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SPENT MUSHROOM SUBSTRATE: A POTENTIAL IN SITU BIOSTIMULANT IN A HERBICIDE-POLLUTED SOIL

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Abstract: This study investigated the usefulness of sterilized spent mushroom substrate (SMS) from Pleurotus ostreatus as biostimulant in a field study to enhance the bioremediation of atrazine-impacted soil. The herbicide, Atraforce (brand name) and the sterilized (autoclaved) SMS (380 g) were applied on microcosms of 0.25m2in situ. The soil was treated with spent mushroom substrate only (SMS), spent mushroom substrate and atrazine (SMS+ATZ), atrazine only (ATZ), and Control (without any treatment). The effect of the various treatments on the soil physicochemical condition and soil bacteria was monitored. The soil was also assayed for the presence of potential atrazine-degraders. The results showed that the organic matter used in the study improved the nutritional condition of the soil, which ameliorated the effect of the pollutant (atrazine) on soil bacteria. The herbicide reduced soil heterotrophic culturable bacterial diversity (week 2: ATZ = 28.57 %, Control = 71.43 %; week 4: ATZ = 42.86 %, Control = 85.71 %; Week 6: ATZ = 57.14 %, Control = 85.71 %) but allowed the proliferation of naturally selected degraders inherent in the perturbed soil. Thus, the reuse of spent mushroom substrate as a bio-fertilizer to stimulate the enzyme activities and proliferation of degraders for the biodegradation of atrazine in atrazine-polluted soil in order to mitigate its entry into the environment is feasible.

Key words: Atrazine, Spent mushroom substrate, bacteria, soil, biostimulation

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Introduction

Pesticide application with the aim of improving productivity of agricultural products is a common practice in recent times. In particular, herbicides which are used for the control of weed in farmlands, gardens and fields around inhabited buildings enter into the environment after application. Most of these herbicides are afterward found in the soil, water, and atmosphere or in the harvested plant produce (Kudsk and Streibig, 2003). Upon entry into the environment, these agro-chemicals may persist in the biosphere (e.g. soil), whereby becoming immobile on soil particles and become components of the food chain (Guthrie and Davis; 1985). As a result of the general use of these herbicides over a long period of time, their effects have been observed as a result of the accumulation of the residues in the environment, where they cause severe pollution in the ecosystems and harmful disruption of the biota (Parsons and Witt, 1989).

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) is a pre or post-emergence herbicide which has been confirmed to have deleterious effect on none target organisms like soil macro and microorganisms (Stanley et al., 2013), aquatic organisms, man and other animals (Atrazine Risk Characterization Document, California Environmental Protection Agency, 2001). When atrazine and its degradation products get into living tissues of animals either through ingestion, inhalation or through the food chain, clinical signs of acute toxicity which may be encountered include muscle weakness, facial swelling, constricted pupils, nasal discharge, decreased activity, salivation, weight loss, e.t.c.Chronic toxicity observed in experimental animals like dogs, mice, and rats showed reduced body weight and food consumption, haematological abnormalities, inflammatory changes in mammary gland, kidney, urinary bladder, heart, skeletal muscle, prostate or eye. Changes in reproductive hormone levels and estrus cycles were also detected (Atrazine Risk Characterization Document, California Environmental Protection Agency, 2001). Thus, improved techniques to increase atrazine removal from the soil through biodegradation become imperative in other to reduce its natural half-life in the soil and 'leaching window'.

In 2014, Delgado and Eymer studied the use of spent mushroom substrate (SMS) as a biostimulant to improve microbial activities which enhanced the bioremediation of polluted soil. Earlier researchers have also proved that the SMS has the potential to enhance the bioremediation of several grades of agro-chemicals due to its rich organic matter, near neutral pH, moderate nutrient composition, presence of beneficial microorganisms, ability to improve soil structure, ability to reduce soil surface crusting and compaction, and increase microbial activity (Pill

et al., 1993; Ahlawatet al., 2010; 2011). Studies have confirmed the possibility of herbicide (atrazine) degradation by bacteria. Degradation of atrazine has been associated with bacteria in the genera Agrobacterium (Devers et al., 2007), Pseudomonas (Hernandez et al., 2008), Chelatobacter, Arthrobacter (Rousseaux et al., 2001), Rhodococcus (Behki et al., 1993), Nocardia (Giardi et al., 1985), Acinetobacter (Mirgain et al., 1993) and Rhizobium (Bouquard et al., 1997). Some of these organisms degrade atrazine partially (Wang et al., 2011; Strong et al., 2002), while others have the capability to mineralize it (Wackett et al., 2002; Yang et al., 2010). In most mushroom farms in Nigeria, the spent substrate is dumped as waste after their use as substrate for growing mushroom (e.g Pleurotus ostreatus). In order to manage the waste, its use in bioremediation as a biostimulatory agent becomes necessary. Therefore, this research work was aimed at improving the soil condition of atrazine-polluted soil through biostimulation using spent mushroom substrate and monitoring their effect on the total culturable heterotrophic bacterial population, diversity and isolation of atrazine degraders within the impacted soil.

MATERIALS AND METHODS

Spent mushroom substrate and Herbicide used

The spent mushroom substrate used for this study was collected from the Faculty of Agriculture Demonstration Farm, Choba Park, University of Port Harcourt, Nigeria where the edible mushroom, Pleurotus ostreatus is cultivated. The basic proximate mineral element composition of the SMS usedwas determined using standard laboratory techniques. The herbicide (atrazine) used in this study was purchased from an agro-chemical store in Port Harcourt, Rivers State, Nigeria. It contains 50% SC atrazine as the active ingredient. The herbicide was prepared according to manufacturer's guidelines and the protocol proposed by Pal and Das Gupta (1994) was also adopted.

Figure 1: Dried spent mushroom substrate

Experimental site description

The experimental site was situated at the Research Farm of the Faculty of Agriculture, University of Port Harcourt. The total area of the experimental site was 123.5m2. The study area was further divided into twelve (12) smaller square blocks of 0.5m (microcosms) at 3m apart from each other (3 rows; 4 columns). The global positioning system (GPS) of the site is Latitude 4.908428 (4°54‘ 30.34‘‘ N) and Longitude 6.923089 (6°55‘ 23.11‘‘ E).

The temperature of the soil ranged between 26 and 27°C throughout the study period in all the treatments. This study was carried out during the rainy season.

Experimental design and soil treatment

Each of the microcosms (area of 0.25m2 each) were first treated with the dried, sterilized (to prevent microbial interference from the organic wastes) SMS. After seven (7) days, atrazine was applied at manufacturer's recommended rate. The treatments applied in this study were: atrazine only (ATZ); spent mushroom substrate (380g) and atrazine (SMS+ ATZ); spent mushroom substrate (380g) only (SMS). There was "CONTROL" in which no treatment was applied. Each treatment and the control were made in three replicates. Plants/weed were completely removed from each of the microcosms to avoid phytoremediation. The completely randomized block design (CRBD) was used in this study.

Samples collection

A composite soil sample (10cm depth) was first taken to determine the bacterial and physicochemical condition of the soil within the study area before treatment. Samples from each treatment and control were collected fortnightly for 6 weeks. Samples collected were properly mixed and characterized to determine the soil physicochemical properties and bacterial population/ diversity response to atrazine treatment and amendment with the organic waste.

Isolation and characterization of total culturable bacteria

The isolation and enumeration and of total culturable bacteria were achieved by using the spread plate technique on Nutrient agar (NA) medium. Isolated bacteria were characterized based on cultural characteristics, staining and biochemical reactions with reference to the Bergey's manual of systematic Bacteriology (1984).

Growth and degradation studies

Citrate-Atrazine (Cit-Atz) medium which contained 1.0g sodium citrate ($C_6H_5Na_3O_7$), 0.5g Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$), 0.5g Calcium chloride Anhydrous ($CaCl_2$), 1.0g Di-Potassium Hydrogen Orthophosphate 3Hydrated (K_2HPO_4), 1.0g Manganous sulphate H_2O ($MnSO_4$), 0.4g Cupric sulphate ($CuSO_4.5H_2O$), 100mg/L atrazine, and 1L distilled water was used for the degradation study (modified Czapek Dox Agar;Udikovic et al.2003). The Sodium citrate was the sole carbon source while atrazine was the only nitrogen source in the medium. The agar medium of the above composition was made by adding 16g agar per litre to form Cit-Atz agar. To isolate atrazine-degrading bacteria, 0.1g of nystatin was added into 1L of media to knock-off fungal contamination (Hui et al., 2013). One gram of soil sample from each microcosm treated with atrazine was added to 10ml of mineral salt medium (MSM) (with no atrazine, and antibiotics) in sterile test tubes and vortexed for 1

minute. Equal volume of 1ml slurry was taken and was added into 250ml Erlenmeyer flask containing 50ml of Cit-Atz medium containing nystatin. The inoculated flasks were incubated at 30°C and isolation of degraders was made at 2 days interval for 10 days.

Isolation of atrazine degrading bacteria

Atrazine degrading bacteria were isolated with the Cit-Atz agar using the spread plate method. Aliquot (0.1ml) of each culture from the degradation study was inoculated in Cit-Atz agar appropriately. Isolation of atrazine degrading bacteria was realized by inoculating the cultures on Cit-Atz agar containing nystatin. The inoculated plates were incubated at 30°C and were observed daily for growth for ten days.

Soil pH

The soil pH of the treated and untreated soil was analyzed using standard analytical technique.

RESULTS

Characterization of study site and the organic waste (SMS) used

Table 1 revealed the prevailing physicochemical status of the experimental site before the commencement of the study. This is necessary in order to ascertain possible alteration in soil condition as a result of the treatments.

The basic proximate mineral element composition of the spent mushroom substrate used for the study was also determined (Table 2).

Table 1:Soil Physicochemical condition of study site before treatments

Parameters	**Quantity**
Moisture content (%)	69.46
Organic carbon (%)	3.46
Total nitrogen (%)	0.16
Available phosphate (ppm)	373.70
pH	6.05
Particle size of soil	
Sand (%)	46.80
Silt (%)	26.00
Clay (%)	27.20
Soil type	Sandy loam
Exchangeable cations (cmol/kg)	
Ca	14.44
K	6.07
Mg	5.07
Na	5.80

Table 2: Basic proximate mineral element composition of the spent mushroom substrate used

N (%)	C (%)	P (ppm)	K (mg/kg)	Ca (mg/kg)	Mg (mg/kg)	Na (mg/kg)	Zn (mg/kg)	Cu (mg/kg)	Fe (mg/kg)	Mn (mg/kg)
9.26	70.02	267.25	67.34	22.61	6.47	2.79	0.20	0.17	4.34	0.42

The mean organic carbon content, total nitrogen content and available phosphorusof the soil under studywas assessed after the treatments. This was done to determine observable alterations caused by the treatments as compared with the control (untreated) microcosm (Figures 2 and 3).

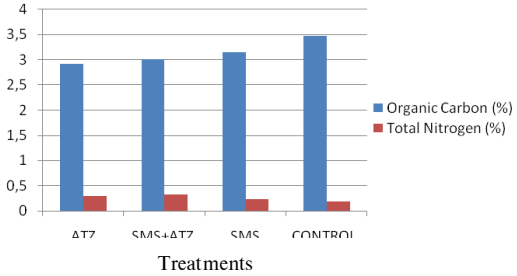

Figure 2: Effect of the treatments on the mean organic Carbon content and total Nitrogen content of the soil

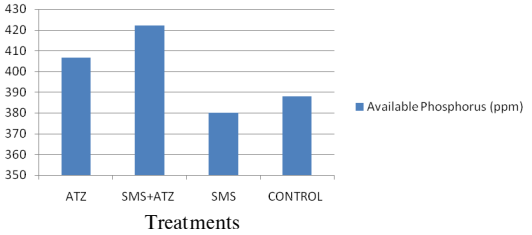

Figure 3: Effect of the treatments on the mean available phosphorus of the soil

A total of four treatments including the control (SMS, SMS+ATZ, ATZ, CONTROL; n=4) were made in this study. Different bacterial species were isolated from each of the treatments. The occurrence of each of these isolates in each treatment based on the sampling period was as shown in Table 3.

Treatment	Week 0 (composite)	Week 2	Week 4	Week 6
SMS	*Bacillus* sp. *Pseudomonas* sp. *Proteus* sp. *Enterobacter* sp. *Micrococcus* sp. *Lactobacillus* sp.	*Bacillus* sp. *Pseudomonas* sp. *Proteus* sp. *Enterobacter* sp. *Micrococcus* sp. *Lactobacillus* sp.	*Bacillus* sp. *Pseudomonas* sp. *Proteus* sp. *Enterobacter* sp. *Micrococcus* sp. *Lactobacillus* sp.	*Bacillus* sp. *Pseudomonas* sp. *Proteus* sp. *Enterobacter* sp. *Micrococcus* sp. *Lactobacillus* sp.
SMS+ATZ	*Bacillus* sp. *Pseudomonas* sp. *Proteus* sp. *Enterobacter* sp. *Micrococcus* sp. *Lactobacillus* sp.	*Bacillus* sp. *Pseudomonas* sp. *Proteus* sp. *Micrococcus* sp.	*Acinetobacter* sp. *Bacillus* sp. *Pseudomonas* sp. *Enterobacter* sp *Micrococcus* sp	*Acinetobacter* sp. *Bacillus* sp. *Pseudomonas* sp. *Enterobacter* sp *Micrococcus* sp *Proteus* sp.
ATZ	*Bacillus* sp. *Pseudomonas* sp. *Proteus* sp. *Enterobacter* sp. *Micrococcus* sp. *Lactobacillus* sp.	*Acinetobacter* sp. *Pseudomonas* sp.	*Acinetobacter* sp. *Bacillus* sp. *Pseudomonas* sp.	*Acinetobacter* sp. *Bacillus* sp. *Pseudomonas* sp. *Micrococcus* sp
CONTROL	*Bacillus* sp. *Pseudomonas* sp. *Proteus* sp. *Enterobacter* sp. *Micrococcus* sp. *Lactobacillus* sp.	*Bacillus* sp. *Pseudomonas* sp. *Proteus* sp. *Enterobacter* sp. *Micrococcus* sp.	*Bacillus* sp. *Pseudomonas* sp. *Proteus* sp. *Enterobacter* sp. *Micrococcus* sp. *Lactobacillus* sp	*Bacillus* sp. *Pseudomonas* sp. *Proteus* sp. *Enterobacter* sp. *Micrococcus* sp. *Lactobacillus* sp

A total of seven different bacteria were isolated from the various treatments in this study. The number of times each of the organisms occurred in the treatments across the four sampling periods (wk 0, wk 2, wk 4 and wk 6) was also represented in Table 4.

Table 4: The effect of the treatments on the total culturable heterotrophic bacterial diversity (species richness) within the sampling period.

Treatments	Week 0	Week 2	Week 4	Week 6
SMS	6(85.71)	6(85.71)	6(85.71)	6(85.71)
SMS+ATZ	6(85.71)	4(57.14)	5(71.43)	6(85.71)
ATZ	6(85.71)	2(28.57)	3(42.86)	4(57.14)
CONTROL	6(85.71)	5(71.43)	6(85.71)	6(85.71)

Diverse atrazine-degrading bacterial species were isolated from each of the two treatments containing atrazine (SMS+ATZ and ATZ). The atrazine degradation study revealed that not all the isolated heterotrophic bacteria possessed the necessary enzyme repertoire for the degradation of atrazine. Table 5 showed the bacterial diversity that were able to utilize atrazine as a nitrogen source, thus were isolated from Cit-Atz agar after 10 days incubation at 30°C.

Table 5: The frequency (%) of the isolated culturable atrazine-degrading bacterial species within the sampling period

Bacterial isolates	Week SMS+ATZ			Week ATZ			Total frequency (%)
	2	4	6	24	6		
Acinetobacter sp.	-	+	+	+	+	+	5(83.33)
Bacillus sp.	+	+	+	-	-	+	4(66.67)
Enterobacter sp.	+	-	+	-	-	-	2(33.33)
Lactobacillus sp.	-	-	-	-	-	-	0(0.00)
Micrococcus sp.	-	-	+	-	-	-	1(16.67)
Proteus sp.	-	-	-	-	-	-	0(0.00)
Pseudomonas sp.	+	+	+	+	+	+	6(100.00)
Diversity	3	3	5	2	2	3	

Key: '+' = Isolated; '-' = Not isolated

Soil pH being an important factor of soil condition was monitored fortnightly till six weeks. The effect of the treatments on soil pH was recorded. The mean pH of the various treatments was as recorded in Figure 4.

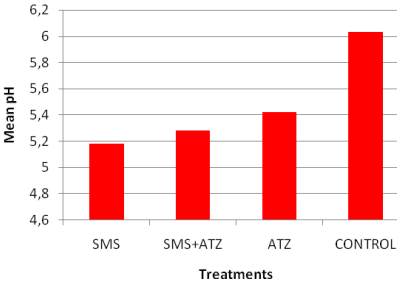

Figure 4: Effect of the treatments on soil pH

The response of the culturable heterotrophic bacteria to the various treatments introduced into the soil was as shown in Figure 5. It also revealed the population dynamics with time.

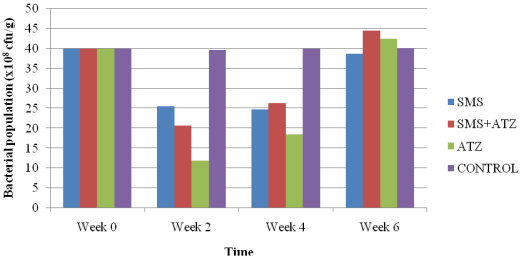

Figure 5: Total culturable heterotrophic bacterial population response to organic amendment and atrazine treatment

DISCUSSION

After amendment with SMS and spiking with atrazine, it was observed that the mean of the soil pH was significantly reduced at $P<0.05$ (Figure 4). The ANOVA showed significant ($P<0.05$) reduction in soil pH in all the treatments when compared with the value obtained in the control (6.03). In all the treatments; SMS (5.18), SMS+ATZ (5.28) and ATZ (5.42) the soil pH declined by 14.10 %, 12.44 % and 10.12 % respectively. This observation concurred with the work of Ayansina and Oso (2006) and Stanley et al. (2013) which reported a reduction in soil pH after atrazine application. Similarly, in 2015 Garcia-Delgadoet al., reported changes in soil chemistry due to the addition of sterilized spent Agaricus bisporus substrate (SSAS) which overtly resulted in a strong reduction in soil pH .Also, the decrease in soil pH as observed in this study is in line with Angelova et al. (2013) who recorded a significantly declined soil pH after treatment with organic material (compost). The decrease in soil pH after treatment with hydrothermal sterilized organic matter was as a result of increased soluble organic matter which culminated in the production of low molecular weight(LMW) organic acids such as acetic acid (Quitainet al., 2002). However, contrariwise, Kaddous and Morgans (1986) reported an increase in soil pH after incorporation of spent mushroom substrate.

The treatments also caused significant ($P<0.05$) decline in the mean of the total organic carbon (TOC) content of the soil. Similarly, as observed by Sangodoyin and Amori (2013) in a degradation process during composting, the total organic carbon was affected which resulted in a decrease in carbon content in treatments when compared with the initial carbon content. However, there were other

studies where soil amendment with organic materials resulted to increased organic carbon (Swer et al., 2011; Abdelhafid et al., 2000a). The total nitrogen content of the soil under study was increased as a result of the treatments. Both atrazine and SMS contain nitrogen component. Therefore it is believed that their introduction to the soil must have improved the soil nitrogen content. This finding is consistent with Swer et al. (2011) who recorded significant increase in soil total nitrogen when an agricultural soil was amended using organic materials.

Phosphorus being a limiting nutrient during the breakdown of atrazine by bacterial degraders in the soil (Qiu et al., 2009) was also monitored.All the treatments but one (SMS only) indicated increase in the soil available Phosphorus (Figure 3). However the rise in Phosphorus level in the soil treated with SMS+ATZ and ATZ only, were not significant at $P<0.05$, Angelova et al. (2013) also recorded a similar effect (increased extractable phosphorus) when soil contaminated with heavy metal was treated with compost.

The culture dependent techniques used for the characterization of culturable heterotrophic bacteria in the study site revealed the presence of three gram positive (Bacillus sp., Lactobacillus sp., Micrococcus sp.) and four gram negative (Acinetobacter sp., Enterobacter sp., Proteus sp., Pseudomonas sp.)bacteria in the study site (Tables 3). Earlier study by Busswel (1994) showed that SMS has the potential to stimulate the catabolic activities of indigenous bacteria by optimizing theirin situ growth conditions. In this study, soil treated with SMS only, gave the highest occurrence of these bacteria (85.71%). A decline (28.57%) in the total culturable heterotrophic bacterial diversity was observed in the microcosm treated with ATZ only, while the microcosm amendment with SMS (SMS+ATZ) ameliorated the perturbed soil by improving the bacterial diversity by 21.43% (Table 3 and 4). Oliver (2005) and Barer (1997) reported that xenobiotics (like herbicides) when applied to the soil have the tendency to exert bactericidal effect on microorganisms, make them dormant, or become viable but none culturable (VBNC). The isolation of Pseudomonas sp. in all the treatments was found outstanding among all the other isolates because it occurred at all the sampling periods, having 100% occurrence. Table 3 showed that Acinetobacter sp. occurred only in microcosms treated with atrazine. This suggests that atrazine has the potential to stimulate the culturability of latent bacteria that possess the ability of its utilization in the perturbed environment. In the other hand, as shown in Table 5, Micrococcus sp., Proteus sp., Enterobacter sp., and Lactobacillus sp. were not isolated in the soil microcosm treated with atrazine only (ATZ) after two weeks of application till week 6. This clearly

supports the effect of atrazine on soil bacterial community as reported by earlier studies (Stanley et al., 2013; Ayansina et al., 2003). However, Enterobacter sp. and Micrococcus sp. were isolated at weeks 2 & 6, and week 6 respectively in the amended soil (SMS+ATZ). Soil amendment using organic wastes as stated by Coleres, (2005), has the capability to restrict contaminant availability to microorganisms leading to the evolution of microbial population that specialize in accessing bound contaminants. Although there was a progressive improvement in bacterial diversity with time in the impacted soil (ATZ) (n = 7; week 2 = 2, week 4 = 3, week 6 = 4) however, in the impacted soil amended with SMS (SMS+ATZ) showed a higher rate of recovery in bacterial diversity (n = 7; week 2 = 4, week 4 = 5; week 6 = 6) (Table 4).

An initial significant ($P<0.05$) reduction in the total culturable heterotrophic bacterial population was observed in all the treatments. From the results obtained in this study, it is evident that both atrazine application and amendment with the sterilized SMS has the ability to thrust an initial decline in soil pH (Stanley et al., 2013; Angelova et al., 2013; Quitain et al., 2002; Garcia-Delgadoet al., 2015). In a study, 'Effect of pH on soil bacterial diversity', Cho et al. (2016) reported that the pH values ranging between 6 and 8 are optimum for bacterial growth. Fernandez-Calvino and Baath (2010) had a similar report in their study. Therefore, the observable cause of the reduction in bacterial population was the drop in soil pH being the resultant effect of the treatments. However, a gradual recovery with time in bacterial population was observed (week 2: SMS = 25.50×10^8 cfu/g, SMS+ATZ = 20.75×10^8 cfu/g, ATZ = 11.90×10^8 cfu/g; week 4: SMS = 24.80×10^8 cfu/g, SMS+ATZ = 26.30×10^8 cfu/g, ATZ = 18.50×10^8 cfu/g; week 6: SMS = 38.65×10^8 cfu/g, SMS+ATZ = 44.50×10^8 cfu/g, ATZ = 42.40×10^8 cfu/g). No significant ($P<0.05$) difference was observed in the mean bacterial population between weeks 2 and 4, but the mean bacterial population at week 6 was significantly higher (at $P<0.05$) than those obtained at weeks 2 and 4 (Figure 5). The high bacterial population observed at week six in ATZ was as result of the proliferation of naturally selected atrazine degraders within the soil. Soil treated with SMS+ATZ had the highest bacterial population at week six. This was as result of the numerous effect of the organic material on the soil. Firstly, the SMS improved the nutritional condition and water-holding capacity of the perturbed soil which encouraged bacterial activity. Nutrition is an important factor in the synthesis and growth ofcells in enzyme activities, which when enhanced will lead to multiplication of pollutant degraders, and culminate in the dissipation of pollutants from the environment (soil). Also, earlier

researchers (Garcia-Delgadoet al., 2015; Coleres, 2005) have reported that organic matters when incorporated into a polluted soil have the ability to adsorb/bind contaminants, thus limiting their interactions and effect on immediate soil microorganisms.

CONCLUSION

The study showed that soil amendment with SMS had more positive effect on the inherent soil microbiota (bacteria) towards achieving the goal of atrazine degradation. Similarly, The addition of organic amendments such as fly ash, pig manure, sewage sludge, have been proved to be effective in lowering the pollutant (metal) toxicity of soil and provides slow release of nutrient sources such as N, P, K (Wong, 2003;Chiu et al., 2006) which at the long run promote bacteria activity. Our earlier study (Stanley et al., 2013) reported of reduced bacterial population in atrazine impacted soil. From the light of this study, the reduction was because the soil do not possess bacteria with necessary enzyme repertoire to utilize the pollutant as a source of nutrient. However, from this study and other studies (Maduike and stanley 2018; Moorman et al., 2001), the reduction in bacterial diversity due to herbicide application was evident, but a natural selection process occured which allowed the multiplication of the few bacteriathat possess the ability to utilize atrazine even in the impacted soil (ATZ). This was succinctly manifested in the result shown in Figure 5.The reuse of spent mushroom substrate as a bio-fertilizer to stimulate the enzyme activities and proliferation of degraders for the biodegradation of atrazine in atrazine-polluted soil is feasible.

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