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Bioremediation Potentials of *Heterobasidion annosum* 13.12B and *Resinicium bicolor* in Diesel Oil Contaminated Soil Microcosms

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ABSTRACT: The remediation potentials of diesel oil by two white rot fungi (Basidiomycota; Hymenomycetes) *Heterobasidion annosum* 13.12B and *Resinicium bicolor* were tested in two artificially contaminated experimental matrices: Norkran's liquid medium and Insch - Pitcaple soil collected from Aberdeen, United Kingdom. Gravimetric measurements showed reductions in the weight of the hydrocarbon extract from soil inoculated with *H. annosum* 13.12B and *R. bicolor*. The reduction in diesel extract was observed to be higher in the treatments with *H. annosum* 13.12B compare with other treatments in soil microcosm amended with 3.5 and 7 % diesel oil. Significant increase in extractable hydrocarbon was detected in Norkran's liquid medium amended with 1 and 3.5 % diesel oil. These results showed that white rot fungi could be harnessed to remediate soil contaminated with diesel oil in the field. © JASEM

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***KEYWORDS*:** bioremediation, diesel oil, *Heterobasidion annosum*, *Resinicium bicolor*, pollution, gravimetric measurement

Introduction

Soil is a major reservoir for organic contaminants such as petroleum hydrocarbons, which include diesel oil. The extent to which these contaminants are sustained in the soil is influenced by both the physical and chemical properties of the contaminants and soil properties (Semple *et al.,* 2003). Organic contaminants with lipophilic and hydrophobic properties tend to persist in the soil. Considering the ever - increasing oil pollution problems around the globe, creating an efficient remediation technology remains a need (Kristanti *et al.,* 2011). Soil contamination by oil spills is a serious environmental problem that may affect human health (Kingston, 2002). For example, multi-ringed polycyclic aromatic hydrocarbons (PAHs) are toxic and carcinogenic (Semple *et al.,* 2003; Son *et al.,* 2003; Jon *et al.,* 2010; Lundstedt 2013). Polychlorinated aliphatic hydrocarbons have been recognized to have carcinogenic properties (Igbinosa *et al.,* 2013; Dario *et al.,* 2013). Moreover, the impacts of these contaminants on other ecological receptors such as fauna, flora, and water bodies still remain an issue. The United States Environmental Protection Agency (USEPA) recorded 16 polycyclic aromatic hydrocarbons (PAHs) as "pollutants of concern", a decision endorsed by the European Union (Semple *et al.,* 2003; Igbinosa *et al.,* 2013). These known problems have prompted research on the identification of microorganisms with specific oil degrading potential.

Mycoremediation utilises fungi capable of breaking down harmful contaminants for the reclamation of a contaminated environment, unlike conventional remediation technologies such as thermal desorption that employs physical and chemical strategies (Nilanjan and Preethy 2010). Seeding of oil contaminated sites with hydrocarbon degrading fungi has been applied as a remediation approach and the significance of indigenous hydrocarbon degraders has been studied (Hamme and Ward 2000; Barathi and Vasudevan 2001; Rahman *et al.,* 2002; Verma *et al.,* 2006). However, nutrient deficiency and low bioavailability of hydrocarbons and other contaminants are among the major factors militating against hydrocarbon biodegradation (Kristanti *et al.,* 2011). Thus, there is the need for the development of strategies that would allow intimate contact of the fungi with the contaminants of concern and, possibly, an effective method of nutrients addition. Other factors such as moisture, pH, temperature and oxygen also play an important role in the breakdown of contaminants by fungi (Boopathy 2000; Nilanjan and Preethy 2010; Venosa and Xueqing 2003). In soil and marine environments, reduced oxygen tensions can drastically decrease biodegradation processes. Oxygen can be introduced into the substrate in gaseous form using bioventing technology (Boopathy 2000). Biodegradation of naphthalene can occur under anoxic conditions however (Meckenstock *et al.,* 2000).

White rot fungi are hymenomycetes (Basidiomycota) and unlike bacteria have the advantage of making

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better contact with poorly bio-accessible contaminants through the production of extracellular enzymes, such as lignin peroxidase, manganese dependent peroxidase and laccases capable of oxidising multi-ringed aromatic compounds including a wide range of harmful environmental xenobiotics (Natalia *et al.,* 2006). Extracellular release of oxidase enzymes has an important function in soil mycoremediation. These enzymes can penetrate surfaces of soil particle where recalcitrant contaminants have a tendency to bind (Harbhajan 2006). Consequently, exploring strains of white rot fungi for diesel oil clean-up potentials is extremely significant. The fundamental question that is urgently demanding for an answer is: do species of *Heterobasidion* and *Resinicium* have oil degradation capabilities? It has been recorded that many ligninolytic and non-ligninolytic fungi are able to degrade petroleum hydrocarbon. For example, *Phanerochaete chrysosporium* can breakdown acenaphthene and benzo-(a)-pyrene; whilst *Irpex lacteus* and *Bjerkandera adjusta* degrade chrysene, fluoranthene and pyrene (Valentin *et al.,* 2007). These findings illustrate the capacity of white rot fungi to bioremediate diesel oil in sites contaminated with hydrocarbons and support the exploration of other fungi in this group for these properties (Kristanti *et al.,* 2011). The aim of this study therefore was to test the ability of two species of white rot fungi, *Heterobasidion annosum* and *Resinicium bicolor* to degrade diesel oil in liquid medium and in soil.

MATERIALS AND METHODS

Fungal isolates: Cultures *Heterobasidion annosum* (strain 13.12B), isolated from Sitka spruce on Bennchie, Aberdeenshire, and *Resinicium bicolor* also isolated from Sitka spruce stump were used.

Medium preparation*:* Two percentmalt extract agar (MEA) was prepared by mixing 8 g of malt extract powder (Oxoid LP0039) and 6 g (1.5 %) of bacteriological agar (Oxoid LP0011) into 400 ml of reverse osmosis (RO) water in 500 ml Duran bottles. The medium was autoclaved at 105 kPa for 15 min and dispensed into 9 cm diameter Petri dishes.

Fungal subculturing: H. annosum 13.12B and *R. bicolor* were subcultured into freshly prepared MEA using aseptic techniques. Cultures were wrapped in Parafilm and incubated at 22 ^{o}C in SANYO incubator for 16 d.

Norkrans medium: One liter (1 L) of Norkran's medium [glucose 20.000g; potassium phosphate (KH_2PO_4) 0.6000g; potassium hydrogen orthophosphate (K_2HPO_4) 0.4000g; ferric citrate ($C_6H_5O_7Fe.5H_2O$) 0.0050g; maganese (II) sulphate tetrahydrate ($MnSO_4.4H_2O$) 0.0050g; zinc sulphate heptahydrate ($ZnSO_4.7H_2O$) 0.0044g; cobalt (II) chloride hexahydrate ($COCl_2.6H_2O$) 0.0010g; calcium chloride dihydrate ($CaCl_2.2H_2O$) 0.1320g; thiamine hydro-chloride ($C_{12}H_{17}CIN_4OS.HCl$) 0.0001g; magnesium suphate heptahydrate ($MgSO_4.7H_2O$) 0.5000g; asparagine ($C_4H_8N_2O_3$) 1.2000g] was prepared by weighing the medium recipes into 1000 ml conical flask containing RO water. The medium was mixed on an electrically driven stirrer machine. Norkran's medium was dispensed in 50 ml volumes into 250 ml conical flasks, corked with foam bungs, covered with aluminium foil and autoclaved at 105 kPa for 15 min. The flasks were kept on the laboratory bench for 12 d before inoculation with two plugs of fungal mycelia.

Addition of autoclaved rice to fungal culture: Rice grains (125 g; Safeway American Long Grain) was weighed into eight 1000 ml plastic beakers containing 2 g sucrose and 200 ml of RO water. The beakers were capped with aluminium foil and autoclaved at 105 kPa for 15 minutes. Autoclaved rice (15 g) was aseptically added to the 16 days old fungal cultures and distributed evenly on the culture surface.

Soil treatment: Twelve 12 kg ofsoil of the Insch Pitcaple (Aberdeenshire) type (Table 1) was sieved through a 3.35 mm stainless-steel sieve and kept in a sealed polythylene bag at 4 ^{o}C for three days before use. Eighty gram (80 g) of soil was weighed into 41 glass Kilner jars. The jar lids were applied without the rubber seal and covered with aluminium foil before autoclaving three times at 105 kPa for 30 min with a 24 h interval between the first and second autoclaving and a 48 h interval between the second and third autoclaving (Tyndallisation process) Autoclaved soils were kept on the laboratory bench for 15 d prior to adjusting soil moisture content by adding autoclaved RO water to each Kilner jar. Soils were left overnight to equilibrate, before treatment. One gram (1 g) of soil was defined as 1 ml of water added to the soil.

Bioremediation assay with H. annosum and R. bicolor in Norkran's liquid medium: Ten flasks of autoclaved Norkran's medium were inoculated with either *H.annosum* 13.12B, or *R. bicolor*, or uninoculated (control) and incubated at 22 ^{o}C with re-randomizing at 5 d after inoculation to account for temperature variations within the incubator. After 10 d of incubation, 1 ml of sterile Tween 20 was added to the 30 flasks and shaken, followed by immediate aseptic introduction of 1% (0.5 ml), and 3.5% (1.75 ml) of sterile diesel. These concentrations of diesel oil were accepted after a prelimanary diesel oil and Norkran's medium emulsification experiment was carried out.The diesel oil was confirmed sterile by sterility test; two commonly used media (potato dextrose and malt extract agar) for fungal cultivation were used whilst nutrient agar was used for bacterial identification. Control treatments included 1% and

3.5% diesel oil without fungi, Norkran's liquid medium and Tween 20 only, or Norkran's medium inoculated with *H. annosum* 13.12B or *R.bicolor*. All treatments were conducted with five replicates. Flasks were shaken at 60 rev/min at 22 ^{o}C on a GALLENKAMP shaker incubator.

Bioremediation assay with H. annosum and R. bicolor in soil: Sterile diesel oil [7 % (5.6 ml) and 3.5 % (2.8 ml)] was added to hydrated soil in 2 sets of 15 kilojars following the method of Kristani et al. (2011).These diesel oil concentrations were adopted upon modification of the method of Kristani et al. (2011). Soils were left overnight for the diesel oil to equilibrate. Thirty gram (30 g) of rice colonised with either isolated *H. annosum* 13.12B or *R. bicolor* was aseptically transferred to Kilner jars and mixed into the soil with a sterile spatula. Control Kilner jar microcosms contained autoclaved soil supplemented with 7% and 3.5 % diesel oil without fungal inocula, autoclaved rice only, or the fungi but no diesel oil. Five replicates were prepared per treatment.

Quantification of hydrocarbons: Gravimetric method was used to determine the degradation effects of these fungi. Samples from the Norkrans liquid medium were collected on days 0, 12, 21, 30 and 35. For the soil assay, samples were taken on days 0, 11, 22, 31 and 36 after addition of diesel oil. Using aseptic technique, 2.5 ml of Norkrans medium or 2.5 g of soil were transferrred to Wheaton vials in a laminar flow cabinet. The samples in the Wheaton vials were shaken in a SK-71 Lab Companion shaking incubator at 120 rev/min for 2 h after adding 20 ml of dicloromethane (DCM). After the phases had separated, 10 ml of the clear supernatant was transferred to pre-weighed aluminium foil trays. Ten ml DCM was pipetted to pre-weighed trays as a negative control. Trays were left for 12 h for the DCM to evaporate and weighed on a METTLER AT261 Delta Range fine balance.

Statistical analysis: Since each treatment was performed with five replicates per sample, mean values and standard errors were computed for all the data. Graphs were plotted with SigmaPlot version12.5 (Systat software inc., Chicago, USA). Statistical analyses were carried out using Minitab16 software and Microsoft excel. A normality test (Anderson-Darling) was done for data from grametric method. Data were then subjected to analysis of variance (ANOVA) and Regression analyses. Probability values (*P*-values) of less than 0.05 were regarded as significant.

RESULTS AND DISCUSSION

The sterility test for diesel oil showed no bacterial or fungal growth indicating that microorganisms find it difficult to survive high concentrations of diesel oil. Fig. 1 and 2 show the graphical presentations of the weights of extractable diesel-derived hydrocarbon by gravimetric method. Tables 2 and 3 give the results of ANOVA analyses of the data obtained by gravimetric method. Significant decrease was observed for treatment inoculated with *H. annosum* between days 0 and 11 in soil treated with 3.5 % and 7 % diesel oil ($P < 0.05$). Subsequently, as the experiment progressed, there was a slight decrease in *H.annosum* treatment with simultaneous increase in *R. bicolor* and control treatments for the 3.5 % diesel amended soil s shown in Fig. 1 (a) and (b). There was significant increase in the weight of extractable hydrocarbon from the Norkran's liquid medium in all the treatments as shown in Fig. 2 (a) and (b)

Several genera of white rot fungi have revealed a high potential to breakdown xenobiotics (Juan *et al.,* 2008) such as hydrocarbon contaminants. Inoculation of diesel-contaminated soil microcosms with *H. annosum* 13.12B led to a reduction in extractable hydrocarbons between days 0 and 11 at both concentrations of diesel as shown in Fig. 1 (a) and (b) r^2=20.7%, b=-0.1350, P=0.022; r^2=13.7%, b=-0.3346, P=0.008). Over subsequent days, there was no significant decrease in hydrocarbon content. It is likely that the hydrocarbon compounds became more sorbed to soil particles with time as suggested by Semple *et al.,* (2003) and Gevao *et al.,* (2000). This sorption may have reduce biodegradation of the hydrocarbon contaminant during incubation. The weights of DCM-extractable compounds in the 3.5 % diesel treatment, however, were higher in the *H. Annosum* inoculated soil than in the *R. bicolor* or control treatments at all-time points. This is in agreement with the suggestion of Adinarayana (1995) who indicated that preference is given to white rot fungi in remediation of contaminants because the filamentous growth forms of the fungi can easily spread in polluted soil by hyphal extension. Boopathy (2000) also reported that degradation is influenced among other factors by biodiversity (cellular biomass). It is also possible that hydrocarbons were sorpted to fungal mycelium as it grows in the soil and soil surfaces since according to Bosma et al. (2007), sorption is a major hindrance to bioremediation. Surprisingly, *H.annosum* treatment for the 3.5 % diesel oil treatment almost exactly matches that for the control treatment with 7% diesel oil. This is an indication that loss of hydrocarbon extract occurred at the same rate in both treatments. No significant changes occurred in weights of extracted compounds in the *R. bicolor* and control treatments with time in soil treated with 3.5 % diesel. Changes in DCM-extractable organic compounds in the 7 % diesel treatment were less clear over time. In control microcosms, weights of DCM-extractable compound fell by approximately 35 % over the 36 days incubation period, possibly due to abiotic factors such as additional volatilisation of the diesel at this concentration compared with the 3.5 % diesel

treatment. In contrast, the weights of compounds extracted from the *H. annosum* and *R. bicolor* treatments varied with time. Although losses of DCM - extractives over 36 days incubation period were more obvious in the *H. annosum* treatments than in *R. bicolor*. Semple *et al.,* (2003) stated that, in addition to biodegradation and volatilisation, hydrocarbons could be lost through sequestration. No effort was made in this work to evaluate the portion removed via abiotic routes, for instance by the use of internal biomarkers (Bragg *et al.,* 1994; Wang *et al.,* 1998) which would have been useful for the validation of bioremediation effectiveness (Bundy *et al.,* 2004). The increases in weights of hydrocarbons extracted from the two fungal treatments on day 22 in the 7 % diesel treatment, at the same time as quantities from the control treatment continued to decrease are also difficult to explain. It is possible that the fungi did not mineralise the diesel oil but degraded it to persistent intermediates that added to the weight of the hydrocarbon extract. This possibility requires further research to determine the mechanisms and pathways of hydrocarbon degradation by these fungi.

Tween 20 (surfactant) was added to Norkran's liquid medium in an attempt to improve bioavailability of the diesel oil due to its ability to reduce surface tension and increase mass transfer (Franzetti et al. 2008). In all treatments in the Norkran's medium, significant increases in the quantities of DCM-extractable organic compounds occurred with time (Fig. 2 (a) and (b); $P > 0.05$; Table 3). Apart from days 0, 21 and 30, the weights of DCM-extractable organic compounds obtained from cultures amended with 3.5 % diesel were significantly higher in the fungal treatments than in the control (Fig. 2a). This may be as result of reduction in volatilisation rate in the control treatment during these days. Moreover, by day 30, the quantities of extractives obtained from the *H. annosum* treatment were higher than in the other two treatments ($p>0.05$) possibly because the fungus was able to break down more quantity of Tween 20 than *R. Bicolor.* Quantities of DCM extractives also increased significantly with time in the 1 % diesel treatment in Norkrans medium (Fig. 2b). From day 12 onwards, however, significantly higher quantities of extractives were obtained from the *H. annosum* treatment ($p>0.05$).

In an effort to boost the degradation levels of contaminants in soil, contaminated soils have been inoculated with different species of fungal immobilised on different substrates such as woodmeal and wheat straw (Kennedy *et al.,* 1990; Andersson and Henrysson 1996; Kristansti 2011). Such immobilisation resulted in efficient degradation of organic contaminants. Plant substrates not only serve as sources of support but also provide nutrients needed for fungal growth and soil penetration. In addition, such substrates stimulate wood degrading enzymes production (Castillo *et al.,* 2001; Fujian *et al.,* 2001). Thus, *H. annosum* and *R. bicolor* degradation potential could be enhanced through immobilisation on plant substrates such as rice grains to stimulate the production of ligninolytic enzymes (e.g. laccases). These enzymes can be applied to contaminated sites to initiate remediation of organic contaminants including hydrocarbons.

Conclusion: The reduction in diesel derived hydrocarbon was observed to be higher in the treatments with *H. annosum* compare with other treatments in soil microcosm experiments amended with 3.5 and 7 % diesel oil. Currently, there is limited information about these fungal hydrocarbon remediation properties, this investigation is an attempt to explore the hydrocarbon degradation potential of white rot fungi *H. annosum* 13.12B and *R. bicolor.*

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Table 1: Characteristics of Insch-Pitcaple Soil

Soil properties	Values
Percentage carbon	6.33
Percentage total nitrogen	0.112
Ph	4.4
CEC meq/100 g	41.62
Ca meq/100 g	1.36
Magnesium meq/100 g	0.2
Sodium meq/100 g	0.12
Potassium meq/100 g	0.05
Iron meq/100 g	4.8
Zinc meq/100 g	0.31
Texture	Loam

Legend: CEC-Cation-exchange capacity; Ca-Calcium

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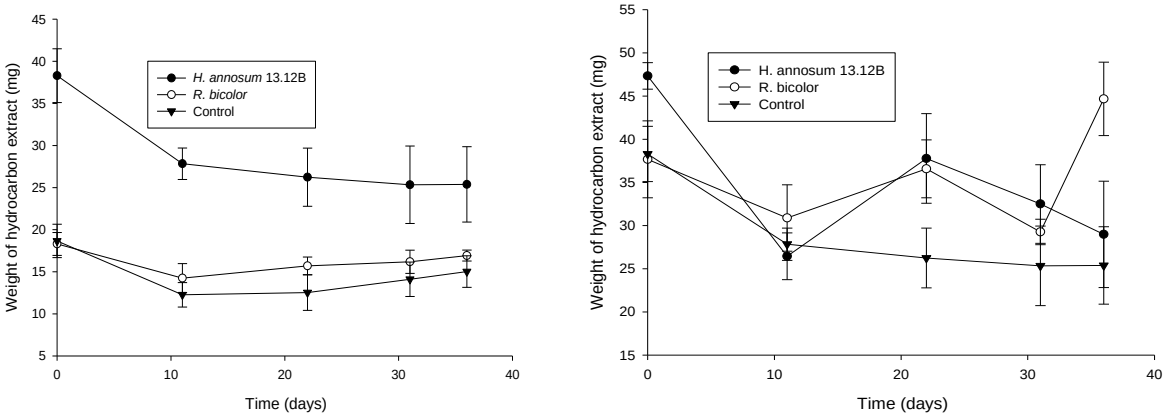

Fig.1. Ch weight of hydrocarbon extract with time from soil artificially contaminated with diesel oil and inoculate *Heterobasidion annnosum* or *Resinicium bicolor.* Error bars represent standard error of the mean (a) 3.5% diesel oil contaminated microcosm; (b) 7% diesel oil contaminated microcosms

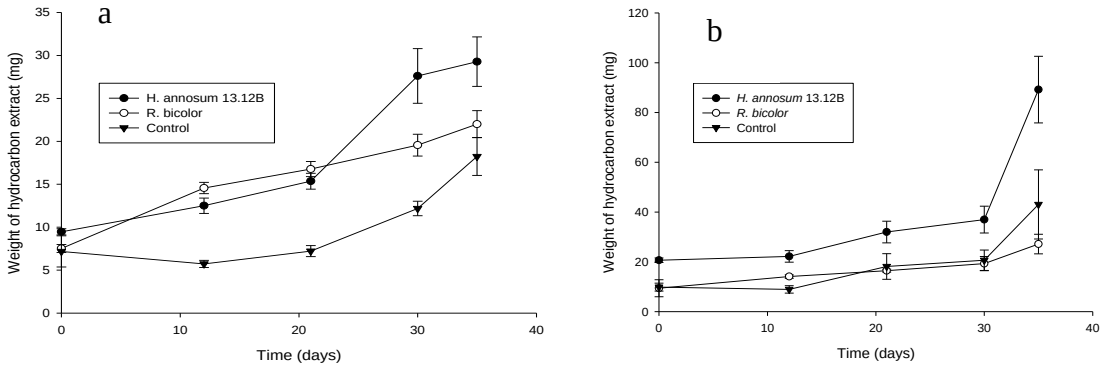

Fig.2. Changes in weight of hydrocarbon extract with time from Norkran's liquid medium artificially contaminated with diesel oil and inoculated with *Heterobasidion annosum* or *Resinicium* bicolor. Error bars represent standard errors of the mean (a) 1% diesel added (b) 3.5% diesel added

Table 2: Summary of ANOVA results for the diesel contamination of soil in microcosms

Treat-ment	Day	df	Sum of Squares	Mean squares	*F* value	*p* value	Significance at 95 %
7 % diesel oil							
H, R,C	0	2	29.5	14.8	0.73	0.502	ns
H,R,C	11	2	75.8	37.9	3.60	0.059	ns
H,R,C	22	2	66.35	33.17	3.40	0.068	ns
H,R,C	31	2	12.8	6.4	0.47	0.634	ns
H,R,C	36	2	9.8	4.9	0.39	0.685	ns
3.5 % diesel oil							
H,R,C	0	2	292.4	146.2	2.63	0.113	ns
H,R,C	11	2	51.5	25.8	0.62	0.62	ns
H,R,C	22	2	402.5	201.3	2.09	0.166	ns
H,R,C	31	2	128.5	64.3	0.89	0.436	ns
H,R,C	36	2	1053	527	4.36	0.038	*

Legend: H-*Heterobasidion annosum* treatment; R-*Resinicium bicolor* treatment
C-Control treatment; df-Degrees of freedom; *-Significant difference; ns-No significant difference

Table 3: Summary of ANOVA results for effects of fungal inoculation on diesel concentrations in Norkran's liquid medium

Treat-ment	Days	Df	Sum of squares	Mean Squares	*F* value	*p* –value	Significance at 95 %
1 % diesel oil H,R,C	0	2	15.13	7.56	1.60	0.241	Ns
H,R,C	12	2	213.93	106.96	46.54	0.000	*
H,R,C	21	2	266.12	133.06	35.33	0.000	*
H,R,C	30	2	596.4	298.2	13.49	0.001	*
H,R,C	35	2	315.0	157.5	5.26	0.023	*
3.5 % diesel oil H, R,C	0	2	405	203	1.97	0.182	Ns
H,R,C	12	2	446.6	223.3	7.19	0.009	*
H,R,C	21	2	725.4	362.7	4.94	0.027	*
H,R,C	30	2	968.0	484.0	6.13	0.015	*
H,R,C	35	2	104.4	5202	7.35	0.008	*

Legend: H-*Heterobasidion annosum* treatment; R-*Resinicium bicolor* treatment
C-Control treatment; df-Degrees of freedom; *-Significant difference; ns-No significant difference

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