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Evaluating Different Soil Amendments as Bioremediation Strategy for Wetland Soil Contaminated by Crude Oil

Raphael Butler Jumbo, Frédéric Coulon, Tamazon Cowley, Ikeabiama Azuazu, Emmanuel Atai, Imma Bortone and Ying Jiang *

School of Water Energy and Environment, Cranfield University, Cranfield MK43 0AL, UK
* Correspondence: y.jiang@cranfield.ac.uk

Abstract: This study evaluated the efficacy of using Tween 80 surfactant (TW80) and food-waste anaerobic digestate fibre (FWAD) as soil amendments for the remediation of wetlands contaminated by crude oil. A 112-day mesocosms experiment was carried out to simulate hydrocarbon degradation under typical acidified wetland conditions. Soil was spiked with 50,000 mg kg^{-1} crude oil and TW80 and FWAD were added to mesocosms at 10%, 20% and 30% *w*/*w*. The soil basal respiration, microbial community dynamics, environmental stress, alkanes, and PAHs degradation were monitored throughout the mesocosm experiment. Amending the mesocosms with FWAD and TW80 enabled the recovery of the soil microbial activities. This was evidenced by soil basal respiration which was the highest in the 30% FWAD and 30% TW80 mesocosms and translated into increased degradation rate of 32% and 23% for alkanes, and 33% and 26% for PAHs compared to natural attenuation, respectively. Efficient total hydrocarbon degradation was achieved in soil mesocosms with 30% FWAD and 30% TW80 at 90% and 86.8%, respectively after 49 days. Maize seed germination results showed significant improvement from 29% to over 90% following the FWAD and TW80 treatment.

Keywords: remediation; wetlands; food-base digestate; surfactant; hydrocarbons

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1. Introduction

Wetlands are poorly drained areas subject to permanent or periodic water saturation [1]. Wetlands (WLs) are both ecologically and economically important because of their high agricultural productivity, complex biogeochemistry, and nutrient cycling ability [2,3]. Studies has shown that acidification is ongoing on account of low pH, high sulphate, and nitrate concentration in the WLs of Niger Delta [4,5], that is one of the most important and biodiverse WLs ecosystems in the world [6]. Acid rainfall caused by oil field gas flaring and continual industrialization has been linked to the acidification of the wetlands [7]. The acidification of wetlands is of primary concern because of its effects on WLs ecosystems, dissolved metal ions, water quality, and agriculture [8]. The wetlands of the Niger Delta house most of the crude oil fields in Nigeria. The exploration and exploitation of crude oil in the WLs have led to contamination through spillages and subsequent alteration in the wetland's ecosystems [9].

The consistent occurrence of the petroleum hydrocarbons (HCs) spillages on acidified wetlands is of public concern for the severe public health, economic, and ecological risks correlated [10,11]. This is a particularly severe and widespread problem in the Niger Delta Region, which has been well documented in several previous studies [12–14]. Certain groups of HC contaminants in the acidified WLs, mainly medium and heavy molecular weight alkanes and polycyclic aromatics hydrocarbons (PAHs), are of concern due to their high mobility, availability, recalcitrant and carcinogenic nature [15–17]. The remediation of these contaminants in acidified WLs using conventional remediation methods including soil excavation and physiochemical treatments were not suitable [18–20].

Previous studies showed that biostimulation using nutrient rich soil amendments can increase the soil microbial activities and subsequently improve the biodegradation of contaminants [21]. Cipullo et al. [22] reported how using compost as nutrient amendment in a bioremediation of soil contaminated by petroleum HCs resulted in a 46% degradation efficiency after 180 days, from their initial concentration of 9163 mg/kg. Typically, biostimulation as a remediation strategy is less intrusive to the environment. Especially when using non-commercial nutrient supplement, including compost, and farmyard manure, the overall remediation costs, and carbon emissions during the treatment process is significantly reduced [23–27].

Digestate from anaerobic digestion (AD) of organic feedstock is a by-product of the AD process [28]. It contains high nutrients including nitrogen, phosphorus, and potassium. When applied to land, it is a high-quality bio-fertilizer which provides readily available nutrients to the soil [29–31]. The solid fraction of the digestate (digestate fibre) are better bio-fertilizers, more hygienic, and stabilized when compared with the whole or liquid fraction of digestate [28,32]. The feedstock to produce digestate includes biodegradable materials such as food waste, farmyard manure, municipal waste, and sewage [33–35]. Sewage digestate has been successfully applied as biostimulant for the remediation of diesel-contaminated soils [32]. However, sewage digestates are known to have high available metal and metalloids content and introduces pathogenic bacteria into the remediated soils [36,37]. Therefore, the land application of sewage digestate has caused environmental and public health concerns [32,33]. Food waste (FW) digestate possess higher nutrients contents when compared to the digestate of other feedstocks [36,38]. FW digestate are known to have low metal and metalloids contents, high nitrogen, phosphorus, and potassium content, which could increase soil nutrient value after hydrocarbons remediation [28,38]. Bacteria from FW digestate can easily adapt to degrade various organic contaminants (such as hydrocarbons), and grow exponentially, by showing the wide metabolic capacities of this digestate [32]. The increased bacteria growth subsequently leads to an increased HC degradation rate at a reduced time.

While the addition of anaerobic digestates, composts, or biochar have all shown promising results in enhancing the degradation of petroleum hydrocarbons, the extent of degradation can be highly variable. This is often related to the accessibility of the hydrocarbon compounds to the microbes, such as to the bioavailability of the hydrocarbons [39,40]. Enhancing the bioavailability of the contaminants by using a surface-active substance like non-ionic surfactant have been demonstrated to be more suitable for soil remediation than cationic and amphoteric surfactants [41]. The non-ionic surfactants are also cost-effective with minimal toxicity to the soil microbial communities [42]. Tween 80 (TW80) is a non-ionic surfactant with a low ecological toxicity, that increases the solubility and mass transfer of hydrophobic organic compounds including hydrocarbons [43]. Despite these benefits, Tween 80 primarily has been used for ex situ soil washing [41], and has rarely been considered as a supplement during in situ hydrocarbon remediation. In this context, this research focuses on evaluating the potential of TW80 and digestate as soil amendments for remediating petroleum HC contaminants in acidified WLs, by establishing their corresponding remediation endpoints.

2. Materials and Methods

2.1. Mesocosm Soil and Experimental Design

Pristine soil with no record of petroleum hydrocarbons contamination was collected from a construction site in Cranfield University (52.0746 N, 0.6283 E). An amount of 160 kg of soil was collected from top to 30 cm soil depth using trowels and shovels. After the soil collection, the soil was air dried at room temperature, sieved through a 2 mm aperture sieve and the sieved soil was stored in a soil cupboard for 4 days at 20 °C before use for the experiment. Triplicate soil mesocosms were set up using 1 kg soil in 2.5 L transparent polytetrafluoroethylene (PFTE) containers. Nine different mesocosms conditions were evaluated as summarised in Table 1.

Table 1. Overview of the biostimulation treatments evaluated along with the controls.

Mesocosm Conditions	Abbreviations
Pristine soil (freshly collected from field)	Control
Pristine soil acidified at pH 5.8 (using HNO_3)	Acidified
Pristine soil acidified at pH 5.8 and spiked at 50,000 mg/kg with crude oil	Crude oil
Pristine soil acidified at pH 5.8 and spiked at 50,000 mg/kg with crude oil + 10% (*w/w*) FW digestate	10% FWAD
Pristine soil acidified at pH 5.8 and spiked at 50,000 mg/kg with crude oil + 20% (*w/w*) FW digestate	20% FWAD
Pristine soil acidified at pH 5.8 and spiked at 50,000 mg/kg with crude oil + 30% (*w/w*) FW digestate	30% FWAD
Pristine soil acidified at pH 5.8 and spiked at 50,000 mg/kg with crude oil + 10% (*w/w*) Tween 80	10% TW80
Pristine soil acidified at pH 5.8 and spiked at 50,000 mg/kg with crude oil + 20% (*w/w*) Tween 80	20% TW80
Pristine soil acidified at pH 5.8 and spiked at 50,000 mg/kg with crude oil + 30% (*w/w*) Tween 80	30% TW80

The mesocosms except the pristine soils (control) were all acidified to pH of 5.8 by using HNO_3 (PrimarPlus-trace analysis grade, supplied by Fisher Scientific UK, Limited). HNO_3 was added as rainfalls in most part of the Niger Delta wetlands are weak nitric acid with pH between 5.7–5.9 [44,45]. The acidified soil mesocosms were spiked with 60 mL of Crude oil (<0.5% sulphur) to achieve a target hydrocarbons concentration of 50,000 mg/kg. The adopted concentration fell within the range of most HC contaminated wetlands in the Niger Delta [46,47]. The mesocosms were incubated at 28 °C to mimic the mean temperature of the Niger Delta, Nigeria [48].

FWAD was air dried and particles larger than 2 mm were removed using a 2 mm aperture sieve (model: BS410 manufactured by: Endecotts, London, England). Dried FWAD were mixed with crude-oil-spiked acidified soil at 10, 20 and 30% (*w*/*w*) in triplicates following the methods described by Nwankwo [49].

TW80 (Polyoxyethylene (20)sorbitan monooleate) was applied at 10, 20 and 30% (*w*/*w*) to the crude oil spiked acidified soil samples in triplicates. The application of the non-ionic surfactants (TW80) was as described by Trinchera and Baratella [50]. Three controls (pristine soil, acidified soil (without HCs) and crude oil spiked acidified soil with no treatment (natural attenuation) were maintained through the experiment. The soil moisture content of 13.75% was increased and maintained at saturation with 54.54% moisture increase. Moisture at saturation was maintained to depict the wetland condition in all mesocosms. Deionised water was used to maintain moisture in all mesocosm. The deionised water was added at 7 days intervals to maintain the soil saturation.

2.2. Soil and FWAD Physicochemical Properties Determination

Physical characteristics of the samples such as moisture content, dry matter, organic matter, water holding content and particle size distribution were analysed. The soil moisture and dry matter content were measured according to BS 7755: Section 3.1 [51]. A quantity of 50 g of air-dried soil samples were oven dried at 105 °C for 24 h. The organic matter content was determined through the loss on ignition (LOI) according to BS EN 13039 [52]. The water holding capacity at saturation and field capacity were determine by using BS 7755 Section 5.5 [53]. The particle size distribution was carried out by sedimentation according to BS-ISO 11277 [54] and ISO 11277 [55] using a Laser Analyzer Master-sizer (MS3000, Malvern Instruments Ltd., Worcestershire, UK), equipped with a hydro adaptor. The soil textural classification was based on percentage clay, silt, and sand using the United State Department of Agriculture soil textural classification scheme.

The chemical characteristics analysed were pH, phosphorus, potassium, magnesium, total nitrogen, total and organic carbons. pH values of the samples were measured with a pH meter (Jenway 3540, Cole-Palmer, Staffordshire, UK). Soil was diluted with deionised water, with a 1:5 soil to water ratio. The mixture was shaken for 60 min at 300 rpm by using an orbital shaker and then it was left to settle for further 60 min before measuring the pH values. The phosphorus content was determined according to BS 7755: Section 3.6:1995 [56], whilst potassium and magnesium were determined according to BS 3882 [57]. The total and organic carbons and total nitrogen were determined by using BS 7755 Section 3.8 [58].

2.3. Soil Biological Properties Determination

Soil basal respiration being the measurement of the steady rate of microbial respiration in soil was used to quantify the CO_2 generation rate [59]. Soil basal respiration was measured at the onset of the experiment and, respectively on days 7, 14, 28, 49, 77, and 112 of the mesocosm experiment. The soil samples basal respiration was determined by using the Rapid automated bacterial impedance technique (RABIT) (Don Whitley Scientific, Bingley, UK) as a respirometer. An amount of 1 g of soil samples moistened to saturation in a glass boat was used as described by Pawlett et al. [60] for the determination of soil basal respiration. The microbial respiratory response ran for 48 h at 25 °C. The changes in conductivities (micro siemens) were determined and quantified to CO_2 according to [61]. The RABIT software (RABIT version 2.31, 01-1999) was used for quantification of the conductivities.

The soil microbial community profiles and dynamics were determined based on phospholipid fatty acids (PLFA) analysis using a modified method from Frostegård and Bååth [62]. The PLFA were measured at 30-day intervals. Lipids were extracted from 10 g of freeze-dried soil samples using 1:2:0.8 ($v/v/v$) of chloroform: methanol: citrate buffer and 30 mg of Butylhydroxytoluene (BHT). The extracted lipids were fractionated by solid phase extraction, and the phospholipids fraction was derivatized by mild alkaline methanolysis according to Dowling and White [63]. The resulting fatty acids methyl esters (FAMEs) were analyzed by gas chromatography as explained in Pawlett et al. [60]. The fatty acids were used as an indicator of the presence of groups of microbes (biomarkers). The biomarkers were categorized into Gram-positive bacteria, Gram-negative bacteria, actinobacteria and fungi according to Quideau et al. [64] and Frostegård and Bååth [62].

The soil microbial count was determined through the use of colony forming unit (CFU) plate counting technique [65]. Soil suspensions were prepared by 10-fold serial dilutions with 1 g of soil in triplicates, using deionized water as diluents. The plates were incubated for a period of 24 h in an incubator (Heraeus Incubator, Thermos Scientific, Germany) at 37 °C.

2.4. Hydrocarbons Analysis

The total petroleum hydrocarbons (TPHs) were extracted and analysed by using the procedure described by Risdon et al. [66]. The readily available hydrocarbons fraction was extracted using 15 mL of methanol (HPLC grade, Merck Life Science Limited, Gillingham, UK) while the bioavailable hydrocarbons fraction was extracted using 50 mL of 50 mM of 2-Hydroxypropyl-β-cyclodextrin (HPCD; Merck Life Science Limited, UK) according to Cipullo et al. [22]. The hydrocarbons fractions of each extraction were analysed using a Shimadzu TQ8040 gas chromatography–mass spectrometer (GCMS) equipped with an AOC 6000 auto-sampler (Shimadzu UK, Milton Keynes, UK) and operated in positive ion mode at +70 eV. Quality control and assurance procedures were carried out with the whole procedure blank, clean soil matrix spike recovery and comparison with reference materials. TPHs and readily available hydrocarbons were analysed, respectively, on days 0, 7, 14, 28, 49, 77 and 112 while the bioavailable hydrocarbons were measured on days 0, 28 and 112. Alkanes were grouped into C11–C18 which are prominently liquids and are medium molecular weight, made up of undecane, dodecane to octadecane. The other group was C19–C37, which are prominently wax and heavy molecular weight, made up of nonadecane, octadecane to heptatriacontane. The petroleum PAHs were similarly grouped into C10–C18 and C19–C27.

2.5. Metal(Loid)s Analysis

Metal(loid)s including molybdenum, chromium, nickel, arsenic, cadmium, lead, and mercury of the soil samples were determined using US EPA Method 3051 [67] and BS7755 Section 3.13 [68]. The soil acid digestion was carried out with 0.5 g of the soil samples added with 6 mL of hydrochloric acid (1.18 specific gravity) and 2 mL of nitric acid (1.42 specific gravity) in the liner of a pressure vessel. The vessels were loaded into a microwave machine

(model: Mars 240/50, manufacturer: CEM corporation, Charlotte, NC, USA) for digestion. After that, 10 mL of the filtrate were used for a flame atomic absorption spectrophotometer analysis (model: Jenway 6850, manufacturer: Jenway, Staffordshire, UK).

2.6. Ecotoxicity Assay

Germination assay for this experiment was carried out by using maize crop (*Zea mays*). Maize crop was chosen for the ecological risk assessment since it exhibits high toxicity sensitivity to high and low molecular weight hydrocarbons based on shoots, and germination delays and root biomass [69,70]. Maize is the second most important cereal crop in Nigeria ranking behind sorghum and is the most-consumed cereal crop within the Niger Delta region of Nigeria [48]. The maize crop was planted using the dibbling method which requires less seeds and gives rapid and uniform germination and good yield [71]. This method is most suited for laboratory-based experiments, and it is commonly practiced among local farmers of maize crops [72]. Five seeds of maize were planted per cell and the germination response and days of germination after planting were recorded at the onset and on day 112.

2.7. Statistical Analysis

Descriptive statistical analysis was carried out including mean, standard deviation, standard error by using Microsoft Excel (Version 2111 Build 16.0.14701.20278). The standard error was used to evaluate the variability across germination assays and the applied environmental stress while the standard deviation was used to ascertain the variability within sample measurements and applied to the metal(loid)s data. The JMP pro (version 16) software was used for spearman correlation. Differences in respiration, hydrocarbons, and concentrations between treatments were compared using spearman correlation at a 99.99 percent confidence level. The difference was significant if $p < 0.01$.

3. Results and Discussion

3.1. Soil Characterisation

The pristine soil is a sandy silt loam soil with a pH of 8.7 and moisture content of 13.75% (Table 2). The C: N: P ratio of soils are important indicators of soil fertility and soils with high C: N: P ratios are referred to as organic-rich soils [73]. The optimal soil C: N: P ratio for effective biodegradation of contaminants by microbes has been recommended at 100:10:1 [74]. The C: N: P ratio of 60:2:1 for the pristine soil samples suggests low carbon and nitrogen (Table 3) in the soil. When soil C: N: P ratios are below the optimal value, it can result in limited microbial activities [75], therefore, supplementation of C and N can aid in stimulating soil microbial activities. FWAD with C: N: P ratio of 250:13:1 indicated that the digestate has a higher quantity of nitrogen and organic carbons to improve the C: N: P ratio in the pristine soil and potentially stimulate microbial activities. This was confirmed by the higher degradation rates of petroleum HCs (shown in Tables 4 and 5) after addition FWAD. The soil total and organic carbons for the pristine soil were 3.09% and 2.25%, respectively (Table 2). These were increased by the spiking of the soil with crude oil to 5.08% and 1.93%, respectively. Acidifying the soil increased the availability of the soil metals and metalloids (Table 6). This observed increment in availability of the metals and metalloids agreed with the findings of Chintala et al. [76] and Ning et al. [77]. The researchers concluded that the availability of metals increased as the soil becomes more acidic.

Table 2. Physical characteristics of the pristine soil used in the mesocosm experiment.

Soil Physicochemical Characteristics	
Soil Moisture content (%)	13.75
Loss on ignition (%)	3.66
Dry matter content (%)	86.25
Water holding capacity (%)	54.54
TOC (%)	3.09
Org C (%)	2.25
TN (%)	0.12
TP (mg/kg)	5.58
TK (mg/kg)	236.00
Soil Particle size distribution	
Sand (%)	46.67
Silt (%)	45.89
Clay (%)	7.44
FWAD characteristics	
TOC (%)	17.22
Org C (%)	4.97
TN (%)	0.98
TP (mg/kg)	300.25
TK (mg/kg)	8107.50
C:N: P	250:13:1

Table 3. Mean chemical properties and bacteria count of soils in the various triplicate mesocosms.

Mesocosm	**Treatment**	**K (mg/kg)**	**C: N: P**	**Bacteria Count ($\times 10^5$ CFU/g)**
FWAD	10% FWAD	1310.00 ± 2.1	128:9:1	20 ± 0.48
	20% FWAD	1694.17 ± 2.3	167:10:1	10 ± 0.4
	30% FWAD	1806.67 ± 2.9	180:9:1	30 ± 0.47
TW80	10% TW80	224.75 ± 1.6	60:2:1	1 ± 0.32
	20% TW80	151.58 ± 1.98	65:2:1	3 ± 0.48
	30% TW80	141.50 ± 1.7	75:3:1	4 ± 0.48
Controls	Control	236.00 ± 2.2	60:2:1	102 ± 0.8
	Acidified	243.83 ± 3.1	58:4:1	2 ± 0.4
	Crude oil	157.08 ± 1.6	60:2:1	7 ± 0.48

Table 4. Soil basal respiration versus PAHs degradation models and degradation rates.

Mesocosms	**Treatment**	**Slope equation**	**R^2**	**Degradation Rates ($mgCO_2$/mg PAHs/Day)**
FWAD	10% FWAD	y = −0.2741x + 513.83	0.98	−0.27
	20% FWAD	y = −0.3282x + 688.55	0.97	−0.33
	30% FWAD	y = −0.45x + 819.33	0.97	−0.45
TW80	10% TW80	y = −0.3672x + 721.46	0.92	−0.37
	20% TW80	y = −0.412x + 726.83	0.97	−0.41
	30% TW80	y = −0.5206x + 867.58	0.98	−0.52
Control	Acidified HCs	y = −0.2433x + 469.52	0.90	−0.24

Where y = basal respiration rate and x = PAH degradation rate.

Table 5. Soil basal respiration versus alkanes degradation models and degradation rates.

Mesocosms	Treatment	Slope Equation	R^2	Degradation Rates ($mgCO_2$/mg Alkanes/Day)
FWAD	10% FWAD	y= −0.2x + 744.6	0.97	−0.2
	20% FWAD	y= −0.31x + 1182.3	0.97	−0.31
	30% FWAD	y= −0.416x + 1363.7	0.91	−0.42
TW80	10% TW80	y= −0.18x + 789.1	0.95	−0.18
	20% TW80	y= −0.32x + 1022.4	0.94	−0.32
	30% TW80	y= −0.34x + 1421.1	0.96	−0.34
Control	Acidified HCs	y= −0.15x + 752.4	0.96	−0.15

Where y = basal respiration rate and x = alkanes degradation rate.

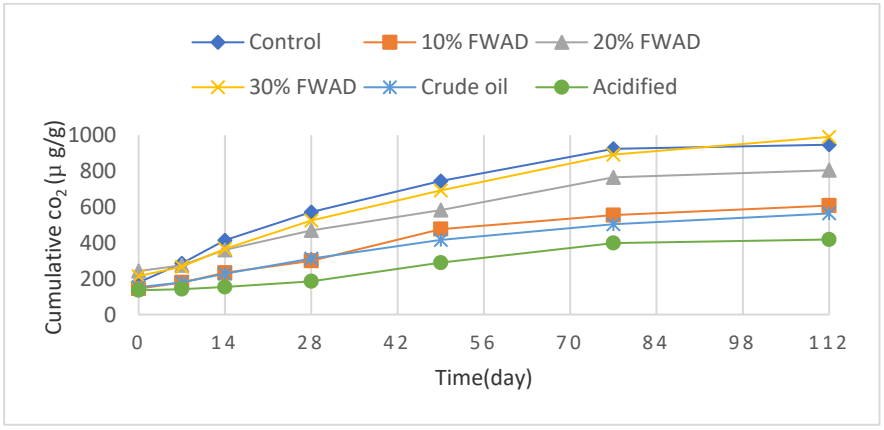
Table 6. Baseline mean concentrations of metal and metalloid and standard deviation in triplicates soil samples.

Metal (Loid) (mg/kg)	Control	Acidified	Crude Oil	FWAD
Mo	0.93 ± 0.08	1.27 ± 0.13	1.21 ± 0.07	1.20 ± 0.49
Cr	45.24 ± 1.23	59.56 ± 2.94	50.87 ± 1.50	36.05 ± 2.17
Ni	29.65 ± 1.24	42.60 ± 0.93	35.25 ± 0.79	23.79 ± 1.58
As	14.83 ± 0.98	20.21 ± 0.74	17.18 ± 0.78	10.13 ± 0.52
Cd	0.59 ± 0.09	0.83 ± 0.09	0.73 ± 0.07	0.72 ± 0.10
Pb	17.00 ± 0.91	23.31 ± 0.45	21.41 ± 0.8	15.62 ± 0.94
Hg	0.29 ± 0.01	0.12 ± 0.01	0.12 ± 0.02	0.14 ± 0.04

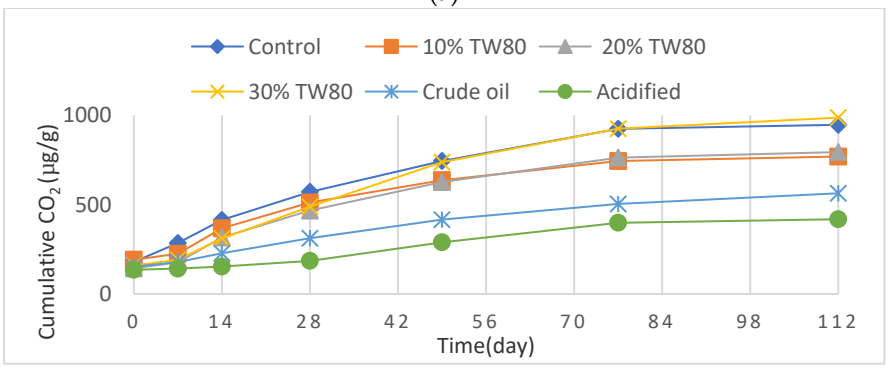
3.2. Soil Respiration and Its Relationship with Hydrocarbons Degradation

The pristine soil cumulative respiration rate resulted to be about 946 μg CO_2/g soil by day 112. The acidification of the pristine soil reduced its cumulative respiration rate by 56% (Figure 1). The poor respiration can be attributed to the stress induced by the acidification on the soil microbial community. Similar conditions have been reported in several studies in the Niger Delta [78,79]. Kaur et al. [80] reported that environmental stresses such as soil acidification can limit microbial communities' performance. The acidified soil with crude oil (crude oil mesocosm) has a better respiration than that acidified pristine soil (Figure 1a,b). The slight increment observed could be linked to the biodegradable HCs by the surviving soil microbes.

The application of digestate and Tween 80 surfactant to the spiked soils caused an increase in the soil respiration (Figure 1). This indicated that the TW80 and FWAD resuscitated the microbial communities by providing the required nutrients (shown in the C:N:P ratio) for improved microbial activities. The 30% FWAD and 30% TW80 mesocosms showed 44% and 43% increment in respiration rate, respectively if compared to the crude oil mesocosms. Similar trends were observed with the other digestate and Tween 80 mesocosms (Figure 1). The reported increments in CO_2 production rate can be linked to increased activities of hydrocarbons degrading microbial communities that used the hydrocarbons as carbon and energy source under the thriving environment provided by the digestate. This finding agreed with the research of Sándor [81], where the researcher posited that when the soil nutrients quality is improved, it stimulates the activities and stability of the soil microbial community. The level of evolution of biogenic CO_2 (CO_2 from biomass or organic matter) is an indication of the organic level in soil after effective remediation of organic contaminants from the soil and indicates the extent of crop germination, growth and yield [82,83]. This hypothesis corroborates the high germination percentages recorded in the highly remediated samples of TW80 and FWAD mesocosms which showed higher cumulative CO_2 values. The Tween 80 surfactant aided in changing the microbial cell surface hydrophobicity and improving the cell surface absorbing ability of the available hydrocarbons [41]. This subsequently caused more petroleum HCs to be degraded and led to increment in the CO_2 generation rate [41,84].



(**a**)



(**b**)

Figure 1. Cumulative respiration (CO_2 µg/g soil) per day for various mesocosms. (**a**) Soil cumulative respiration per day for FWAD mesocosms; (**b**) Soil cumulative respiration per day for TW80 mesocosms.

The interrelation of the soil basal respiration and the hydrocarbons content showed that an inverse relation was established for all the mesocosms spiked with crude oil (Tables 4 and 5). Negative degradation rates were established for the FWAD and TW80 mesocosms, which implied that the higher the gradients (that is the degradation rates) the more CO_2 that are produced and the more reduction in the soil PAHs and alkanes. The reduction in PAHs and alkanes allowed for improvement of the remediated soil economic value, whilst the increasing CO_2 generation implied that a habitat was gradually restored on the soil. 30% FWAD and 30% TW80 mesocosms showed the highest degradation rates for both alkanes and PAHs (Tables 4 and 5) indicating the fastest HC degradation. A strong positive correlation was established in all the mesocosms between the respiration rate and hydrocarbons degradation rates using spearman correlation techniques at a probability $p < 0.01$. At $p < 0.01$, spearman coefficient (χ) is considered significant if it is greater than absolute p but less than 1 (Table 7). This strong correlation shown implied that the more the respiration rates, the more the hydrocarbons that are degraded by the active microbial communities. These suggestions were supported by Jiang et al. [85] stating that the more the hydrocarbons degraders, the more the CO_2 produced in mesocosms.

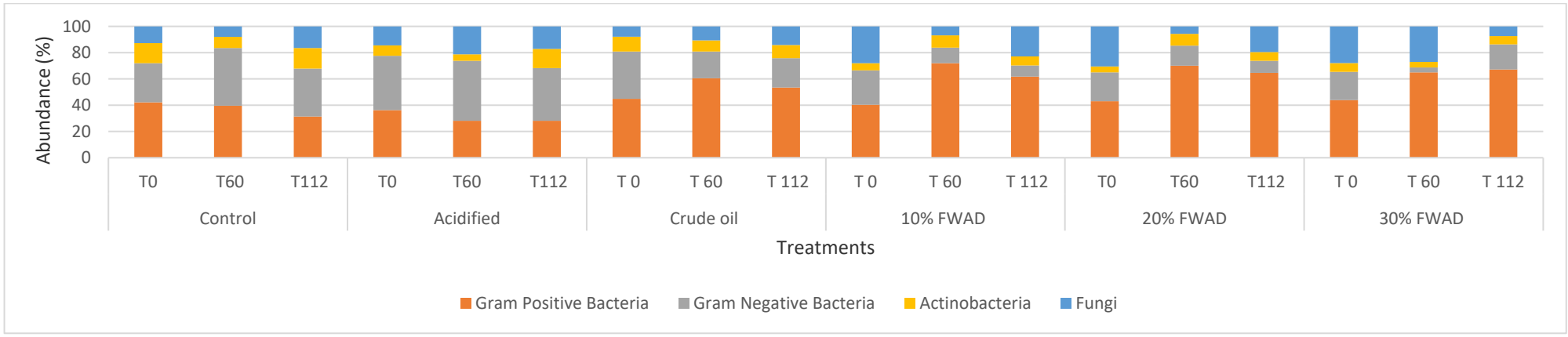
Table 7. Correlation between basal respiration and TPH degradation.

Treatment	Spearman Coefficient (γ)	Prob > |p|	Correlation Strength
Control	0.8104	<0.0001	++++++++
Acidified	0.87	<0.0001	+++++++++
Crude oil	0.8805	<0.0001	+++++++++
10% TW80	0.8395	<0.0001	++++++++
20% TW80	0.8732	<0.0001	+++++++++
30% TW80	0.8949	<0.0001	+++++++++
10% FWAD	0.8588	<0.0001	+++++++++
20% FWAD	0.8358	<0.0001	++++++++
30% FWAD	0.8327	<0.0001	++++++++

3.3. Soil Microbial Community Dynamics, and Environmental Stress

At the onset of the experiments, the soil microbial community was composed of 42% Gram-positive bacteria, 30% Gram-negative bacteria, 15% actinobacteria and 13% fungi (Figure 2). Crude oil contamination and acidification induced a shift in the soil microbial community towards the Gram-positive and Gram-negative bacteria. The application of FWAD and TW80 to the soils further induced the shift towards the Gram-positive bacteria (Figure 2). The observed dominance by the Gram-positive and negative bacteria could be linked to the degradation of the long chains and recalcitrant PAHs and alkanes [86,87]. Studies by Cipullo et al. [22] correlated hydrocarbon degradation to PLFA specific to the microbial communities that survived the stress from the hydrocarbons' contamination. The observed dominant microbial communities (Figure 2) survived and adjusted to the applied stress from both the acidification and crude oil spike. Dunfield [88] and Lewe et al. [89] stated that resistant microbial groups can survive severe environmental stresses.

The applied environmental stress was examined using a trans/cis ratio (Figure 3) from PLFA of Figure 2. The high environmental stresses observed on day 30 of remediation dropped across the mesocosms on day 112 of remediation. The conversion from cis to trans unsaturated fatty acids causes a reduction in microbial membrane fluidity, which counteracts against induced stress [80,90]. However, the trans/cis ratio (Figure 3) for the acidified and crude oil mesocosms were greater than 10. This implied that the microorganisms in the acidified and crude oil mesocosms experienced nutrient starvation. This is in agreement with the research of Zhang et al. [87] on the characteristics analysis of PLFA in sediments. The researchers concluded that at a trans/cis ratio >10, the sediments bacteria were in an unhealthy situation and were experiencing severe starvation due to the applied environmental stress. The reduction in environmental stress (trans/cis ratio) at the FWAD and TW80 mesocosms could be linked to the observed shift in the dominant Gram-positive microbial communities. This subsequently implied that there was a drop in soil toxicity and improvement in the soil ecological quality [87,91], which can be attributed to the FWAD and TW80. The trans/cis ratio has higher predicted efficiency for environmental stress when compared with percentage actinobacterial PLFA and G+/G- ratio [92].



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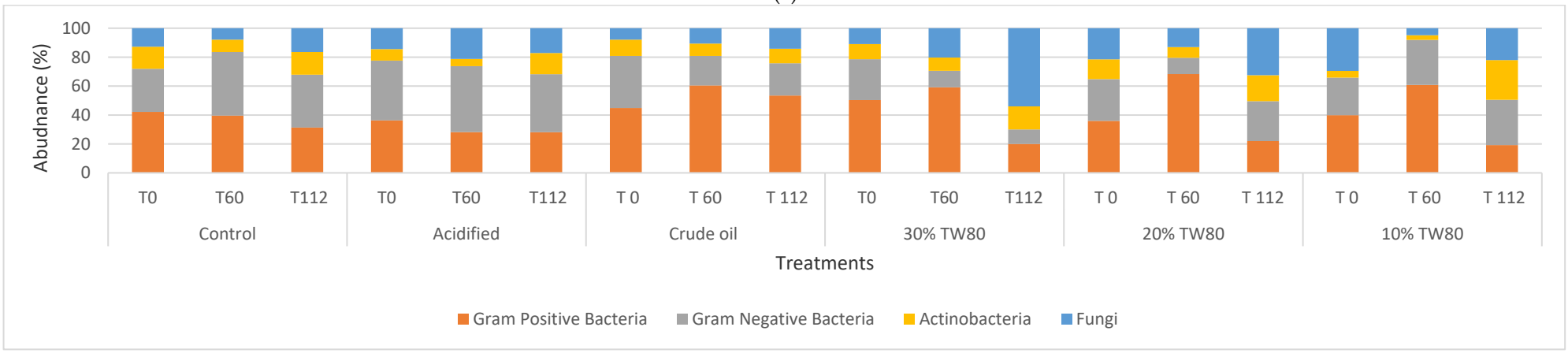


(**b**)

Figure 2. Soil microbial communities' dynamics based on PLFA abundance changes. (**a**) FWAD mesocosms; (**b**) TW80 mesocosms.

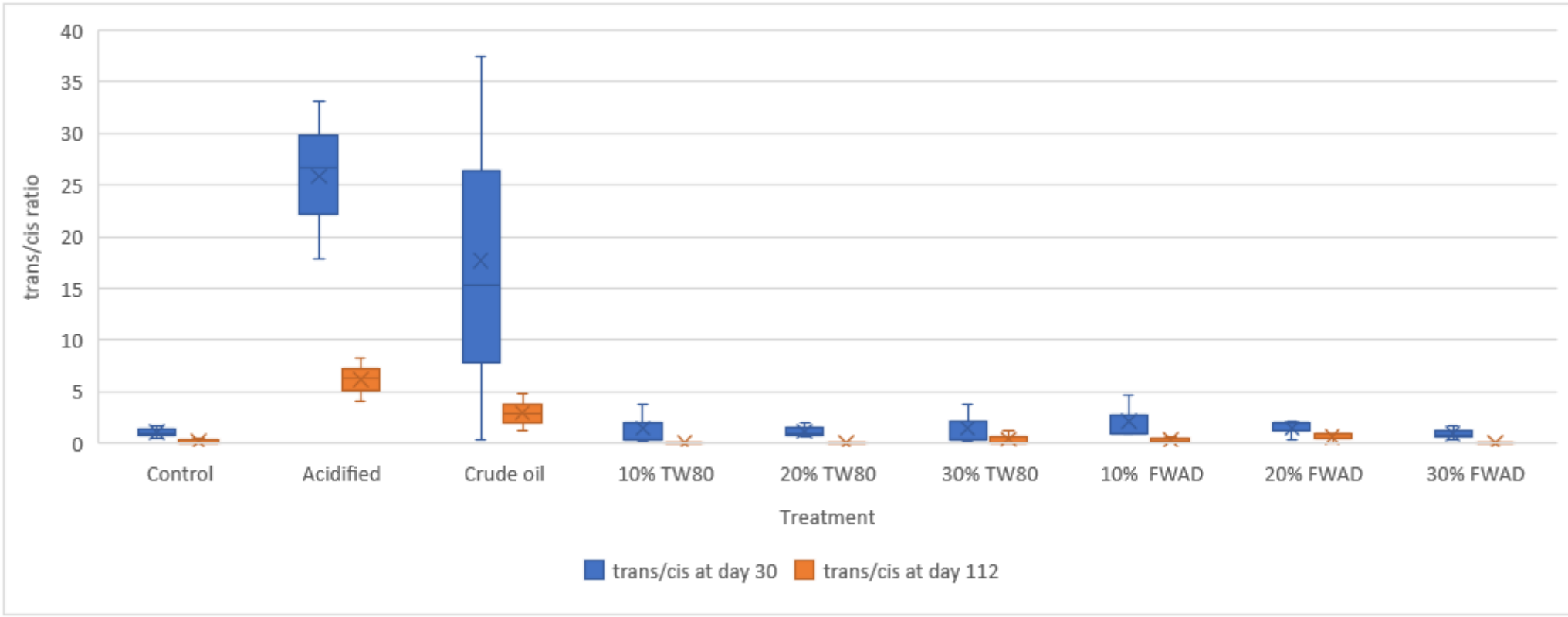


Figure 3. Cis/trans ratio for FWAD and TW80 mesocosms.

3.4. Crude Oil Degradation

In the Tween 80 mesocosms (TW80), the extent of alkanes and PAHs degradation was greatest in soil mesocosms with 30% TW80 in comparison with 20% and 10% TW80 (Figure 4a). On day 49, 75.5% of alkanes and 98% of PAHs in the 30% TW80 mesocosm were degraded (Figure 4a). These results agreed with the study of Feng et al. [93], in which the researchers observed that surfactant increases dissolution of PHCs in the aqueous phase which aids in bioaccessibility of the contaminants to the microbes for degradation. Ceschia et al. [43] confirmed that surfactants in wet soils reduced the interfacial tension and attraction between the contaminants, soil particles and soil moisture. This subsequently makes the contaminants more accessible to the cell walls of the bacteria leading to the mineralisation of the hydrocarbons. It was observed that the bioavailability of the contaminants in the TW80 mesocosms decreased following the degradation of the hydrocarbons (Figure 4a,c). A more significant degradation for medium molecular weight hydrocarbons (C11–C18) was observed compared with heavy molecular weight hydrocarbons (C19–C37) (Figure 4a,c).

The medium molecular weight alkanes which include undecane, dodecane, tridecane to octadecane showed more than 99% degradation by day 112 of remediation for the 30% TW80 mesocosms (Table 8 and Figure 4a). Previously reported hydrocarbons contaminant degradation on wetlands using biochar showed reduced degradation, with 50% degradation of 500 mg/kg alkanes at the same period [94]. The heavy molecular weight hydrocarbons (C19–C37) which include pristane, phytane, nonadecane, hexatriacontane, and heptatriacontane degraded at a reduced rate with the heavier molecular weights showing lesser degradation and availability (Table 9). On day 112, about 85% of the total C19–C37 alkane degradation was achieved at the 30% TW80 mesocosms. Other TW80 mesocosms showed similar degradation but with reduced degradation rates. Wartell et al. [95] stated that medium weight alkanes are more easily degraded by microorganisms if compared to the heavy molecular weight alkanes. The fast degradation observed can be linked to the TW80 which changed the soil bacteria cell surface hydrophobicity by absorbing the surfactants molecules to the bacteria cell surface which subsequently improved the transmembrane transport of the hydrocarbons to the bacterial cell [41]. The degradation pattern observed with the alkanes were similar to that of the soil PAHs. The medium molecular weight compounds degraded on the average 1.25 times faster than the heavier molecular weight PAHs for the TW80 mesocosms. Naphthalene, Fluorene, Phenanthrene, Benz[a]anthracene and Chrysene (that is C10–C18) on day 49 showed about 99% degradation for 30% TW80 (Figure 4a and Table 10). The heavier molecular weight PAHs such as Benzo[b]fluoranthene, Benzo[k]fluoranthene to Indeno(123)[cd]pyrene showed 96% degradation (Figure 4a and Table 11) for 30% TW80. On day 112, both medium and heavy molecular weight PAHs showed more than 99% degradation. Wang et al. [40] researched on surfactant-enhanced remediation of PAHs in farmlands. The researchers concluded that surfactant weakens soil contaminants' sorption, thereby enhancing PAHs desorption from soil.

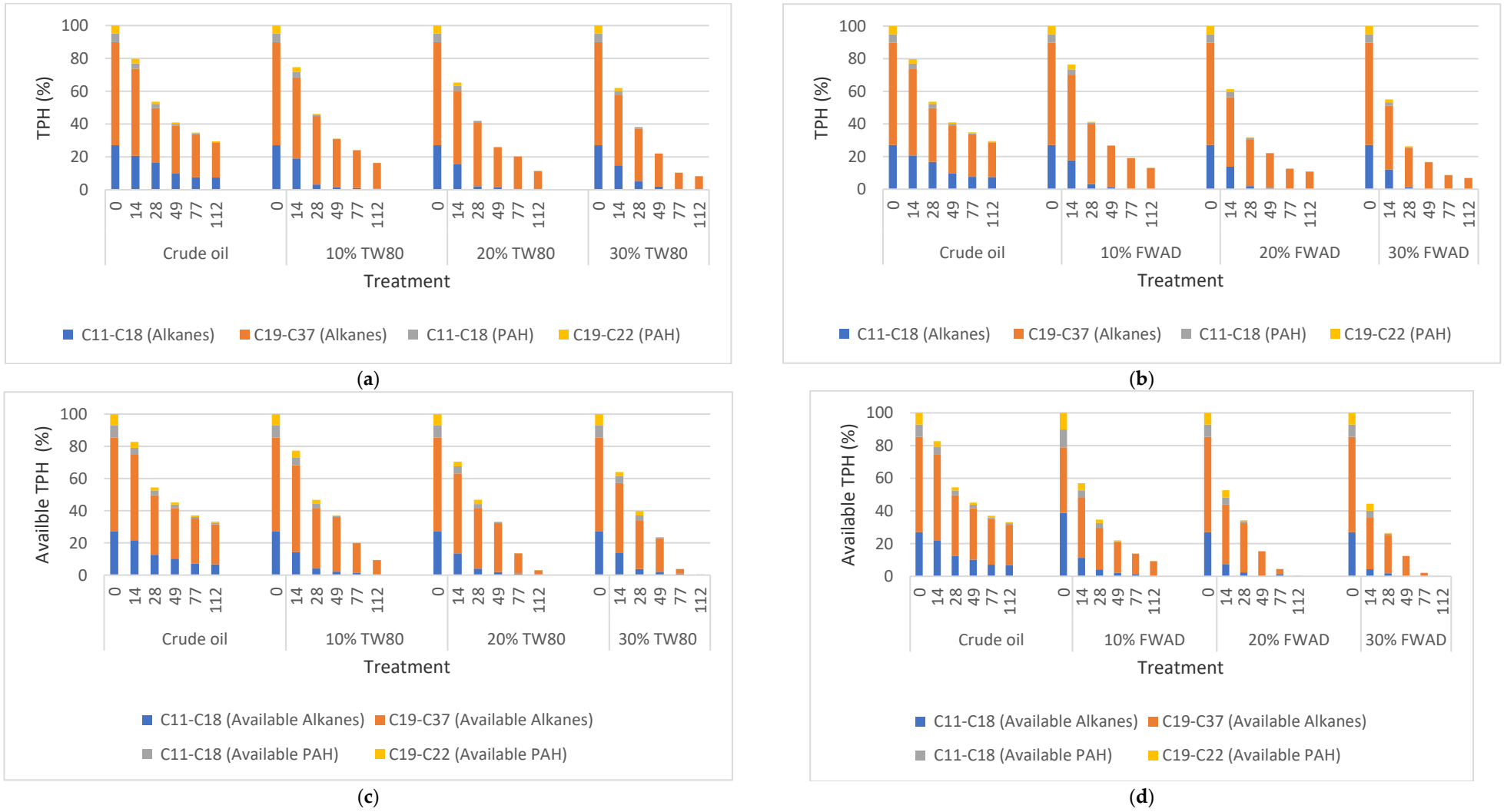


Figure 4. Total and bioavailable hydrocarbon fractions degradation. (**a**) Total TPH degradation for TW80 mesocosms; (**b**) Total TPH degradation for FWAD mesocosms; (**c**) Available TPH for TW80 mesocosms; (**d**) Available TPH for FWAD mesocosms.

Table 8. Mean alkanes concentrations (c) and percentage degradations for C11–C18 alkanes.

Alkane Group	Initial Alkanes	Percentage Degradation (%) on Day 112					
C11–C18	(mg/kg)	Crude Oil	10% FWAD	20% FWAD	30% FWAD	10% TW80	20% TW80	30% TW80
Undecane	2339.0	93.7	99.7	99.8	99.8	99.7	99.7	99.8
Dodecane	1226.2	59.8	99.3	99.5	99.7	99.3	99.4	99.6
Tridecane	1710.5	74.8	99.7	99.7	99.8	99.6	99.7	99.8
Tetradecane	1658.9	68.5	99.6	99.6	99.7	99.5	99.5	99.6
Pentadecane	1669.2	67.4	99.5	99.5	99.5	99.3	99.4	99.6
Hexadecane	1597.1	62.8	99.2	99.2	99.3	98.2	99.2	99.3
Heptadecane	1648.6	75.1	98.7	98.7	99.3	98.7	98.2	98.8
Octadecane	1576.5	65.2	98.2	98.2	98.5	98.4	98.4	98.5
Overall % degradation		70.9	99.2	99.3	99.5	99.1	99.2	99.4

Table 9. Mean alkanes concentrations and percentage degradations for C19–C39 alkane groups.

Alkane Group	Initial Alkanes	Percentage Degradation (%) on Day 112					
C19–C37	(mg/kg)	Crude Oil	10% FWAD	20% FWAD	30% FWAD	10% TW80	20% TW80	30% TW80
Pristane	1100.0	80.0	90.8	91.7	96.4	91.8	94.9	96.3
Phytane	1151.8	82.5	91.0	92.2	96.4	90.4	95.5	96.6
Nonadecane	1296.9	75.8	91.9	94.6	96.1	91.4	93.8	96.7
Eicosane	1416.3	75.5	91.7	92.3	95.6	91.3	94.3	97.2
Heneicosane	1714.9	81.2	93.0	93.6	95.3	89.5	94.5	96.5
Docosane	1808.8	75.4	90.4	93.2	96.6	90.6	94.3	95.0
Tricosane	1798.9	79.4	90.2	92.4	96.1	90.0	94.4	95.0
Tetracosane	1775.7	77.7	89.8	92.6	93.1	89.3	95.0	96.5
Pentacosane	1743.9	79.3	89.6	93.0	94.5	89.1	94.2	95.9
Hexacosane	1890.3	77.3	88.7	93.2	94.2	86.5	94.4	94.9
Heptacosane	1070.9	69.7	71.8	87.8	90.5	68.9	90.5	92.3
Octacosane	1936.8	79.5	83.1	91.7	94.1	80.0	89.8	94.9
Nonacosane	1953.8	78.4	83.5	87.7	89.7	74.5	89.3	94.9
Triacontane	1612.5	67.7	74.8	79.2	87.3	67.3	86.2	93.8
Hentriacontane	1569.9	66.9	73.9	75.3	81.0	65.0	77.0	84.7
Dotriacontane	1313.9	45.2	60.6	62.6	81.8	55.4	61.6	81.0
Tritriacontane	1058.0	22.3	51.1	52.8	72.7	43.5	51.6	78.0
Tetratriacontane	1183.6	38.6	56.5	58.7	81.2	44.6	48.5	65.0
Pentatriacontane	1245.7	35.6	58.6	60.0	81.1	45.7	50.3	55.9
Hexatriacontane	1176.4	32.3	56.2	57.7	78.9	42.3	43.9	44.9
Heptatriacontane	1254.6	20.4	59.3	61.7	68.4	44.3	46.6	48.1
Overall % degradation		63.8	77.9	81.1	88.6	72.9	80.0	85.4

Table 10. Mean alkanes concentrations and percentage degradations for C10–C18 PAH groups.

PAH Group	Initial PAHs	Percentage Degradation (%) on Day 112					
C10–C18	(mg/kg)	Crude Oil	10% FWAD	20% FWAD	30% FWAD	10% TW80	20% TW80	30% TW80
Naphthalene	224.1	91.4	99.9	99.9	99.9	99.9	99.9	99.9
Fluorene	458.5	91.6	99.1	99.9	99.9	99.9	99.9	99.9
Phenanthrene	931.1	95.5	99.9	99.9	99.9	99.9	99.9	99.9
Anthracene	297.2	96.7	99.7	99.9	99.9	99.3	99.1	99.9
Pyrene	67.9	87.9	98.5	99.9	99.9	97.0	98.1	99.9
Benz(a)anthracene	212.3	90.4	98.6	99.9	99.9	98.1	98.9	99.5
Chrysene	356.6	88.8	98.6	99.9	99.9	98.0	99.4	99.0
Overall % degradation		91.8	99.2	99.9	99.9	98.9	99.1	99.7

Table 11. Mean alkanes concentrations and percentage degradations for C19–C22 PAH groups.

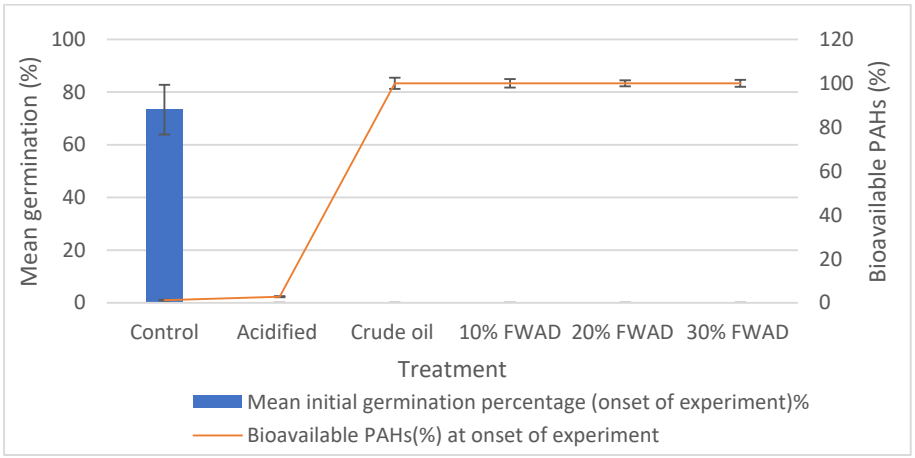
PAH Group	Initial PAHs	Percentage Degradation (%) on Day 112						
C19–C22	(mg/kg)	Crude Oil	10% FWAD	20% FWAD	30% FWAD	10% TW80	20% TW80	30% TW80
Benzo[b]fluoranthene	334.8	93.0	99.1	99.9	99.9	99.0	99.9	99.9
Benzo[k]fluoranthene	186	93.5	99.3	99.6	99.7	99.0	99.2	99.9
Benz(a)pyrene	176.7	87.3	99.1	99.7	99.9	98.4	99.4	99.9
Benzo(ghi)perylene	818.4	89.4	99.1	99.7	99.7	98.1	99.1	99.6
Benzo[b]triphenylene	604.5	89.8	99.3	99.4	99.7	98.0	99.1	99.7
Indeno(123)[cd]pyrene	344.1	86.5	98.5	99.2	99.8	97.9	98.9	99.1
Overall % degradation		89.9	99.1	99.6	99.8	98.4	99.3	99.7

The fastest hydrocarbon degradation for the FWAD mesocosms was at the 30% FWAD mesocosms (Figure 4b). 82% of alkanes and 98% of PAHs were degraded by day 49 compared to the natural attenuation (crude oil mesocosms), which has less than 65% for both alkanes and PAHs. Gielnik et al., [96] hypothesized that the metabolic potential of soils can be enriched by the bacteria contained in digestate which can provide new HCs degrading taxa and increase the *alkB* gene content. *AlkB* genes encoding alkane hydroxylases belonging to monooxygenases are effective in alkanes degradation [60,97,98].

This hypothesis corroborates with the high CFU/g count in the FWAD mesocosms (Table 4), which can be linked to the high metabolization of the petroleum HCs by the dominating Gram-positive bacteria communities. On day 49, it was observed that the undecane, dodecane, tridecane to octadecane (medium molecular weight HCs) degraded faster than the heavy molecular weight hydrocarbons. The medium molecular weight HCs (C11–C18) showed about 99.5% degradation on day 112 for the 30% FWAD mesocosms (Figure 4b and Table 9). This increased degradation could be linked to the availability of the medium weight hydrocarbons and the increased microbial activities caused by the availability of nutrients (supplied by FWAD) needed for optimal performance of the microbes (Table 4 and Figure 4d). It was observed that the heavy molecular weight hydrocarbons which include pristane, phytane, nonadecane to hexatriacontane and heptatriacontane on day 112 showed reduced degradation as the molecular weight increases to achieve 88.6% degradation (Table 9). The PAHs in the FWAD mesocosms degraded faster than the alkanes (Figure 4b,d). On day 112, more than 99% of the PAHs were degraded (Tables 10 and 11) [99] and [100] stated the application of stimulants (such as FWAD) could cause an increase in surface area of the samples which could allow for increased microbial attacks on the PAHs.

3.5. Remediation Endpoint

At the onset of the experiment, germination was only recorded at the control (pristine soil). The acidification of the soil to pH of 5.8 and spiking with crude oil increased the soil toxicity and inhibited the germination of the maize crops (Figure 5). This result corroborates the research of [101] on the response of crops to soil acidity. The researchers concluded that soil acidity severely affects crops' root development and germination. The bioavailability of the PAHs and alkanes (from the spiked crude oil) to the maize crops may have increased the soil toxicity level leading to the no-germination recorded at the onset of the experiments (Figure 5). Bioavailability, the freely available fraction of contaminants in soil is an important feature in risk assessment as it explains contaminants partitioning and degradation in the environment [22]. Seed germination bioassays alongside bioavailability, despite being cost effective, have the potential to evaluate the establishment of the remediation endpoint [22,102,103].



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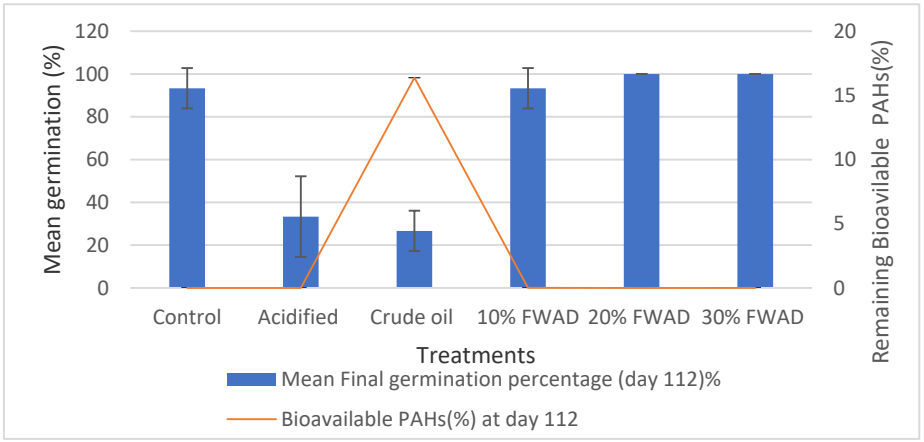

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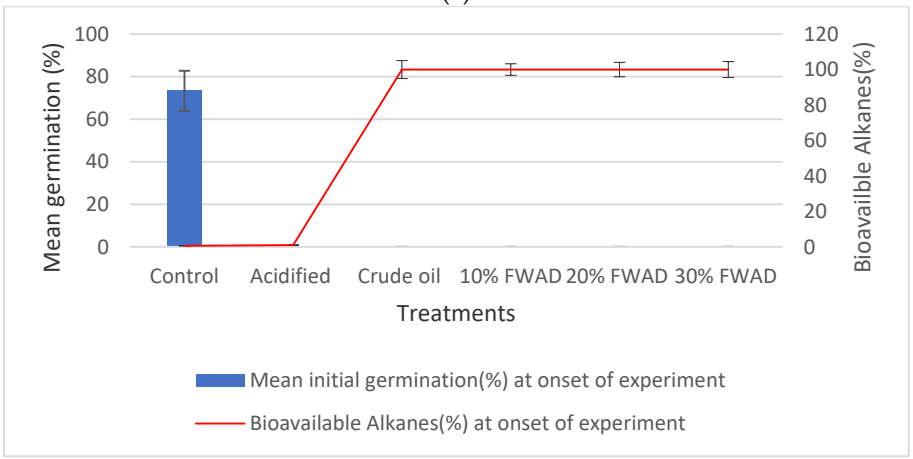

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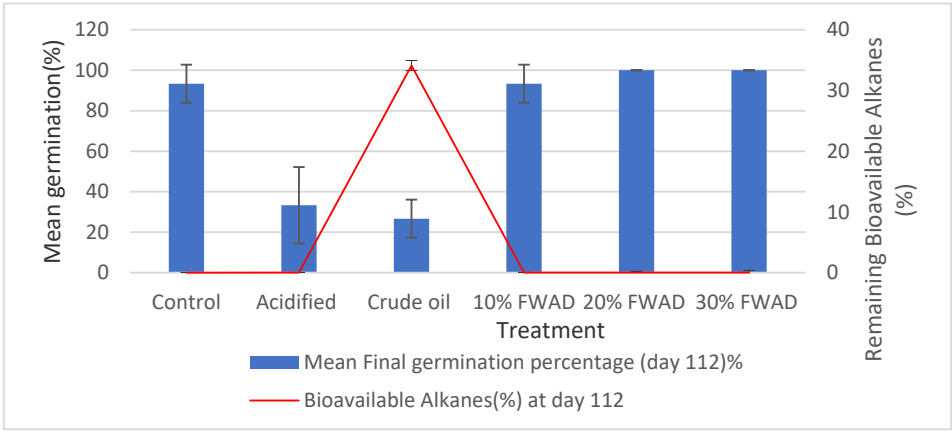
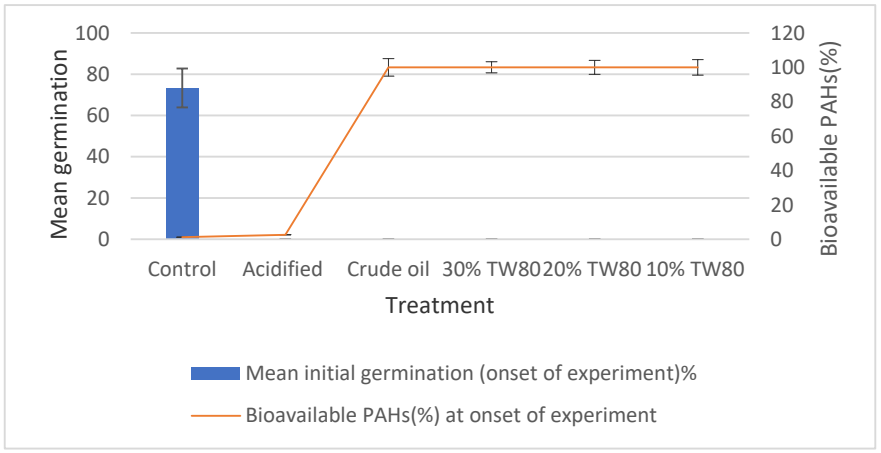


(**d**)

Figure 5. *Cont*.



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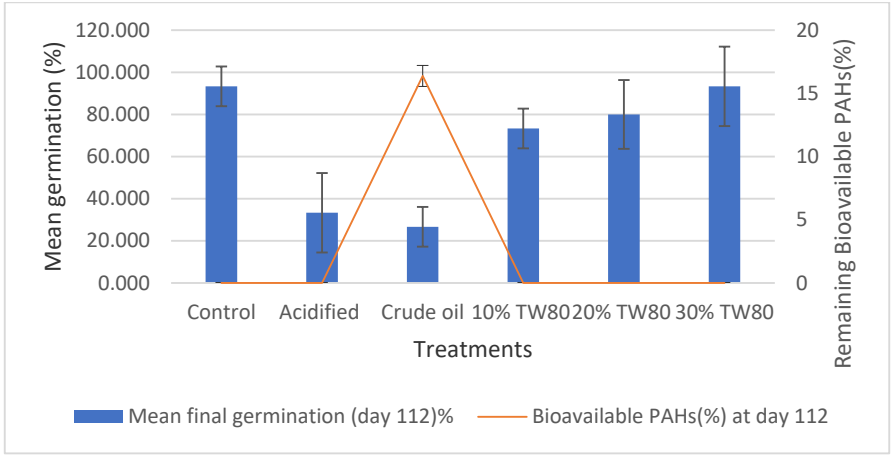

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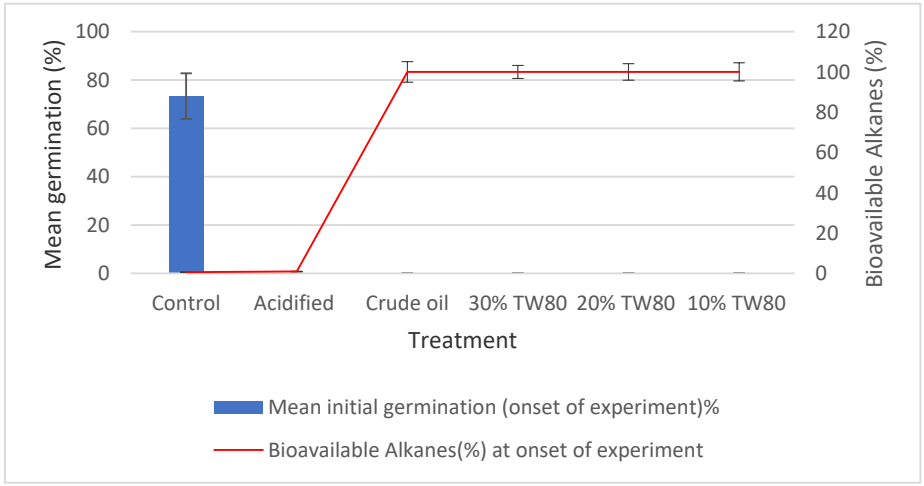

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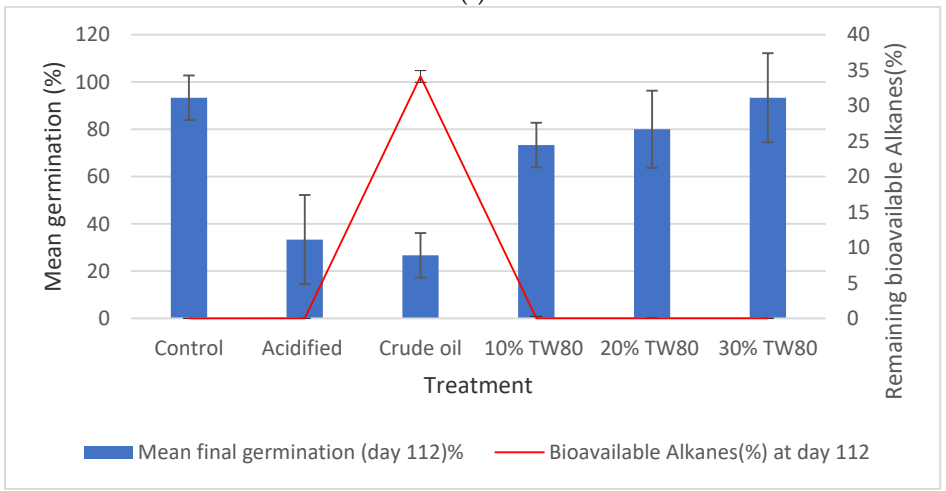

(**h**)

Figure 5. Mean germination of maize crops and bioavailable HCs for various mesocosms. (**a**) Mean initial germination and bioavailable PAHs for FWAD; (**b**) Mean final germination and bioavailable PAHs for FWAD; (**c**) Mean initial germination and bioavailable alkanes for FWAD; (**d**) Mean final germination and bioavailable PAHs for FWAD; (**e**) Mean initial germination and bioavailable PAHs for TW80; (**f**) Mean final germination and bioavailable PAHs for TW80; (**g**) Mean initial germination and bioavailable alkanes for TW80; (**h**) Mean final germination and bioavailable alkanes for TW80.

On day 112 of the experiment, the highest germination was recorded at the 30% and 20% FWAD with 100% germination, while the 30% TW80 and the control had 93% germination and the crude oil mesocosms had 26% germination (Figure 5a,b,f,h). The above 90% germination in the various remediated mesocosms corroborates the low bioavailable PAHs and alkanes in the mesocosms. These agreed with the ecotoxicity evaluation research of [104] who stated that the response of crops to germination on polluted soils varies with ability of the nutrient to remediate contaminants from the soil. This implied that the FWAD and TW80 treatments aided in the recovery of the soil contaminated with crude oil. Overall, the extent of recovery shown by the soils through the maize germination and the low bioavailable alkanes and PAHs was an indication that remediation endpoint was achieved on day 112.

4. Conclusions

This research has shown that acidified wetlands contaminated by petroleum HCs can be effectively remediated using low carbon stimulants such as FWAD and TW80 surfactant. The Gram-positive bacteria were the dominant microbial group in the FWAD and TW80 surfactant mesocosms. The application of 30% FWAD, and 30% TW80 degraded the HCs contaminants in the acidified wetlands by 90% and 86.8% of TPH in 49 days, respectively. The 30% FWAD were the least metabolically stressed mesocosms, followed by 30% TW80 at the end of remediation when compared with the other mesocosms. Therefore, 30% FWAD and 30% TW80 mesocosms showed the least environmental toxicity to the soil ecosystems and achieved remediation endpoints faster. This conclusion was further confirmed by the more than 90% maize crops germination alongside no bioavailable HCs recorded at the end of the experiment in the 30% FWAD and 30% TW80 mesocosms. The extent and rate of HCs degradation was dependent on the CO_2 generation rate from the basal respiration of the soil microbial communities since the HCs were mineralized by the microbes to generate the CO_2.

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