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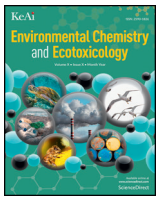

# Enhanced hexazinone degradation by a *Bacillus species* and *Staphylococcus species* isolated from pineapple and sugarcane cultivated soils in Kenya

Boniface Mbithi Muendo [a,e], Victor Odhiambo Shikuku [b,*], Zachary Moranga Getenga [c], Joseph Owuor Lalah [a], Shem Oyoo Wandiga [d], Geoffrey Muriira Karau [e], Michael Rothballer [f]

[a] Department of Chemical Science and Technology, Technical University of Kenya, P.O Box 52428-00200, Nairobi, Kenya
[b] Department of Physical Sciences, Kaimosi Friends University College, P.O.Box 385, Kaimosi, Kenya
[c] Department of Physical Sciences, Machakos University, P.O.Box 109-60400, Machakos, Kenya
[d] Institute for Climate Change and Adaptation, College of Biological and Physical Sciences, University of Nairobi, P.O.Box 30197-00100, Nairobi, Kenya
[e] Kenya Bureau of Standards, P.O. Box 54974-00200, Nairobi, Kenya
[f] Helmholtz Zentrum München, German Research Center for Environmental Health (GmbH), Department for Environmental Sciences, Research Unit Microbe-Plant Interactions, D-85764 Neuherberg, Germany

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ABSTRACT

In this work, hexazinone-degrading bacterial strains from Kenyan tropical soils with long term application history were isolated and identified. Non-sterile soils from sugarcane and pineapple cultivated fields with over 15 years' hexazinone application history degraded 82.2% and 93.4% initially applied hexazinone under laboratory conditions after 146 days of incubation, respectively. In contrast, non-sterile soils without history of application degraded 48.8% and 36.8% of hexazinone after 146 days, respectively. From liquid culture tests, using soils with prior application history from sugarcane and pineapple cultivated soils, two hexazinone-degrading bacterial strains were isolated and identified as *Staphylococcus gallinarum* and *Bacillus toyonensis* and *Bacillus thuringiensis*, respectively. The pure isolates rapidly degraded hexazinone up to 38.4% and 53.2% of 50 mg/L in 46 days, respectively, with microbial hexazinone metabolite B previously reported detected. These bacterial strains from Kenyan soils have been identified for the first time as prospective hexazinone-degraders from pineapple and sugarcane-cultivated soils.

## 1. Introduction

Pesticides are hazardous to the environment and human health; hence their usage must be strictly regulated to ensure that maximum residual levels in diverse environmental compartments are satisfied [1]. Chemical composition, soil texture, weather, and agricultural management approaches all affects the extent of pesticides contaminating various ecological compartments, among other factors [2].

Hexazinone (3-cyclohexyl-6-dimethylamino-1-methyl-1,3,5-triazine-2,4-dione), is a robust herbicide that is used to manage a wide range of annual and perennial weeds as well as woody plants in agricultural plantations, roadway and railway rights of way, and industrial plant sites [3,4]. Because of its high solubility, hexazinone is commonly detected in rivers and groundwater systems [5]. Hexazinone has been found to hydrolyze very slowly in buffered solution, with a half-life of more than a year at pH values (5–9) [6]. The average half-life for hexazinone has been recorded as 18.3 days [7] and 144 days [8], indicating a moderate to lengthy half-life in soil and water, respectively.

Several investigations on pesticide residues, including hexazinone, have been conducted in Kenya in regions where commercialized agricultural farming is undertaken, with results showing concentrations over the maximum residue limits detected [9,10]. Hence there are growing concerns regarding the potential effects of pesticides on non-target organisms [11]. High concentrations of hexazinone in the aquatic environment have been to have long-term negative impacts and toxicological threats to aquatic life forms [12].

Sugarcane (*Saccharum officinarum*) and pineapple (*Ananas comosus*) production are both widespread in the Nzoia and Thika areas, supporting a combined population of nearly 6 million people. These two sugarcane and pineapple agricultural zones in Kenya are about 600 km apart [13].

* Corresponding author.
*E-mail address:* vshikuku@kafuco.ac.ke (V.O. Shikuku).

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Following the Kenyan government's restriction on atrazine imports, hexazinone, marketed under the trade name Velpar and applied at a rate of 0.5–1 kg/ha, is frequently used in sugar cane and pineapple fields to control pre- and post-emergence broad leafed and grassy weeds.

Hexazinone was discovered to be slowly biodegradable after it was introduced to the market [12,15]. However, research in fields with a long history of application have demonstrated an accelerated degradation accompanied by the formation of hexazinone metabolites over the last 10 years [14–16]. Repeated application of hexazinone can thus result in adaptation of soil microbial communities able to degrade the compound.

The utilization of microbes for bioremediation of pesticide-contaminated soils has become a popular subject of investigation, with the goal of determining pesticide environmental fate and discovering degraders [17]. This technique has been described as one of the most environmentally friendly methods of decontaminating the environment, while chemical methods have proven to be more expensive and even worsen the situation by introducing new chemicals into the ecosystem [18]. Studies have reported hexazinone is primarily degraded through microbial metabolism [15]. A strain related to *Enterobacter cloacae* was identified through biochemical and molecular characterization as a capable hexazinone degrader [16]. This isolate from sugarcane cultivated soils in Nzoia sugar belt was found to degrade up to 27.3% of the initially applied 40 μg/mL hexazinone after 37 days of incubation in liquid culture. Studies from contaminated tropical soils identified bacterial strains related to *Enterobacter cloacae* and *Pseudomonas species* which were capable of degrading hexazinone [15]. The two isolates alone could rapidly degrade hexazinone with half-lives of 2.95 days and 3.08 days, respectively, while a 2–3-fold increase in hexazinone degradation rates was observed in their mixed bacterial culture. The strains *Microbacterium foliorum, Paenibacillus illinoisensis* and *Rhodococcus equi* were isolated from temperate regions and confirmed as hexazinone degraders [19]. Its degradation has been reported to proceed *via* demethylation and hydroxylation of the cyclohexyl ring [14,15,20,21]. Most of the studies on biological degradation of hexazinone have been reported in the temperate regions. To date, only one study on hexazinone degradation in Kenya (Nzoia River Basin) using a sugarcane growing soil with repeated application history has been reported [16]. These studies have demonstrated that microbial communities vary with soil properties and may even vary over time. The objective of this study was to investigate degradation ability of adapted microbes in a pineapple and sugarcane soil with long hexazinone history, enrich the adapted microbial community from these soils and to isolate and identify the key degraders. The findings of this study will add to the list of strains identified from sugarcane cultivated soils as well as strains isolated from pineapple cultivated soils that are capable of degrading hexazinone and thereby remediating the pollutant, which is a subject of concern. Because this is the first investigation from such a cropping system in Kenya, any strains from the pineapple grown soils are of significant interest in this study.

## 2. Materials and methods

### 2.1. Soil sampling and characterization

Soil samples were collected in June 2014 from fields within Nzoia sugarcane nucleus estate at an altitude of 1788 M, (0° 53′2″ N, 35, 4′ 0″ E), and Thika pineapple nucleus estate at an altitude of 1815 M, (0° 4′ 55″ N 34° 50′ 49″ E), where hexazinone has been extensively applied at a rate of 0.5–1.0 kg/ha (active ingredient) for over 15 years. Fields for sampling were identified through random stratified sampling technique and soil samples were collected from 0 to 15 cm depth. All the soil samples were placed in sterile amber bottles then placed in a cooler at 4 °C for transport to the laboratory for degradation studies and isolation of hexazinone-degrading bacteria. The soil samples were thoroughly prepared through crushing, homogenization and sieving. Soil samples from Nzoia sugarcane field contaminated with hexazinone were characterized as having organic carbon 2.52%, carbon content 1.06%, nitrogen content 0.18%, clay content 17.6% and sand content 55.3%. The non-contaminated soils from Nzoia had organic carbon 2.04%, carbon content 0.99%, nitrogen content 0.21%, clay content 22.1% and sand content 58.7%. The soil samples from pineapple fields in Thika contaminated with hexazinone had clay soils composed of organic carbon 1.98%, carbon content 0.87%, nitrogen content 0.13%, clay content 90% and sand 5%. The non-contaminated soils from Thika had organic carbon 2.31%, carbon content 1.07%, nitrogen content 0.22%, clay content 87% and sand 9%. Samples were aseptically transferred on ice to the laboratory.

### 2.2. Reagents and chemicals

Hexazinone (purity 99.9%) and its metabolites (purity 99.5%) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Mineral salt medium (MSM) for hexazinone liquid culture experiments was prepared by dissolving 1.5 g $K_2HPO_4$, 1.5 g $KH_2PO_4$, 0.5 g KCl, 0.5 g $MgSO_4.7H_2O$, 1 g each of $FeSO_4$ and $ZnSO_4$ in 1 L of de-ionized and distilled water [22]. The medium was sterilized by autoclaving at 121 °C for 20 min and pH adjusted to 7.0. Hexazinone dissolved in methanol was aseptically added until final concentration of 50 mg/L was attained. All other Analytical grade reagents including Agar, anhydrous sodium sulphate and HPLC grade solvents were purchased from Sigma Aldrich through a local supplier Kobian Kenya Limited and prepared according to manufacturer instructions. Stock solutions of both hexazinone and its metabolites were prepared in methanol and working solutions prepared by appropriate dilution of the stock solutions. Both the stock solutions and the working solutions were stored at 4 °C for use.

### 2.3. Hexazinone and its metabolites residual determination in soils

The residue levels of hexazinone and its metabolites in soils were determined prior to laboratory degradation experiments. A sub-sample of 30 g of the well ground, air-dried, homogenized and sieved soil was weighed in triplicates and exhaustively extracted in soxhlet extractors with 150 mL of pure methanol for 8 h [23]. Extracts of 150 mL were filtered, dried by the use of anhydrous sodium sulphate and reduced by vacuum evaporation to approximately 2 mL. Clean up procedure was done using solid phase extraction (SPE) method by percolating the sample through a $C_{18}$ SPE cartridge that had already been conditioned by passing 10 mL methanol and 10 mL double distilled water successively. Without allowing the cartridges to dry, extracts were loaded and analytes eluted with 5 mL HPLC grade methanol. The clean extracts were dried over anhydrous sodium sulphate before filtration through 0.2 μm filters. A further concentration of the eluate to 0.5 mL was done through a gentle stream of nitrogen. Extracts were analysed by HPLC (LC-10AT VP Shimadzu) equipped with SPD-20A UV-detector at 254 nm and 125 × 4 mm Crom Saphir 110 $C_{18}$ 5 μm column. The HPLC measurement conditions included a pressure of 107 bar, a mobile phase of (isocratic) acetonitrile: water: methanol (40: 40: 20), a flow rate of 1 mL/min and an injection volume of 5 μL. Hexazinone and its metabolite standards were used to compare the retention times and preparation of the calibration curves. These detailed clean-ups and HPLC analysis procedures were standardized for analysis of hexazinone and its metabolites residues. Prior to this experiment, a recovery study was undertaken. This involved spiking of 30 g soil sample with 10 mg/L hexazinone and extraction to establish the efficiency of extraction and clean up procedures.

### 2.4. Enhanced degradation of hexazinone studies

Two different types of soils from sugarcane and pineapple cultivated fields were screened for their capability to degrade hexazinone following the protocol described by [24] with a slight modification. One type of soil had been exposed to hexazinone for more than fifteen (15) years, while the other had never been exposed to hexazinone. One set of experiments was conducted using non-sterile (NS) soils and the other set which acted as a control was sterilized (S) using 1% (*w/v*) of sodium azide [25]. The soils without prior exposure were sampled near the sugarcane and pineapple main plants which was a distance of about 2 km and 2.5 km,

respectively, from where soils with history of exposure were sampled. The soil characterization exhibited minimal differences when comparing soils with prior and without prior exposure histories. The moisture content of the soil was determined through heating 1 ± 0.01 g in an oven at 105 °C for 24 h. To achieve a final concentration of 5 mg/g, hexazinone dissolved in methanol was drop-wise added to 3.5 ± 0.01 g of previously dried and ground soil. The flasks were placed in a fume hood for 24 h for the solvent to evaporate and the moisture content restored to 60% using deionized water. 46.5 ± 0.01% dry soil equivalent aliquots were added to each brown amber bottle, homogenized, and loosely covered with perforated aluminum foil before incubation at 25 °C [24]. Throughout the experiment, the soil moisture content was kept at 60% of the water holding capacity using gravimetric measurements. All the samples were incubated in triplicates for 146 days. The soils that demonstrated increased hexazinone breakdown were then utilized as inoculants in liquid culture enrichment tests.

### 2.5. Liquid enrichment culture experiment

With minor adjustments, the liquid enrichment procedure was used as previously demonstrated by other methods [16]. Mineral salt medium (MSM) for hexazinone liquid culture experiments was prepared by dissolving 1.5 g $K_2HPO_4$, 1.5 g $KH_2PO_4$, 0.5 g KCl, 0.5 g $MgSO_4.7H_2O$, 1 g each of $FeSO_4$ and $ZnSO_4$ in 1 L of de-ionized and distilled water [22]. The medium was sterilized by autoclaving at 121 °C for 20 min. An aliquot of 49 mL of sterile MSM was added to the flask and swirled thoroughly to completely dissolve the hexazinone. This was carefully calculated to ensure the final concentration of the herbicide in the MSM solution was 50 mg/L. A total of 16 sets of Erlenmeyer flasks were prepared for inoculation. An aliquot of 1 g of soil was dissolved in 9 mL of 0.85% NaCl in distilled sterile water and vortexed for 2 min. The mixture was diluted to $10^{-3}$ in 10-fold dilution steps. Dilutions of the soil slurry (aliquot of 1 mL) were used for inoculating the fresh 49 mL MSM with hexazinone as a substrate in 100 mL Erlenmeyer flask. The liquid cultures were incubated in a Wisecube rotational shaker at 30 ± 1 °C. For the first 10 days, turbidity (optical density) and hexazinone residue concentrations were tested every 2 days, and then every 4 days for the remainder of the sampling period using a UV/VIS spectrophotometer at 600 nm and HPLC, respectively. A plot of optical density and degradation expressed as % of initial concentration of hexazinone remaining against time was obtained.

Fresh 49 mL of MSM was inoculated with 1 mL aliquots of the selected previous liquid cultures with enhanced hexazinone degradation capacity and subjected to liquid enrichment tests. A series of new liquid cultures were prepared till after the tenth enrichment, a fully enriched mixed liquid culture was expected to be achieved [19]. An aliquot of 100 L was collected from the 10th enrichment of the mixed liquid culture and diluted to 1 mL with fresh MSM. In sterile Eppendorf tubes, serial dilutions were prepared up to the fifth dilution. 100 μL aliquots were obtained from the 1st and 4th dilutions and dispersed in triplicates on agar plates (agar 3%, hexazinone 50 mg/L) and incubated at 30 °C. To obtain pure isolates, the individual colonies grown on the agar plates were streaked on another set of agar plates and incubated at 30 °C. MSM inoculated with soil from sugarcane planted fields yielded a total of 28 isolates. Using soil from pineapple cultivated field, a total of 17 isolates were obtained with hexazinone as the substrate. To monitor hexazinone degradation, the 45 pure isolates with the ability to digest it were individually suspended as pellets in fresh MSM modified with 50 mg/L hexazinone and cultured for 46 days at 30 ± 1 °C under aerobic conditions (in triplicates). The control was a sterile MSM medium containing hexazinone but no inoculant. Cell density and hexazinone and metabolite residues were determined using a 3 mL aliquot every 2 days for the first 10 days and every 4 days for the remaining sampling period, as described above.

### 2.6. Analysis of hexazinone and its metabolites residues from MSM

Hexazinone and its metabolites were extracted and cleaned before HPLC analysis as described earlier. An aliquot of 3 mL withdrawn from the liquid cultures was centrifuged at 5000 rpm for 10 min at 25 °C. The supernatant was extracted using agitation with 10 mL of dichloromethane. This was repeated thrice followed by a final washing with dichloromethane. The extracts were pooled, dried over anhydrous sodium sulphate, filtered and concentrated in a rotary evaporator to 3 mL. Clean up procedure was as earlier explained using SPE cartridges. HPLC analysis was undertaken as explained after reconstituting the samples into HPLC methanol.

### 2.7. Characterization of isolates

The bacterial isolates were examined physically by noting the color, texture, consistency and elevation of the colony. Colony morphology was inspected using an epi-fluorescent microscope. Biochemical tests which included nitrate reduction, citrate utilization and catalase test were determined as described elsewhere [27]. The strain's salt tolerance, optimum temperature and optimum pH for growth were also determined [28]. The identity of the isolates was further determined through a molecular characterization procedure [17]. High molecular weight chromosomal DNA was extracted from the isolated strain by use of the fast DNA Spin kit for soil (MP Biomedicals, Eschwege, Germany). The universal primer pair 616F, 5`-AGAGTTTGATYMTGGCTCAG-3 and 630R, 5`-CAKAAAGGAGGTGA TCC-3 [24,29,34] corresponding to nucleotide positions 8–27 and 1528–1544 in the 16S-rDNA from *Escherichia coli*, [15,16,30,32] was used for amplification of a 1.4 kb region of the 16S rRNA coding genes.

The amplified 16S rRNA gene fragments were further gel-purified and subsequently cloned with the TOPO TA Cloning kit containing PCR 2.1 vector (Invitrogen, Paisley, UK). Insert-positive clones (blue-white selection) were isolated and purified with the Nucleo spin plasmid miniprep isolation kit (Macherey-Nagel, Dueren, Germany). The presence of inserts with correct size was controlled as described in the manufacturer's protocol by *Eco*RI restriction endonuclease digestions. 16S rDNA plasmid inserts were sequenced using the Big-Dye-Terminator sequencing kit (Applied Biosystems, Darmstadt, Germany) and an ABI-prism-377 automated sequencer (Applied Biosystems, Darmstadt, Germany) as it was previously described [24,31]. The resulting 16S rDNA-sequences were aligned with the *Sina Aligner V1.2.11*on the Silva website (www.arb-silva.de) and the software package ARB was used for further phylogenetic analysis (http://www.arb-home.de) [32].

## 3. Results and discussion

### 3.1. Recoveries and residual concentration of hexazinone and its metabolite from Nzoia and Thika fields

The average recoveries of hexazinone from spiked MSM were in the range 85.0 ± 4.05% to 94.8 ± 2.01%. The soils from Nzoia and Thika fields had hexazinone residue concentrations of 0.23 ± 0.02 mg/g and 0.14 ± 0.05 mg/g, respectively, with no residues of metabolite detected. The limit of detection (LOD) for hexazinone was 0.009 mg/g.

### 3.2. Screening soils for enhanced degradation of hexazizone

Fig. 1 shows the enhanced degradation of hexazinone in Nzoia and Thika soils with repeated application history of the compound. The non-sterile soils with over 15 years of continuous application of hexazinone in Nzoia (NS15-NZ) and Thika (NS15-TH) degraded 82.2% and 93.4% of 5 mg/g initial concentration of hexazinone, respectively. In comparison, non-sterile soils without history of application in Nzoia (NS0-NZ) and Thika (NS0-TH) degraded 48.8% and 36.8% of 5 mg/g initial concentration of hexazinone after 146 days, respectively (Fig. 1). In Thika and Nzoia, there was a significant difference ($p < 0.005$) in hexazinone degradation between non-sterile soils with a 15-year history of application and non-sterile soils with no history of treatment. The indigenous microbial community had adapted to use of hexazinone for carbon and energy source and was able to degrade the compound as demonstrated by our results. This finding

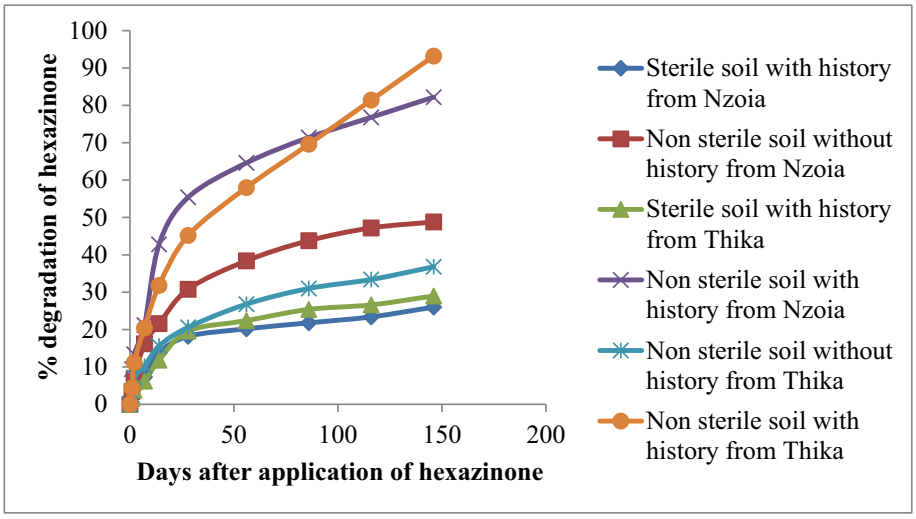


**Fig. 1.** Degradation of hexazinone using non-sterile and sterile soils from sugarcane and pineapple cultivated fields with and without history of hexazinone application (NS15-non-sterile soils with over 15 years' application history, S15-sterile soils with over 15 years' application history, NS0-non-sterile soils without application history, NZ-Nzoia, TH-Thika).

backs up previous findings that revealed adaptive degrading bacteria develop in soils that have been repeatedly treated with pesticides, resulting in increased breakdown [24]. In the sterile set of soils with history of application from Nzoia (S15-NZ) and Thika (S15-TH), the percentage of degradation was 26% and 29% after 146 days, respectively (Fig. 1). Hexazinone degradation in the sterile soils from Thika (S5-TH) and Nzoia (S5-NZ) suggests that hexazinone was chemically decomposed; nonetheless, total sterility of the sterilized soils is difficult to affirm in the absence of empirical evidence on microbial activity. The faster decomposition of hexaznone in non-sterile soil compared to sterile soil indicates that long-term use of hexazinone resulted in ad This initial study using hexazinone compound at the concentration of 5 mg/L was critical in our study as it gave the researchers some insight information on microbe adaptation and degradation of hexazinone. The enhanced degradation was monitored for a longer period to establish that degradation was purely as a result of adapted microbes with minimal degradation resulting from other factors like volatilization and chemical degradation. A higher degradation rate was always recorded at the initial stages of the experiment due to availability of hexazinone in higher concentration and also the compound had not adsorbed to soil matrix. This allowed complete degradation of hexazinone by the indigenous microbes. Opportunistic microbes could have also utilized hexazinone for carbon and energy source. Other studies using atrazine, which belongs to the same triazine family as hexazinone, found that indigenous microbes degraded 90% of the initially applied atrazine within 98 days of incubation [17].

### *3.3. Degradation of hexazinone by liquid enrichment cultures from Thika and Nzoia*

Fig. 2 shows the degradation of hexazinone by the liquid culture from pineapple and sugarcane cultivated soils in Thika and Nzoia, respectively. By the end of the incubation period, the average degradation of hexazinone in the control, comprised of sterile MSM and hexazinone without the inoculum, was found to be insignificant (1.01 ± 0.001%). This was attributed to chemical degradation. By the 46th day of the incubation period, liquid enrichment cultures from Thika and Nzoia soils had decomposed 70.0 and 66.0% of the applied 50 mg/L of hexazinone, respectively (Fig. 2). The degradation of hexazinone by the two liquid cultures from Nzoia and Thika were comparable with liquid culture from Thika soil exhibiting slightly higher degradation potential. The microorganisms utilized

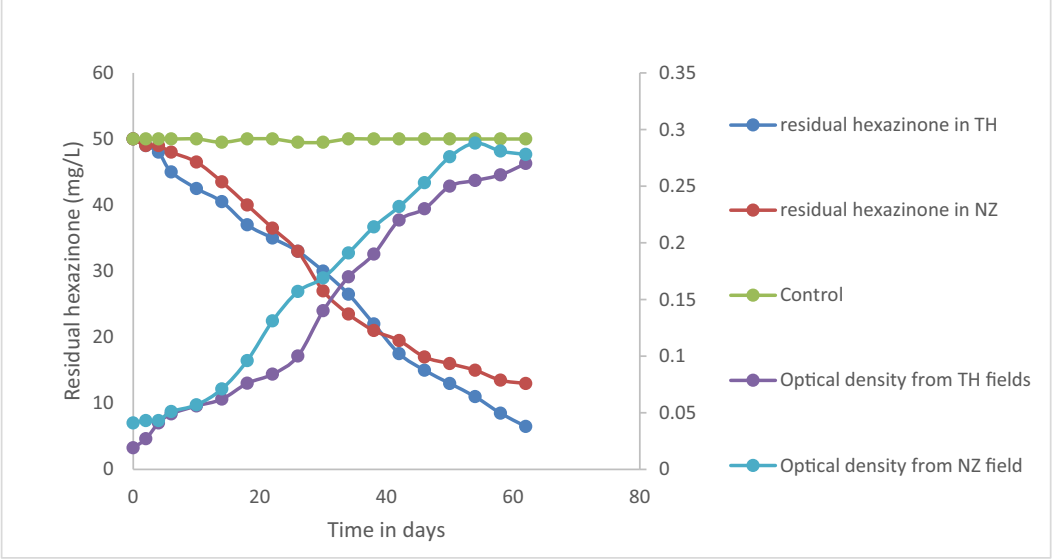


**Fig. 2.** Bacterial growth and residual hexazinone curves in liquid cultures using sugarcane and pineapple cultivated soils (NZ-Nzoia soil, TH-Thika soil).

hexazinone as a carbon and energy source, as evidenced by the drop in hexazinone content as the optical density increased with time, as seen in Fig. 2. Microbial degradation is therefore responsible for the disappearance of hexazinone. The enriched microbial community from pineapple and sugarcane cultivated soils registered a degradation rate of 0.064 mg/L and 0.057 mg/L per day, respectively. The enrichment cultures from Thika and Nzoia showed a lag phase of 1 day presumably, because the microbes had adapted to the utilization of hexazinone and hence, did not take long to adapt to the utilization of the compound under laboratory conditions. There was a positive relationship between the degradation of hexazinone and growth of the cultures. The growth of the strains from Nzoia and Thika soils reached the highest cell mass of 0.288 and 0.273 $OD_{600}$ on the 54th and 62nd day of incubation respectively. Studies using a different Kenyan sugarcane cultivated soil on the same herbicide (approximately 100 km away), demonstrated that the enrichment culture had a lag phase of 7 days [16]. The degradation data was subjected to first order kinetics with the enrichment culture from pineapple and sugarcane cultivated soils registering half-live values of 10.8 days and 12.2 days.

### 3.4. Degradation of hexazinone by pure hexazinone-degrading strains

Fig. 3 shows the degradation of hexazinone by the two isolates from pineapple and sugarcane cultivated fields. Liquid enrichment culture for hexazinone using pineapple and sugarcane cultivated soils yielded eleven (11) and twenty-six (26) strains, respectively. To assess their individual hexazinone breakdown capabilities, the isolates were re-transferred to liquid culture with a 50 mg/L concentration of hexazinone. Out of the isolated 37 pure isolates, two (2) strains abbreviated H/T (Hexazinone/Thika with pineapple) and H/N (Hexazinone/Nzoia with sugarcane) were able to degrade hexazinone completely. After 46 days of incubation, the isolates from Thika and Nzoia soils decomposed up to 52.3 and 38.4% of the 50 mg/L hexazinone initially administered, respectively (Fig. 3). The strains H/T and H/N registered a degradation rate of 0.017 mg/L and 0.010 mg/L per day, respectively, which was less relative to the degradation by microbial communities. The strains H/T and H/N registered a degradation rate of 0.017 mg/L and 0.010 mg/L per day, respectively, which was less compared to the degradation by microbial communities. When the data was subjected to first-order kinetics, half-live values of 40.2 and 69.3 days were calculated for the pure strains from Thika and Nzoia soils, respectively.

The results suggest that total degradation of hexazinone was higher when the microbes acted together as a consortium but lower when microbes acted as single strains. Complete and faster bioremediation is through a consortium of microbes. This is consistent with studies by other researchers who have reported that, the mixed bacterial culture degraded hexazinone at a higher rate compared to individual isolates [16,24]. Other studies [15], reported half-life of 1.38 days for a consortium of bacterial strains against half-life of 3.08 days when using a single strain for degradation studies. The H/T strain capacity to degrade hexazinone was slightly higher than H/N, indicating that hexazinone biodegradation efficiency is species-specific.

### 3.5. Phylogenetic and taxonomic characterization of the isolates

Figs. 4 and 5 show the phylogenetic tree after molecular analysis of the 16S rRNA gene sequence clustering the isolated strains to the respective genus. The sugarcane cultivated soil from Nzoia harbored the isolate strain designate H/N which clustered under the genus staphylococcus with NCBI GenBank accessions D83366 identified as *Staphylococcus gallinarum* [33]. The pineapple cultivated soil from Thika also harbored the isolate designate H/T which clustered under the genus Bacillus with NCBI GenBank accessions CP006863 and AB426479 identified as *Bacillus toyonensis* and *Bacillus thuringiensis,* respectively. The H/N isolate had 16S rRNA gene sequence similarity of 97.5% to *Staphylococcus gallinarum* D83366 (type strain) while the H/T isolate showed a 99.6% similarity to both *Bacillus toyonensis* CP006863 and *Bacillus thuringiensis* AB426479.

The large size of gene on 16S rRNA gene code made it ideal choice for the isolates morphological analysis. In Kenya, isolation of the adapted microbes due to repeated application has not been reported extensively although the compound hexazinone has been in use for over 20 years. Only one study has been undertaken in Kenya and reported *Enterobacter cloacae* as a capable degrader of hexazinone from sugarcane cultivated soil [16]. No strains have been isolated from a pineapple cultivated soil though the compound is widely used in pineapple growing areas in Kenya (Thika and Kisii highlands) for control of weeds. Studies from the temperate regions, isolated *Enterobacter cloacae* and *Pseudomonas species* as hexazinone degraders [15]. Other studies from temperate regions identified *Microbacterium foliorum, Paenibacillus illinoisensis and Rhodococcus equi* as hexazinone degraders [19]. However, studies on biodegradation of atrazine using sugarcane cultivated soils in Kenya reported members belonging to

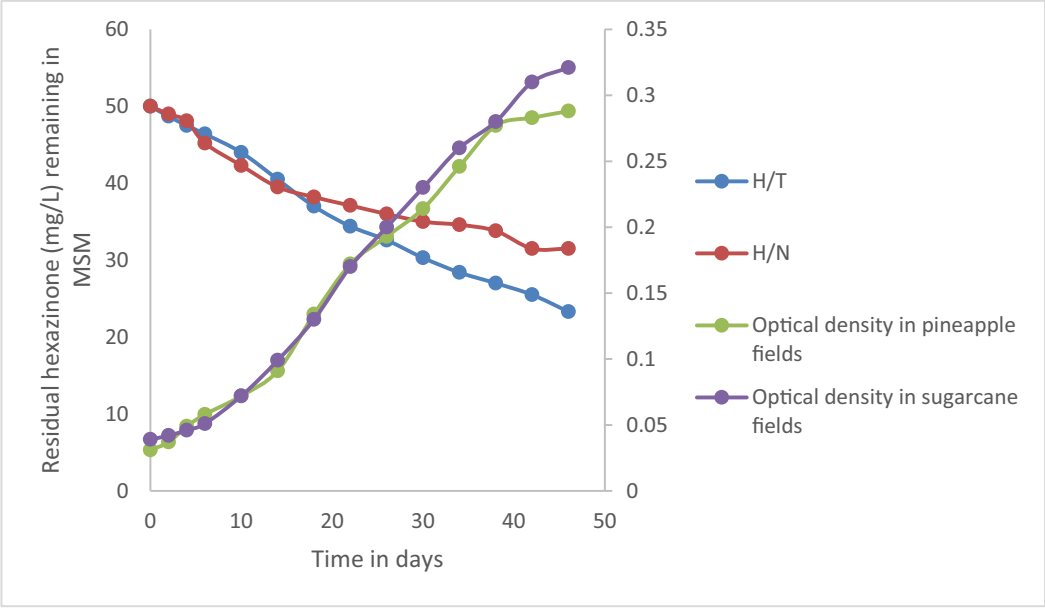


**Fig. 3.** Bacterial growth and hexazinone degradation curves in liquid culture by pure isolates from pineapple and sugarcane cultivated soils (H/T strain-Hexazinone/Thika with pineapple and H/N strain-Hexazinone/Nzoia with sugarcane).

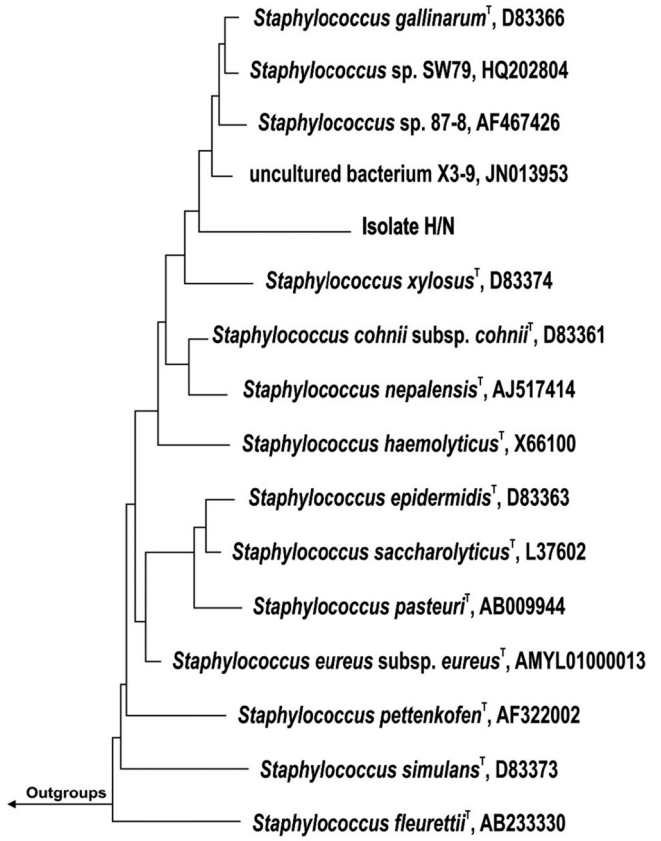


**Fig. 4.** Phylogenetic dendrogram showing the position of the hexazinone degrading isolate H/N closely affiliated with *Staphylococcus gallinarum*. The bar indicates the estimated substitution per nucleotide position.

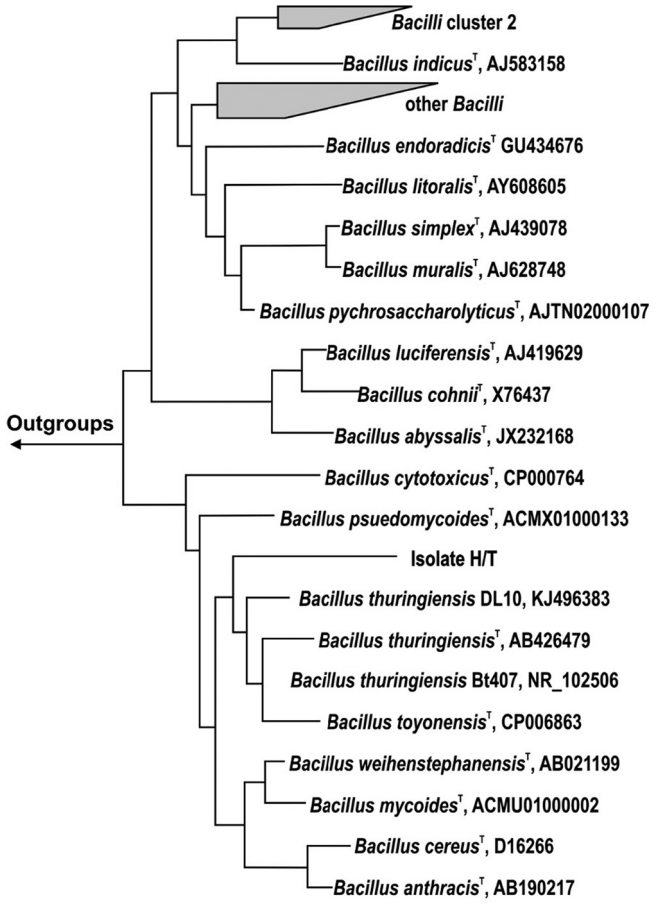


**Fig. 5.** Phylogenetic dendrogram showing the position of hexazinone degrading isolate H/T closely related to *Bacillus thuringiensis* and *Bacillus toyonensis*. The bar indicates the estimated substitution per nucleotide position.

the same bacterial family as the degraders of hexazinone, namely, *Burkholderia cepacia* and *Enterobacter cloacae* [34]. In another study on atrazine and terbuthylazine, a strain belonging to a member of the genus *Arthrobacter* was isolated as a capable degrader [17]. Hexazinone, atrazine and terbuthylazine belong to the group of triazine pesticides. This signals the need to carry out more studies to isolate and characterize the bacterial strains that have become adapted to the chemicals applied in the area and hence can be used for bioremediation of the contaminants.

The genetic distance between the isolate H/N and the strain with accession number D83366 was 0.0000 while the genetic distance between the isolate H/T and strains with accession numbers KJ496383 and CP006863 was 0.0001 confirming the isolates as *Staphylococcus gallinarum* and *Bacillus thuringiensis, Bacillus toyonensis,* respectively (Table S1, supplementary material). Ngigi and co-workers [16,34] identified and characterized *Enterobacter cloacae* as hexazinone and triazine degraders from sugarcane-cultivated soil from the Nzoia sugar region, which is the same one from where the various species herein reported were separated. This demonstrates the abundant biodiversity found in Kenyan soils, which can be used for bioremediation of polluted soils. The *Enterobacter cloacae* isolate degraded hexazinone up to 27.3% of the initially applied concentration of 40 mg/L after 37 days of incubation in a liquid culture medium [16], suggesting the isolates reported in the present work are better hexazinone degraders though reference [16] did not report the degradation half-life. Wang et al. [15] reported 86.7% degradation of 40 mg/L hexazinone after 10 days by WFX-1 bacterial strain indicating better degradation capability than the stains reported in this work. Cumulatively, these findings confirm that the synergistic effect of a bacterial community or consortium results to a higher activity to hexazinone degradability than an individual strain. *Staphylococcus gallinarum* and *Bacillus thuringiensis/Bacillus toyonensis* are herein identified for the first time as prospective hexazinone degraders from sugarcane and pineapple-cultivated soils, respectively.

### 3.6. Morphological and biochemical characterization of the isolates

Table 1 shows that both isolates were rod shaped, motile and had smooth texture as determined by observation through an epi-fluorescent microscope. Both strains grew in nutrient broth supplemented with 0–7% (*w/v*) NaCl but optimum growth occurred in media with 0–0.5% NaCl. When the strains were tested for optimal growth under different pH values, optimal growth was observed at pH range 5.0–8.0, with weak growth occurring at pH 10.0 and no growth occurring at pH 4.0. Optimal growth of the strains also occurred at temperature between 27 °C and 37 °C. Both isolates were aerobic and showed positive results for citrate utilization, catalase test and nitrate reduction.

**Table 1**
Biochemical characteristics of Hexazinone-degrading Isolates H/N and H/T from Kenyan Soils.

| Test/Observation | Isolate H/N | Isolate H/T |
|---|---|---|
| Physical Characteristics | Texture-smooth; Color-Whitish cream; Elevation-Flat; Margin-Entire | Texture-smooth; Color-Whitish cream; Elevation-Flat; Margin-undulate |
| Morphological Characteristics | Rod shaped | Rod shaped |
| Citrate utilization | − | − |
| Nitrate reduction | + | + |
| Motility at 37 °C | + | + |
| Protease activity | + | − |
| Salt tolerance | + | + |
| Catalyst test | + | + |
| Gram stain | − | − |

### 3.7. Metabolites formation in soils inoculated with pure isolates

The liquid cultures included just the hexazinone peak amounting to 1.4% and 2.1% of the chemical degraded during the first 2 days of incubation utilizing isolates from sugarcane and pineapple grown soils. This meant there were no hexazinone metabolites produced. However, on the 4th day of incubation using the liquid culture with inoculants from the pineapple cultivated soil, a peak of metabolite (3-(4-hydroxycyclohexyl)-6-(methylamino)-1-methyl-1,3,5-triazine-2,4(1H,3H)-dione (B), was detected [20]. The same metabolite was detected in the liquid culture with inoculants from sugarcane cultivated soil on the 6th day of incubation. The amount of (3-(4-hydroxycyclohexyl)-6-(methylamino)-1-methyl-1,3,5-triazine-2,4(1H,3H)-dione (B), formed from the degradation of hexazinone in the pineapple and sugarcane cultivated soils by the 18th day of incubation amounted to 13.2 and 10.7%, respectively.

## 4. Conclusions

The present study confirms that fields with continuous exposure to pesticides develop adapted microbes capable of utilizing these pesticides as carbon and energy sources. The study reports for the first time the degradation of hexazinone by a close relative of *Staphylococcus gallinarum* from sugarcane cultivated soil in Nzoia and a strain closely affiliated to *Bacillus toyonensis* and *Bacillus thuringiensis* from pineapple cultivated soil in Thika, Kenya. The *Bacillus* species strain's capacity to degrade hexazinone was higher than *Staphylococcus* species strain, indicating that hexazinone microbial degradation efficiency is species-specific. Additionally, microbial communities were more effective in mineralizing hexazinone than the single strains. The identified native bacterial strains can be developed and used for bioremediation of hexazinone-contaminated soils.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.enceco.2022.02.002.

## Conflic of interest

The authors declare no competing interests.

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