Remediation of soil polluted with spent oil using cow dung

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Remediation of soil polluted with spent oil using cow dung

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Abstract. Spent oil is any petroleum-based or synthetic oil that contain impurities or loss of major properties thus affecting its unique purpose. In this study, we selected and contaminated a site in Covenant University with spent oil. This site was amended with cow dung and studied for 56 days. Spent oil contaminated soil without stimulation with cow dung served as the control. This study was done during the dry season period and the sites left to natural edaphic factors. Physicochemical parameters such as pH, moisture content were monitored to determine the influence of cow dung as biostimulatory agent when compared with control (untreated) site. Within the period of this investigation, we recorded decrease in the pH value on the amended site, the value ranged between 8.60-7.77 while the control increased from 8.30 -8.42. The moisture content (%) were 95.0- 82.1 and 90.8-103.0 for the amended and control sites respectively. Following these obtained dynamics, we isolated organisms using enrichment technique from the contaminated sites and the control. We characterized the isolates using phenotypic characteristics and comparison with standard reference organisms. The bacterial isolates obtained include: *Arthrobacter Mycobacteria Pseudomonas* and *Corynebacterium*. The axenic cultures ability to utilize spent oil was monitored via indirect estimation using pH and Optical density dynamics for 240hrs. All the organisms exhibited growth in the MS medium supplemented with spent oil. The pH and optical density (OD) from bacterial species obtained from the control sample ranged between 6.91 ± 0.20- 6.56± 0.29 and the OD 0.278±0.150-0.826 ± 0.33. For the amended sample, the bacterial species showed decline in pH that ranged between 7.13±0.30 - 6.33±0.10 while the recorded OD values ranged between 0.190±0.03 - 0.621±0.50. Comparing the results obtained for the control and the amended soil. It was obvious that organisms from the amended soil (either in-situ or in-vivo) showed more metabolic activities on the spent oil. This study suggests that use of cow dung in appropriate concentrations could be very useful in bioremediation of soil contaminated with spent oil.

1. Introduction

Spent oil or waste lubricating oil is oil obtained during servicing of machines and automobiles [1]. Spent oil is similar to unused oil, except that impurities such as trace metals have been added to the oil due to high temperature and pressure of the operating engines, thus contributing significantly to chronic hazards of the spent oil [2,3]. Most companies that operate in Nigeria use machines that requires lubricating oil, thus the release of the spent oil into the environment is one major source of environmental pollution. This challenge is more worrisome where the regulatory framework/ law for citing of companies and mechanic workshop are not obeyed. Most mechanic workshops are manned by ignorant artisans that may not be aware of the environmental implications of indiscriminate discharge of spent oil. When spent oil are released into the environment it could affect many species of flora and fauna in the environment [4,5]. Spent oil could be characterized in appearance as black to brown liquid mixture. It could contain low to high molecular weight (C15-C18) aliphatic and aromatic hydrocarbon, polychlorinated biphenyls, lubricative additives and decomposition products.

Hydrocarbons could be removed from the soil environment via several ways. These methods include: biosparging, bioventing, composting bioaugmentation, biostimulation, mycoremediation and phytoremediation. Furthermore, other conventional technique used for remediation include excavating

up contaminated soil and moving it to landfill sites or capping the contaminated areas of soil. Among these, bioremediation is utmost in terms of hydrocarbon removal [6]. It is effective, economic and eco-friendly and could lead to the complete mineralization of hydrocarbon [7]. One promising method is the application of animal manure, chemical fertilizer that will stimulate soil microbes. Chemical fertilizers have the disadvantage in that it lowers the soil fertility. An alternative is to use organic fertilizers (poultry/ goat manure), to biostimulate the polluted areas for a clean-up or remediation process[8].

In the reports of [9-10] organic nutrients such as chicken droppings, periwinkle shells have been used for the bioremediation of petroleum polluted soils in Nigeria.

Cow dung is composed of reservoir of nutrients and energy that could support microbial growth and subsequently remediation of various organic pollutants [11]. Cow dung can improve the soil structure and fertility by contributing diverse species of microorganisms which include but not limited to *Acinetobacter , Bacillus* and *Pseudomonas* that are essential for natural biogeochemical processes [12,13]. Numerous bacteria genera have exhibited great potentials in using hydrocarbon substrates [14-18]. These bacterial species are common to pristine and oil polluted environments [19-21]. Based on the need to evolve a cost effective method of reclamation of sites polluted with spent oil, this study was set out to use cow dungs in stimulating soil polluted with spent oil for reclamation purposes. In addition, we herein report that bacterial species associated with the cow dung were competent in removing the spent oil via slurry experimentation.

2. Materials And Methods

Chemicals, Reagents and Instrumentation

The nutrient agar, nutrient broth were sourced from Micro master, India. The reagents $(NH_4)_2SO_4$, $MgSO_4.7H_2O$, $Ca(NO_3)_2.4H_2O$, K_2HPO_4, KH_2PO_4, and NaCl are of analytical grade and obtained from Merck, Germany. The Voges Proskauer medium, urease base agar, starch agar, methyl red, peptone water were generously provided by the Microbiology laboratory of Covenant University, Ota. The spent oil was obtained from a mechanic workshop in Ota, Ogun State, Nigeria. Mechanical/orbital shaker (Model HZQ-X300), Centrifuge (Model 80-2), pH meter, UV spectrophotometer (Genesys 10 UVS spec model) were used in this investigation.

Media Preparation

Chloride-free minimal salts (MS) medium as described by (Nwinyi *et al*., 2014; 2016) was used for the e degradation experiments . The medium consists of 0.5g $(NH_4)_2SO_4$, 0.1 g $MgSO_4•7H_2O$, 0.076 g $Ca(NO_3)_2.4H_2O$ and 40mM phosphate buffer (pH 7.25). Solid MS medium was made by the addition of 1.8% Agar (Difco Laboratories, Detriot, MI USA). MS medium was supplemented with the spent oil for the enrichment and degradation assay.

Study site

The selected site for this study was soil surrounding the College of Science and Technology. The site was condoned off and marked out in two transect. The two sections of the soil were contaminated with spent oil collected from a motor mechanic shop in Ota. A section of the marked study site was inoculated with cow dung while the other section without the cow dung served as a control. Prior to the contamination with the spent oil, the soil samples were collected using a hand trowel and placed in a black nylon bag for physicochemical evaluation. The samples were then transported to the laboratory for further analysis.

The pH fluxes and moisture content analysis of soil samples

The collected soil samples were sieved, removing stones and sent for analysis on some physicochemical parameters such as conductivity, pH and moisture content.

Moisture determination

The crucible were washed and dried. The weight of each crucible was measured and referred to as (W1). 5g of each soil sample were weighted into two sterile crucible dish (W2) and placed in the oven

at 105˚C for 3hours. This was allowed to cool before it was reweighed as (W3). The $W3_1$, were placed in the oven again for 30 minutes and allowed to cool and reweighed as ($W3_2$). This was repeated until similar weight value is obtained (W3*). The difference in weight between the initial weight and constant weight gained represents the moisture content. The mathematical calculation of moisture content is given below.

$$\textit{Moisture Content (\%)} = \frac{W3 - W1 \quad X \quad 100}{W2 - W1}$$

Where W1 = initial weight of empty crucible,
W2 = weight of crucible + soil before drying,
W3= final weight of crucible + soil after drying

pH determination

Five (5g) of soil samples were dissolved in distilled water. However since it was not deionised water, the pH of the distilled water was measured following the calibration of the pH meter. Then soil samples were homogenised using glass rod. This was allowed for about 5 minutes before taking readings.

Isolation and characterization of spent oil degrading bacterial species

Following the physiological changes obtained from the physicochemical analysis, liquid culture spent oil degradation was conducted. For this, 20g each of the soil samples collected (soil biostimulated with cow dung) were sieved and inoculated into 250ml conical flask containing 100ml MS media. This was homogenized by gently stirring to allow soil to evenly spread and mix with the medium. Aliquots of spent oil were added to supplement as carbon and energy source. The bioreactor was incubated in an orbital shaker (Model HZQ-X300) for 2weeks. Following the end of the incubation period, 0.1ml of the enrichment broth was pipette and streaked over minimal salt agar (MS-Agar) with spent oil sprayed on the surface. This was incubated in the dark for 5days at ambient temperature. Colonies that appear were further selected and periodically transferred to obtain a pure culture. The pure isolates were maintained on MS agar sprayed with spent oil to keep the isolates stressed in other to continue to express the catabolic plasmids required for spent oil degradation. Using morphological examinations, notable bacterial strains from distinct colonies were selected for identification and degradative potentials. They selected bacterial strains were maintained on agar slant and placed in the refrigerator at 4˚C. The pure cultures were tentatively named as C1, C2, C3, S1, S2, S3 classified phenotypically using standard methods and comparison with standard reference organisms. The following tests were carried out Gram stain, morphology, catalase, oxidase, colony motility, methyl red, Voges Proskauer, indole, nitrate reduction, gelatin hydrolysis, spore test, starch hydrolysis, Urease activity, citrate, sugar utilization , growth in 5%NaCl and growth at pH 6.0

Inoculum preparation for degradation studies

The obtained pure cultures were incubated on a freshly prepared sterile nutrient broth in cotton wool stoppered Balch tubes and incubated at 30˚C for 120h. This was done to produce critical mass of cells required for the degradation studies. The cells were harvested by centrifugation at 35 x 100 rpm for 50 minutes, washed two times in phosphate buffer saline at pH of 7.25, transferred into a sterile eppendorf tubes and re-suspended in MS medium. The inocula were used for the growth and degradation assay.

Biodegradation studies

The biodegradation studies were performed in sterile 160mL serum bottles tubes containing MS medium (20 mL) supplemented with the spent oil. These were seeded with pure cultures at 10^5 cells/ml. Tubes were labelled, stoppered with sterile cotton and incubated in a rotary shaker (Model H2Q-X 300) at 65 rpm at 30.4˚C for 12 days. For the abiotic control, MS medium and spent oil devoid of organism was incubated at the similar conditions as samples. Measurements of optical density (OD) and pH were carried out at 0, 72, 192, 240 hrs. Optical density was measured at 600 nm using (Genesys 10 UVS spec model).

Statistical analysis
Statistical tests (mean and standard deviation) were performed using the EXCEL spreadsheet

3. Results And Discussion
The pH fluxes of the amended soil (cow dung) was 8.6-7.8; control 8.2-8.4 for the 56 days of monitoring of the soil samples (See Figures 1and 2). The figures 3a and b, show the biodegradation potential of the obtained isolates. The pH and optical density fluxes shows the behavior of the organisms in spent oil when incubated at 240hrs.The moisture content within this period of investigation ranged between 95-82.1 for the amended soil while the control had 90.8-103.
Six bacterial species (C1, C2, C3, S1, S2 and S3) were isolated, characterized and selected for further biodegradation studies on spent oil. The isolates C1, C2 and C3 are non-motile, S2 and S3 are motile. S3 and C1 does not have pigmentation C3 are gram negative while others are gram positive. The data of Table 1a and 1b showed that isolates were similar to members of the genus *Corynebacterium spp, Pseudomonas, Arthrobacter* and *Mycobacteria* species.

Table 1a. Morphological characteristic of bacterial isolates

Distinguishing features	C1	C2	C3	S1	S2	S3	
Colony Shape	Round	Irregular	Irregular	Round	Irregular	Irregular	
Colony egde/margin	Entire	Undulate		Lobate	Entire	Filiform	Filiform
Pigmentation No	Green	Green	Yellowish green		No	No	
Opacity of Colony	Translucent		Opaque	Opaque	Opaque	Opaque	Opaque
Surface of Colony	Smooth	Smooth	Smooth	Smooth	Rough	Rough	
Consistency or Texture	Buttery	Brittle	Mucoid	Mucoid	Brittle	Brittle	

Table 1b. Cultural and Biochemical Features of Bacterial isolates capable of Degrading Spent oil

Distinguishing features	C1	C2	C3	S1	S2	S3
Gram's Reaction & Morphology	+ Short rods	+ Short rods	- Long rods	+ Short rods	+ Short rods	+ Short rods
Motility	Non-motile	Non-motile	Non-motile	Non-motile	Motile	Motile
Citrate	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Indole	-	-	+	+	-	-
H_2S Production	-	-	-	-	+	+
Methyl Red	-	-	-	-	-	-
Starch Hydrolysis	+	+	+	+	+	+
Urease	-	-	+	-	-	-
Oxidase	+	+	+	+	+	+
Glucose	-	+	+	+	+	+
Gas Production	-	-	+	-	+	+
Lactose	-	-	-	-	-	+
Gas Production	-	-	-	-	-	-

Sucrose	-	-	-	-	+	+
Gas Production	-	-	-	-	+	+
Growth at pH 6.0	+	+	+	+	+	+
Growth at 35˚C	+	+	+	+	+	+
Growth in 5% NaCl	+	+	+	+	+	+
Acid Fast Stain	-	-	-	-	-	-
Spore Stain	-	-	-	-	-	-

+: Positive Reaction
-: Positive Reaction
C1> Control 1
C2> Control 2
C3> Control 3
S1> Stimulated soil 1
S2> Stimulated soil 2
S3> Stimulated soil 3

From the results of the biochemical tests and colonial morphology features, the most probable microorganisms include:
C1> *Corynebacterium spp*
C2> *Corynebacterium spp*
C3> *Pseudomonas spp*
S1> *Arthrobacter spp*
S2> *Mycobacterium spp*
S3> *Mycobacterium spp*

Plate 1.0 shows conical flasks containing 200ml of Nutrient Broth with the bacterial isolates. The turbidity and colour change depicts microbial growth.

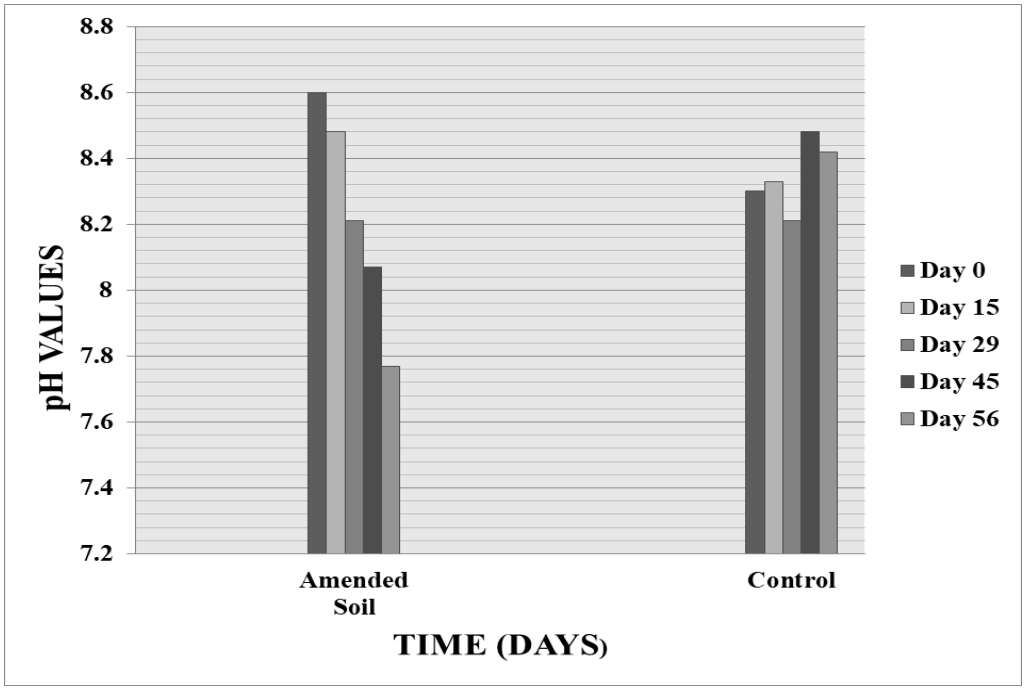

Fig. 1.0 shows pH values of soil contaminated with spent oil against different day intervals

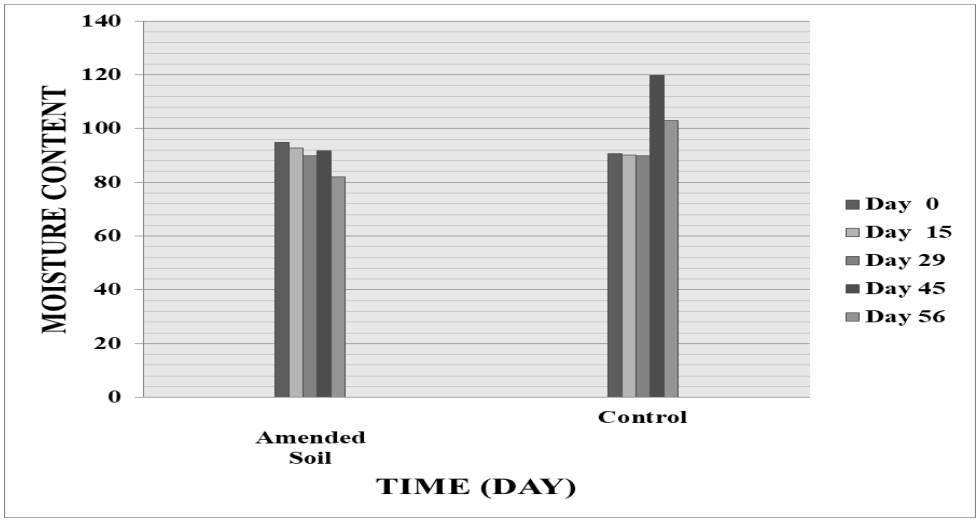

Fig. 2.0 shows the moisture content of contaminated soil samples against time (day)

3.

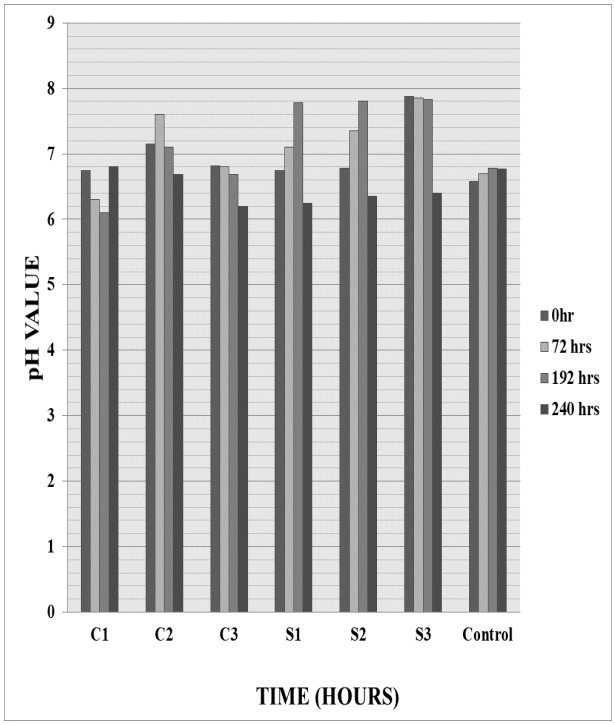

Fig. 3a. pH values for the bacterial isolates against time during biodegradation of spent oil

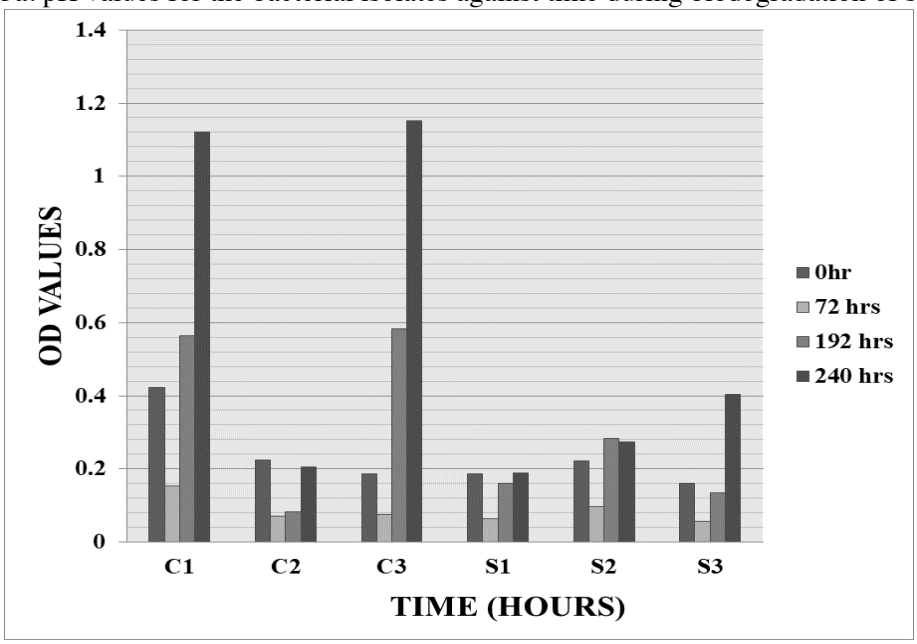

Fig. 3b. Optical densities of bacterial isolates against time during biodegradation of spent oil

Culture-dependent methods using the organisms' morphological and physiological behaviours have been used in characterizing these bacterial species, which include: Gram-negative bacteria, *Pseudomonas* and other gram positive *bacteria* genera *Corynebacterium*, *Arthrobacter* and *Mycobacterium* genera. These organisms are among some notable bacteria species competent in degrading hydrocarbon. Some specific organisms that are capable of degrading the hydrocarbons include *Bacillus subtilis, Bacillus* sp., *Alcaligenes* sp., *Flavobacterium sp.,Micrococcus roseus, Corynebacterium* sp., *Citrobacter freundii, Klebsiella, Arthrobacter, Acromobacter, Alcaligenes, Nocardia* and actinomycetes [22-24]. This study reports the effects of cow dung on the microbial

utilisation of spent oil on polluted soil in-situ. The samples (control and stimulated soil) which were collected at different dimensions of collection site were analysed weekly for various pH and moisture content. pH refers to the hydrogen ion concentration in soil and moisture content refers to the maximum water content that does not restrict oxygen diffusion in the soil. From the results shown in Fig 1.0, there is a decrease in moisture content from 95 at day 0 to 82.1 at day 56; while in Fig 2, fluctuations where observed in moisture content from 90.8 at day 0 to 89.9 at day 29 and increases back to 103 at day 56. The decrease in pH shown in Fig.1 of the stimulated soil signifies the presence of organic acids produced as a result of microbial utilisation of hydrocarbons [25] while the fluctuations in pH shown in Fig.2, may have resulted from high metabolic activities possibly resulting in the production of intermediate metabolites by indigenous microbes [26].

In this study, enrichment methods were used to isolate the bacterial species where the spent oil served as carbon and energy source [27]. From the enrichment culture, certain bacterial strains were isolated successfully. They were tentatively named *Arthrobacter*, *Corynebacterium spp*, *Mycobacterium spp* and *Pseudomonas spp* (See Table1a,b). The pH and optical density (OD) were taken at different time intervals; 0hr, 72hrs, 192hrs and 240hrs. From results obtained there were fluctuations in pH and OD, with pH showing significant decrease from 6.75 to 6.1 and a gradual increase to 6.8 in C1 tentatively identified as *Corynebacterium spp* and OD reads 0.422, 0.153, 0.565, 1.120 from 0hr – 240hrs.The increase could be from high metabolic activities possibly resulting in the production of intermediate metabolites produced by organisms [26]. The decreases in pH observed in Fig. 4.1 were attributed to the degradation of spent oil which could lead to a release of organic acids and final products that probably lowered pH of the mixture [26]. The result shown in Fig. 4.3 and Fig. 4.4 showed an increase in OD which indicates an increase in the number of degradative cells signifying the isolates' ability to utilize spent oil as a sole carbon source. This ability may be possibly due to production of biosurfactants by the organisms making the hydrocarbons more available for microbial utilisation [28-29]. The dominance of gram positive bacteria in this study is due to their stronger cell wall envelope than gram negative bacteria and a higher tolerance to hydrocarbon hence their ability to thrive in highly variable environment contaminated with spent oil [30].

[31] reported that cow dung contain a vast reservoir of nutrients and energy capable of supporting microbial growth, thereby enhancing microbial degradation of various pollutants These isolates *Arthrobacter*, *Pseudomonas*, *Corynebacterium* and *Mycobacterium* species with proper nutrient supplementation such as the stimulation of organic manure could be used for enhancement of bioremediation of oil polluted sites.

The results of this study have shown that there is potential in using cow dung in the bioremediation of contaminated sites.

4. Conclusion

This study has shown that the spent oil spilled sites could be remediated using cow dung, overall, the soil fertility and structure could be improved using this eco-friendly means of bioremediation.

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