Perennial crop growth in oil-contaminated soil in a boreal climate

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Abstract

Soil contamination by petroleum hydrocarbons is a global problem. Phytoremediation by plants and their associated microorganisms is a cost-effective strategy to degrade soil contaminants. In boreal regions the cool climate limits the efficiency of phytoremediation. The planting of oil-tolerant perennial crops, especially legumes, in oil-contaminated soil holds promise for great economic benefits for bioenergy and bio-fertilizer production while accelerating the oil degradation process. We established a multi-year field experiment to study the ecological and agronomic feasibility of phytoremediation by a legume (fodder galega) and a grass (smooth brome) in a boreal climate. In 40 months, soil oil content decreased by 73% - 92%, depending on the crop type. The oil degradation followed first-order kinetics with the reduction rates decreasing as follows: bare fallow > galega-brome grass mixture > brome grass > galega. Surprisingly, the presence of oil enhanced crop dry matter and nitrogen yield, particularly in the fourth year. The unfertilized galega-brome grass mixture out-yielded the N-fertilized pure grass swards over years by an average of 33%. Thus, a perennial legume-grass mixture is both ecologically and agronomically sustainable as a cropping system to alleviate soil contamination in the boreal zone, with considerable potential for bioenergy and bio-fertilizer production.

1. Introduction

Soil pollution by petroleum hydrocarbons (PHCs) is an increasing problem around the world. In Finland, for example, the number of contaminated sites grew from 10,400 in 1994 to 23,850 in 2013 (Pyy et al. 2013). *In situ* bioremediation using indigenous microbes is an effective and low-cost strategy to degrade contaminants, but this process is limited by microbial activities, the biochemistry of enzymes, the resistant nature of the contaminants, and site-specific factors (Balba et al. 1998, Boopathy 2000). Plants are able to enhance the bioremediation of oil-polluted soil by stimulating soil microflora (Radwan et al. 1995, Suominen et al. 2000, Acharya et al. 2014). The efficiency of this phytoremediation relies on the establishment of healthy plants with sufficient shoot and root biomass growth to support the activities of a flourishing microbial consortium at the rhizosphere (Wenzel 2009). Dense cultivation of suitable crops in polluted sites was thus suggested as a promising approach for bioremediation (Radwan et al. 1995).

Nutrient deficiency, however, particularly that of nitrogen and phosphorus, often limits biodegradation in contaminated sites (Wenzel 2009). Legumes, due to their capacity for symbiotic biological nitrogen fixation, can do without N fertilizer input, thus assisting in the bioremediation of soils contaminated with petrochemical waste (Kamath et al. 2004, Chiapusio et al. 2007). Since bioremediation is a slow process that does not allow many disturbances of the contaminated soil, the use of perennial legumes with proper field management holds promise for accelerated oil degradation. Fodder galega (*Galega orientalis* Lam.), a fast-growing perennial forage legume, and smooth brome (*Bromus inermis* L.), a cool-season perennial sod-forming grass, are both persistent in boreal and nemoral zones and have been shown to grow well together in crop mixtures (Jasinskas et al. 2008, Kryževičienė et al. 2008). The oil tolerance and rhizoremediation potential of *G. orientalis* and its microsymbiont *Neorhizobium galegae* to remediate oil-contaminated soils have been demonstrated at microcosm and mesocosm scales (Suominen et al. 2000, Lindstrom et al. 2003, Jussila et al. 2006, Mikkonen et al. 2011).

For these reasons, we set up a systematic, field-scale study on bioremediation of oil-contaminated soil coupled with plant biomass production in a boreal region. We established a multi-year field experiment to investigate the ecological suitability and potential economic benefits of fodder galega and smooth brome to grow in and bioremediate an oil-contaminated soil, and to develop an integrated and sustainable system for long-term cost-effective bioremediation practice in boreal and nemoral climates.

2. Materials and methods

2.1 Experimental design and climatic conditions

The field experiment was established at the Viikki Experimental Farm, University of Helsinki, Finland (60°14'N, 25°01'E, 8 m AMSL) (Table 1). It was a split-plot experimental design in four replicate blocks, with four crop treatments (pure brome grass, pure galega, galega-brome grass mixture, and bare fallow) as the main plot factor (Figure S1). The sub-plot factor was factorial combinations of oil spiked (7000 ppm) and unspiked treatments with plant growth promoting bacteria (PGPB) inoculated and un-inoculated treatments, providing 64 plots in all (4 crop treatments × 2 oil treatments × 2 PGPB treatments × 4 replicates). The mean temperatures of the growing seasons (May – October) exceeded the long-term (1971-2000) average by 2.1 °C in 2010 and 1.8 °C in 2011, and the precipitation exceeded the average in 2009, 2011 and 2012 (Table 2).

2.2 Treatment preparation and field management

2.2.1 Field management

The site was treated with two herbicides: glyphosate (N-(phosphonomethyl)glycine), a broad-spectrum systemic herbicide, before the establishment of the plots in June 2009 and Basagran® SG (165 g / 50 liter) for post emergence broad-leaved weed control in September 2009. Weeds growing in the experimental plots were removed manually in the growing season, except for a second glyphosate treatment in June 2011 in the bare fallow plots, although this treatment had no visible effect. Buffers between the blocks were maintained as weedy grassland to prevent edge effects and other disturbances between neighbouring plots. Mineral N fertilizer (60 kg ha^{-1} of N as urea) was given to the pure grass plots in the summer of 2009. In May 2012, mineral N fertilizer was given only to the PGPB-treated pure grass plots. The legume plots and legume-grass mixtures received no N fertilizer throughout the experiment.

2.2.2 Oil spike

The spiking experiment was performed to evaluate the biological toxicity of the oil hydrocarbons and to assess the overall bioremediation efficiency. The oil was a mixture of used motor engine oil (Teboil Lubricants Classic Mineral Motor oil, SAE 10W-30, API SF/CD, Finland), with a density of 0.877 kg l^{-1} at 20°C, according to the manufacturer. The target contamination was 7000 ppm (7 g kg^{-1}) of motor oil in soil, assuming a soil bulk density of 1.0 g ml^{-1}. For each oil-spiked plot, 6 kg of oil was mixed with 10 kg of white coarse sand (0.5 - 1.2 mm), spread, and mixed into the top 20 cm of soil in the oil-treated plots with a rotary tiller on 17 June 2009. Ten kg of pure sand without oil was mixed into the top 20 cm of soil in the control plots.

2.2.3 Seed co-inoculation and sowing

Before sowing, commercial seeds of *G. orientalis* cv. 'Gale' (Naturcom Oy, Ruukki, Finland) and *B. inermis* cv. 'Lehis' (Jõgeva Plant Breeding Institute, Estonia) were surface-sterilized before inoculation with bacteria. To ensure biological nitrogen fixation, all galega seeds were inoculated with *Neorhizobium galegae* strain HAMBI 540 (University of Helsinki, Helsinki, Finland). Two plant growth promoting root-colonizing bacteria strains, *Pseudomonas trivialis* 3Re27 (Graz University of Technology, Graz, Austria) and *Pseudomonas extremorientalis* TSAU20 (National University of Uzbekistan, Uzbekistan), were inoculated onto the seeds of both crops as described by Egamberdieva et al. (2010). The PGPB-free seeds were used as controls. The inoculated seeds were mixed with peat prior to sowing. The seeds were manually sown and lightly covered by raking. The first sowing was done on 7 July 2009. Brome grass was sown at 35 kg ha^{-1}, galega at 25 kg ha^{-1}, and the combination at 26 kg ha^{-1} of brome grass and 6 kg ha^{-1} of galega to give a 75:25 ratio. Due to a poor initial growth of the galega, it was resown in May 2010.

2.3 Crop biological measurements and data handling

The crops were cut with a forage harvester twice in a growing season over three successive growing seasons (2010-2012). The first cut was done when flowering began in late June and the second cut was done in late August, these being typical harvesting times for hay or silage. The total fresh biomass of crops (W) was weighed on the day of harvesting and the species in the mixtures were separated. The proportion of galega (G%) was estimated on the basis of fresh weight in each mixture plot. Crop dry matter content (DM%) was determined by drying to the constant mass at 105 °C. The DM yield (t ha^{-1}) of each harvest was calculated as follows: DM yield = DM% × W. The total DM yield in the mixture plots was the sum of both crops. The annual DM yields for the crops were the sum of the DM yields of two harvests per year.

For chlorophyll measurement, the youngest fully expanded blades were selected. The mean of 10-20 readings per plot from the portable chlorophyll meter (SPAD-502, Minolta, Japan) was obtained per plot on every 15 June and 15 July each year from 2010 to 2012 (20 readings per monoculture plot, and 20 readings from brome grass and at least ten from galega per mixture plot). We did one additional SPAD reading on 15 August 2012. SPAD readings for mixture plots was estimated by: mixture SPAD reading = $SPAD_B \times (1 – G\%) + SPAD_G \times G\%$, where the subscripts G and B refer to galega and brome grass, respectively. The annual SPAD reading of each plot was calculated as the average of readings measured in June and July in each year.

The $\delta^{15}N$ (^{15}N natural abundance) technique was used to determine biological N fixation (BNF) in the galega, according to Unkovich et al. (2008). The brome grass in the same soil was used as the non-N-fixing reference plant to determine the $\delta^{15}N$ of the plant-available N in the soil. About 100 grams of each crop shoot sample were dried at 60 °C overnight before the determination of total carbon (C%) and nitrogen content (N%) by the Dumas combustion method with a VarioMax CN-analyzer (Elementar Analysensysteme GmbH, Hanau, Germany), followed by the stable isotope abundance (atom% ^{15}N) by mass spectrometry at the James Hutton Institute, Scotland, UK. Four replicated dry grass shoot samples taken from the mixture plots were pooled before determination of the atom% ^{15}N. The atom% ^{15}N of the PGPB-treated legume shoot samples harvested in 2010 was determined. The total N yield (kg ha^{-1}) of monoculture plots was calculated as N yield= N% × DM% × W. The total N yield (kg ha^{-1}) of crops in mixture plots per cut was estimated as follows: mixture N yield = N_B% × DM_B% × W_B × (1 – G%) + N_G% × DM_G% × W_G × G%. Since the N% of each crop in mixture plots was not measured separately in 2010, the total N yield (kg ha^{-1}) per cut in this year was therefore calculated as follows: mixture N yield in 2010 = N% × DM_B% × W_B × (1 – G%) + N% × DM_G% × W_G × G%. Because the second harvests were not done on the same dates in each year, the annual crop C content, N content and C:N ratio of each plot were calculated for data analysis, as the average of values measured from two harvests per year. The sample natural abundance expressed as parts per thousand relative to atmospheric N_2 was calculated as follows: $\delta^{15}N$ (‰) = (sample atom%^{15}N – atom% ^{15}N in the atmosphere) / atom% ^{15}N in the atmosphere × 1000, where the atom% ^{15}N in the atmosphere is 0.3663 (Unkovich et al. 2008). The proportion of legume shoot N derived from atmospheric N_2 (%Ndfa) was calculated as follows: %Ndfa = ($\delta^{15}N$ of reference plant - $\delta^{15}N$ of N_2-fixing legume) / ($\delta^{15}N$ of reference plant – 'B' value) × 100, where 'B' value, the $\delta^{15}N$ of shoots of the *Neorhizobia galegae*-inoculated galega that is fully dependent upon N_2 fixation and sampled at the same growth stage as the field plants, is the correction factor to adjust for isotopic fractionation by the legume (Unkovich et al. 2008). However, a proper 'B' value for the shoot of fodder galega is lacking. Carlsson et al. (2006) proposed that the variation of 'B' value in relation to plant age and overwintering with the ^{15}N natural abundance method was small compared to the variation that occurred between different *Rhizobium* strains. We therefore used the same 'B' value (-1.40) averaged from the 'B' values of all *Rhizobium* strains and plant age for shoots of three temperate forage legume species (-1.2 in *Trifolium hybridum*, -1.3 in *T. pratense* and

-1.7 in *T. repens*) grown in northern Scandinavia to estimate the %Ndfa in galega shoots in successive growing seasons, according to the recommendations from Unkovich et al. (2008). The seed N content was negligible in the long period cultivation of the legume (Carlsson et al. 2006). The biologically fixed N (BNF) yield (kg ha^{-1} of N) in the legume per legume-cropped plot was calculated as BNF yield = DM yield of the legume × N% of the legume × %Ndfa / 100. The annual N yields and the annual BNF yield were the sum of the two harvests per year. The annual proportion of legume shoot N derived from atmospheric N_2 (annual %Ndfa) was calculated as follows: annual %Ndfa = annual BNF yield / annual N yield in the shoot of the galega × 100.

2.4 Soil sampling and physiochemical analysis

2.4.1 Soil sampling

The soil was sampled six times (Table 3). On each occasion, sixteen sub-samples were taken from the topsoil (0-25 cm) in each plot using an auger with a diameter of 2 cm. The sampling was designed so as not to disturb the plants. Since the germination of galega in the first year was poor, the samples were taken under the canopy in the monoculture plots rather than from bare soil. In the mixture plots, two of sixteen sub-samples were taken under the galega canopy. The 16 sub-samples of each plot were combined to one composite sample, mixed, sieved through a 5 mm mesh, put in a plastic bag and stored at -20°C until the analysis.

2.4.2. Soil chemical analysis

After thawing, the samples were air-dried at room temperature (1 week), ground by hand and sieved through a 2 mm mesh before analysis. Soil properties (electrical conductivity, pH in water, and total C and N) were measured from three repeated sample sets in July 2009, November 2010 and May 2012 respectively. Electrical conductivity (EC) and soil pH were measured in a soil-water suspension 1:2.5 (v:v). EC was measured from the solution part of the suspension using a conductivity meter (MeterLab™ CDM210, Radiometer Analytical), and the pH was measured by a pH-meter (SCHOTT CG842, SI Analytics) after mixing the suspension with a glass rod. Soil dry matter content (DM_S) was determined by drying to the constant mass at 105 ºC. Soil total C and N contents were measured using the VarioMax CN-analyzer and corrected to the oven-dry basis.

2.5 Determination of oil concentration

To monitor the oil degradation over time, we estimated the oil concentration of each oil spiked plot based on the difference of total solvent extractable material (TSEM) concentration between the plot and the average of 4 to 5 randomly selected control plots at each sampling time. The hydrocarbons were extracted from thawed soil samples according to the modification of the ISO 16703:2004 method by Jørgensen et al. (2005). Ten grams of homogenized moist soil samples were weighed (G_S, 0.001 g accuracy) and dissolved in 10 ml of retention time window solution (1 L HPLC-pure *n*-heptane + 30 µl *n*-decane + 30 mg *n*-tetracontane) and 20 ml HPLC-pure acetone by ultrasonication for 30 min. A blank sample (without soil) was prepared on every extraction day. Acetone was removed by duplicate washing of the extract with deionized water at the ratio of 1:1, followed by centrifugation (1600 × g) for 5 min. The upper organic phase was removed to a new glass tube and dried with 0.1 g of water-free Na_2SO_4 (dried at least 4 h at 550 °C). The water-free hydrocarbon extract was removed to a new 10 ml storage glass tube and carefully sealed and stored at -20 °C.

TSEMs, consisting of all the hydrocarbons extracted from soil samples (Wang and Fingas 1997), were measured gravimetrically immediately after extraction according to Mikkonen et al. (2012). Generally, TSEM values of the control plots remained rather stable over time with an average of 0.83 g kg^{-1} soil dry matter.

2.6 Statistical analysis of data

All soil chemical and plant data were analyzed using SPSS (version 20, IBM Inc., Armonk, NY, USA). We used repeated measures split-plot analysis of variance (RM ANOVA) with the sampling time as the repeated factor (within-subject factor) to test the overall between- and within- subjects effects (sphericity assumed) on soil properties, oil concentration and crop physiological characters.

Crop treatment as the main plot factor, and oil and PGPB treatments as the subplot factors were fixed factors, while block (replicate) was a random factor. Thus, crop was tested against the interaction term crop × block to take out the effect of the main plot from the residual variance so it does not skew the error variance of the subplot stratum. Oil, PGPB and their remaining interactions were tested against the subplot error mean square.

Since PGPB resulted in no significant impact on soil parameters and oil concentration for the first four sampling times (data not shown), we sampled soils only from PGPB-untreated plots afterwards. Similarly, the $\delta^{15}N$ was measured only from PGPB untreated plots in 2011 and 2012. Therefore the analyses of variance for soil parameters, oil concentration, annual %Ndfa and annual BNF yield in the legume shoot were carried out and reported only for the PGPB-untreated plots.

To further test the between-subjects effects of 1) the oil treatment and crop treatment on soil chemical properties, 2) the crop treatment on oil concentration and 3) the oil treatment and PGPB treatment on crop physiological characteristics in separate years, the split-plot univariate analysis of variance (UV ANOVA) was applied. Means of oil concentration between different crop treatments and means of physiological properties between different crops were compared using the Tukey HSD multiple pairwise comparison test, whereas means in relation to different sampling times were compared using Bonferroni multiple pairwise comparison test in SPSS. All differences were concluded significant at $p \leq 0.05$. Kinetic modelling was performed to estimate the rates of oil degradation in different cropping systems, according to Snoeyink and Jenkins (1980).

3. Results

3.1 Crop performance in bioremediation cropping systems

3.1.1 Crop growth in clean soil

Crop physiological properties (SPAD readings, C, N content, C:N ratio) were species-dependent and were not affected by oil treatment (Table 4). The time × crop interaction on crop physiological properties was evident (Table 4). SPAD readings of galega rose from year to year, while those of brome grass decreased (Table 4). Within each year, SPAD values of galega fluctuated, while those of the brome grass decreased (data not shown). Crop N content dropped from 2010 to 2011 in brome grass and increased each year in galega, leading to opposite changes in the C:N ratio (Table 4).

Crop dry matter and N yield differed in different cropping systems and in different years (Table 5). Each year over the three years, the legume-grass mixture out-yielded (DM basis) the pure stands of galega by an average of 2.31 Mg ha^{-1} (32%) and of brome grass by 2.41 Mg ha^{-1} (34%) in clean soil (Table 5, Tukey HSD test, $p < 0.01$). Brome grass yield in the mixture plots exceeded the yields of the corresponding pure grass plots in each year (data not shown). The galega yield more than doubled in 2011 and 2012 over that in 2010. Yield of the brome grass was higher in the June cut than in the August cut, but that of the galega showed little difference between cuts (data not shown). An average of 233, 100 and 156 kg ha^{-1} of N (Table 5) was harvested annually from the above-ground parts of pure galega, pure brome grass and their mixture, respectively from the oil-unspiked plots, over three years. The N yields of both brome grass and mixture remained stable over years, whereas that of pure galega more than doubled after the first year (Table 5).

The parameters of $\delta^{15}N$, %Ndfa, BNF yield and BNF yield / galega DM yield were measured to evaluate the biological nitrogen fixation efficiency of the legume *Galega orientalis* in the bioremediation experiment. Neither the crop (monoculture or mixture) nor the oil treatment had any significant influence on the $\delta^{15}N$ of galega in PGPB-free plots (data not shown). Nevertheless, the $\delta^{15}N$ values of the galega changed with time, with the values of 0.61, 2.10, 0.65, -0.43, -0.08 and -0.50 from the six harvests, respectively. The $\delta^{15}N$ in the pooled shoot sample of the brome grass was 4.68, 5.53, 4.20, 3.08, 3.84 and 3.30 in the six harvests in the oil-untreated plots and 6.38, 6.42, 3.94, 1.54, 4.09 and 3.68 in the oil-treated plots. The proportion of N derived from the atmosphere (%Ndfa) was higher in the galega in the mixture than in the pure stands over years (RM ANOVA, $p = 0.051$), especially in 2012 (Table 6). The annual values of %Ndfa did not differ between 2010 and

2011, and significantly increased in 2012 (Bonferroni multiple pairwise comparison test, $p < 0.01$). The annual BNF yield of pure galega continuously and greatly increased from 2010 to 2012 (Table 6), whereas the BNF yield of galega in mixture significantly decreased in 2011 before it achieved its highest value in 2012 (Table 6). Galega in mixture plots fixed more atmospheric N per unit of dry matter yield than its monoculture counterparts (26.3 vs 20.5 g kg^{-1} in clean soil), especially after it achieved its stable growth in 2012.

3.1.2 Toxicity assessment of oil contamination on crop growth

Oil spiking significantly enhanced the overall crop performance, in spite of a slight decrease of total C content in brome grass in 2010 (UV ANOVA, $p < 0.05$, data not shown) and a reduction of %Ndfa in galega and mixture in 2011 (Table 6). Oil contamination was associated with an increase in the annual crop dry matter yield of 494 kg ha^{-1} (6.7%) in brome grass-, 992 kg ha^{-1} (13.8%) in galega- and 580 kg ha^{-1} (6.1%) in mixture-cropped plots throughout the experiment (Table 5). The positive effect of oil treatment on crops was also seen in the elevated N yield, up by 19.0 kg ha^{-1} (11.7%), annually (RM ANOVA, $p < 0.01$). The oil enhancement of N yield was greatest in pure galega plots, 39 kg ha^{-1} (16.7%) higher in oil-spiked plots than in clean controls (Table 5). In 2011, although the %Ndfa showed reductions by oil contamination (Table 6), the N yield of the legume was still 34 kg ha^{-1} (11.7%) higher in oil-spiked plots than in clean controls (Table 5). The oil enhancement of crop growth was more obvious in 2012 than the previous two years, characterized by the significant increase of DM yield, %Ndfa, BNF and N yield, especially in the pure galega plots (Table 5 and Table 6).

3.1.3 PGPB effect on crop growth and BNF

Generally, the effect of PGPB on crop physiological properties (Table 4) and DM yield (Table 5) was minor, but it enhanced BNF in the legume-cropped plots (Table 7). The PGPB enhancement was by the large shown as the increase of %Ndfa, especially in the mixture plots. The %Ndfa in PGPB-treated plots was 13.5% (19%) higher than in PGPB-untreated plots in the first cut in 2010 and 16.1% (31%) higher in the second (Table 7). PGPB treatment increased the BNF yield in legume shoots by 25.6 kg ha^{-1} (37%) in pure galega plots and 8.3 kg ha^{-1} (45%) in mixture plots relative to the corresponding PGPB-untreated plots in 2010 (Table 7). The positive effect of PGPB treatment was also found in crop N yield in 2012 (Table 5), leading to a lower crop C:N ratio (data not shown); however, due to the fertilization of the PGPB-treated pure grass plots in that year, the results were not comparable.

3.2 Impacts of oil and crop treatment on soil chemical properties

Oil contamination significantly affected soil total C content, C:N ratio and electrical conductivity, but had no influence on total N content and pH (Table 8). In July 2009, oil addition significantly increased the soil total C by 3.69 g kg^{-1} (16.6%) and C:N ratio by 1.6 (14.7%) relative to the control soil. Soil total C significantly decreased with time as the oil was degraded, but was still higher than in the control plots in May 2012 (univariate ANOVA, $p < 0.01$). The average soil C:N ratio in the field was generally low (<11:1 in control plots, <12:1 in oil treated plots), and was significantly affected by the oil × crop interaction. The galega soil showed the lowest C:N ratio (10.5:1) in clean control plots, but the highest value (12.1:1) in oil-treated plots. Oil treatment significantly decreased soil EC by 11.7 µS cm^{-1} (17.6%) (Table 8). The EC values dropped significantly in the control plots over time, but remained stable in oil-treated plots (time × oil interaction). Soil pH decreased slightly with time with an average value of 6.4. None of the treatment factors or their interactions affected soil total N content, which averaged 2.21 g kg^{-1}. Crop treatment had no impact on soil chemical properties, but the interactions between crop treatment and other factors on soil properties were evident (Table 8). For example, the time × crop × oil interaction had a significant effect on soil EC. In oil treated soils, EC values increased in bare fallow and brome grass plots but decreased in galega and mixture plots. In clean soils, EC values in galega and mixture plots decreased sharply in 2010 before a slight increase in 2012 whereas EC values in brome grass plots decreased continuously with time (data not shown).

3.3 Oil degradation pattern

The oil concentration decreased over time (Figure 1). At the end of the experiment, 92% of the initial input of 7 g oil kg^{-1} soil was lost from the bare fallow, 75% from the pure grass, 73% from the pure legume and 77% from the mixture plots. Oil degradation was most intense during the first month, as shown by a reduction of oil concentration by 43% in bare fallow, 40% in the pure grass plots, 34% in the pure galega plots and 52% in the mixture plots. The second significant loss of oil occurred during the second growing season, 2010 (months 12-18). Afterwards, oil concentration remained relatively stable with slight fluctuations until the end of the experiment. Oil degradation differed among crop treatments. The average oil concentration in the bare fallow plots was lower than that in the pure legume plots by 0.90 g kg^{-1} soil DM (25%) throughout the experimental period (Tukey HSD test, $p < 0.05$).

The oil degradation in all plots followed a typical first-order kinetic pattern, in which the oil degradation rate was proportional to the oil concentration. The coefficients of determination (R^2), ranging from 0.379 to 0.571 (Table 9), were all statistically significant. The first-order degradation rate constants (k) in different treatments followed the order bare fallow > mixture > brome grass > galega (Table 10). According to this model, 15 to 24 months are required to halve the hydrocarbon concentration in this region, depending on the crop type (Table 10). Nevertheless, the initial oil concentrations estimated in the first-order kinetic models were lower than the designed oil input value (7 g kg^{-1}) by an average of 2.02 g kg^{-1} (29%).

4. Discussion

4.1 Evaluation of the values of legume-grass mixture cultivation in a boreal climate

We studied the ecological suitability and agronomic productivity of fodder galega and brome grass through the assessment of adaptation ability, BNF efficiency and DM production in a boreal region. Both the legume *G. orientalis* and grass *B. inermis* are suitable to grow in boreal regions as potential bioenergy swards. The slow initial growth of the galega, reaching stable production in the second or third year after sowing as indicated by the increasing values of N, chlorophyll, C content, DM yield and BNF yield, agrees with previous studies (Adamovich 2001, Halling et al. 2002, Singer et al. 2003, Kryževičienė et al. 2008, Zolotarev 2010). In addition to the plant's intrinsic growth pattern, the cool environment might have limited the nodulation and BNF activities of the microsymbiont *Neorhizobium galegae* in the first year. The rapid germination of the brome grass in the seedling year in this boreal field accorded with expectation (Global Invasive Species Database 2010). The continuous stable growth of the grass demonstrated its excellent adaptation to the boreal conditions.

The unfertilized galega-brome mixture out-yielded the N-fertilized pure grass swards over years by an average of 34%, although fewer seeds were sown. This finding is in accordance with previous field studies (Halling et al. 2002, Adamovich et al. 2007, Kryževičienė et al. 2008), where it was attributed to the substantial BNF capacity of fodder galega. Our data demonstrated that BNF was more efficient in the mixture, with a higher %Ndfa and a higher ratio of BNF yield to legume DM yield than in pure legume stands, bearing in mind that no alterations of soil total N content occurred between different crop treatments. This result was attributable to an effective legume-rhizobium symbiosis, where BNF is a product of the interaction between soil N environment and overall legume growth (Lindström 1984, Unkovich and Pate 2000). Assuming that soil was the only N source for grass uptake without directional N transfer from the legume to the grass, the $\delta^{15}N$ values of the reference grass would be about the same throughout the experimental period. Nevertheless, we observed decreasing $\delta^{15}N$ values and higher yield of the brome grass in the mixture plots over time, indicating N translocation from the legume to the grass, probably via litter decomposition. Fodder galega increased the amount of bioavailable nutrients in soil after it achieved its stable growth, indicated by the elevated soil electrical conductivity in pure galega plots in 2012. Similarly, a fallow field turfed with fodder galega was found to accumulate bioavailable nutrients P, K and

Mg in soil, due to dissolution of nutrients from less soluble compounds and subsequent nutrient mobilization by soil microbes (Sienkiewicz et al. 2011).

Given its adaptation ability, substantial BNF capacity, productive longevity and effects on soil fertility, the galega-brome grass mixture has potential for bioenergy production in boreal regions. In addition, its C:N ratio (27:1) was close to the known optimal value (25:1) for microbial decomposition (Ndegwa and Thompson 2000). Together with the substantial N yield, the legume-grass mixture residues can be converted into high-value fertilizer by soil fauna and microflora. Thus, the legume-grass mixture has both agronomic and ecological merits, indicating its suitability as a bioenergy cropping system for boreal regions.

4.2 Overall assessment of the legume-cropping bioremediation system

4.2.1 Evaluation of soil chemical composition and oil dissipation

The aim of this work was to evaluate the effectiveness of the bioremediation cropping systems, complex interactions between crops, soil and contaminants during oil contamination and degradation. Soil quality is dependent on the interactions between soil physical, chemical and biological properties (Dexter 2004). Oil contamination has a great impact on soil quality and in turn, soil quality directly affects the effectiveness and efficiency of bioremediation. The amount of oil used was sufficient to be detected in clear increases in total soil C, C:N ratio and TSEMs. Soil electrical conductivity, which is influenced by properties of the pore-filling contaminants at the interface region of a soil (Börner et al. 1993), decreased in oil-spiked plots. The lack of effect of oil on other soil properties such as pH and total N content is attributable to the effective buffering of the soil system.

Most laboratory or field experiments spiked with different types of hydrocarbon mixtures are modelled by first-order kinetics (Jørgensen et al. 2000, Nocentini et al. 2000, Van Gestel et al. 2003), whereas only a few studies (Sarkar et al. 2005) have found second-order kinetics necessary. The coefficients of determination (R^2) were modest but all statistically significant, indicating that our oil degradation data fit reasonably to the first-order kinetic model, although there was wide variation in oil concentration between replicates. The kinetic parameters based on the first-order degradation model revealed a higher oil reduction rate in bare fallow and mixture and a lower rate in monocultures. The high degradation rate constant of oil degradation in the bare fallow demonstrated that the indigenous microorganisms were capable of degrading hydrocarbons in soil. Crop treatments had a negative effect on oil dissipation, particularly the pure galega, under which oil reduction was slower than in the bare fallow, throughout the experiment. This phenomenon contrasts with other studies where crops were found to stimulate microbial oil-degradation activities at the rhizosphere, because root exudates comprised of organic acids, sugars, amino acids and aromatics with changeable composition or amount are excellent primary C and energy substrates for organisms to degrade organic pollutants (Miya and Firestone 2001, Singer et al. 2003, Rentz et al. 2005, Kawasaki et al. 2012). The soil microorganisms may favour easily degradable plant derived compounds over oil hydrocarbons as energy and C substrates. Another possible reason for this result lies in the oil determination method. A no-till management system had the greatest seasonal fluctuation of soil organic carbon (Wuest 2014). The solvent co-extracted natural, carbon-rich compounds such as waxes and chlorophyll from the plants might have contributed to soil TSEMs, elevating the estimates of oil concentration in the vegetated plots. The fluctuation in TSEM values after 18 months of oil degradation may have been due to seasonal inputs of plant residues and root exudates. The determination of the composition of different hydrocarbon groups, as presented by Wang and Fingas (1997), is needed to further confirm this assumption.

The initial rapid loss of approximately ~42% of oil in the first month is in line with previous field studies, where removal of motor lubricating oil was most rapid during the first month and then declined with time (Jørgensen et al. 2000). Joner et al. (2004) attributed the rapid oil dissipation to priming effects following soil excavation and homogenization at the initial stage when neither readily available C (root-derived organic materials) nor mineral nutrients were limiting for biodegradation of hydrocarbons. Nevertheless, abiotic loss of a large proportion of light volatile hydrocarbons was likely to account for the initial rapid oil dissipation and the experimental area smelled strongly of oil for several weeks after spiking. Margesin and Schinner (1997) demonstrated that about 30% of diesel oil was eliminated due to abiotic loss at 10 °C in newly contaminated soils. Similarly, 30% of hydrocarbon was assumed to be lost by physical weathering (vaporization or dissolution) in the first year after the Exxon Valdez spill in Alaska (Bragg et al. 1994). In the first order-kinetic models the estimated initial oil concentrations were lower than the real input value. Plausibly the estimated concentrations were the starting points of the biodegradation process after evaporation. In the present experiment, the second significant oil dissipation occurred during the 2010 growing season, probably due to the biodegradation of easily available hydrocarbons by indigenous soil microorganisms. Afterwards, oil degradation slowed down.

We achieved a more than 70% reduction of oil in the vegetated plots and more than 90% in bare fallow in the three-year field experiment. Incomplete biodegradation of complex hydrocarbon mixtures with a stabilized residual concentration prevails in bioremediation projects (Nocentini et al. 2000). When the biodegradation has reached the residual concentration, further intensive treatment is rarely useful even when optimal biodegradation conditions are provided (Huesemann 1997, Nocentini et al. 2000). The residual oil in the soil was expected, as the resistance of hydrocarbons to biodegradation and extraction increases with time in soil (Hatzinger and Alexander 1995). There are two major recognized causes of incomplete biodegradation, both of which emphasize the poor bioavailability of aged hydrocarbons to microbes: 1) the sequestration of hydrocarbons within the pores of soil aggregates, and 2) the inherently recalcitrant characteristics of residual fractions (Huesemann 1997). Only the latter aspect poses a chronic threat to the environment. Although the exact composition of hydrocarbon fractions in the newly spiked motor oil and the residues was not determined, one would expect to find the heavier distillates among the remaining compounds at the end of a bioremediation experiment (Sarkar et al. 2005). The different petroleum hydrocarbon fractions could be quantified (Wang and Fingas 1997, Mikkonen et al. 2011), but it was suggested that the total petroleum hydrocarbons (TPHs) an insufficient measure to monitor soil remediation due to accumulation of polar and complexed degradation products (Mikkonen et al. 2012). Since we were interested in monitoring the whole remediation process, we did not attempt to quantify individual fractions.

Furthermore, some plants are able to directly remediate oil-contaminated soils by several mechanisms, including hydraulic control, volatilization, stabilization, transformation, degradation and rhizodegradation (Kamath et al. 2004). We lack evidence to identify the direct oil-phytoremediation capacity of these two crops in the field, although both galega and brome grass remained healthy and productive under oil stress.

4.2.2 Crop growth under oil stress

Limitations to the growth of plants and metabolic activities of microbes may arise from the severity of contamination and the heterogeneous nature of soil (Acharya et al. 2014). Certain annual plants were able to survive in moderately to weakly contaminated sites with oil content below 10% by weight (Radwan et al. 1995). Phytotoxicity assessment of oil contamination should thus take place prior to the implementation of a bioremediation project. Previous greenhouse experiments found that the growth, nodulation and BNF of fodder galega were normal in soil contaminated with either fuel or diesel oil (3000 ppm), except that biomass production was reduced in the presence of fuel oil (Suominen et al. 2000, Mikkonen et al. 2011). Our field data demonstrated that used motor oil (7000 ppm) was insufficient to stress the growth of the both crops despite a minor loss of crop total C in brome grass in 2010 and of %Ndfa in galega in 2011. The normal growth and symbiotic functions of the galega under oil stress in the field validated the applicability of the above mentioned greenhouse findings.

Conversely, the presence of oil, to our surprise, markedly enhanced the crop dry matter yield, BNF efficiency and N yield, especially in 2012. More interestingly, the effect of the oil treatment was particularly significant on the legume. As discussed earlier, BNF, a product of the interaction between soil N environment and overall legume growth (Lindström 1984, Unkovich and Pate 2000), was influenced by the interactions between the legume, the rhizobium, soil and the hydrocarbons in oil contaminated plots. Mikkonen et al. (2011) attributed this positive post-effect of oil addition on the growth of galega to the reduced phytotoxicity following the loss of easily-degradable alkanes that are more toxic to plants than aromatics. The subsequent recovery of soil microbial activities, especially those of the rhizobia, from oil inhibition might have contributed to the increased BNF, DM yield and N yield of the legume. Earlier experiments also found that oil treatment had positive effects on soil in two aspects: 1) enhancement of biochemical and microbial activities with regard to higher microbial biomass C and metabolic activities (Caravaca and Roldán 2003, Mikkonen et al. 2011), and 2) the induction of 15-fold greater soil porosity in the range of transmission pores, which are essential for the growth of plant principal roots, for drainage and for aeration (Caravaca and Roldán 2003). Thus the biological, chemical and physical enhancement of soil quality by oil contamination might have together induced vigorous crop growth, despite the initial inhibition on BNF of the legume-rhizobium symbiotic system. Hence, the combination of these two perennial crops is a good candidate to achieve considerable output while alleviating soil contamination in boreal soils.

4.2.3 PGPB effect

In this experiment, we aimed to evaluate the effects of plant growth promoting bacteria on soil, crop growth and oil reduction. Co-inoculation of fodder galega with its specific rhizobia and plant growth promoting *Pseudomonas* strains improved plant growth, nodulation and N content in a pot experiment (Egamberdieva et al. 2010). In the present study, the enhancement by *Pseudomonas* strains on the BNF of fodder galega in regard to %Ndfa and BNF yield was evident in 2010, but there was little evidence of such an effect on the overall crop DM production. In Estonia, high N fertilization rate reduced the role of fodder galega in the galega-grass mixture swards (Lättemäe et al. 2013). We can therefore hypothesize that the enhancement of N fixation by PGPB was mainly driven by the nutrient requirement of the legume. When there is sufficient soil mineral N for the growth of the legume, BNF would be inhibited. In the presence of resource competition from the grass in the mixture plots, the PGPB took effect to improve the BNF in the legume.

Neither soil properties nor oil concentration responded to the PGPB inoculation (data not shown) in our field, in contrast to other studies where PGPB enhanced the rhizoremediation of polluted soils (Pajuelo et al. 2011, Vershinina et al. 2012, Bhattacharyya and Jha 2012). However, the oil tolerance ability of PGPB strains, the local field factors and the hydrocarbon composition were different from these studies. Long-term monitoring should be applied to reveal the effects of PGPB on perennial crops.

5. Conclusions

Bioremediation by indigenous organisms is considered a cost-effective strategy to reclaim contaminated sites. In our bioremediation field experiment, different cropping systems exhibited the same first-order kinetic pattern of oil degradation. The oil degradation was incomplete 40 months after the oil exposure, with a dissipation of 73% - 92% of oil concentration. The result that the highest oil degradation rates occurred in bare fallow and lowest in fodder galega, disagrees with our hypothesis that the legume galega was expected to stimulate oil degradation owing to its BNF ability. Nevertheless, our results demonstrate the ecological and agronomic feasibility of planting a legume (fodder galega) and a grass (smooth brome) on oil-contaminated soil in a boreal climate, as both crops were oil-tolerant. The presence of oil, unexpectedly, enhanced the overall crop dry matter yield and N yield over years. In addition, oil enhanced the BNF efficiency of the legume. Fodder galega could fully replace N fertilizer for brome grass and enable a high dry matter production of the grass in the mixture plots, due to its substantial BNF ability. The inoculation of plant growth promoting bacteria had a minor effect on crop physiological properties, but significantly increased the BNF efficiency of the legume, especially in the mixture plots. The galega-bromus grass mixture residues can be converted into high-value fertilizer owing to the close-to-optimum C:N ratio via microbial decomposition. Therefore, we suggest that the perennial legume-grass mixture, co-inoculated with appropriate rhizobia and PGPB, is a competitive and sustainable cropping system to alleviate soil contamination in boreal soils, with considerable economic value for bioenergy and bio-fertilizer production.

Factors influencing the efficiency of the boreal legume-cropping bioremediation system remain to be further identified. In subsequent work, molecular techniques such as NGS sequencing, microbial community fingerprinting techniques and functional gene analysis will be incorporated to identify the oil-degrading populations and the specific metabolic pathways with the aim to provide a comprehensive evaluation of the legume-cropping bioremediation system in a boreal region.

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Tables

Table 1. General information about the experimental field

Site properties	Details
Site area	420 m^2
Plot size	3.75 m^2 (2.5 m × 1.5 m)
Soil structure	Clay loam (on average, 32% clay, 36% silt, 32% sand)
Altitude	8 m
Vegetation zone	Boreal
Annual precipitation	650 mm
Annual mean temperature	4.9°C
Farming systems	Integrated
Cropping history	*Salix*

Table 2. Monthly average temperature (°C) and total precipitation (mm) during the growing season in Helsinki for the experimental period May – Oct. (2009–2012) compared with the long term data (1971–2000), provided by Finnish Meteorological Institute.

Month	Average temperature (°C)						Precipitation (mm)				
	1971–2000	2009	2010	2011	2012		1971–2000	2009	2010	2011	2012
May	9.8	11.0	11.5	9.9	10.9		37	45	59	27	65
June	14.8	14.1	14.6	16.7	13.7		57	75	33	49	88
July	17.2	17.2	21.7	20.6	17.7		63	131	49	56	54
August	15.8	16.7	18.1	17.5	16.0		80	49	97	173	39
September	10.9	13.5	12.2	13.6	12.5		56	40	50	88	160
October	6.2	4.2	6.0	8.5	6.7		76	90	29	69	93
Average	12.5	12.8	14.0	14.5	12.9	Total	369	429	312	460	499

Table 3. Information on soil sampling

Year	Growing season	Date	No. of samples
2009	Crop	July 16–17	64
2010	Beginning	May 17–25	64
2010	End	November 12	64
2011	Beginning	May 17–19	64
2012	Beginning	May 21	32 (PGPB-untreated plots)
2012	End	October 11	32 (PGPB-untreated plots)

Table 4. Crop physiological properties during the experimental years, with significance of terms from repeated measures ANOVA

Treatment		SPAD readings				Crop C%				Crop N%				Crop C:N ratio			
		2010	2011	2012	*Mean*	2010	2011	2012	*Mean*	2010	2011	2012	*Mean*	2010	2011	2012	*Mean*
Brome grass		33.6	28.6	27.6	*29.9*[c]	44.5	44.2	43.0	*43.9*[b]	1.6	1.3	1.4	*1.4*[c]	28.3	33.9	32.3	*31.5*[a]
Galega		33.0	38.0	40.1	*37.0*[a]	45.0	46.1	44.7	*45.2*[a]	2.7	3.2	3.5	*3.1*[a]	17.0	14.4	13.0	*14.8*[c]
Mixture		32.9	30.7	30.5	*31.4*[b]	44.5	44.2	43.4	*44.0*[b]	1.9	1.5	1.7	*1.7*[b]	24.1	30.8	26.3	*27.0*[b]
	SEM	0.4	0.3	0.5	*0.3*	0.0	0.3	0.1	*0.1*	0.1	0.02	0.03	*0.02*	0.6	0.3	0.3	*0.2*
Tests of Between-Subjects Effects																	
Source	df	significance level															
crop	2				***				***				***				***
Tests of Within-Subjects Effects (Sphericity Assumed)																	
Source	df	significance level															
time	2				*ns*				***				**				***
time × crop	4				***				*				***				***
time × crop × oil	4				*				*ns*				*ns*				*ns*

SEM standard error of mean, *df* degrees of freedom, *ns* not significant, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. The tests were based on split-plot-based repeated measures (RM) ANOVA model: Y_1 = residue (error) + crop + crop × replicate + oil + PGPB + oil × PGPB + oil × crop + crop × PGPB + oil × crop × PGPB, with time as the repeated factor. The factors or interactions that had no significant effects on soil physiological parameters are not presented in this table. The italic numbers refer to the average values (as well as the corresponding SEM) of the same crop species-treated plots yearly, regardless of oil treatment, since oil treatment had no significant impact on these crop physiological parameters. Different uppercase letters (a, b and c) indicate significant differences ($p < 0.05$) between the means of crop treatments based on Tukey HSD multiple comparison test.

Table 5. Annual dry matter and N yields in the experimental treatments, with significance of terms from the analysis of variance

Treatment			Dry matter yield, Mg ha^{-1}				Crop N yield, kg ha^{-1}			
Crop	Oil		2010	2011	2012	*Mean*	2010	2011	2012	*Mean*
Brome grass	–oil		6.85	7.38	7.02	*7.09*	106	94	100	*100*
	+oil		7.61	7.58	7.55	*7.58*	111	96	102	*104*
Galega	–oil		3.95	9.06	8.55	*7.19*	117	289	293	*233*
	+oil		4.65	10.01	9.87	*8.18*	140	323	353	*272*
Mixture	–oil		7.92	11.25	9.32	*9.50*	149	167	150	*156*
	+oil		8.90	11.12	10.22	*10.08*	182	150	178	*170*
		SEM	0.29	0.37	0.39	*0.28*	12	10	7	*7*
Tests of Between-Subjects Effects										
Source		df	significance level							
crop		2	***	**	**	**	**	***	***	***
oil		1	**	ns	**	**	ns	ns	***	**
PGPB		1	ns	ns	ns	*ns*	ns	ns	**	*ns*
crop × oil		2	ns	ns	ns	*ns*	ns	*	**	*ns*
Tests of Within-Subjects Effects (Sphericity Assumed)										

Source	df	significance level		
time	2		***	***
time × crop	4		***	***
time × oil	2		*ns*	*
time × crop × oil × PGPB	4		*	*ns*

SEM standard error of the means; *df* degrees of freedom, *ns* not significant, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. The tests of Between-Subjects and Within-Subjects Effects were based on split-plot-based repeated measures (RM) ANOVA model: Y_1 = residue (error) + crop + crop × replicate + oil + PGPB + oil × PGPB + oil × crop + crop × PGPB + oil × crop × PGPB, with time as the repeated factor. The results of RM ANOVA are shown under the column of means. Because some of the interactions with time were significant, split-plot univariate (UV) ANOVA was conducted on each year's individual results. The factors or interactions that had no significant effects on the tested parameters are not presented in this table.

Table 6. The %Ndfa, BNF yield and the BNF yield / DM yield in galega above-ground dry matter in 2010–2012, with significance of terms from the analysis of variance

Treatment			Shoot %Ndfa				Shoot BNF yield, kg ha^{-1}				BNF yield / galega DM yield, g kg^{-1}			
Crop	Oil		2010	2011	2012	*Mean*	2010	2011	2012	*Mean*	2010	2011	2012	*Mean*
Galega	–oil		65.4	61.2	70.6	*65.7*	69.8	177.1	186.6	*144.5*	18.8	19.4	23.4	*20.5*
	+oil		59.5	52.8	72.0	*61.4*	68.6	159.7	249.4	*159.2*	17.0	16.9	26.0	*20.0*
Mixture	–oil		69.7	70.3	83.4	*74.5*	19.3	7.4	23.5	*16.7*	21.5	21.9	35.6	*26.3*
	+oil		68.7	63.4	84.2	*72.1*	17.5	6.2	36.5	*20.0*	22.8	20.7	45.5	*29.7*
		SEM	4.4	1.9	0.2	*1.2*	5.9	7.6	8.5	*5.7*	2.1	1.0	1.0	*1.2*
Tests of Between-Subjects Effects														
Source		df	significance level											
crop		1	ns	ns	*	*p=0.051*	ns	ns	*	***	ns	ns	**	*
oil		1	ns	**	**	*	ns	ns	**	*ns*	ns	ns	ns	*ns*
crop × oil		1	ns	ns	ns	*ns*	ns	ns	**	*ns*	ns	ns	ns	*ns*
Tests of Within-Subjects Effects (Sphericity Assumed)														
Source		df	significance level											
time		2				**				***				**
time × crop		2				*ns*				***				*ns*

time × oil	2	*ns*	***	*
time × crop × oil	2	*ns*	**	*ns*

%Ndfa Proportion of shoot N derived from the atmosphere, *ave.* average, *SEM* standard error of mean, *df* degrees of freedom, *ns* not significant, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. All results were obtained from PGPB-untreated plots. The tests of Between-Subjects and Within-Subjects Effects over years were based on split-plot-based repeated measures (RM) ANOVA model: Y_1 = residue (error) + crop + crop × replicate + oil + oil × crop, with time as the repeated factor. The results of RM ANOVA are shown under the column of means. Because some of the interactions with time were significant, split-plot univariate (UV) ANOVA was conducted on each year's individual results.

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Table 7. PGPB effect on the nitrogen fixation efficiency of the legume-cropping systems in both cuts of 2010, with significance of terms from the analysis of variance

Treatment			%Ndfa			BNF yield, kg ha^{-1}		
PGPB	Crop		1st cut	2nd cut	*2010*	1st cut	2nd cut	*2010*
PGPB-	Galega		69.9	38.5	*62.4*	58.7	10.6	*69.2*
	Mixture		70.1	52.3	*69.2*	18.0	0.4	*18.4*
PGPB+	Galega		70.4	42.6	*66.0*	84.8	10.0	*94.8*
	Mixture		83.6	68.4	*83.0*	26.1	0.7	*26.7*
		SEM	2.7	4.4	*2.6*	3.4	0.7	*3.8*
Source		df	significance level					
crop		1	ns	***	*ns*	***	***	***
oil		1	ns	*	*ns*	ns	**	*ns*
PGPB		1	*	*	**	***	ns	***
crop × oil		1	ns	*	*ns*	ns	**	*ns*
crop × PGPB		1	*	ns	*ns*	*	ns	*

%Ndfa the proportion of N derived from atmospheric N_2, *BNF yield* the N yield that was biologically fixed into the legume shoot from atmospheric N_2, *SEM* standard error of mean, *df* degrees of freedom, *ns* not significant, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. The italic numbers refer to the calculated annual values (not the average values between both cuts) as well as the corresponding SEM under the same PGPB treatment, regardless of oil treatment. Tests of Between-Subjects Effects were performed based on the split-plot-based univariate (UV) ANOVA model: Y_1 = residue (error) + crop + crop × replicate + oil + PGPB + oil × PGPB + oil × crop + crop × PGPB + oil × crop × PGPB. The factors or interactions that had no significant effects on the test parameters are not presented in this table.

Table 8. Changes of soil chemical properties in regard to oil contamination over years, with significance of terms from the repeated measures ANOVA based on a split-plot experimental design

Treatment	Sampling time	pH		EC, μS cm^{-1}		Total C, g kg^{-1}		Total N, g kg^{-1}		C:N ratio	
–oil	July 2009	6.48(0.02)	*6.35(0.02)*	79.9(1.96)	*66.6(1.4)*	23.89(0.36)	*23.78(0.21)*	2.21(0.05)	*2.22(0.02)*	10.9(0.2)	*10.8(0.1)*
	Nov. 2010	6.35(0.02)		63.7(2.06)		24.34(0.40)		2.28(0.04)		10.7(0.2)	
	May 2012	6.22(0.03)		56.2(1.84)		23.11(0.16)		2.15(0.02)		10.7(0.1)	
+oil	July 2009	6.53(0.02)	*6.39(0.02)*	51.6(1.96)	*54.9(1.4)*	27.85(0.36)	*26.07(0.21)*	2.23(0.05)	*2.21(0.02)*	12.5(0.2)	*11.8(0.1)*
	Nov. 2010	6.39(0.02)		54.1(2.06)		25.67(0.40)		2.21(0.04)		11.7(0.2)	
	May 2012	6.26(0.03)		54.4(1.84)		24.68(0.16)		2.19(0.02)		11.2(0.1)	
Tests of Between-Subjects Effects											
Source	df	significance level									
crop	3	*ns*		*ns*		*ns*		*ns*		*ns*	
oil	1	*ns*		***		***		*ns*		***	
oil*crop	3	*		*ns*		*ns*		*ns*		*	
Tests of Within-Subjects Effects (Sphericity Assumed)											
Source	df	significance level									
time	2	***		***		***		*ns*		**	

time × crop	6	*ns*	*	*	*ns*	*ns*
time × oil	2	*ns*	***	***	*ns*	**
time × crop × oil	6	*ns*	***	*ns*	*ns*	*ns*

df degrees of freedom, *ns* not significant, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, *EC* electrical conductivity. All mean values were averaged from PGPB-untreated plots with standard error of the means (SEM) presented in brackets. The italic numbers refer to the mean values (as well as SEM) of the parameters averaged between years in the presence or absence of oil treatment, regardless of cropping systems.

Table 9. Comparison of correlation coefficients for first-and second-order linear models for oil degradation in bare fallow, pure brome grass-, pure galega- and mixture-cropped soils

Matrix	First-order linear model R^2	df	Second-order linear model R^2	df
Bare fallow	0.461***	25	0.000	27
Brome grass	0.571***	26	0.066	27
Galega	0.521***	27	0.436***	27
Mixture	0.379***	27	0.146*	27

df degrees of freedom, * $p < 0.05$, *** $p < 0.001$

Table 10. Degradation coefficient (*k*), half-life and regression equations for oil degradation in bare fallow, pure brome grass-, pure galega- and mixture-cropped soils

Crop treatment	*k* ($month^{-1}$)	Half-life (months)	Regression equations
Bare fallow	0.048	15	$Y_{BF} = 4.51e^{-0.048t}$
Brome grass	0.030	23	$Y_{B} = 5.26e^{-0.030t}$
Galega	0.029	24	$Y_{G} = 5.15e^{-0.029t}$
Mixture	0.042	16	$Y_{M} = 4.99e^{-0.042t}$

Supplementary materials

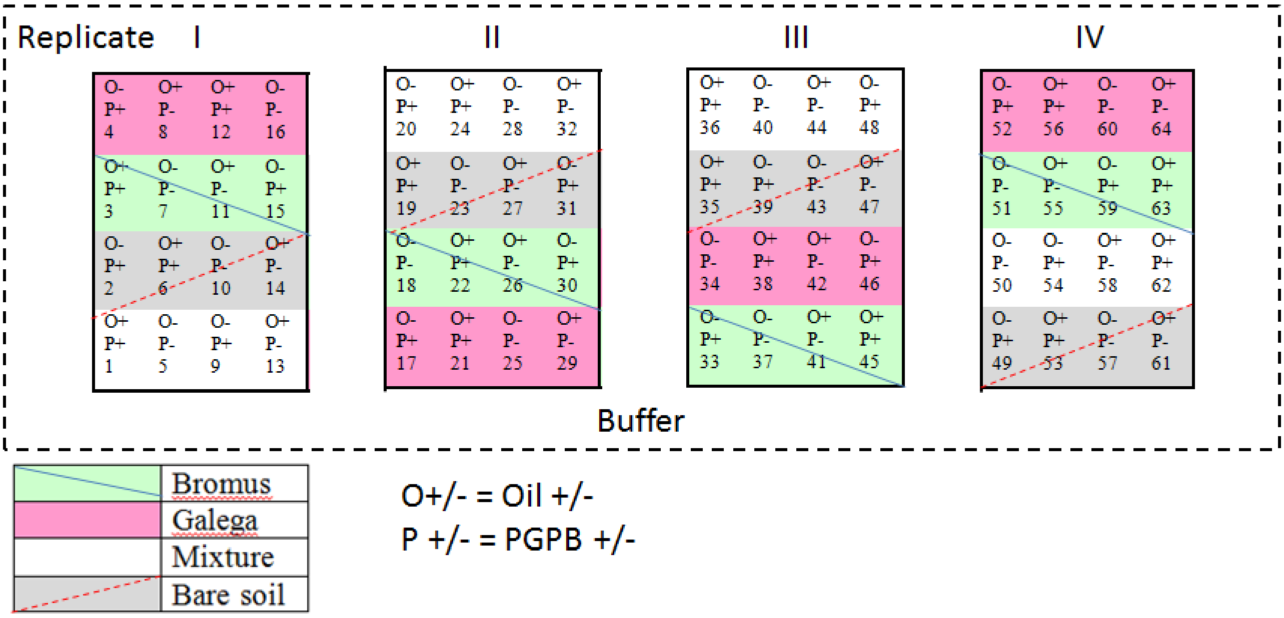

Figure S1. Layout of the field experiment, showing species, oil and PGPB treated plots. Numbers are plot codes.