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MOLECULAR CHARACTERIZATION OF INDIGENOUS ATRAZINE DEGRADERS FROM AGRICULTURAL FIELDS IN LAGOS, SOUTHWEST NIGERIA

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Abstract: *Two composite soil samples were obtained from Lagos state agricultural field at Ikorodu and Lagos State University (LASU) agricultural field, both fields having over 10 years history of Atrazine application. The soil samples were obtained from 0 - 30cm depth with sterile soil auger. The control soil samples with no previous history of Atrazine application were obtained from Badagry beach at the same depth. Physico-chemical analysis of both composite soil samples (mean pH 4.93) and composite water samples obtained from nearby freshwater stream (mean pH 4.55) at the same depth were determined. Analysis of variance (ANOVA) showed there was significant relationship among the parameter and across the locations the result was not significantly different at 0.05 level of significance. Serial dilution technique was engaged to obtain indigenous microbial population while bacterial atrazine - degraders were obtained using minimal salt medium supplemented with 3% atrazine and fungal species with Sabouraud Dextrose agar (SDA) supplemented with atrazine at 5% respectively. Biodegradation / Time-Course study revealed Atrazine concentration reduction using Spectrophotometric analysis. DNA of some selected isolates were extracted and sequenced using 16S rRNA and ITS methods. The genetic data of these isolates were submitted to the NCBI GeneBank database. The selected Atrazine degraders identities were confirmed as follows; Pseudomonas alcaligenes (MT355448.1), Bacillus mycoides (JX144699.1) and Aspergillus aculeatus (LC496490.1) in the NCBI database.*

Keywords: *Atrazine, bioremediation, DNA, herbicide, microorganisms, xenobiotic*

1. Introduction

Atrazine (2-chloro - 4-ethylamino - 6-isopropylamino-1,3,5-triazine) is one of the widely used herbicides worldwide in the control of broadleaf weeds in sugarcane, sorghum, and maize plantations [1, 2]. It has been reported to be persistent, with a half-life of around 41–231 days, high solubility as well as mobility in soils with growing public health concerns, particularly groundwater contamination. Consequently, it has been detected in the food chain, affecting biodiversity and mammals [3]. Atrazine is a triazine herbicide and its molecular formula is $C_8H_{14}ClN_5$, (Fig. 1). It is a white powdery substance, unstable at high temperature. It has a melting point range from 173°C – 175°C. Its solubility in water is 33 mg/L at 20°C and it dissolves easily in organic solvents [4]. Atrazine is a halogenated aromatic compound with its dealkylated and deaminated metabolites being very toxic, carcinogenic and having capacity to disrupt the functioning of the endocrine system, however, its mineralization by microbes in natural environment have been established [5,6].

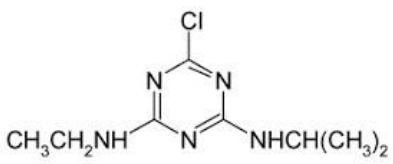

Fig. 1: Chemical Structure of Atrazine [4].

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Persistence of atrazine in soils and subsurface environment induces its biodegradation by microorganisms. It has been detected in surface and groundwater at levels exceeding the Environmental Protection limits. Consequently, its high-water solubility, long residual time, and being difficult to decompose, enhances its capacity to contaminate groundwater easily and inflict damage to the succession crops as well as animals [7]. Researchers have demonstrated that atrazine has toxic effects on algae, aquatic plants, aquatic insects, fishes and mammals [8].

Biodegradation is considered as the foremost, safe, and effective bioremediation process since microorganisms have been found to modify amino group on Atrazine, this includes: *Aspergillus niger* AN 400, *Achromobacter sp., Arthrobacter sp., Delftia sp., Pseudomonas sp*.ZXY-1 [9,10]. The degradation pathway of Atrazine has been studied at molecular level; the first stage catalyzed by hydrolytic enzyme encoded by genes atzA and trzN, to hydrolyze and dechlorinate to produce hydroxyatrazine; then, hydroxyatrazine hydrolytic enzyme (encoded by atzB) catalyzes hydroxyatrazine to transfer to N-isopropyl amide, which is thereafter hydrolyzed by N-isopropylamide hydrolase (encoded by atzC) to generate cyanuric acid [10]. In addition, some other intermediate degradation products have also been reportedwhich include 2-hydroxyl-4-ethylamino-6-isopropylamino-1,3,5-triazine (HEIT), 2-hydroxyl-4,6-bis(ethylamino)-1,3,5-triazine (MEET), and 4,6-bis(ethylamino)-1,3,5-triazin-2(1H)-one (AEEO) [11]. The incontrovertible evidence of atrazine mineralization has been provided using gas chromatographic (GC) and high-performance liquid chromatography (HPLC) tools, this also showed the efficacy of co-culture method at mineralizing atrazine over pure culture. This is a new biostimulation strategy which is also synergistic, this method enhanced atrazine mineralization [12]. Atrazine degraders are diverse in soil and the culture condition for the isolation of bacterial atrazine-degrader consortium often determines the predominating bacterial population among the consortium [13].

The objectives of the current study were to isolate the indigenous microbial consortium with the metabolic capability to mineralize Atrazine in tropical environment and characterize these isolates with the molecular techniques to have their specific identity.

2. Materials and methods

History of the sampled fields

Lagos state University agricultural field

The experiments were carried out at the Lagos State University, (LASU) Nigeria. The agricultural field was sandy/loam comprising two hectares cultivated to *Cassava manihoti* and two hectares to *Zea mays*, the remaining being unarable land. Atrazine application was repeated on the field in October 2020 and soil samples were collected from the field a month after its introduction.

Imota farm settlement

This has been in existence since 1973 and it spans up to ten (10) Hectares arable crops were cultivated on this farm such as cucumber, orange, carrot, cassava, pumpkin leaves (ugwu), pawpaw, maize e.t.c. “Mega Premestral”, a pre-emergence herbicide was applied on the farm thrice between June 1996 and January 1998. Premestral contains atrazine and metalachlor as the active ingredients which were previously applied on the farm. The herbicide “Atrazine” has been in use on the farm since 1980.

Sample collection.
Soil samples (recently treated with atrazine) and water samples from nearby stream were collected aseptically at 0-30 cm depth. Two core soil samples were collected randomly with a sterile soil auger (2.5 cm inner diameter) at 0 - 30 cm depth from each plot (20 cm x 20 cm) from Imota farm settlement, Lagos and LASU Agricultural field. They were kept in black polythene bags and air-dried for five days before sieving with 0.02 mm mesh, after which isolation of microbial consortium suspected to be involved in degradation of Atrazine commenced. Soil samples were collected from Badagry beach with no previous history of atrazine application for control. Overall, six soil samples were collected; ten grams of soil and 100ml of composite water samples were taken into seed envelopes from each of the composite soil samples and sterile glass bottles respectively, thereafter kept in a cool place prior to the determination of their physico-chemical properties. Water samples were collected at 0-30cm depth with sterile bottles from each of the nearby stream to each of the farms and each made into composite samples independently.

Chemicals
The different chemicals of analytical grade used for this study include Atrazine (Sigma-Aldrich Co. U.K.), Cycloheximide (Sigma-Aldrich Co. U.K.), Nutrient agar (Biotech.Lab. U.K.). Sabouraud Dextrose agar (Biomark, India.), molecular biology reagents (Zymo research chemicals, U.S.A.).

Sterilization of media and equipment
All glasswares were sterilized by dry heat using hot-air oven at 170°C for 30 minutes and microbial media used were sterilized in the autoclave at 121°C.

Media preparation
Nutrient agar, Minimal salt agar and Sabouraud Dextrose Agar (SDA) were prepared following Manufacturer's instruction. All microbiological media were sterilized at 121ºC for 15 minutes [14].

Minimal salt medium
The media was prepared by dissolving the following: Agar 20g, KH_2PO_4 2.7g, Na_2HPO_4 2.8g, $(NH_4)_2SO_4$ 0.3g, $MgSO_4.7H_2O$ 0.5g, Ca $(NO_3)_2$ 0.1g and 0.25ml of trace element in 1000ml of distilled water at pH 7.3. Then it was supplemented with Atrazine at 0.03g/ml concentration and autoclaved at 121℃ for 15 mins [15,16] with modifications.

Enrichment cultures for Atrazine-degraders
Selectively enriched cultures with the ability to use Atrazine as carbon and nitrogen energy source were obtained by spiking a field with previous history of atrazine application with the herbicide a month before sample collection. The native microbial consortium degrading atrazine on the agricultural field were then isolated using soil dilution technique [17] and Nutrient agar (pH 7.3) supplemented with atrazine at 0.03 g/L, incubation was at 28±2 ºC for 12 days to isolate bacterial consortium while Sabouraud dextrose agar (SDA) supplemented with atrazine at 0.05 g/L was used to isolate native fungal population, the plates were incubated at ambient temperature (28±2ºC) in the dark to preclude photolysis reaction for 5 - 7 days. Cycloheximide (50 mg/L) was added to media used for isolation of bacteria [18, 16]. The atrazine stock solution was prepared in methanol (50 mg/ml) and was shaken vigorously for several hours prior to incorporation into the medium. The atrazine stock solution was stored unfiltered at room temperature in the dark.

The controls were uninoculated plates containing the media used.

Determination of physio-chemical properties of soil and water samples.

The composite soil and water samples obtained were taken to the laboratory for proximate analysis to determine pH, Nitrogen content, NO_3^-, PO_4^{-3}, SO_4^{2-}, Cl^-, $Hg^{2+}$and NH_4^+ [19, 20].

Extraction of soluble salts

Transfer 100g of soil into a 750ml reagent bottle. Approximately, 500ml of distilled water was added and was shaken in an "Endover and shaker" for about one hour. The resulting suspension was filtered through a sinter filter. The first few extracts were rejected, and the rest collected for analysis. The soil extract was then used to determine the nitrate, phosphate, sulphate and ammonium ions in samples and other components according to Bareither *et al*. [20] and AOAC [21].

Atrazine-degraders assay

Several aseptic transfers of bacterial isolates on Nutrient medium and Minimal salt medium each supplemented with atrazine at 0.05 g/L and 0.03 g/L respectively were incubated at 28±2^{o}C for 12 days to develop subcultures which culminated in preparation of pure cultures of each isolate [18]. Native fungal population were assayed using SDA supplemented with 0.05 g/L of atrazine several aseptic transfers of fungal mycelia were done on this medium and the plates were incubated at ambient temperature for 5 - 7 days. The controls were uninoculated plates containing the media used [14,18].

Identification of fungal Atrazine-degraders

SDA supplemented with Atrazine at 0.05 g/L for 5 - 7 days at ambient temperature was used for preliminary isolation and identification of fungal atrazine - degraders [18]. Pure cultures of fungal isolates were thereafter subjected to molecular characterization.

Soil microcosm Atrazine degradation / Time study

A biodegradation / time study was carried out using spectrophotometer Surgifield (Surgispec SM-23D Spectrophotometer, SURGIFIELD Medicals England) at 652nm to monitor changes in growth of individual isolates inoculated into broth cultures of minimal salt medium and sabouraud dextrose agar supplemented with Atrazine at 0.03g/L and 0.05 g/L respectively [18, 22].

DNA extraction and amplification of the ITS gene

DNA extraction

A measurement of 80mg (wet weight) of fungal cell that had been re-suspended in up to 200µl of deionized water to a ZR BashingBead™ Lysis Tube was obtained. It was secured in a bead beater fitted with a 2.0ml tube holder assembly (Scientific Industries' Disruptor Genie**™** , Cat. No. S6001-2 from Zymo Research Corp.) and processed at maximum speed for 5 minutes. The ZR BashingBead**™** Lysis Tube was centrifuged in a micro-centrifuge at ≥10,000 x g for 1minute. Up to 400µl supernatant was transferred to a Zymo-Spin**™** IV Spin Filter (orange top) in a Collection Tube and centrifuged at 7,000 rpm (7,000 x g) for 1 minute. The base of the Zymo-Spin**™** IV Spin Filter was snapped off prior to use. 1,200µl of Fungal DNA Binding Buffer was added to the filtrate in the collection tube of step four. Thereafter, 800µl of the mixture from step five was transferred to a Zymo-Spin**™** IIC Column in a Collection Tube and centrifuged at 10,000 x g for 1minute. The flow through was discarded from the Collection Tube and step six was repeated. Then, 200µl DNA Per-Wash Buffer was

added to the Zymo-Spin™ IIC Column in a new Collection Tube and centrifuged at 10,000 x g for 1minute. Thereafter, 500µl Fungal DNA Wash Buffer was added to the Zymo-Spin™ IIC Column and centrifuged at 10,000 x g for 1 minute. The Zymo-Spin™ IIC Column was transferred to a clean 1.5ml micro-centrifuge tube and 100µl DNA Elution Buffer was added directly to the column matrix. It was then centrifuged at 10,000 x g for 30 seconds to elute the DNA [23, 24].
The sequence information was analyzed using NCBI Blast.

Sequencing
The extracted DNA was subjected to amplification with a thermal cycler (Helena Biosciences, Sunderland, United Kingdom) and the primers listed in Table 3 were used. All the primers, synthesized by Sigma Aldrich House, Suffolk, United Kingdom, were used in four sets of PCR procedures [25, 26, 24] as follows. The first set of PCRs was standardized to amplify ITSI by using primers ITS1 and ITS2. The 25-µl reaction mixture contained 100 µM deoxynucleoside triphosphates, 0.1 µM each primer, 1µl PCR buffer with 2.0 mM $MgCl_2$, 2µl of template DNA sample, and 1 U of Taq polymerase (Qiagen). The reaction involved initial denaturation at 96°C for 10 min, followed by 30 cycles in series of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min, with a final step of one cycle at 72°C for 10 min to final extension. The second set of PCRs was done to amplify ITSII with primer pair ITS3 and ITS4. The reaction mixture and conditions were the same as those used for the first set of PCRs, with the exception that the annealing temperature was 56°C. The third set of PCRs was performed to amplify both ITSI and ITSII along with the 5.8S rRNA gene by using primer pair ITS1 and ITS4 (Table 1).
The reaction mixture was of the same composition as described above. The first cycle of initial denaturation was performed at 95°C for 10 min, followed by 30 cycles in series of 95°C for 1 min, 55°C for 1min, and 72°C for 90 s, with a final cycle at 72°C for 10 min. The fourth and final set of PCRs involved primers invSR1R and LR12R, specific for the IGS region along with the 5S rRNA gene. The reaction mixture contained all of the components at the same concentrations described above, except that $MgCl_2$ was used at 3.0 mM and the condition for annealing was fixed at 55°C for 1 min and extension was at 72°C for 150 s. For all the PCR protocols, a reaction mixture without sample DNA was used as a negative control and the products were analyzed and stored as described above. Single – strand conformation polymorphism (SSCP) analysis; The amplified products were denatured at 95°C for 10 min and snap-cooled on ice before they were mixed with denaturing buffer (80% [wt/vol] deionized formamide, 10 mM EDTA [pH 8.0], 1 mg of xylene cynol per ml, 1 mg of bromophenol blue per ml). The samples were then electrophoresed on a 5% acrylamide gel. The gels were then silver stained and analyzed.

Similarity-dissimilarity analysis: SSCP profiles were generated by using all of the samples from the PCRs and the four sets of primers (Table 1). Sequencing of PCR products; to determine the complete sequences of ITSI and ITSII, the amplified products obtained with primer pair ITS1 and ITS4 were sequenced with an ABI Prism automated DNA sequencer (model 3100, version 3.0; Applied Biosystems, Warrington, United Kingdom) with the single primer ITS1. These sequences were used to identify the fungi and bacteria with the help of the BLASTn program (www.ncbi.nlm.nih.gov/BLAST) [24 - 26].

Data Analysis

In order to identify organisms, DNA sequence data generated from this study was blasted against ITS/16S rRNA type strain database on NCBI. Threshold for identification was set according to [27] with modification to accommodate species with high congeneric sequence divergence range. An isolate was assigned to species level if the best matching reference species showed ≥ 90% homology and the next best matching reference species showed at least 0.8% less sequence homology. A strain or isolate was assigned to genus level when there was 90% to 95% homology to the best matching species, or when more than one sequence entry of several species from the same genus showed ≥90% homology. For sequences with <90% homology to the best match, an assessment of the congeneric sequence divergence range was carried out by blasting an arbitrarily chosen reference sequence of a type organism for the genus of interest against the type organisms' ITS/16S rRNA gene sequence database. The sequence divergence observed in this blast hits was used to adjudge the congeneric sequence divergence range and this range was used to determine the plausibility of the identification for sequences with <95% homology to blast's best match. Where homology fell below 95% and without the support of congeneric sequence divergence value, Identification was considered unsuccessful. Phylogeny was constructed to support the identification by ITS sequence using Fast Minimum Evolution algorithm as deployed on NCBI and visualized on MEGA11 [28]. Counts of species and genus were utilized as an estimate of species and genus diversity respectively. In the estimation of the species diversity, unidentified isolates were considered as different from the identified species and therefore counted as different species. In the estimation of the genus diversity, as opposed to the treatment in the estimation of species diversity, unidentified isolates were removed from the analysis. Other descriptive and inferential statistics were carried out on R using the follow R packages: base [29], reshape2 [30], dplyr [31], ggplot2 [32], ggpubr [33], qpcR [34], knitr [35, 36, 37]. The data wrangling was achieved using Reshape2 and dplyr; inferential statistics were carried out on R base; and data was visualized using ggplot2, ggpubr, qpcR and knitr.

Table 1

Primers used for amplification

Primer	Sequence (5′– 3′)	Direction Tma (°C)	Position	Reference
ITS1	TCCGTAGGTGAACCTGCGG	Forward 68.4	ITS Region	23
ITS2	GCTGCGTTCTTCATCGATGC	Reverse58.1	ITS Region	23
ITS3	GCATCGATGAAGAACGCAGC	Forward 68.1	ITS Region	23
ITS4	TCCTCCGCTTATTGATATGC	Reverse 61.5	ITS Region	23
LR12R	GAACGCCTCTAAGTCAGAATCC	Forward 62.9	IGS Region	22
invSR1R	ACTGGCAGAATCAACCAGGTA	Reverse 63.4	IGS Region	22

Source: [26].

Results

The physio-chemical analysis of composite soil and water samples from the two agricultural fields revealed comparatively that the soil samples were less acidic to water samples (Table 2 and 3). Although, Imota soil was more acidic (Mean pH 4.7 - 5.1) than that of LASU farm soil (Mean pH 5.0) (Table 2). Phosphates (PO_4^{3-}) ion was relatively higher in LASU soil (6.10mg/Kg) than that of Imota soil (4.9 - 5.50mg/Kg). Nitrate-nitrogen (NO_3 -N) was higher in LASU soil(1.28mg/Kg) than Imota soil (0.55- 0.97mg/Kg). Unusually high concentration of Cl^{-1} and SO_4^{2-} ions were detected in the composite soil samples analysed from the two agricultural fields. This observation might be due to the impact of wastewater of manufacturing plant in the vicinity of the two farms and application of treated water in some segments of the two farms (Table 2). The degree of acidity of the freshwater stream in the middle of the two farms impacted on the farm soil. Generally, the nutrient level of the composite water samples were low; NO_3-N (0.53-2.5mg/L), NH4-N(0.16 - 0.38mg/L),PO_4^{3-} (0.20- 0.22mg/L), organic C(1.30 -1.45mg/L) (Table 2.1, 2.2 and 3.1, 3.2).

Table 2.1

Physico-chemical analysis of soil samples

Parameters	Lasu farm soil (d)	Imota farm Soil (b)	Imota farm soil (c)	Beach soil (e)
NH_4 –N (mg/kg)	ND	ND	ND	ND
pH	5.0	5.1	4.7	6.3
PO_4^{3-} (mg/kg)	6.10	5.50	4.9	4.7
NO_3-N (mg/kg)	1.28	0.97	0.55	0.33
SO_4^{2-} (mg/kg)	56.60	108.91	165.05	39.6
Cl^- (mg/kg)	301.89	118.81	77.69	118.81
THC (mg/kg)	ND	ND	ND	ND
CEC meq 100g	2.53	2.40	2.58	3.2
K^+	0.132	0.145	0.136	0.173
Mg^{2+}	0.314	0.330	0.247	0.164
Ca^{2+}	1.313	0.517	0.338	0.519
Na^+	0.375	0.198	0.1940	0.395
C (%)	1.45	1.33	1.44	1.31

Keys: ND = Not Determined

Table 2.2

Analysis of Variance with ND as 0

Tests of Between-Subjects Effects

Dependent Variable: Values

Source		Type II Sum of Squares	df	Mean Square	F	Sig.
Location	Hypothesis	1617.437	3	539.146	.508	.679
	Error	38177.797	36	1060.494[a]		
Parameters	Hypothesis	108368.652	12	9030.721	8.516	.0001
	Error	38177.797	36	1060.494[a]		

a. MS(Error)

Table 3.1

Physico-chemical analysis of composite water samples

PARAMETERS	IMOTA	LASU
pH	4.3	4.8
Ca^{2+} (mg/L)	36.0	30
Total Nitrogen (mg/L)	0.12	0.51
PO_4^{3-} (mg/L)	0.22	0.20
NO_3-N (mg/L)	0.53	2.5
NH_4 – N (mg/L)	0.16	0.38
SO_4^{2-} (mg/L)	5.0	4.3
Cl^- (mg/L)	20.0	10.2
THC (mg/L)	ND	ND
SALINITY (%)	0.45	0.48
CONDUCTIVITY (ms/cm)	0.12	0.11
TURBIDITY (FTU)	12	2.02
C (%)	1.30	1.45
CEC	2.40	2.45

Keys: FTU = Formalin turbidity units. ND = Not Determined.

Table 3.2

T- Test with ND as 0

T-Test Paired Differences

		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	Imota - LASU	1.65714	3.90308	1.04314	-.59643	3.91071	1.589	13	.136

The analysis test for the significant relationship between Imota and LASU data, the result showed that the data from the two locations are not significantly different from each other since the p-value of 0.136 is greater than significant value of 0.05.

Selection of atrazine-degrading microorganisms

All the 24 isolates were tested on the atrazine-supplemented minimal salt medium, only 6 (12.5%) showed atrazine-degradation potential. However, the best three (one fungus, two bacteria) were further selected for molecular identification and microcosm biodegradation experiment.

Microcosm biodegradation / time course study

The biodegradation experiment as at Day 3, there were significant differences in the optical density obtained from the atrazine-biodegradation experiment, with the medium containing *Pseudomonas alcaligenes* MT355448.1 having the highest optical density (Table 5). At Day 5, the least optical density (0.480 OD) was recorded in medium containing *Aspergillus aculeatus* LC496490.1 and this was significantly different from the other two biodegradation experiments. On Day 14, the least optical density (0.469) was recorded in medium containing *Aspergillus aculeatus* LC 496490.1 and this was significantly different from other experiments.

Table 4

Molecular identity of test isolates using Basic Local Alignment Search Tool (BLAST)

Isolate code	Scientific name	Accession number	% Identity
NAW1	*Pseudomonas alcaligenes*	MT355448.1	92.61
NAL1	*Bacillus mycoides*	JX144699.1	94.45
SDAL1	*Aspergillus aculeatus*	LC496490.1	98.51

Table 5

Residual atrazine concentration (OD) following biodegradation / Time course experiment.

Isolate	Day		
	3	**7**	**14**
Pseudomonas alcaligenes **MT 355448.1**	0.275±0.00[a]	0.553±0.00[a]	0.514±0.00[a]
***Bacillus mycoides JX144699*.1**	0.265±0.00[b]	0.540±0.00[b]	0.488±0.00[b]
Aspergillus aculeatus **LC496490.1**	0.216±0.00[c]	0.480±0.00[c]	0.469±0.01[c]

Values are Means ± Standard deviation of duplicate observations. Means with different superscript down each column are significantly different from each other at p≤0.05 using the New Duncan Multiple Range test.

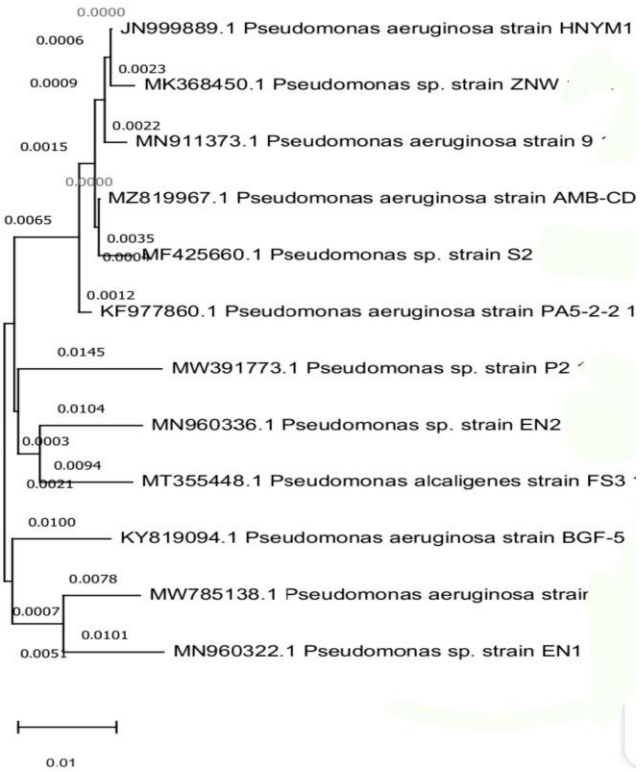

Fig. 2: *Pseudomonas alcaligenes* phylogenetic tree

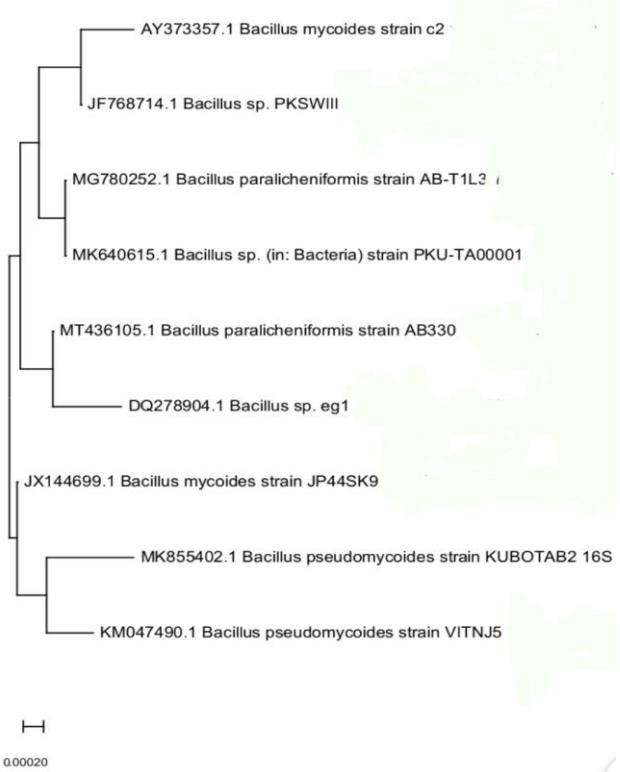

Fig. 3: *Bacillus mycoides* phylogenetic tree

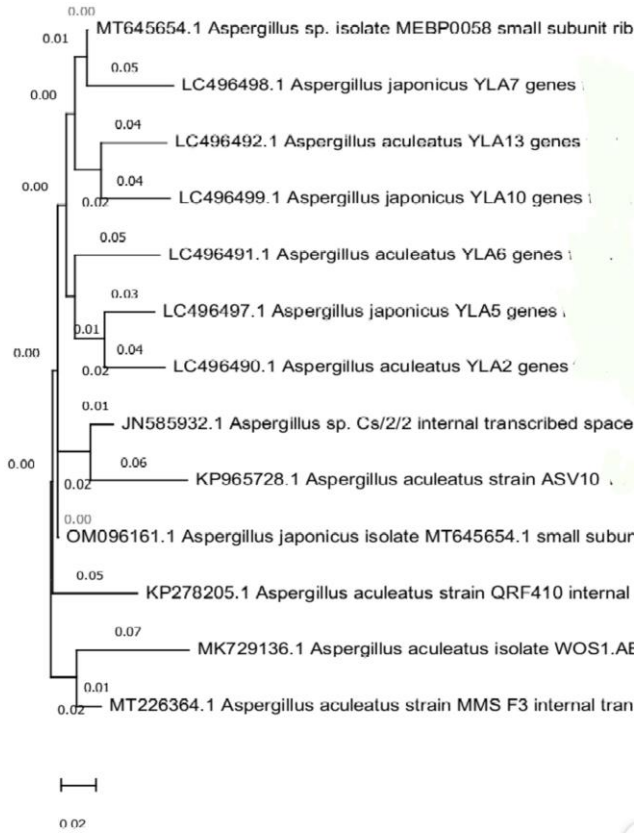

Fig. 4: *Aspergillus aculeatus* phylogenetic tree

Discussion

Generally, the soil and water samples used in the current study were acidic, low in organic Carbon (C) and Nitrogen (N) nutrient, this was as a result of the repeated annual cultivation of the land which is common in sub-saharan Africa. However, the adequate presence of cations and anions for sustainable agricultural practice were observable from both ecosystems from which the samples were obtained. C and N have been previously reported to be important factors that influence, if not regulate, the rate of mineralization of Atrazine in soil and water [18, 22]. However, the Imota and LASU soils as well as the nearby streams were relatively low in C and N content, as well as being acidic, this was unable to hinder the biodegradation of the herbicide (Table 2 & 3). ANOVA carried out on the physico-chemical parameter from the two locations showed there was significant relationship among the parameter since the p-value of 0.001 is less than 0.05 level of significance. Although, investigations across the sampling locations showed that the result from each of the locations was not significantly different since the p-value of 0.679 is greater than 0.05 level of significance. T-test of independence revealed that there was no significant relationship in the two locations from which water samples were obtained, hence, the result of the two locations were not significantly different from each other since the p-value of 0.136 is greater than 0.05 level of significance (Table 2 & 3). It is indicated that C/N ratio had no influence on the activity of the microbial community that degrades atrazine rather an important factor is the availability of microbial consortium with the metabolic capability to utilize both the hydrocarbon and N components present in Atrazine for growth and biomass production [38, 39], this was evident in the spectrophotometric study (Table 5). There was strong evidence to

support the fact that low N in the soil and water ecosystems enhanced the mineralization of Atrazine as C and N energy source, hence the proliferation of microbial atrazine degraders in both ecosystems. This corroborated the findings of Alvey & Crowley [16]. The acidic pH of both the water and soil samples from the tropical agricultural fields is suggestive of the fact that the atrazine-degraders encountered in the present study were acidophiles. Although, previous studies have reported atrazine – degraders from alkaline pH environment [40]. Atrazine degradation has been reported to be pH and temperature dependent only at higher atrazine concentrations [41, 42] and that no additional sources of carbon and nitrogen is required for microbial mineralization of atrazine, this is a report from tropical environment with similar temperature and pH range [43, 44]. Comparatively, it thus becomes incontrovertible from the current discovery that atrazine degradation by microbial community from tropical environment is not pH dependent, rather microbial community with metabolic capacity to mineralize atrazine is diverse in tropical soils. This is corroborated by the report of Douglass *et al*. [13]. So many bacterial species have shown high efficiency of atrazine degradation with a broad optimum pH and temperature ranges and this capability has been found to be enhanced by cooperating with other microorganisms suggesting that microbial consortium has demonstrated huge potential for remediation of atrazine-contaminated environment [27, 45]. The persistence and mobility of atrazine in the soil were found to be very high, hence, it has frequently been identified in surface and groundwater at concentrations well over the reasonable limits and hence considered as a potential environmental contaminant and absolutely considered as most noticeable groundwater contaminant [46,47, 8]. The presence of metal ions as well as physico-chemical properties and soil - type are principal contributory factors that enhanced rate of degradation of atrazine in agricultural fields, this has played out in the current study, and it is in alignment with the findings of Chen *et al*. [48].

In this study, a number of microorganisms were discovered to grow on the atrazine contaminated soil and water, this was visible by their growth on atrazine-supplemented minimal salt medium and sabouraud dextrose medium, hence, showed their ability to utilize atrazine as a source of C and N energy in this process, some microbes metabolize atrazine and convert it to cyanuric acid [49, 42], while others cleave the *s*-triazine ring and thereafter mineralize it [50]. This observation corroborated other reports of *Pseudomonas* sp. [51, 52], *Arthrobacter* sp. [52], *Citrococcus* sp. [53], *Rhodococcus* sp. [41], *Pleurotus ostreatus* INQS 40310 [5] being atrazine- degraders. *Paenarthrobacter ureafaciens* ZY has a unique degradation efficiency of 12.5mgL^{-1} in liquid media (at pH 7, 30 °C and atrazine concentration of 100mgL^{-1}). This novel organism was found to exhibit synergistic relationship with other indigenous atrazine-degraders at optimal pH range (5.0 - 11.0) and temperature range (20°C to 40°C) for atrazine degradation [42]. The current study has affirmed that atrazine is biodegraded both in soil and water by microorganisms, this is corroborated by the findings of Arar *et al*. [53] that quantitative PCR analysis and Gas chromatographic analysis attest to the fact that atrazine is biodegraded in groundwater and soil. The significance of the current study is that the atrazine degraders were natives and acidophiles from tropical environment with mean temperature range of 25°C to 35°C. This suggested that atrazine degradation in the tropical environment was not pH dependent rather microbial consortium

with metabolic capacity for atrazine utilization for growth and biomass production were diverse in the environment.

Particularly in this study, three isolates showed great potential in atrazine degradation as they utilized the atrazine as a source of nitrogen and carbon energy. These isolates identified as *Pseudomonas alcaligenes* MT355448.1, *Bacillus mycoides* JX144699.1 and *Aspergillus aculeatus* LC496490.1 (Table 4). This study corroborated the findings of Martins *et al*. [54], that *Pseudomonas alcaligenes* exhibited the fastest growth on culture medium supplemented with high concentration of atrazine. The isolation of *Bacillus mycoides* also corroborated the study by Ayansina & Oso [55] in which several species of *Bacillus* spp. (Fig. 3) were isolated on different atrazine-treated soils, while the characterization and identification of a novel isolate *Aspergillus aculeatus* further substantiate the incontrovertible evidence of fungal participation in the consortium that degrades Atrazine in natural environment. Furthermore, Sebiomo *et al*. [56] isolated twelve *Bacillus* spp. including *Bacillus licheniformis*, *Bacillus subtilis*, e.t.c., based on biochemical characterization which this study also identified by the same method. However, molecular characterization identified these isolates as *Bacillus mycoides*, hence highlighting the great potential of this species of bacteria in atrazine degradation as well as the reliability and precision of molecular methods for characterization of microbial isolates. The efficacy of the metabolic capabilities of bacterial species at mineralizing atrazine in aquatic environment and wastewater at three elevated concentrations was investigated with the incontrovertible evidence of gas chromatography.

The outcome was reported and bacterial species were found to be efficient at clean-up of atrazine-contaminated environment particularly the bacterial strain *Bacillus paramycoides* [57], which has gene sequence almost as that of *Bacillus mycoides* isolated in this study.

In the present study, there was significant increase in the optical density of the enrichment media, thus indicating active growth or utilization of the substrate, atrazine. Several studies have reported these isolates as active atrazine degraders; according to a study by Ojo [51], *Pseudomonas* sp. (Fig. 2) isolated from an atrazine-contaminated tropical soil in Southwest Nigeria showed significant atrazine-degrading ability, indicated by increase in the optical density from the start of the biodegradation experiment till the 15th day. In a farmland in Ijebu-Ode, southwest Nigeria deliberately treated with atrazine, Sebiomo *et al*. [56] reported active degradation of atrazine by several fungal species including *Aspergillus* sp. (Fig. 4) and *Fusarium* sp. up to 71.8%.

It is worth note that the optical density of the media increased till the 7th day until when a decrease set in on the 14th day of the experiment and this suggested exhaustion of required nutrients, hence the microbes declined in growth (Table 5). This observation is similar to observations by Sebiomo *et al*. [56], in which there was a significant decline in the optical density of the biodegradation medium after the 20th hour of experiment. The specificity and reliability of molecular methods for identification of microbial isolates and the fact that atrazine is biodegradable in tropical environment was affirmed.

4. Conclusion

This study has shown that repeated application of atrazine as herbicides on agricultural soils makes it to become persistent in the environment and native

microbial population get acclimatized to the xenobiotic compound while simultaneously activating atrazine – metabolizing genes. However, the previous believe that atrazine is a non-biodegradable xenobiotic compound has been quashed since different microorganisms such as *Pseudomonas alcaligenes* MT355448.1, *Bacillus mycoides* JX144699.1 and *Aspergillus aculeatus* LC496490.1 isolated in this study have great potential to mineralize atrazine, hence are candidates for integration into reclamation of atrazine-polluted environment. These microbes are cheap natural resource that enhance sustainable development as well as being cost-effective means of remediating polluted natural environment and they are environment - friendly.

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Dataset: All Dataset are available in GenBank and NCBI repository, the Accession numbers for isolates are displayed and trackable. (*www.ncbi.nlm.nih.gov/genome*).

Ethical Consent: Ethical approval is not required for this type of work in Nigeria, since we did not test any material on human and animal subjects.

Consent to Participate: The consent to participate in this study was given by each author.

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7. References

[1]. SANCHEZ, V., LOPEZ-BELLIDO, F. J., CANIZARES, P. & RODRIGUEZ, L. Assessing the phytoremediation potential of crop and grass plants for atrazine-spiked soils," *Chemosphere*, 185, 119-126 (2017).
[2]. LIN, Z., ZHEN, Z., LIANG, Y., LI, J., YANG, J., ZHONG, L., ZHAO, L., LI, Y., LUO, C., REN, L., & ZHANG, D. Changes in atrazine speciation and the degradation pathway in red soil during the vermiremediation process. *Journal of Hazardous Materials*, 364, 710-719 (2019).
[3]. Singh, S., Kumar, V., Chauhan, A., Datta, S., Wani, A. B. & Singh, N. (2018) Toxicity, degradation and analysis of the herbicide atrazine. *Environmental Chemistry Letters*, 16, 211-237 (2018). https://doi.org/10.1007/s10311-017-0665-8
[4]. MARTINS, E. C., DE FREITAS MELO, V., BOHONE, J. B. & ABATE, G. Sorption and desorption of atrazine on soils: The effect of different soil fractions. *Geoderma*, 322, 131-139 (2018).

[5]. LOPES, R. D. O., PEREIRA, P. M., PEREIRA, A. R. B., FERNANDES, K. V., CARVALHO, J. F., DE FRANÇA, A. S., VALENTE, R. H., MANUELA DA SILVA, M. & FERREIRA-LEITÃO, V. S. Atrazine, desethylatrazine (DEA) and desisopropylatrazine (DIA) degradation by *Pleurotus ostreatus* INCQS 40310, *Biocatalysis & Biotransformation*, 38(6), 415- 430(2020). DOI: 10.1080/10242422.2020.1754805

[6]. HONG, J., BOUSSETTA, N., ENDERLIN, G., MERLIER, F. & GRIMI, N. Degradation of residual herbicide Atrazine in Agri-food and washing water. *Foods* 11(6), 2416 (2022). *https://doi.org/10.3390/foods11162416*.

[7]. QU, M., LI, H. & LI, N. Distribution of atrazine and its phytoremediation by submerged macrophytes in lake sediments," *Chemosphere*,168,1515-1522(2017).

[8]. REHAN, M., EL SHARKAWY A. M. L. & El FADLY, G. Microbial biodegradation of S-triazine Herbicides in soil. *Journal Crop Research & Fertilizers*, 1, 1-6 (2016).

[9]. HONG, R., GUO, Z., GAO, J. & GU, C. Rapid degradation of atrazine by hydroxyl radical induced from montmorillonite templated subnano-sized zero-valent copper. *Chemosphere*, 180, 335-342 (2017).

[10]. ZHAO, K., QUAN, X., CHEN, S., YU, H. T., ZHANG, Y. B. & ZHAO, H. M. Enhanced electro-Fenton performance by fluorine-doped porous carbon for removal of organic pollutants in wastewater. *Chemical Engineering Journal*, 354, 606-615 (2018).

[11]. ZHAO, X. C., OUYANG, W., HAO, F. H., LIN, C. Y., WANG, F. L., HAN, S. & GENG, X. J. Properties comparison of biochars from corn straw with different pretreatment and sorption behaviour of atrazine. *Bioresource Technology*, 147, 338-344 (2013).

[12]. JIANG, Z., ZHANG, X. Y., WANG, Z. Y., CAO, B., DENG, S. J., BI, M. C. & ZHANG, Y. (2019) Enhanced biodegradation of atrazine by Arthrobacter sp. DNS10 during co-culture with a phosphorus solubilizing bacterium: *Enterobacter* sp. P1. *Ecotoxicology and Environmental Safety* 172, 159-166 (2019).

[13]. DOUGLASS, J. F., RADOSEVICH, M. & TUOVINEN, H. O. Molecular analysis of atrazine – degrading bacteria and catabolic genes in the water column and sediment of a created wetland in an agricultural/ urban watershed. *Ecological Engineering*, 83, 405–412 (2015).

[14]. GERHARDT, P., MURRAY, R. G. E., COSTILOW, R. N., NESTER, E. W., WOOD, W. A., KRIEG, N. R. & PHILLIPS, G. B. *Manual of methods for General Bacteriology*. American Society for Microbiology Pp. 524-526 (1981).

[15]. KOHLER, K. P. E., KOHLER-STAUB, D. & FOCHT D. D. Co-metabolism of polychlorinated biphenyls: Transformation of Aroclor 1254 by growing bacterial enhanced cell. *Appl. Environ. Microbiol*. 54(8), 1940-1945 (1988).

[16]. ALVEY, S. & CROWLEY, D. E. Influence of organic amendments on biodegradation of atrazine as Nitrogen source, *Journal of Environmental Quality*, 24, 1156-1162 (1995).

[17]. KRIEG, N. R. Enrichment and Isolation In: Manual of methods for General Bacteriology (eds.) Gerhardt, P., Murray, R.G.E., Costilow, R.N., Nester, E.W., Wood, W.A., Krieg, N.R. & Phillips, G.B. Publ. Am. Soc. for Microbiology. Pp.126–130 (1984).

[18]. MANDELBAUM, R. T., WACKETT, L. P. & ALLAN, D. L. Mineralization of the s-triazine ring of atrazine by stable bacterial mixed cultures. *Applied and Environmental Microbiology*, 59, 1695-1701 (1993).

[19]. American Public Health Association (APHA) Standard Methods for the examination of water and wastewater, 18th Ed. American public Health Association (APHA), Washington, D. C. (1992).

[20]. BAREITHER, C. A., BREITMEYER, R. J., MEYER, L. L., BENSON, C. H., EDIL, T. B. & BARLAZ, M. A. Physical, Chemical, and Biological Characterization of Solid Waste Samples In: *Proc-Global Waste Management Symp. Penton Media*, New York, Pp.1-9 (2010).

[21]. AOAC. Official Methods of Analysis of the Association of Official's Analytical Chemists 14th Ed. Association of Official Analytical Chemists, Washington D.C. Pp. 223 - 225, 992 - 995 (1990).

[22]. MANDELBAUM, R. T., ALLAN, D. L. & WACKETT, L. P. Isolation and characterization of a *Pseudomonas* sp. that mineralizes the s-triazine herbicide atrazine. *Applied and Environmental Microbiology*, 61, 1451-1457 (1995).

[23]. GUTER, R. L. & KOSHLAND JR., D. E. The molecule of the year. *Science*, 246, 1543-1544 (1990).

[24]. SOLANKI, G. POLYMERASE Chain Reaction. *International Journal of Pharmaceutical Research* 2(3), 98-102 (2012).

[25]. QIAGEN. QIAamp DNA mini and Blood Mini Handbook. Fifth Edition, *HB-0329-004-1102728-HB-QIAamp –DNA-Mini-Blood-Mini-0516-WW*. Pp. 55-57 (2016).

[26]. KAMUSOKO, R., JINGURA, R. M., PARAWIRA, W. & CHIKWAMBI, Z. Purification and Amplification of DNA from cellulolytic Bacteria: Application for Biogas production from crop residues. *Methods Mol. Biol*. 2290, 187–201 (2021). *Doi:10.1007/978-1-0716-1323-8_13.*

[27]. WANG, X., FU, Y. F., WANG, R. Y., LI, L., CAO, Y. H., CHEN, Y. Q., ZHAO, H. Z., ZHANG, Q. Q., WU, J. Q., WENG, X. H., CHENG, X. J. & ZHU, L. P. Identification of clinically relevant fungi and prototheca species by rRNA gene sequencing and multilocus PCR coupled with electrospray ionization mass spectrometry. *PLoS One*, 9(5), e98110. (2014). *https://doi.org/10.1371/journal.pone.0098110*
[28]. TAMURA, K., STECHER, G., & KUMAR, S. MEGA11: Molecular Evolutionary Genetics Analysis version 11. *Molecular Biology and Evolution* 38, 3022-3027 (2021).
[29]. R CORE TEAM. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/. (2021).
[30]. WICKHAM, H. RESHAPING Data with the reshape Package. *Journal of Statistical Software*, 21(12), 1-20 (2007). URL http://www.jstatsoft.org/v21/i12/.
[31]. WICKHAM, H., FRANÇOIS, R., HENRY, L., & Müller, K. dplyr: A Grammar of Data Manipulation. R package version 1.0.7. (2021). https://CRAN.R-project.org/package=dplyr
[32]. WICKHAM, H. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York (2016).
[33]. KASSAMBARA, A. ggpubr: 'ggplot2' Based Publication Ready Plots. R package version 0.4.0. https://CRAN.R-project.org/package=ggpubr (2020).
[34]. SPIESS, A. qpcR: Modelling and Analysis of Real-Time PCR Data. R package version 1.4-1. https://CRAN.R-project.org/package=qpcR (2018).
[35]. XIE, Y. knitr: A Comprehensive Tool for Reproducible Research in R. In: *Victoria Stodden, Friedrich Leisch and Roger D. Peng, (editors) Implementing Reproducible Computational Research.* (2014). Chapman and Hall/CRC. ISBN 978-1466561595.
[36]. XIE, Y. Dynamic Documents with R and knitr. (2nd edition). Chapman and Hall/CRC. ISBN 978-1498716963 (2015).
[37]. XIE, Y. knitr: A General-Purpose Package for Dynamic Report Generation in R. R package version 1.36. (2021).
[38]. CRAWFORD, J. J., SIMS, G. K., MULVANEY, R. L. & RADOSEVICH, M. Biodegradation of atrazine under denitrifying conditions. *Applied Microbiology and Biotechnology*, 49(5), 618-623 (1998).
[39]. WACKETT, L. P., SADOWSKY, M. J., MARTINEZ, B. & SHAPIR, N. Biodegradation of atrazine and related s-triazine compounds: from enzymes to field studies. *Applied Microbiology and Biotechnology*, 58, 39-45, (2002).
[40]. SINGH, B. K., WALKER, A., MORGAN, J. A. W. & WRIGHT, D. J. Role of soil pH in the development of enhanced biodegradation of fenamiphos. *Appl. & Env. Microbio*. 69(12), 7035-7043 (2003).
[41]. KOLEKAR, P. D., PHUGARE, S. S. & JADHAV, J. P. Biodegradation of atrazine by *Rhodococcus* sp. BCH2 to N-isopropylammelide with subsequent assessment of toxicity of biodegraded metabolites. *Environmental Science & Pollution Research*, 21(3), 2334-2345 (2014). *https://doi.org/10.10007/s 11356-013-2151-6*
[42]. ZHAO, Y., LI, X., LI, Y., BAO, H., NAN, J. & XU, G. Rapid biodegradation of atrazine by a novel Paenarthrobacter ureafaciens ZY and its effects on soil native microbial community dynamic. *Front. Microbiol*. 13, 1103168 (2023). *https://doi.10.3389/fmicb.2022.1103168*
[43]. MUELLER, T. C., STECKEL, L. E. & RADOSEVICH, M. Effect of soil pH and previous Atrazine use history on Atrazine degradation in a Tennessee field soil. *Weed Science*, 58(4), 478 - 483(2010).
[44]. OMOTAYO, A. E., ILORI, M. O., OBAYORI, O. S. & Amund, O. O. Influence of pH, temperature and nutrient addition on the degradation of atrazine by Nocardiodes spp. Isolated from agricultural soil in Nigeria. *Malaysian Journal of Microbiology*, 12(4), 270 – 278.
[45]. YANG, Z. C., YU, A. Q., SHAN, C., GAO, G. D. & PAN, B. C. Enhanced Fe (III)-mediated Fenton oxidation of atrazine in the presence of functionalized multi-walled carbon nanotubes. *Water Research*, 137, 37-46 (2018). *https://doi.org/10.1016/j.watres.2018.03.006*
[46]. GHOSH, P. K. & PHILIP, L. I. G. Y. Environmental significance of atrazine in aqueous systems and its removal by biological processes: an overview. *Global NEST Journal*, 8(2), 159-78 (2006).
[47]. KAUSHIK, G., SATYA, S. & NAIK, S. N. Food processing a tool to pesticide residue dissipation - A review. *Food Research International*, 42, 26-40 (2009).
[48]. CHEN, L. W., HU, X. X., YANG, Y., JIANG, C. L., BIAN, C., LIU, C., ZHANG, M. Y. & CAI, T. M. Degradation of atrazine and structurally related s-triazine herbicides in soils by ferrous-activated persulfate: Kinetics, mechanisms and soil-types Effects. *Chemical Engineering Journal*, 351, 523-531 (2018).

[49]. WANG, Q. F. & XIE, S. G. Isolation and characterization of a high-efficiency soil atrazine-degrading *Arthrobacter* sp. strain. *International Journal of Biodeterioration and Biodegradation*, 71, 61-66 (2012).
[50]. YANG, C., LI, Y., ZHANG, K., WANG, X. & MA, C. Atrazine degradation by a simple consortium of *Klebsiella* sp. A1 and *Comamonas* sp. A2 in nitrogen enriched medium. *Biodegradation*, 21(1), 97–105 (2010). DOI: 10.1007/s10532-009-9284-9
[51]. OJO, O. A. Microbial utilization of the hydrocarbon components of Atrazine in a tropical environment Southwest, Nigeria. *African Journal Infectious Diseases* 1(1), 25-29 (2007).
[52]. DESITTI, C., CHERUTI, U., BELIAVSKI, M., TARRE, S. & GREEN, M. Long-term atrazine degradation with microtube-encapsulated *Pseudomonas* sp. strain ADP. *Environmental Engineering Science*, 33, 3 (2016).
[53]. ARAR, M., BAKKOUR, R., ELSNER, M. & Bernstein, A. Microbial hydrolysis of atrazine in contaminated groundwater. *Chemoshere*, 322 (2023) 138226. DOI: *10.1016/j.Chemosphere.2023.138226*. E pub 2023 Feb 22
[54]. MARTINS, P. F., MARTINEZ, C. O., CARNEIRO, P. I. B., AZEVEDO, R. A., PILEGG, S. A. V. & PILEGGI, M. Selection of microorganisms degrading S-Metolachlor herbicide. *Brazilian Archives Biol. Technol.*, 50(1), 153-159 (2007).
[55]. AYANSINA, A. D.V. & OSO, B. A. Effect of two commonly used herbicides on soil microflora at two different concentrations. *Afri. J. Biotechnol.*, 5(2), 129-132 (2006).
[56]. SEBIOMO, A., OGUNDERO, V. W. & BANKOLE, S. A. Utilisation and biodegradation of atrazine and primextra. *Journal of Microbiology and Antimicrobials*, 3(3), 64-76 (2011).
[57]. ALATTAS, S.G. ZABERMAWI, N. M. & BESTAWY, E.E. Biodegradation of atrazine using selected marine bacteria: bacteria: Possibilities for treating pesticide-contaminated wastewater. *Journal of King Saud University-Science* 35, 102721(2023)
[58]. *https://doi.org/10.1016/j.jksus.2023.102721*
https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch