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«Οι σύγχρονες τεχνικές βιο-ανάλυσης στην υγεία, τη γεωργία, το περιβάλλον και τη διατροφή»

Applied and Environmental Microbiology, Feb. 2010, p. 810–819 Vol. 76, No. 3
0099-2240/10/$12.00 doi:10.1128/AEM.00534-09

Effect of Biostimulation and Bioaugmentation on Degradation of Polyurethane Buried in Soil[▽]

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Received 5 March 2009/Accepted 19 November 2009

This work investigated biostimulation and bioaugmentation as strategies for removing polyurethane (PU) waste in soil. Soil microcosms were biostimulated with the PU dispersion agent "Impranil" and/or yeast extract or were bioaugmented with PU-degrading fungi, and the degradation of subsequently buried PU was determined. Fungal communities in the soil and colonizing buried PU were enumerated on solid media and were analyzed using denaturing gradient gel electrophoresis (DGGE). Biostimulation with yeast extract alone or in conjunction with Impranil increased PU degradation 62% compared to the degradation in untreated control soil and was associated with a 45% increase in putative PU degraders colonizing PU. Specific fungi were enriched in soil following biostimulation; however, few of these fungi colonized the surface of buried PU. Fungi used for soil bioaugmentation were cultivated on the surface of sterile wheat to form a mycelium-rich inoculum. Wheat, when added alone to soil, increased PU degradation by 28%, suggesting that wheat biomass had a biostimulating effect. Addition of wheat colonized with *Nectria haematococca*, *Penicillium viridicatum*, *Penicillium ochrochloron*, or an unidentified *Mucormycotina* sp. increased PU degradation a further 30 to 70%, suggesting that biostimulation and bioaugmentation were operating in concert to enhance PU degradation. Interestingly, few of the inoculated fungi could be detected by DGGE in the soil or on the surface of the PU 4 weeks after inoculation. Bioaugmentation did, however, increase the numbers of indigenous PU-degrading fungi and caused an inoculum-dependent change in the composition of the native fungal populations, which may explain the increased degradation observed. These results demonstrate that both biostimulation and bioaugmentation may be viable tools for the remediation of environments contaminated with polyurethane waste.

The polyester polyurethanes (PU) are a diverse group of synthetic polymers with many industrial and commercial applications, including insulating and packaging foams, fibers, fabrics, and synthetic leather goods (20). These polymers contain intramolecular bonds analogous to those found in biological macromolecules (such as ester and urethane linkages), making them susceptible to enzymatic degradation and assimilation by environmental microbial communities (17, 42). The susceptibility of plastics to biodegradation is of increasing importance as the generation of plastic waste material continues to increase and plastics now comprise more than 30% of household waste in the United States (32). By exploiting the biodegradability of plastics such as PU, bioremediation by microorganisms in the environment shows great potential for reducing the burden of plastic waste.

Although the diversity of natural microbial populations often means that the potential for waste remediation exists at polluted sites, factors such as absence of electron acceptors or donors, low nitrogen or phosphorus availability, or a lack of induction of the metabolic pathways responsible for degradation can inhibit waste remediation. In these cases, addition of exogenous nutrients can enhance the degradation of waste, a process known as biostimulation. Biostimulation of *in situ* microbial communities has been used to enhance the degradation of crude oil (22, 29), tetrachloroethene (19), diesel fuel (24, 28), and polyaromatic hydrocarbons (41).

If communities native to polluted sites lack significant populations of waste degraders, microbes with the desired phenotypes can be added exogenously in a process known as bioaugmentation. This approach has been successfully used to remediate a wide range of waste products, from hydrocarbons (8, 34) to heavy metals (15, 16). Numerous PU-degrading organisms have been isolated from a range of environments (6, 9, 26, 30), and this has provided a large reservoir of organisms for potential bioaugmentation of PU waste.

This study was the first study to assess the potential of biostimulation and bioaugmentation as methods for accelerating the degradation of PU waste in the environment. The response of fungal communities in soil microcosms to (i) addition of nutrients or (ii) a large influx of PU-degrading fungi was investigated using culture-based and molecular techniques, and the effect of these treatments on the degradation of PU coupons buried in these microcosms was determined.

MATERIALS AND METHODS

Fabrication of PU coupons for burial. Sheets of 1.5-mm-thick PU were prepared as described previously (9). Rectangular coupons that were 4 by 7 by 0.15 cm were cut, resulting in a total surface area of 59.3 cm^2. The coupons were sterilized by immersion in 70% (vol/vol) ethanol prior to burial in soil.

Soil used in microcosms. Garden soil recovered from a site in Greater Manchester, United Kingdom, was used in this work. This soil was described previously during *in situ* soil PU burial experiments (9). Previous analysis revealed that this soil is a sandy gley soil of the Blackwood series (27). The soil had a pH of 5.5 and contained 43.4 g kg^{-1} organic carbon and 3 g kg^{-1} nitrogen (analysis performed by Adas Laboratories, United Kingdom).

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▽ Published ahead of print on 30 November 2009.

TABLE 1. Origins and identities of the putative PU-degrading fungal isolates used for bioaugmentation

Putative PU-degrading fungus	Origin[a]	ITS sequence accession no.	Reference
Penicillium ochrochloron	PU buried in John Innes compost	AJ509865	6
Geomyces pannorum	PU buried in sandy soil	DQ779788	9
Penicillium viridicatum	PU buried in sandy soil	DQ779779	9
Isolate 19n	PU	NA[b]	This study
Penicillium inflatum	PU buried in organic soil	DQ779783	9
Nectria hematococca	Sandy soil	DQ779785	9
Isolate 11n	Soil	NA	This study
Mucormycotina sp.	Soil biostimulated with Impranil and YE	FJ379796	This study

[a] "PU" indicates that the isolate was recovered from the surface of PU buried in soil; "soil" indicates that the isolate was recovered from soil in which PU was buried.
[b] NA, not applicable.

Soil microcosms for biostimulation studies. To test the effect of biostimulation on the degradation of buried PU, 12 microcosms containing 330 g of soil were prepared in plastic boxes that were 16 by 12 by 5 cm. The moisture content of the soil was adjusted to 40% of the maximum water-holding capacity (defined as the maximum amount of water that the soil could hold at saturation) using either sterile distilled water ("control microcosms"), a solution containing 200 g liter^{-1} yeast extract (YE) in water ("YE-treated' microcosms"), the colloidal PU dispersion Impranil DLN (Bayer GmbH, Dormagen, Germany) mixed with an equal volume of sterile distilled water ("Impranil-treated' microcosms"), or an aqueous solution containing 200 g liter^{-1} YE plus 50% (vol/vol) Impranil ("Impranil- and YE-treated microcosms"). Three replicates of each type of microcosm were prepared in this way. The amount of water lost through evaporation was determined by weighing the microcosms every 2 to 3 days, and the water was replenished by using the solutions described above. This regimen was followed for 12 weeks. After this time, sterile PU coupons (six coupons in each microcosm) were buried in the soil and incubated for a further 12 weeks, during which time the soil water content was maintained as described above. PU coupons were then recovered, the communities colonizing their surfaces were investigated, and the extent of PU degradation was determined. A sample of soil from each microcosm was also taken and used for analysis.

Fungal strains used for bioaugmentation. Putative PU-degrading fungi isolated from the surfaces of buried PU along with strains isolated in a previous study (6, 9) (Table 1 describes of the isolates used and their origins) were used for bioaugmentation. All of the fungi used for bioaugmentation were confirmed to be able to degrade solid PU during growth as monocultures on the surface of PU coupons (data not shown).

Preparation of inoculum for bioaugmentation. PU-degrading fungal isolates were cultivated on the surface of sterile wheat grains to produce large quantities of inocula. Wheat (100 g) was placed into 10 250-ml Erlenmeyer flasks and sterilized by autoclaving (121°C, 15 min). The sterilized grain was moistened using 50 ml mineral salts medium [containing (per liter of H_2O) 7 g K_2HPO_4, 3 g KH_2PO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, and 1 g $(NH_4)_2SO_4$ and supplemented with 2 g liter^{-1} of D-glucose). Each flask was inoculated with 100 μl of a suspension containing 1×10^4 spores ml^{-1} and incubated at 25°C for 4 weeks in the dark with occasional mixing. One flask was left uninoculated as a control.

Soil microcosms for bioaugmentation studies. The effect of bioaugmentation on the degradation of PU was determined by inoculating soil with wheat colonized by PU-degrading fungal isolates and then mixing the preparations. The soil was divided into 200-g samples, and each sample was mixed with 50 g of wheat colonized with a monoculture of an isolate. An additional 200 g of soil was combined with 50 g of a mixture of all of the isolates in equal proportions. Petri plates were filled with these soil-wheat mixtures, and a single sterile PU coupon was buried in each plate. PU was also buried in control microcosms containing only soil or soil to which sterile wheat alone had been added. The plates were sealed with Parafilm and incubated at 25°C for 4 weeks. Six replicates of each microcosm were prepared. After 4 weeks, the PU coupons were recovered and analyzed.

Recovery of biomass from buried PU. Biomass was recovered from the surface of PU as previously described (6) following burial in order to analyze the microbial communities growing on the surface. Three of the six PU coupons from each microcosm were used for biomass recovery, and the remaining coupons were used for determination of the tensile strength. PU coupons were submerged in 20 ml sterile phosphate-buffered saline (PBS), and biomass was scraped off each side using a sterile scalpel blade. The biomasses recovered from the three coupons from each microcosm were pooled, and 1 ml of the resulting biomass suspension was used for viable counting. The remaining biomass was centrifuged at $3,000 \times g$ for 30 min, and the biomass pellet was used for DNA extraction and denaturing gradient gel electrophoresis (DGGE) analysis.

Microbial viable counts. The total viable counts of fungi in the soil and on the surface of buried PU were determined by dilution plating preparations in PBS onto soil extract agar (2). Colonies were counted after 5 to 7 days of incubation at 25°C. Putative PU degraders were enumerated by counting colonies producing zones of clearance on Impranil agar plates (10) following 5 to 7 days of incubation at 25°C. The medium included 50 μg ml^{-1} of chloramphenicol to inhibit bacterial growth. Three replicates were used for each microcosm. In bioaugmentation studies, the ability to recover the added isolates was tested at the end of the 4-week burial period by plating biomass from the surface of the PU coupons onto potato dextrose agar (Formedium, United Kingdom) and comparing the macroscopic and microscopic morphologies of the isolates with those of the original strains.

DNA extraction. A FastDNA SpinKit for soil (Q-Biogene, CA) was used to extract DNA from 0.4-g soil samples or 0.5-g (wet weight) samples of biomass recovered from the surface of buried PU. To remove all traces of PCR-inhibiting compounds, 20 μl of extracted DNA was electrophoresed for ca. 15 min on a 1.0% (wt/vol) agarose-Tris-acetate-EDTA gel. Bands of genomic DNA were then excised, and DNA was recovered using a Nucleospin Extract II gel extraction kit (Machery-Nagel, Düren, Germany).

DGGE analysis of fungal communities in the soil and on the surface of buried PU. PCR was used to generate DNA fragments for fungal community DGGE analysis as previously described (9) in order to amplify the fungal ITS1 spacer region using the JB206c/GM2 primer set. The fragments generated were run on a 25 to 55% parallel DGGE gel, and fungal community profiles were visualized under UV light after staining with Sybr gold (Molecular Probes, The Netherlands). In order to determine if inoculated fungi could be detected by DGGE in the soil or on colonized buried PU, the DGGE analysis of bioaugmented microcosm samples also included marker lanes containing PCR products from inoculated isolates. Each of the triplicate microcosms was subjected to DGGE, and UTHSCSA image tool v3.0 (http://ddsdx.uthscsa.edu/dig/itdesc.html) was used to compare band alignments in DGGE profiles. Principal component analysis was conducted using the PAST program v1.92 (14), and it revealed >95% similarity for the presence and position of the bands for the three replicate gels, indicating that there was minimal variation and a high degree of reproducibility. Therefore, for presentation purposes, the PCR products from the three replicate samples in this study were pooled prior to DGGE.

Identification of cultivable fungal isolates. Isolates were grown in malt extract broth (Oxoid, United Kingdom) at 25°C for approximately 1 week, and genomic DNA was extracted as described previously (4). The ITS1-5.8S-ITS2 region of the fungal rRNA gene complex was then PCR amplified using the ITS1/ITS4 primer set as described previously (9), and PCR products were sequenced using in-house facilities. Sequences were used to interrogate the National Center for Biotechnology (NCBI) nucleotide database using the blastn algorithm (http://www.ncbi.nlm.nih.gov) and were subjected to phylogenetic analysis to determine the reliability of the identification. For each fungus identified, the most probable closely related species were determined using the Taxonomy Browser provided by the NCBI. Sequences from these closely related species were obtained and aligned with the sequences recovered in this study using ClustalW implementation in the MEGA 3.1 software package. Neighbor-joining trees (bootstrap corrected using 1,000 samples) were constructed using the aligned sequences. The sequences obtained in this study were deemed to be reliable if they were found to cluster with other, putatively closely related fungi.

TABLE 2. Total numbers of viable and Impranil-degrading fungi in soil treated with YE and/or Impranil and numbers of fungi on PU buried in treated soil

Treatment	Soil communities[a]		PU communities[a]	
	Mean fungal viable counts (CFU g^{-1})	Mean Impranil-clearing fungal viable counts (CFU g^{-1})	Mean fungal viable counts (CFU cm^{-2})	Mean Impranil-clearing fungal viable counts (CFU cm^{-2})
Distilled H_2O (control)	5.7×10^5 (2.7×10^4) A	2.2×10^5 (8.8×10^3) A	6.6×10^4 (5.2×10^3) A	2.1×10^4 (3.6×10^3) A
Yeast extract	8.9×10^5 (4.0×10^4) B	2.0×10^5 (1.8×10^4) A	7.8×10^4 (3.5×10^3) B	3.0×10^4 (3.8×10^3) B
Yeast extract plus Impranil	6.2×10^6 (1.3×10^6) C	3.4×10^6 (8.2×10^5) B	8.2×10^4 (4.7×10^3) B	3.1×10^4 (2.2×10^3) B
Impranil alone	5.6×10^5 (4.5×10^4) A	2.4×10^5 (1.5×10^4) A	4.9×10^4 (1.4×10^3) C	1.87×10^4 (8.2×10^2) A

[a] The results are expressed per gram of soil and per square centimeter of PU surface ($n = 3$). The values in parentheses are the standard errors of the means. Means in the same column followed by the same letter were not significantly different ($P > 0.05$).

Culture-independent identification of fungi. The identities of fungi whose numbers increased following treatment with Impranil or with Impranil and YE were determined in a cultivation-independent manner by cloning ITS1-5.8S-ITS2 PCR fragments generated from soil and PU community DNA (using the ITS1/ITS4 primer set) into the pGEM-T Easy plasmid (Promega, United Kingdom). Clones of *Escherichia coli* JM109 transformed with the plasmids obtained were then screened by colony PCR using the JB206c/GM2 primer set. PCR products were subjected to DGGE as described above alongside soil and PU community profiles. Clones producing bands that comigrated to the same positions as bands showing increased intensity in these profiles following treatment with Impranil or with Impranil and YE were then selected for sequencing. These sequences were used to interrogate the EMBL fungal database as described above.

Measuring the degradation of buried PU. The tensile strength of PU coupons recovered from the soil microcosms after burial was determined to assess the extent to which the PU was degraded, as previously described (6). The three coupons from each microcosm that were not used for biomass recovery were cut into strips that were 2 mm by 20 mm. Fifteen strips from each treatment were randomly selected and stretched at a rate of 200 mm min^{-1} using an Instron 4301 (Instron Ltd., Swindon, United Kingdom). Tensile strength was defined as the maximum load when the coupon broke and was inversely proportion to the degree of degradation. The tensile strength of unburied control strips was also determined.

Statistical analysis. Where appropriate, data were subjected to analysis of variance to determine statistical significance (SPSS v13.0; SPSS Inc., Chicago, IL), with the significance threshold set at a P value of <0.05.

RESULTS

Effect of biostimulation on the numbers of viable fungi in soil. Plating of microcosm soil samples following biostimulation revealed that putative PU-degrading fungi were common even in nonbiostimulated soil; 39% of the fungal CFU recovered from control soil showed zones of clearance on Impranil agar (Table 2). Conversely, very few Impranil-clearing bacteria were found in this study (data not shown). Treatment of soil with Impranil alone did not significantly ($P > 0.05$) change the numbers of viable or PU-degrading soil fungi (Table 2). Treating soil with YE alone did not significantly ($P > 0.05$) increase the numbers of PU-degrading fungi; however, the total numbers of viable fungi increased 1.6-fold ($P < 0.05$). Treatment with YE and Impranil together increased the numbers of viable fungi 10.8-fold ($P < 0.05$). However, ca. 90% of these fungi were two morphotypes later identified as *Trichosporon multisporum* and an unidentified *Mucormycotina* sp. A 16-fold increase ($P < 0.05$) in the number of PU degraders was seen in soil treated with YE and Impranil. However, much of this increase was due to proliferation of the unidentified *Mucormycotina* sp., which was shown to be a PU degrader, and there was no increase in the numbers of other PU-degrading fungi.

Effect of biostimulation on the numbers of cultivable fungi colonizing the surface of buried PU. Samples of biomass recovered from the surface of buried PU were plated onto solid media to determine the effect of biostimulation on the communities colonizing buried PU (Table 2). Treatment of soil with both YE and Impranil or with YE alone significantly ($P < 0.05$) increased the total numbers of viable fungi on the surface of the PU by 24% and 18%, respectively, compared to the control. Furthermore, there was an approximately 45% increase ($P < 0.05$) in the number of putative PU degraders on the surface of PU buried in soil treated in this way compared to the control. There was no significant ($P > 0.05$) difference between the counts for YE-treated microcosms and the counts for microcosms treated with both YE and Impranil. Treatment of soil with Impranil alone significantly ($P < 0.05$) reduced the numbers of total viable fungi on the surface of the PU by 25% compared to PU buried in untreated control soil, and 11% fewer putative PU-degrading fungi were present, although the reduction was not significant ($P > 0.05$).

DGGE analysis of communities in biostimulated soil. With the exception of soil communities treated with both YE and Impranil, biostimulation had no visible effect on the distribution of morphotypes recovered on solid media. Therefore, DGGE was used to detect changes in fungal populations in the soil and on buried PU following biostimulation without reliance on cultivation (Fig. 1).

Treatment of soil with Impranil alone had a limited effect on the soil populations, and a high degree of similarity was observed in the DGGE profiles. Nevertheless, several bands observed for Impranil-treated soil communities were absent from, or less intense in, the control profile, suggesting that there was possible enrichment of a limited number of fungal species. Bands produced by three of these putatively enriched species were recovered via cloning and were sequenced to determine their identities (numbered bands in Fig. 1, lane IS). Treatment with YE (lane YS) or with YE and Impranil (lane IYS) had a more marked effect on the fungal soil communities, and there was a reduction in the number of bands visible in the community profiles, suggesting that the biodiversity was reduced.

The DGGE profiles of fungal communities in the soil bore little resemblance to those of communities on the surface of the PU (Fig. 1), suggesting that only a subset of the soil fungal community could colonize the surface of the PU. Furthermore, the majority of the most intense bands in the PU profiles were not visible in the soil profiles, indicating that the fungi proliferating on the surface of the PU were probably minor members of the soil community.

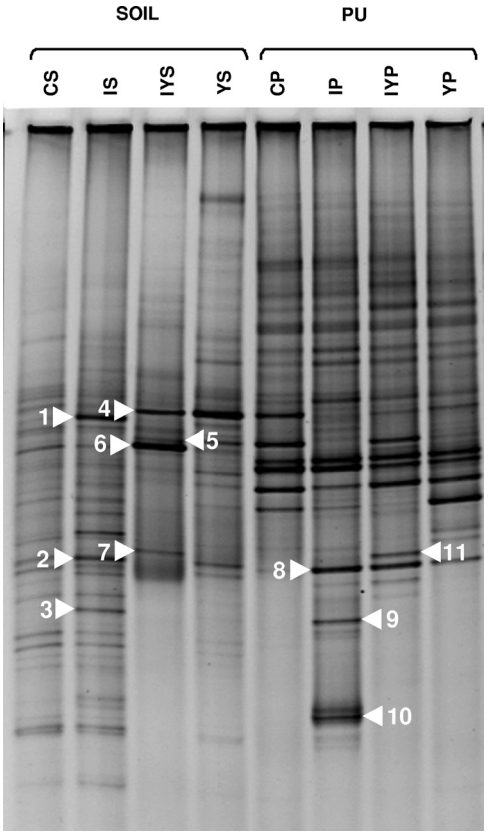

FIG. 1. DGGE profiles of fungal communities in soil and on PU after treatment of soil microcosms with sterile distilled water (lanes CS and CP), yeast extract (lanes YS and YP), Impranil (lanes IS and IP), or both Impranil and YE (lanes IYS and IYP). The numbers indicate bands that were sequenced and identified.

The majority of the bands in the soil profiles whose intensity increased following biostimulation were not readily visible in the profiles of communities colonizing the buried PU (Fig. 1, compare lanes IYS and YS to lanes IYP and YP), suggesting that fungi that were enriched following biostimulation did not colonize the PU surface. Interestingly, even though the different soil profiles were highly heterogeneous at 4 weeks, there was a high degree of similarity between the profiles of the PU communities regardless of the treatment (Fig. 1, compare lanes PU). Although the PU community profiles were similar, a number of bands were unique to individual treatments, indicating that the type of treatment influenced the colonization of buried PU. The DGGE profiles of the replicate coupons were essentially identical ($P < 0.05$) and highly reproducible (results not shown), and the DGGE profiles shown were derived from pooled PCR products from replicate samples.

Culture-independent identification of fungi enriched following biostimulation. DGGE bands with increased intensities following biostimulation (Fig. 1) were sequenced to identify fungi showing putative enrichment following treatment. In this way, *Hohenbuehelia* sp., *Trichosporon gracile*, *Phoma* sp., *Geomyces pannorum*, and *Mortierella hyalina* were identified as fungi with increased band intensities in biostimulated soil. However, none of these species were represented in the fungi that were enriched on the surface of buried PU, which were identified as *Hypocrea virens*, *Zalerion varium*, *Bionectria ochroleuca*, and *Nectria haematococca* (Fig. 2 and Table 3).

Effect of biostimulation on the degradation of buried PU. The tensile strength of PU recovered from biostimulated soil was determined to assess the effect of biostimulation on PU degradation. The tensile strength of PU was found to be greatly reduced even in the control soil (Fig. 3), and PU lost ca. 90% of its tensile strength after 12 weeks of burial. PU buried in soil treated with YE or with YE and Impranil had 45% less tensile strength than PU buried in the control soil ($P < 0.05$), indicating that biostimulation successfully enhanced PU degradation under these conditions. There was no significant ($P > 0.05$) difference between the YE and YE-Impranil treatments. Interestingly, treatment of soil with Impranil alone inhibited PU degradation, and the PU had twice the tensile strength of PU buried in control soil ($P < 0.05$).

Numbers of fungi in bioaugmented soil and on the surface of buried PU 4 weeks after inoculation. Soil and biomass from buried PU were each plated on solid media to determine the numbers of viable and putative PU-degrading fungi 4 weeks after inoculation of the soil with wheat colonized by PU-degrading fungi.

Addition of the sterile wheat control significantly ($P < 0.05$) increased the numbers of viable fungi in the soil 26-fold, from 7.5×10^5 to 2.0×10^7 CFU g^{-1}, and significantly ($P < 0.05$) increased the numbers putative PU degraders 38-fold to 9.1×10^6 CFU g^{-1}. Of the inoculants tested, only *P. ochrochloron*, the unidentified *Mucormycotina* sp., and a mixture of all isolates significantly ($P < 0.05$) increased the numbers of viable fungal CFU in the soil above the level obtained with sterile wheat; these inoculants increased the viable counts 4.5-, 2.7-, and 3.4-fold, respectively. In addition, only *P. ochrochloron* and a mixture of all isolates significantly ($P < 0.05$) increased the numbers of putative PU-degrading fungi in soil compared to sterile wheat alone (10-fold and 3-fold increases, respectively). PU buried in untreated control soil was colonized by 4.5×10^3 viable CFU cm^{-2}, 44% of which were identified as putative PU degraders on Impranil agar plates. Addition of sterile wheat alone significantly ($P < 0.05$) increased both the viable and PU degrader counts on the surface of buried PU 35-fold. As seen in the soil communities, few of the isolates exhibited a significant ($P < 0.05$) increase compared with the sterile wheat control, with the exception of *P. ochrochloron*, for which the number of CFU on buried PU increased 4-fold compared to the sterile wheat control.

Persistence of inoculated fungi in soil after 4 weeks. DGGE was used to determine if the fungi used for bioaugmentation were detectable in the soil 4 weeks after inoculation. Only bands corresponding to the unidentified *Mucormycotina* sp. and *N. haematococca* were clearly visible in the DGGE profiles of the corresponding soil communities (Fig. 4, lanes 4 and 9, arrows). There was also a much fainter band corresponding to *P. viridicatum* (Fig. 4, lane 6). Even though the majority of the isolates were not detectable by DGGE after 4 weeks, each introduced species did have a significant influence on the fungal community profile in the soil (Fig. 4).

Colonization of buried PU by inoculated fungi. DGGE analysis of fungal communities colonizing PU buried in inoculated soil revealed trends similar to those observed in soil

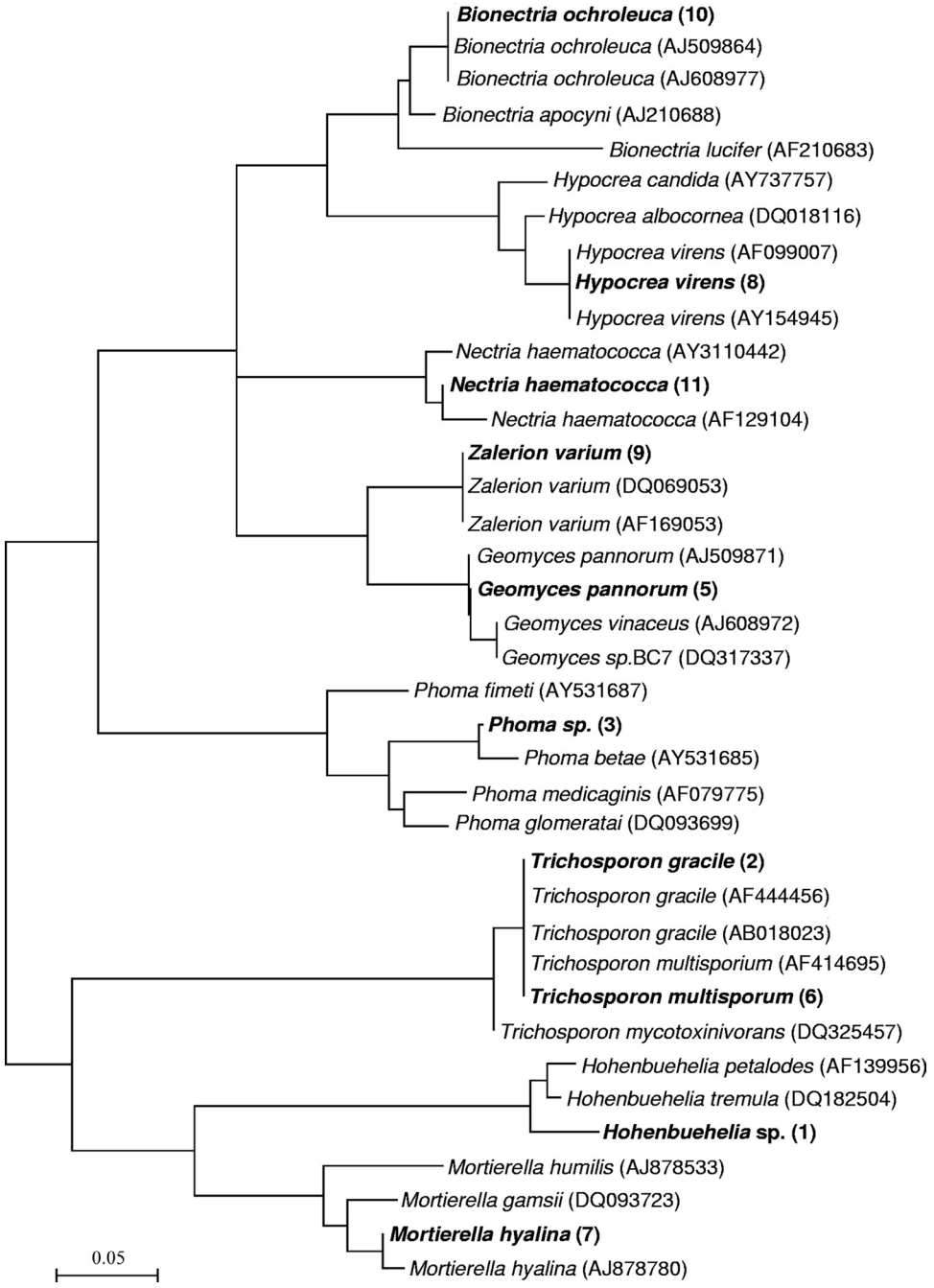

FIG. 2. Phylogenetic analysis of sequences cloned from bands in the DGGE profiles of fungal communities in the soil and on the surface of buried PU whose intensities increased following Impranil treatment. ITS1-5.8S-ITS2 sequences were compared to sequences of putatively closely related species by constructing a neighbor-joining phylogenetic tree (bootstrap corrected with 1,000 samples). Isolates obtained in this study are indicated by bold type, and the numbers in parentheses indicate the corresponding bands in Fig. 1.

communities. In the majority of cases none of the bands in the community profiles for the surface of the PU comigrated with bands in the marker lanes, suggesting that significant numbers of the majority of the isolates had not colonized PU 4 weeks after burial (Fig. 5, lanes 2, 4, 5, 7, 8, 9 and 10). The only isolate with clear evidence of colonization was *P. viridicatum* 5 (lane 6), although a much fainter band corresponding to the unidentified *Mucormycotina* sp. was present (lane 3.).

As seen in the soil communities, the banding pattern produced by each PU community shown in Fig. 5 depended on the type of inoculant, suggesting that, although the isolates were not detectable on the PU coupons themselves, the introduction of the isolates nevertheless had influenced the subsequent composition of fungal communities colonizing the surface of buried PU.

Effect of bioaugmentation on the degradation of buried PU. The tensile strength of PU buried in the control soil decreased significantly ($P < 0.05$) by 24% after 4 weeks of burial. Sterile

TABLE 3. Putative identities of fungal species enriched following addition of Impranil to soil[a]

Community type	Treatment	DGGE band	Strain	Identity determined by phylogenetic analysis
Soil	Impranil alone	1	SIH1	*Hohenbuehelia* sp.
Soil	Impranil alone	2	SITG2	*Trichosporon gracile*
Soil	Impranil alone	3	SIP3	*Phoma* sp.
Soil	Impranil plus YE	4	SIYZ4	*Mucormycotina* sp.
Soil	Impranil plus YE	5	SIYGP5	*Geomyces pannorum*
Soil	Impranil plus YE	6	SIYTM6	*Trichosporon multisporum*
Soil	Impranil plus YE	7	SIYMH7	*Mortierella hyalina*
PU	Impranil alone	8	PIHV8	*Hypocrea virens*
PU	Impranil alone	9	PIZV9	*Zalerion varium*
PU	Impranil alone	10	PUIBO10	*Bionectria ochroleuca*
PU	Impranil plus YE	11	PIYNH11	*Nectria hematococca*

[a] Impranil was added alone or with yeast extract. The intensities of bands selected for sequencing were greater following treatment with YE and/or Impranil (see Fig. 1).

wheat significantly ($P < 0.05$) enhanced PU degradation, and there was a 45% reduction in tensile strength when it was added (Fig. 6). Inoculation of *G. pannorum*, *Penicillium inflatum*, and isolate 19n had no significant ($P > 0.05$) effect compared to addition of sterile wheat alone. The remaining isolates, however, all significantly ($P < 0.05$) enhanced PU degradation compared to sterile wheat. PU buried in soil inoculated with isolate 11n or a mixture containing equal amounts of all isolates reduced the tensile strength by 62%, and *N. haematococca*, *P. viridicatum*, and the unidentified *Mucormycotina* sp. reduced the tensile strength by 77% (there was no significant difference between these strains). The greatest loss of tensile strength occurred when the soil was inoculated with *P. ochrochloron*; under these conditions buried PU lost 85% of its tensile strength over the course of 4 weeks ($P < 0.05$).

DISCUSSION

The aim of this work was to determine if biostimulation and bioaugmentation can be used to enhance PU waste degradation in the environment. Both treatments caused a reduction in the tensile strength of PU coupons buried in treated soil microcosms. Tensile strength is frequently used as a sensitive indicator of plastic degradation, as cleavage of intramolecular bonds leads to changes in mechanical properties (11). Although this method cannot determine if PU is mineralized, the loss of structural integrity observed suggests that bioaugmentation and biostimulation are viable methods for stimulating biodegradation of PU waste in the environment.

Although both biostimulation and bioaugmentation enhanced PU degradation, PU buried in untreated control soil was also affected, losing 24% of its tensile strength after 4 weeks and 90% of its tensile strength after 12 weeks. This was unlikely to be due to an abiotic process, as PU has been proven to be resistant to abiotic degradation in previous studies (6, 7). Rather, this reduction was likely to have been caused by fungi in the soil, as 39% of the fungi recovered were identified as putative PU degraders using Impranil clearance assays; few bacterial degraders were observed. The finding that putative PU degraders were so numerous in soil with no known previous exposure to PU suggests that PU was degraded by enzymes secreted during nutrient acquisition from natural substrates (3). The fact that no significant numbers of bacterial PU degraders were recovered, as has been reported in previous studies (6, 9), suggests that fungi play an important role in PU biodeterioration in aerobic environments.

Biostimulation with YE alone or with YE and Impranil increased PU degradation by 45% compared to control soil, which may be attributed to the observed 45% increase in PU degraders colonizing PU. Buried PU coupons may have been a

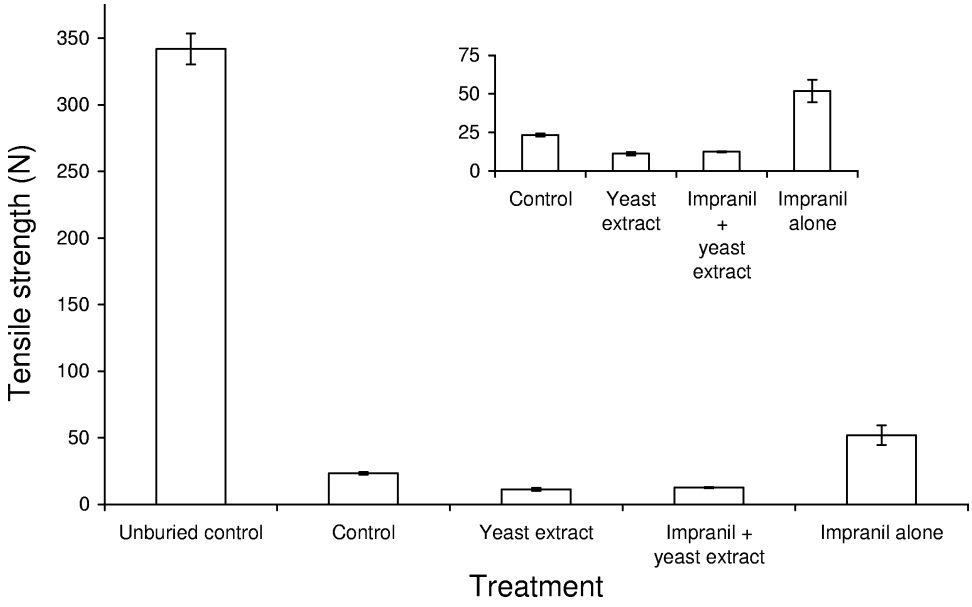

FIG. 3. Tensile strength of PU after 12 weeks of burial in soil treated with YE and/or Impranil. The tensile strength of unburied PU is also shown. The inset shows the values minus the value for the unburied control ($n = 15$).

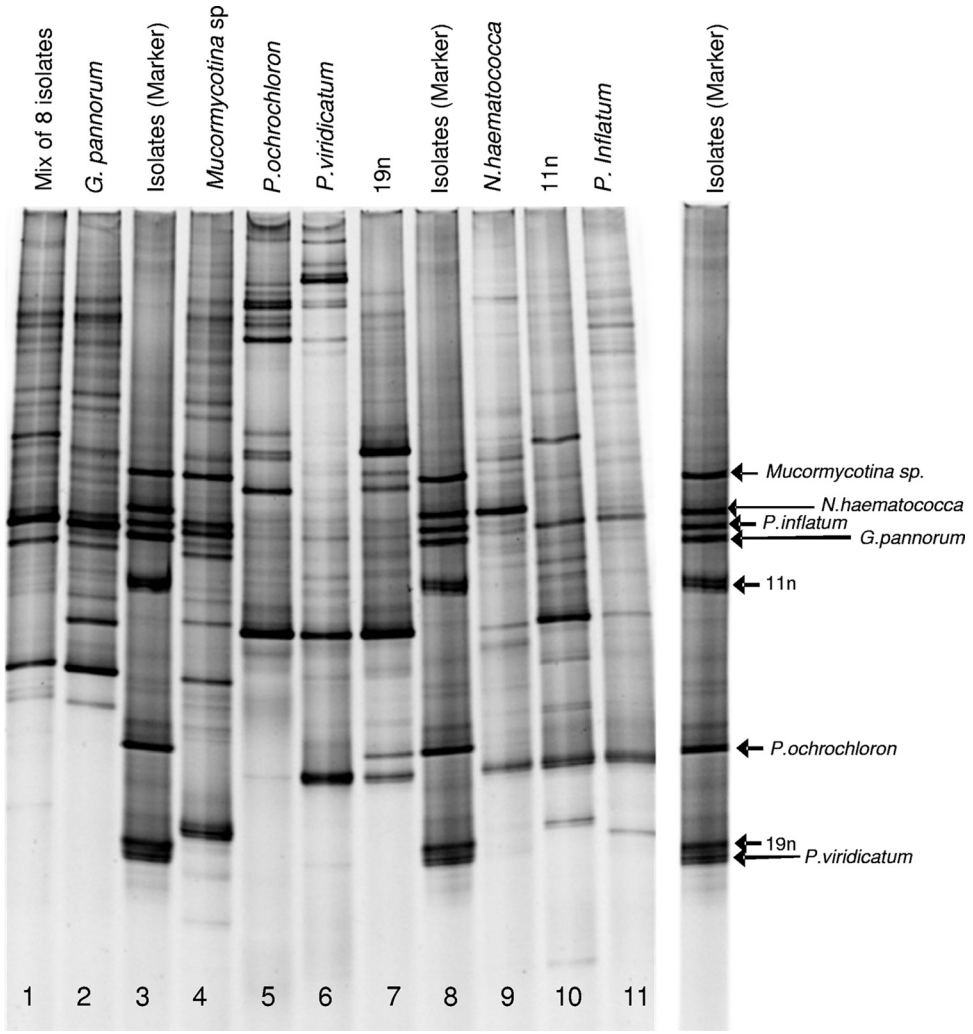

FIG. 4. DGGE of fungal soil communities 4 weeks after inoculation of soil with wheat colonized by PU-degrading fungal isolates. The marker lanes (lanes 3 and 8) contained DGGE products from all eight isolates. The arrows in lanes 4, 6, and 9 indicate bands that comigrated with bands produced by *Mucormycotina* sp., *P. viridicatum*, and *N. haematococca*, respectively.

relatively poor growth substrate, and the influx of nutrients in the form of Impranil and/or YE allowed a larger degrading population to exist on the surface, leading to enhanced degradation. Biostimulation can also enhance degradation by inducing enzymes responsible for degradation. Oceguera-Cervantes et al. (25) observed that the Impranil-like PU dispersion "Hydroform" induced esterase secretion in *Alicycliphilus*. However, treatment with both Impranil and YE did not result in significantly greater degradation than treatment with YE alone, and therefore Impranil may not induce enzymes other than those induced by the presence of YE.

Treatment with both Impranil and YE increased the number of PU degraders in soil 16-fold; however, this increase could be attributed to the proliferation of a single unidentified *Mucormycotina* sp., and there was no evidence of an increase in the level of other PU degraders. This proliferation had no apparent effect on PU degradation even though the unidentified *Mucormycotina* sp. was found to enhance degradation when it was used as an inoculum for bioaugmentation. However, only endpoint measurements after 12 weeks were recorded, and it is therefore possible that the rate of degradation was higher in soil treated with Impranil and YE during the early stages. Numerous studies have demonstrated that there was a higher rate of degradation of various pollutants even when the final extent of degradation was not significantly different for biostimulated and control treatments (5, 22, 28, 29).

Treatment of soil with Impranil alone appeared to reduce the degradation of buried PU, and this may have reflected differences in the hydrophobicity of the two materials, as enzymes capable of degrading Impranil are not necessarily capable of degrading solid PU (1).

Although putative PU-degrading fungi were common in the soil, DGGE and sequencing revealed that only a small subset of fungi, including *Hohenbuehelia* sp., *T. gracile*, *Phoma* sp., *Mucormycotina* sp., *T. multisporum*, *M. hyalina*, and *G. pannorum*, showed increased band intensities following biostimulation. It has been suggested that only a small subset of a given community significantly participates in nutrient flow (13); if this is true, even if Impranil was a suitable growth substrate for

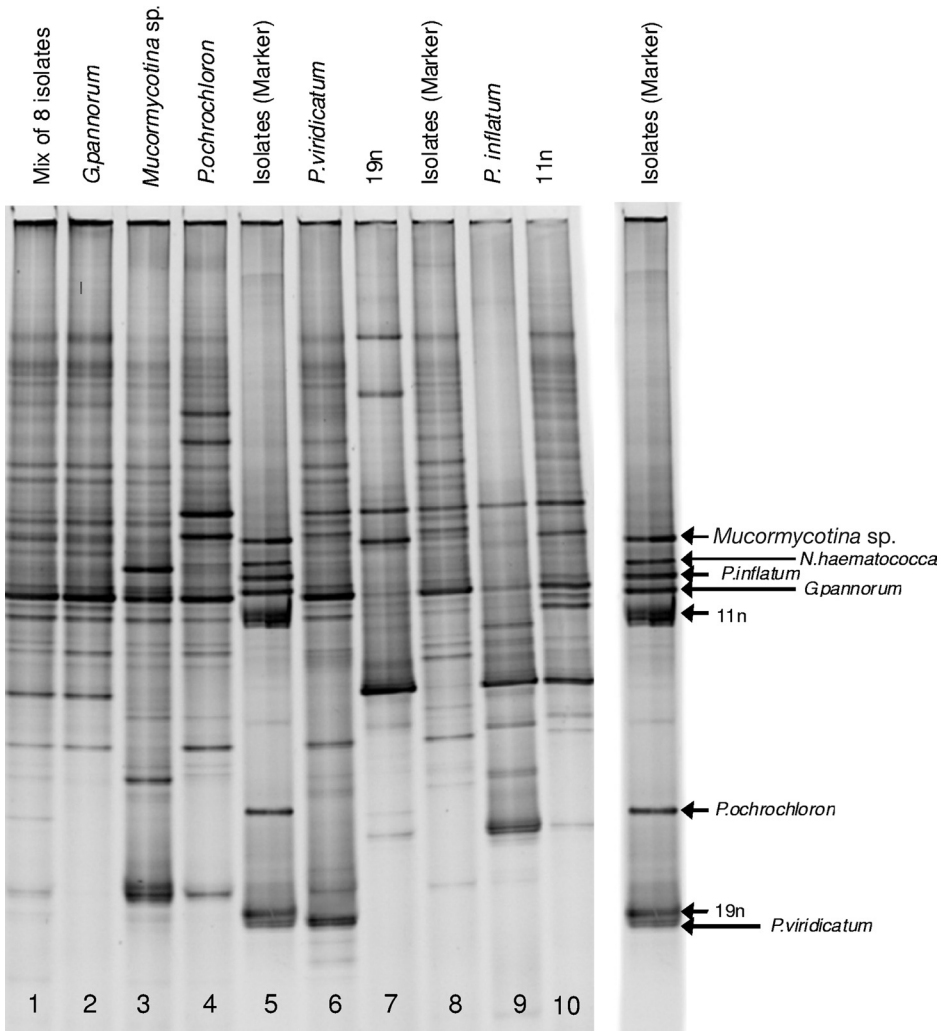

FIG. 5. DGGE of fungal communities colonizing PU after 4 weeks of burial in soil inoculated with wheat colonized by PU-degrading fungal isolates. Marker lane 5 contained DGGE products from all eight isolates. The arrows in lanes 3 and 6 indicate bands that comigrated with bands produced by the *Mucormycotina* sp. and *P. viridicatum*, respectively.

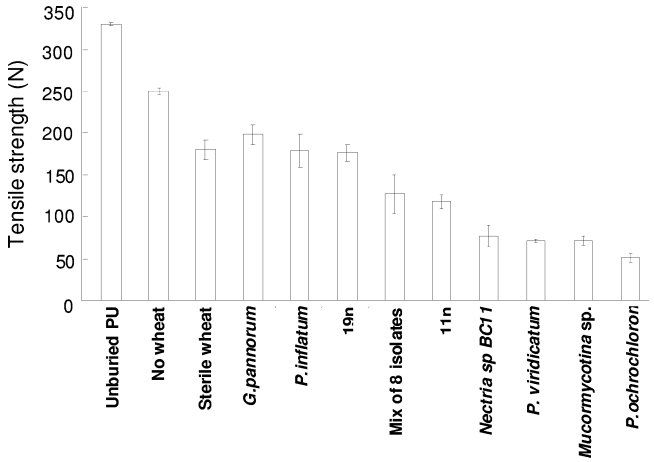

FIG. 6. Tensile strength of PU after 4 weeks of burial in soil inoculated with wheat colonized by putative PU-degrading fungi. Unburied PU, PU buried in soil containing sterile wheat, and PU buried in soil with no wheat were included as controls. The error bars indicate standard errors of the means ($n = 15$).

the majority of the organisms, few members of the community would have proliferated to a significant degree.

Very few of the fungi enriched in the soil following biostimulation were found on the surface of buried PU after 4 weeks; the exception was the unidentified *Mucormycotina* sp. DGGE revealed that colonization was carried out by a different set of soil fungi, including *H. virens*, *Z. varium*, *B. ochroleuca*, and *N. haematococca*. It has been hypothesized that PU-degrading enzymes may require hydrophobic binding domains to degrade solid PU, which may not be necessary for Impranil degradation (1); hence, fungi able to use Impranil as a growth substrate during biostimulation may have been unable to do so with solid PU. Furthermore, the chemical composition of PU strongly influences its degradability and suitability as a growth substrate (32), and Impranil and solid PU do not have identical formulations. However, when isolates identified as putative PU degraders using Impranil clearance assays were inoculated onto the surface of sterile PU coupons, they were able to use the PU as a growth substrate (data not shown). It is possible that PU was initially colonized by fungi enriched by biostimulation that

were then displaced by other soil fungi. Such community succession has been shown to occur on plastic in the environment (31, 39).

The inoculum for bioaugmentation was prepared by cultivating PU-degrading fungal isolates on the surface of sterile wheat grains, which provided a convenient method for generating large quantities of biomass. Furthermore, it has been demonstrated that including a nutritive matrix along with a bioaugmenting inoculum can enhance degradation and survival of the inoculum (21). Addition of uninoculated wheat alone led to a 28% increase in PU degradation, probably due to the nutrients in the wheat, as the numbers of putative PU degraders in the soil and colonizing PU increased more than 35-fold. Wu et al. (40) observed a similar effect on the numbers of native polyaromatic hydrocarbon degraders in contaminated soil after addition of ground corn cobs.

Almost all of the strains used for bioaugmentation were not detected by DGGE in the soil or on the surface of buried PU after 4 weeks even though it has been reported that DGGE is able to detect community members at levels as low as 0.1% of the population (37). Many factors have been found to impact the survival of an inoculant in soil, including moisture content, pH, temperature, oxygen and nutrient availability (36), predation (38), amensalism, and parasitism (18). The species used may have been maladapted to competing in the soil, as several of the isolates used in this study were not originally isolated from the soil used here. Indeed, the isolates that were detected after 4 weeks, namely, the unidentified *Mucormycotina* sp., *N. haematococca*, and possibly *P. viridicatum*, were initially recovered from the soil used in this study. Additionally, the high level of native PU degraders may have negated any selective advantage that PU-degrading phenotypes may have otherwise conferred on the isolates; survival is often much better when bioaugmentation is carried out for populations with much smaller numbers of native degraders (12, 23, 41).

It is also possible that added isolates may have been underrepresented due to biases in DNA recovery or PCR amplification that are common in complex soil environments (33).

With the exception of *G. pannorum*, *P. inflatum*, and isolate 19n, all isolates significantly increased degradation of PU compared to sterile wheat alone. *P. ochrochloron* had the greatest effect, causing an 85% reduction in the tensile strength after 4 weeks. As with biostimulation, community succession may explain how bioaugmentation still enhanced degradation even though colonization of the PU surface was not detected after 4 weeks; PU may have been initially colonized by the inoculum, which was followed by a period of enhanced degradation, after which isolates may have been displaced by native soil fungi. Despite being undetectable either in the soil or on the PU surface, each inoculant had a profound, inoculum-dependent impact on the structure of the fungal communities in the soil and on the surface of buried PU. Since each fungus is likely to have different PU-degrading abilities, such an alteration of community structure due to bioaugmentation would be expected to have an impact on degradation; however, it is unclear why the shift in community structure should favor more efficient PU degraders.

This work showed that biostimulation and bioaugmentation may be used to enhance the degradation of PU in soil. Although neither treatment was required for degradation in the soil used in this work due to the high numbers of native PU-degrading fungi, this work provides proof of principle that biostimulation and bioaugmentation can enhance the degradation of PU in soil. Treatments that enhance PU degradation may be of much greater utility under less favorable conditions, such as those in landfills or at sites with fewer native degraders, or in remediating environments contaminated with PU.

ACKNOWLEDGMENTS

We thank the BBSRC and Arch Biocides for financial support for a CASE studentship to L.C.

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