced in the bottom to allow water circulation and prevent colmatation. The pots were placed in the exterior of the laboratory building in a non-covered area. Each pot was filled with about 660 g of contaminated soil. Three pots with old contaminated soil and three other ones with soil with a mixture of old and recent contamination remained non-vegetated to be used as controls (natural attenuation). The remaining pots received transplants of the selected plants: one foot of *H. portulacoides*, three feet of *S. maritimus*, five feet of *J. maritimus* or four feet of *J. maritimus* together with two feet *S. maritimus* (in association). Three replicates of each experiment were carried out. All the plants were collected in Douro River estuary (near the refinery from where the weathered soil came from). Plants were transplanted to the pots without a previous washing of the roots with the purpose of diminish plant stress.

Biweekly, all the pots were supplied with one quarter-strength modified Hoagland nutrient solution (Hoagland and Arnon, 1950). After 7 and 14 months of exposure soil samples from each pot were collected by using a small shovel, placed in aluminium foil and immediately refrigerated in an ice chest. Only the fraction at the level of the root system was used for analysis, as in studies carried out in a microcosm scale the surface layer of the soil is much influenced by abiotic factors. Samples were conserved in two different ways: frozen at −20 °C for determination of TPHC and refrigerated for 2 h for microbial analysis, which was carried out within a maximum of 24 h to avoid artificial results.

2.2. Material and methods

2.2.1. Reagents, material and equipment

Sampling material was previously washed with Teepol and rinsed several times with deionized water followed by ethanol washing as additional precaution.

The glass material used in the sample’s handling was firstly washed with Teepol and deionised water and then maintained in a 13% (v/v) HNO₃ solution for at least 24 h. After being washed with deionised water, vials and septa were dried and maintained in the oven at 40 °C, and the remaining required material was dried at room temperature.

2.2.2. Total petroleum hydrocarbons analysis

Soil was dried at room temperature and only the fraction <2 mm was analysed, since smaller particles have higher surface area and therefore much greater capacity to adsorb contaminants.

Fourier transform infrared spectrophotometry (FTIR) was used to quantify the level of TPHC in soil. A Jasco FT/IR spectrophotometer (460 Plus model) provided with a quartz cell of 10 mm path length from Starna Scientific was used for this purpose. A method previously optimized (Couto et al., 2011b) was used. Briefly, soil sample (1 g) was homogenized with anhydrous sodium sulphate (1 g) and with 10 mL of tetrachloroethylene and submitted to a one step 30 min ultrasonic extraction (ultrasonic bath Elma, Transonic 460/H). After centrifugation (Mixtase, Selecta) a cleaning step of the liquid phase with 0.3 g of silica gel (deactivated with 2% of water (Wwater/Wsilica gel)) was carried out (silica gel addition, agitation for 10 min and filtration through glass wool) in order to remove interference compounds. An increased number of extraction steps were tested but without any advantage (Couto et al., 2011b).

For estimating TPHC concentration in the extract, calibration standards were prepared from a stock standard solution mixture containing equal volumes of isooctane and hexadecane and the respective absorbance was measured at the maximum near 2926 cm⁻¹ after baseline correction (using pure solvent for background spectra and scanning the range 2700–3200 cm⁻¹). TPHC in the soil extract was quantified by direct comparison with the calibration curve, after suitable dilution with tetrachloroethylene. The quality control of the method was carried out by analysing extracts of soil samples spiked with known amount of hydrocarbons (mean recovery of 102 ± 9% (n = 4) and by analysing the certified reference material (CRM350-100, TPHC in Sandy Loam Soil (C6–C35)) (Couto et al., 2011b). Limits of detection and of quantification were 63 mg kg⁻¹ and 210 mg kg⁻¹, respectively. Soil extract of each replicate were treated separately, each one being analysed in triplicate.

Gas chromatography with flame ionization detection (GC-FID) was used to obtain information on the profile of PHC distribution between C1₀ and C₄₀ in the soil. A Varian 3800 Gas Chromatograph provided with a column VF-5ht (Varian Factor Four)
30 m × 0.25 mm × 0.25 μm. was used for this purpose as well as a protocol adapted from the literature (Saari et al., 2007) but using 1 g of soil and 1 g of sodium sulphate for drying and ultrasonic extraction (30 min) instead mechanical shaking. Six millilitre of the extract (acetone:n-hexane (1:1(v/v))) was solid phase extracted (Florisil). The chromatographic program was the one reported by Saari et al. (2007).

2.2.2.1. Microbial analysis. Culturable hydrocarbon degraders were enumerated using a modified most probable number (MPN) method (Wrenn and Venosa, 1996).

This analysis was carried out (in triplicate for each replicate of the different treatments) at the beginning of the study and after 7 and 14 months of soil exposure in the pots. Sterile 96-well microtiter plates were used and for each plate two rows of five wells served as sterile control. A 180 μL Bushnell–Haas (B–H) solution and 5 μL of crude oil or turbine oil (used as the selective substrate for determination of specific degraders) were added to each well. For each sample, 1 g of soil was mixed with 1 mL of B–H, vortex and allowed to sediment. Tenfold serial dilutions were performed, and the plates were inoculated by adding 20 μL of each dilution to five rows of seven wells.

The samples were incubated, in the dark, at room temperature for two weeks. 50 μL of INT (3 g L⁻¹) were added to each well. Positive results were scored and analysed as described in literature (Wrenn and Venosa, 1996) after overnight incubation.

2.3. Statistics

Both TPHC concentration and MPN data were subjected to analysis of variance (ANOVA) and then compared by Tukey multiple range test at p < 0.05.

3. Results and discussion

GC–FID chromatograms were drawn to get some information on the distribution of PHC in the target interval (between C₁₀ and C₄₀) (Fig. 1A). Chromatograms for crude oil and turbine oil were included for comparison (Fig. 1B). A joint analysis of these two Figs. shows that in the soil with a mixture of old and recent contamination the turbine oil fraction stood out at much higher levels than crude oil, as expected, whereas in the soil with old contamination the presence of turbine oil was not evident, suggesting that most of the contamination with turbine oil that took place 21 months ago had been already removed by natural attenuation. This result is consistent with the fact the initial level of TPHC determined in the soil with old contamination (10.4 ± 0.9 mg g⁻¹) was identical to the level of TPHC in the soil of the refinery before contamination with oil of turbine 21 months ago (about 10 mg g⁻¹).

Effects of the different plants and the association of plants on the levels of TPHC in the soil are shown in Table 1 and the initial and final MPN of culturable hydrocarbon degraders in the different pots are presented in Table 2.

3.1. Remediation efficiency in soil with only old contamination

Table 1 shows that, in the soil with old contamination, after 7 months of exposure (from March to October) statistically significant removal of PHC was observed in the presence of S. maritimus (TPHC concentration was 13% lower than the initial level). A similar tendency was observed for H. portulacoides (contamination 10% lower than the initial one). In contrast, J. maritimus, and the association of J. maritimus and S. maritimus did not have any measurable effect on PHC remediation, which indicates that both the nature of the plant and environmental conditions may condition remediation efficiency.

Natural attenuation was not observed, which suggested that most of the bioavailable PHC fraction had been already degraded by indigenous microorganisms when this work started. PHC degraders are ubiquitous in soil environment (Leahy and Colwell, 1990) and the physiologically adapted microorganisms could use the available PHC as carbon source (Couto et al., 2010), thereby degrading them. Very probably crude oil degraders began to establish when the crude oil spill occurred and, later on, when the field. This result is compatible with a strong reduction of plant activity during fall and winter seasons.

MPNturbine oil was one or two orders of magnitude higher than those observed in non-vegetated soil, similarly to that mentioned in previous works (Glick, 2010). Microorganisms living in soil near the roots can display a greater range of metabolic capacities (Alkorta and Garbisu, 2001), providing different benefits to the plants, which vary from synthesis of compounds that protect plants by decreasing plant stress hormone levels to chelators for delivering important nutrients to plants or degrading contaminants before they can negatively impact the plants (Gerhardt et al., 2009). However, despite the symbiotic plant-microorganisms from the rhizosphere may have favored the development of the microbial community, only S. maritimus (and H. portulacoides) were able to cause, directly or indirectly, PHC bioavailability to allow the occurrence of some PHC degradation.

Higher MPNCrude oil than MPNturbine oil (10⁻⁷ vs. 10⁻⁶) was observed at the beginning of this work, which may be explained by the fact that the crude oil contamination occurred long before (some years ago) than contamination by turbine oil (21 months ago).

Chemical soil analysis was repeated after 14 months of exposure and the found TPHC levels were not significantly different from those observed after only 7 months of exposure (therefore, they are not shown). The absence of natural attenuation was expected by the reasons given above. The absence of remediation efficiency by the plants (particularly S. maritimus), in the period that embraced mainly fall and winter, was probably a result of the reduced activity of the plant. Regarding the number of hydrocarbon degraders, the differences between vegetated and non-vegetated soil were much smaller after 14 months of exposure. This was evident for MPNturbine oil in some cases and for both MPNCrude oil and MPNturbine oil in other cases (Table 2), and is compatible with a strong reduction of plant activity during fall and winter seasons.

3.2. Remediation efficiency in soil with a mixture of old and recent contamination

In contrast with what was observed in the soil with old contamination, in the soil containing also recent contamination significant and marked PHC degradation was observed in all pots after 7 months of exposure (the results are included in Table 1). PHC removal was higher in vegetated pots than in non-vegetated ones (natural attenuation only), indicating that the plants direct or indirectly favoured PHC degradation. This was particularly notorious in the pots that contained S. maritimus or H. portulacoides, whose effectiveness was, respectively, 37% and 22% higher than natural attenuation (Table 1). These two plants were the only that favored PHC removal in the soil with only old contamination, confirming
that the ability of plants to assist contaminant biodegradation varied among plant species, as it has been observed in other cases (Muratova et al., 2003; Olson et al., 2003; Siciliano et al., 2003; Liste and Prutz, 2006). *S. maritimus* was able of causing removal of 79% of the initial TPHC contamination. In addition to the recent contamination, *S. maritimus* could also remove about 40% of the older contamination, which is a remarkable result and corroborated the tendency observed for the soil with old contamination only. Again, association of *S. maritimus* and *J. maritimus* in the same pots (to note that these two species were associated in the site where they were collected – Douro River estuary) did not significantly increase PHC remediation relatively to natural attenuation.

It has been reported that some mixed plant populations may cause synergetic effects, displaying higher total positive effect than individual one (Cheema et al., 2010). However, in the present case, the presence of *J. maritimus* seemed to neutralize the effectiveness of *S. maritimus* by itself. The absence of improvement of hydrocarbons degradation by dual-species planting has also been reported (Phillips et al., 2006; Gaskin and Bentham, 2010), which seems to indicate that association effects will depend on plant nature, characteristics of contamination and soil and/or environmental conditions. The last factor also may explain why in experiments carried out, in parallel, in mesocosm in a refinery environment on soil containing also recent contamination with turbine oil, any positive role of *S. maritimus* was not observed after 12 months of exposure (the plant transplants took place in July, and very probably needed several months to adapt to the new environmental conditions) being, however, observed later (in the measurements carried out after 24 months of exposure, the effectiveness of *S. maritimus* was 15% higher than that resulting of natural

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**Table 1**

Levels of initial TPHC concentrations and those observed in the tested soils after 7 months of exposure at different experimental conditions.

<table>
<thead>
<tr>
<th>Treatment</th>
<th><em>S. maritimus</em></th>
<th><em>J. maritimus</em></th>
<th><em>S. maritimus</em> + <em>J. maritimus</em></th>
<th><em>H. portulacoides</em></th>
<th>Natural attenuation (control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Old contamination (Initial TPHC: 10.4 ± 0.9 mg g⁻¹)</td>
<td>9.1 (0.1) ¹⁺</td>
<td>15.2 (3.4) ²⁺</td>
<td>10.2 (0.4) ³⁺</td>
<td>9.4 (1.2) ⁴⁺</td>
<td>10.1 (2.4)</td>
</tr>
<tr>
<td>Mean TPHC removal (%)</td>
<td>13</td>
<td>0</td>
<td>23</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>Belowground biomass (g m⁻²)</td>
<td>458</td>
<td>29.7</td>
<td>318</td>
<td>36.0</td>
<td></td>
</tr>
<tr>
<td>Mixture of old and recent contamination (Initial TPHC: 30 ± 2 mg g⁻¹)</td>
<td>6.2 (1.8) ⁵⁺</td>
<td>15.6 (1.4) ⁶⁺</td>
<td>16.7 (2.3) ⁷⁺</td>
<td>10.7 (1.6) ⁸⁺</td>
<td>17.4 (2.1) ⁹⁺</td>
</tr>
<tr>
<td>Mean TPHC removal (%)</td>
<td>79</td>
<td>48</td>
<td>44</td>
<td>64</td>
<td>42</td>
</tr>
<tr>
<td>Belowground biomass (g m⁻²) ¹⁺</td>
<td>179.4</td>
<td>92.3</td>
<td>164</td>
<td>64.7</td>
<td></td>
</tr>
</tbody>
</table>

¹⁺ Mean values and respective standard deviation (between brackets, n = 3).

²⁺ Values determined at the end of 14 months of exposure. Same letters indicate statistically significant differences among treatments (p < 0.05).
Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0 month</th>
<th>7 month</th>
<th>14 month</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude oil</td>
<td>Turbine oil</td>
<td>Crude oil</td>
<td>Turbine oil</td>
</tr>
<tr>
<td>Control</td>
<td>J. maritimus</td>
<td>H. portulacoides</td>
<td>J. maritimus</td>
</tr>
<tr>
<td>7.1 ± 0.1</td>
<td>7.14 ± 0.02</td>
<td>7.0 ± 0.4</td>
<td>7.14 ± 0.02</td>
</tr>
</tbody>
</table>

The absence of positive effects on PHC remediation of J. maritimus by itself as well as of an association of S. maritimus and J. maritimus were also observed in the mesocosm study (unpublished results).

To note that at the end of the experiments with S. maritimus alone and with association of S. maritimus and J. maritimus the total belowground biomasses were markedly lower in the soil that contained a mixture of old and recent contamination than in that containing only old contamination (see Table 1). This result suggests that a higher development of root system (and therefore a higher area available for occurrence of synergistic interactions with microorganisms) may not be a determinant factor for improvement of remediation efficiency. Very probably, the smaller root system development observed in the pots that contained S. maritimus in the presence of recent PHC contamination resulted of a toxic effect caused by this contaminant in this plant species.

In non-vegetated soil, 42% of the total initial PHC concentration, which corresponded to 63% of only the more recent PHC contamination, was already removed after 7 months of exposure (natural attenuation). This fact indicated that indigenous microorganisms were able to consume, as a carbon source, most of the available turbine oil in a relatively short period of time. However, PHC degradation was slower than in the presence of plants, as either S. maritimus or H. portulacoides had removed 100% of recent contamination after 7 months of exposure.

In the study carried out, in parallel, in the refinery environment a slightly lower effectiveness of PHC removal, 33% of the total initial concentration (or 66% of only the recent contamination) was observed, in the upper soil layer (down to 10 cm depth), after 9 month of exposure (Couto et al., 2010).

Levels of TPHC in the soil were also measured after 14 months of exposure. In vegetated soil, the levels were not significantly lower than those observed after 7 months of exposure (results not shown), as was also observed for soil with only old contamination and probably by the same reason (absence of plant activity). In non-vegetated soil remediation continued, since the percentage of removal increased to 52% relatively to the initial total contamination. These results are consistent with the arguments put forward in interpreting the results obtained for the soil containing only old contamination. Microorganisms alone were efficient for removal recent PHC contamination, but from the results of this work it cannot be inferred that indigenous microorganisms by themselves will be able to degrade old contamination. The presence of S. maritimus made also possible remediation of less available contamination which seemed recalcitrant for microorganisms. It is worthy to note that the remediation efficiency of the plants for old contamination was much higher in the presence of recent contamination (mixture of old and recent contamination), very probably because the last case was favourable to proliferation of PHC degraders in the rhizosphere of the plants, as the microorganisms could use PHC as a carbon source. Indeed, Table 2 shows that both MPN_{turbine oil} and MPN_{crude oil} were significant and markedly higher after 7 months of exposure than at the beginning which did not happen when only old contamination was present. Soil vegetated by S. maritimus and H. portulacoides displayed the highest values and non-vegetated soil the lowest values. It may be caused by the release of exudates by the roots, which favoured the proliferation of PHC degraders near the roots, the effects being more marked in pots were PHC degradation was more extensive. It is known that the chemical composition of root exudates and rates of exudation differ considerably among plant species (Alkorta and Garbisu, 2001) what could influence the number and the type of microorganisms that proliferated near roots.

To note that in the beginning of the experiment, MPN_{turbine oil} and MPN_{crude oil} presented in soil with mixture of old and recent attenuation (Couto et al., 2011a)).
contamination were significantly lower than those presented in the soil with only old contamination, very probably as a result of toxic effect of the turbine oil added 2 d ago on the number of cultural hydrocarbon degraders.

After 14 months of exposure the MPN were similar or slightly higher than those observed after 7 months of exposure. However these data are hard to interpret based on the TPHC measured in the soil at that time.

4. Conclusions

This work showed that the salt marsh plants S. maritimus and H. portulacoides have potential to be used in remediation of PHC contamination in soil. In the presence of transplants of these plant species PHC removal was faster and more extensive than natural attenuation. In addition, in only 7 months of exposure, S. maritimus (the most efficient plant) was capable of removing not only the recent contamination but also 40% of the older contamination which was refractory to natural attenuation.

Results of this work (carried out in microcosms in open space) corroborate those obtained in other on the same soil that was carried out, in parallel, in mesocosm in a refinery environment (Couto et al., 2011a).

As S. maritimus (and also H. portulacoides) are very common in salt marshes in Atlantic coast of Europe, it is probable they will be also useful for recovering, in situ or ex-situ, coast sediments contaminated with PHC.

In contrast, J. maritimus and association of J. maritimus and S. maritimus did not reveal capability to remove PHC from soil, the presence of J. maritimus inhibiting anyway the capability of S. maritimus when the two plants was associated in the same pot. As the two plants are sometimes associated in the natural environment, the presence of J. maritimus might be a limitation for application of S. maritimus in PHC remediation in situ when the two plants species are associated there.

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