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Full Length Research Paper

Utilisation and biodegradation of atrazine and primextra

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The abilities of twelve bacterial isolates to utilise atrazine and primextra and the degradation dynamics of the two herbicides in soil was determined in this study. Soil treatments were carried out in triplicates using the completely randomized blocked design. Utilisation of atrazine and primextra were determined by monitoring growth rates of the bacteria, actinomyces and streptomyces via viable counts, optical density and pH changes. Chromatographic analysis of soil samples were also done to determine residual concentration of herbicides. The viable count and the optical density values increased significantly (P< 0.001) from the 0 h to the 20th day and latter dropped on the 25th to the 30th day. The pH values dropped significantly (P< 0.001) from the 0 h to the 30th day. *Bacillus subtilis* recorded the highest viable count values of 8.98 and 9.25 on the 20th day and optical density values of 0.934 and 1.631 respectively on atrazine and primextra on the 20th day during growth on atrazine and primextra thus significantly (P <0.001) utilising atrazine and primextra for growth. *Pseudomonas putida* had the lowest pH value of 5.84 on atrazine while *P. stutzeri* recorded the highest pH value of 6.42 during growth on atrazine. *Norcadia farcinica* had the lowest pH of 5.97 during growth on primextra after the 30th day of growth while *Flavobacterium aquantile* had the highest pH value of 6.34 after the 30th day of growth on primextra. The area and height of atrazine and primextra detection peaks decreased from the second to the sixth weeks of herbicide treatment. The decrease in atrazine and primextra peak indicated more atrazine and primextra degradation. *B. subtillis* has shown the best ability to utilise atrazine and primextra respectively. This study has shown that bacterial isolates could be applied to remediate soils polluted with both atrazine and primextra. The progressive biodegradation of atrazine and primextra has also been elucidated in this work.

Key words: Optical density, viable count, biodegradation, herbicide.

INTRODUCTION

Contamination of agricultural soils with organic and inorganic pollutants results from industrial and domestic wastes, agricultural inputs and several other human activities. These pollutants are usually disseminated in soil by repeated flooding. Optimisation of agricultural resources for improved and sustainable agriculture involves the use of pesticides. Various herbicides have been used to control unwanted weeds (Nweke et al., 2007).

Atrazine {2-chloro-4-(ethylamino)-6-isopropylamino-1, 3-5-triazine} is a widely used 5-triazine herbicide. It is used as pre-emergence herbicide in control of broad leaf and grassy weeds in a variety of commercial crop as well as road side and fallow fields (Munier-Lamy et al., 2002). Early studies on the environmental fate of atrazine have shown that it is transformed slowly by fungi (Kaufman and Kearny, 1970). Metolachlor {2-chloro-N-(ethyl-6-methyl (phenyl)-N-(2-methoxy-1-methylethyl acetamide} is a selective herbicide used in control of grassy weeds in the cultivation of corn, soybeans, peanuts, cotton and other crops. Metolachlor is often used in combination with other broadleaved herbicides (example, atrazine, metobromuron and propazine) to extend the spectrum of activity (Ayansina and Oso, 2006).

Biodegradation is one of the natural processes that

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help to remove xenobiotic chemicals from the environment by microorganisms (Singh, 2008). It is one of the most cost effective methods amongst remedial approaches. Several excellent reviews have been published on the biodegradation or bioremediation, both generally (Prescott et al., 2008; Chatterjee et al., 2008) or specifically, of xenobiotic compounds (Austin et al., 1977; Chaudry and Chapalamadugu, 1991; Zhang and Bennet, 2005; Chauhan et al., 2008; Chowdhury et al., 2008).

Microbial degradation of herbicides in soils relies mainly on three key variables; the ability of the microorganisms to degrade the pesticides, the quantity of these microorganisms in the soil and the activity of soil microbial enzyme system (Anderson, 1984; Joshi and Gupta, 2008).

This work was carried out to determine the ability of some bacterial isolates to utilise atrazine and primextra as carbon source and to elucidate the biodegradation rates of atrazine and primextra.

MATERIALS AND METHODS

Soil sampling

Surface layer of soil samples, up to 5 cm depth were collected from cassava farm in Ijebu-Ode (Ogun State, Nigeria) with no prior pesticide treatment and plot that had been deliberately treated with herbicides. Soil samples were collected randomly every 2 weeks for 6 weeks. The soil samples were sieved through a 2.0 mm width mesh to remove stones and plant debris and placed in air tight polythene bags.

Herbicides

The herbicides that were used are atrazine (Atrylone 80WP, trademark of Insis Limited) and primextra (a product of Syngenta, a combination of atrazine and metolachlor).

Soil treatments

The treatments were carried out for a period of 6 weeks; at company recommended rates of 4 L/ha (at 350 ml in 15 L sprayer) for primeextra while recommended rate of 3 kg/ha (atrazine powder) was used for atrazine treatment. Soil treatments were carried out in triplicates using the completely randomized blocked design. The herbicides were applied to the soil surface, using the 15 L sprayer, twice a week. The physico-chemical properties of the untreated soil used in this study are shown in Table 1.

Microbial enumeration

Nutrient agar (NA) was used for the enumeration of total heterotrophic bacteria by the pour plate method. Incubation was done at 30°C for 24 to 48 h. Potato dextrose agar (PDA) was used for enumeration and isolation of fungi. Incubation of fungal isolates was done at 25°C for 48 h. Bacterial and actinomycetes isolates were characterized based on cultural characteristics, staining reactions and biochemical reactions. Identification was thereafter made with reference to Bergey's manual of systemic bacteriology (1984). Starch Casein Agar was used for the enumeration of total actinomycetes count.

Herbicide utilisation patterns of different isolates

The ability of herbicide degrading bacterial isolates to utilise pure herbicide substrates was tested in minimal salt medium in (g/L) (Na_2HPO_4 2.13 g, KH_2PO_4 1.3 g, NH_4Cl 0.5 g, $MgSo_4.7H_2O$ 0.42 g, NaCl 10 g, $NaNO_3$ .42 g and trace element 1.0 ml) (Moneke et al., 2010). Atrazine and primeextra were introduced separately into 100 ml Erlenmeyer flasks, at 1.0v/v (concentration 100 ppm), containing 20 ml of the basal medium. The flasks were then autoclaved and inoculated with 1.0 ml portion of each isolate (except one which serves as control). The flasks were subsequently incubated in an orbital shaker incubator at 150 rev/min at 30°C for 7 days. The growth in each flask was determined based on the turbidity of the medium at 600 nm using spectrophotometer (PG T70 U.V/VIS spectrophotometer). The growth rates (on atrazine and primextra) of the organisms that had the heaviest growth were then determined.

Determination of the growth rates of bacteria, actinomyces and streptomyces isolates

The ability of microbial isolates to degrade herbicide substrates (atrazine and primextra) in pure cultures was determined in minimal salt medium (g/L) (Moneke et al., 2010). The components were dissolved in 1000 ml distilled water, homogenised on hot plate magnetic stirrer to form uniform solution for 30 min. The pH of the basal medium was adjusted to pH 7.2. The basal medium of 150 ml was dispensed into 250 ml Erlenmeyer flasks and herbicide substrates were introduced into each flask respectively at 100 ppm after sterilisation which was done separately in an autoclave at 121°C for 15min and cooled to ambient temperature.

1 ml aliquot of diluted overnight broth cultures of each test organism ($\times 10^4$ cells/ml) were seeded into each flask respectively and the flasks were incubated in a gyratory shaker incubator at 150 rpm for a period of thirty days at 30°C

Utilisation of herbicide fractions by microbial isolates was evaluated by monitoring bacteria, actinomyces and streptomyces growth measured by viable count on nutrient agar. The optical density was determined at 600 nm wavelength with PG T70 U.V/VIS spectrophotometer and changes pH, was determined with pH meter (Model Hama microprocessor P211 pH meter).

Extraction of herbicides

The degraded herbicide fractions from the soil sample were extracted with Acetonotrite and Methanol. Two grams of herbicide treated soil samples was weighed into 100 ml beaker and 25 ml of Acetonitrite and Methanol (4:1) was added to the prepared soil sample. The mixtures were shaken vigorously and then allowed to stand for one hour. The supernatants were filtered using Whatman No 4 filter paper. The extracts were then transferred into a sterile MacCartney bottle for chromatographic analysis.

Gas chromatography assay

The extracted herbicides were analysed by injecting 1 µl of each extract into a Hewlett Packard gas chromatography with flame ionization detector. Separation of the herbicides into components was carried out in a fused silica capillary column programmed for 50 to 300°C at 3.5°C/min. Flame ionisation detector was used to detect and quantitate the compounds eluting from the chromatographic column. The carrier gas was nitrogen. The data

Table 1.Physico-chemical properties of untreated soil used in this study.

Properties	Values
pH	1.74
% organic matter	6.24
% moisture content	1.74
NH-N (ppm)	3.61
Na (ppm)	1124
K^+ (ppm)	1595.8

Table 2. Microorganisms isolated from herbicide treated soil samples and control soil samples.

Treatment	Microorganisms
Control	*Bacillus sphaericus, B. coagulans, B. subtilis, Corynebacterium equi, C. piosum, B. mycoides, Alcaligenes faecalis and Acinetobacter calcoaceticus, A. bovis, A. maeslundii* and *Streptomyces griseomycini*
Atrazine	*Norcadia rhodnii, Corynebacterium fascians, Bacillus licheniformis, P. putida, P. sutzeri, Micrococcus roseus, Acinetobacter mallei, B. subtilis, S. marcescens and Micrococcus varians, A. viscous, A. maeslundii* and *A. israelii*
Primextra	*Bacillus megaterium, B. cereus, S.marcescens, A. eutrophs, Chromobacterium lividium, A. latus, N. farcinica, B. subtilis, Xanthomonas* sp., and *F. aquantile, A. viscous*, *A. bovis* and *Streptomyces nigrifasciens.*

was digitalised using analytical A/D converter.

RESULTS

Microbial enumeration

Presented in Table 2 are the microorganisms obtained from herbicide treated and control soil samples. In all herbicide treated soil samples *Bacillus* sp. *Pseudomonas* sp. and *Actinomyces viscous* were of common occurrence.

Atrazine and primextra utilisation

The heaviest growth on atrazine and primextra were obtained from the following bacteria species; Atrazine treated soils: *Bacillus licheniformis, Pseudomonas putida, Bacillus subtilis, A. viscous, Pseudomonas stutzeri* and *Serratia marcescens.* Primextra treated soils: *Bacillus megaterium, Alcaligenes eutrophs, Norcadia farcinica, B. subtilis, Flavobacterium aquantile* and *Actinomyces bovis.*

The viable count and optical density values of all the bacterial isolates used in this study significantly ($P < 0.001$) increased. The viable count and the optical density values increased from the 0 h to the 20th day and latter dropped on the 25th to the 30th day. The pH values dropped significantly ($P < 0.001$) from the 0 h to the 30th day. *B. subtilis* recorded the highest viable count value of 8.98 on the 20th day and optical density value of .934 on the 20th day during growth on atrazine thus significantly ($P < 0.001$) utilising atrazine for growth (Figures 1 and 3). *S. marcescens* recorded the lowest viable count and optical density values of 7.78 and 0.549 respectively on the 30th day during growth on atrazine (Figures 1 and 3). *P. putida* had the lowest pH value of 5.84 on atrazine while *P. stutzeri* recorded the highest pH value of 6.42 during growth on atrazine (Figure 5).

B. substilis significantly ($P < 0.001$) utilised primextra for growth recording the highest viable count value of 9.25 (Figure 4) and optical density value of 1.631 (Figure 2) on the 20th day. *Norcadia farcinica* had the lowest pH of 5.97 during growth on primextra after the 30th day of growth while *F. aquantile* had the highest pH value of 6.34 after the 30th day of growth on primextra (Figure 6). Meanwhile *A. bovis* recorded the lowest viable count (Figure 4) and optical density values (Figure 2) of 7.83 and .741 on the 30th day of growth on primextra.

Biodegradation of atrazine and primextra

The area and height of atrazine and primextra detection peaks decreased from the second to the sixth weeks of herbicide treatment (Figures 7 to 12) indicating more atrazine and primextra degradation. Atrazine detection peaked at retention times of 15.866 min (Figure 7), 16.066 min (Figure 8) and 15.90 (Figure 9) while

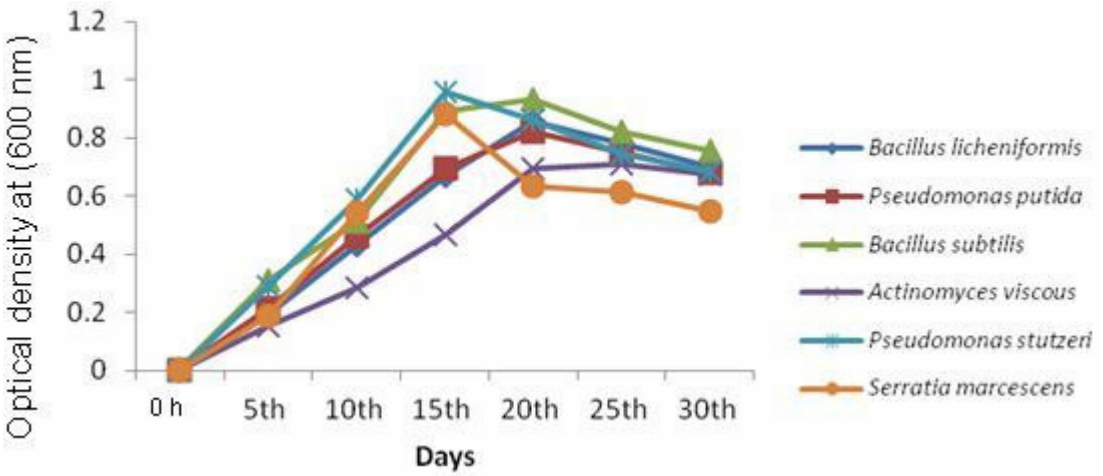

Figure 1. Changes in optical density of during growth (atrazine treated soils).

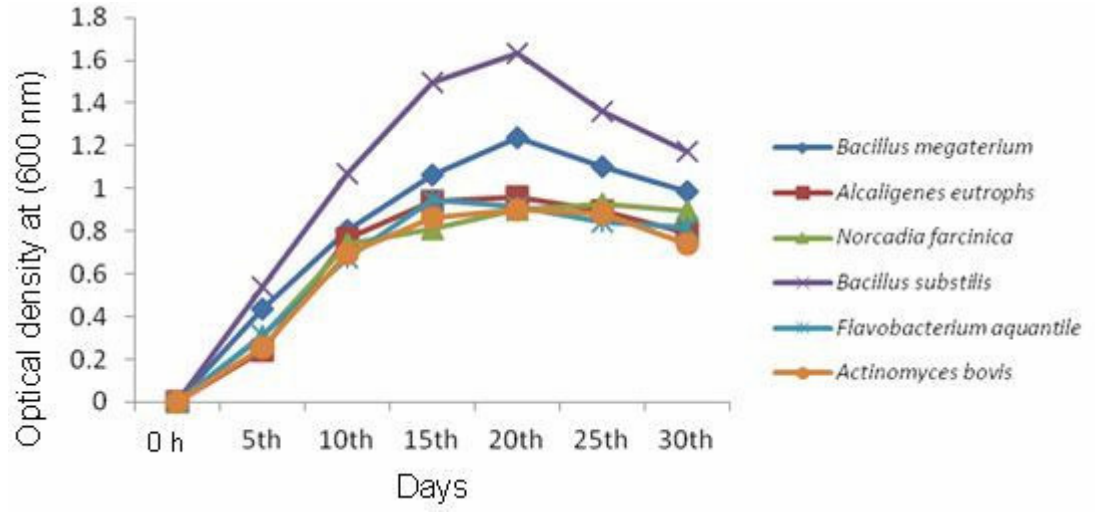

Figure 2. Changes in optical density during growth (primeextra treated soils).

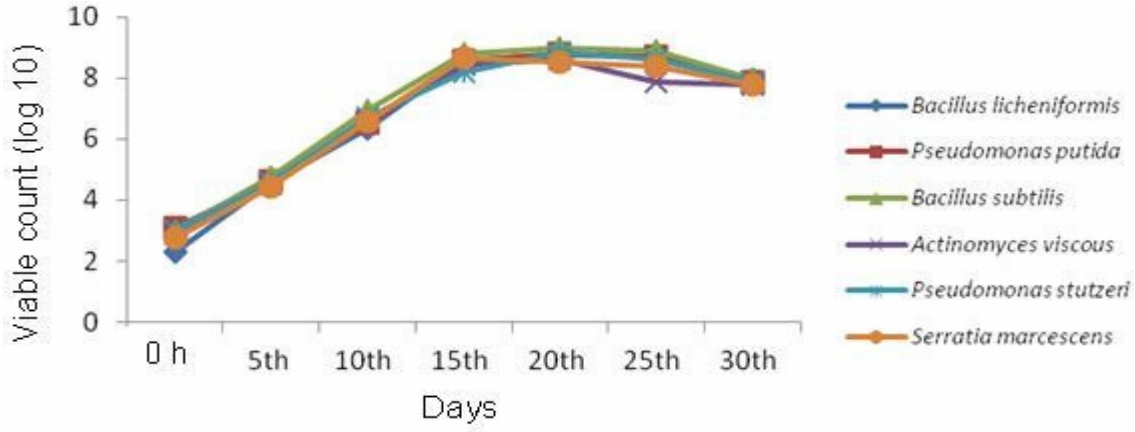

Figure 3. Changes in viable counts during growth (atrazine treated soils).

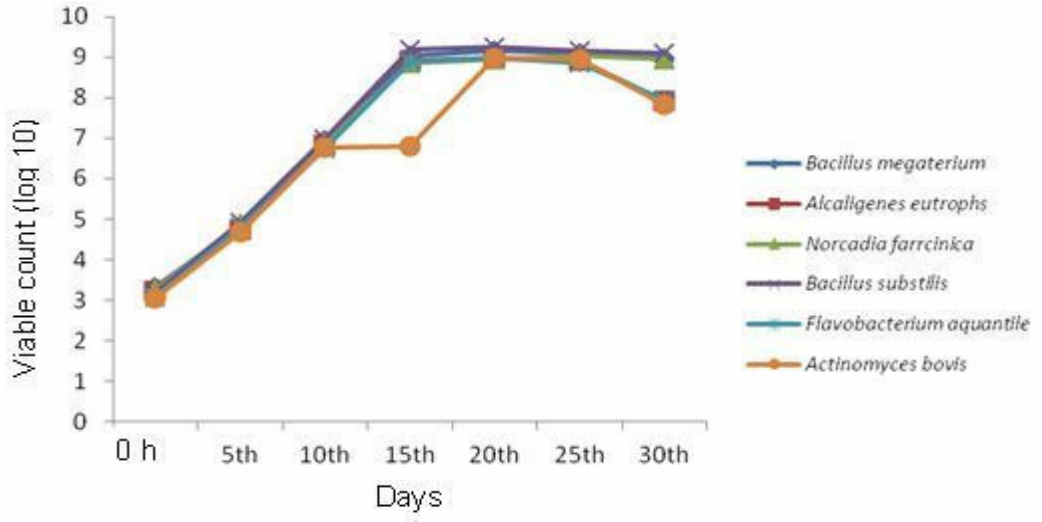

Figure 4. Changes in viable counts during growth (primeextra treated soils).

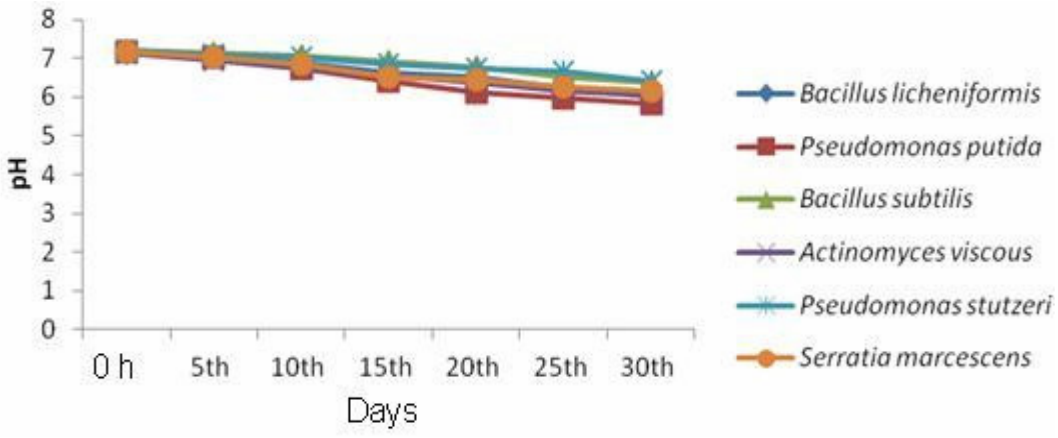

Figure 5. Changes in pH during growth (atrazine treated soils).

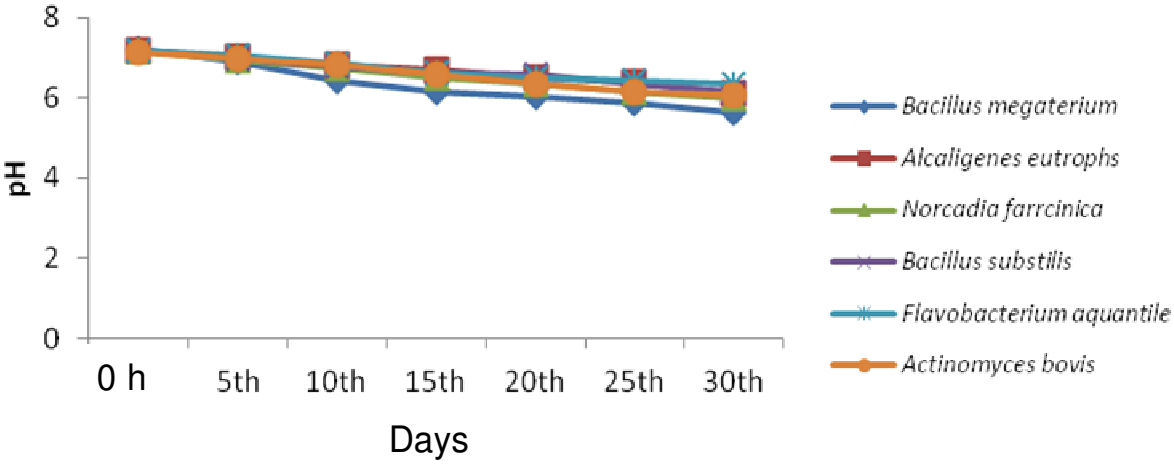

Figure 6. Changes in pH during growth (primeextra treated soils).

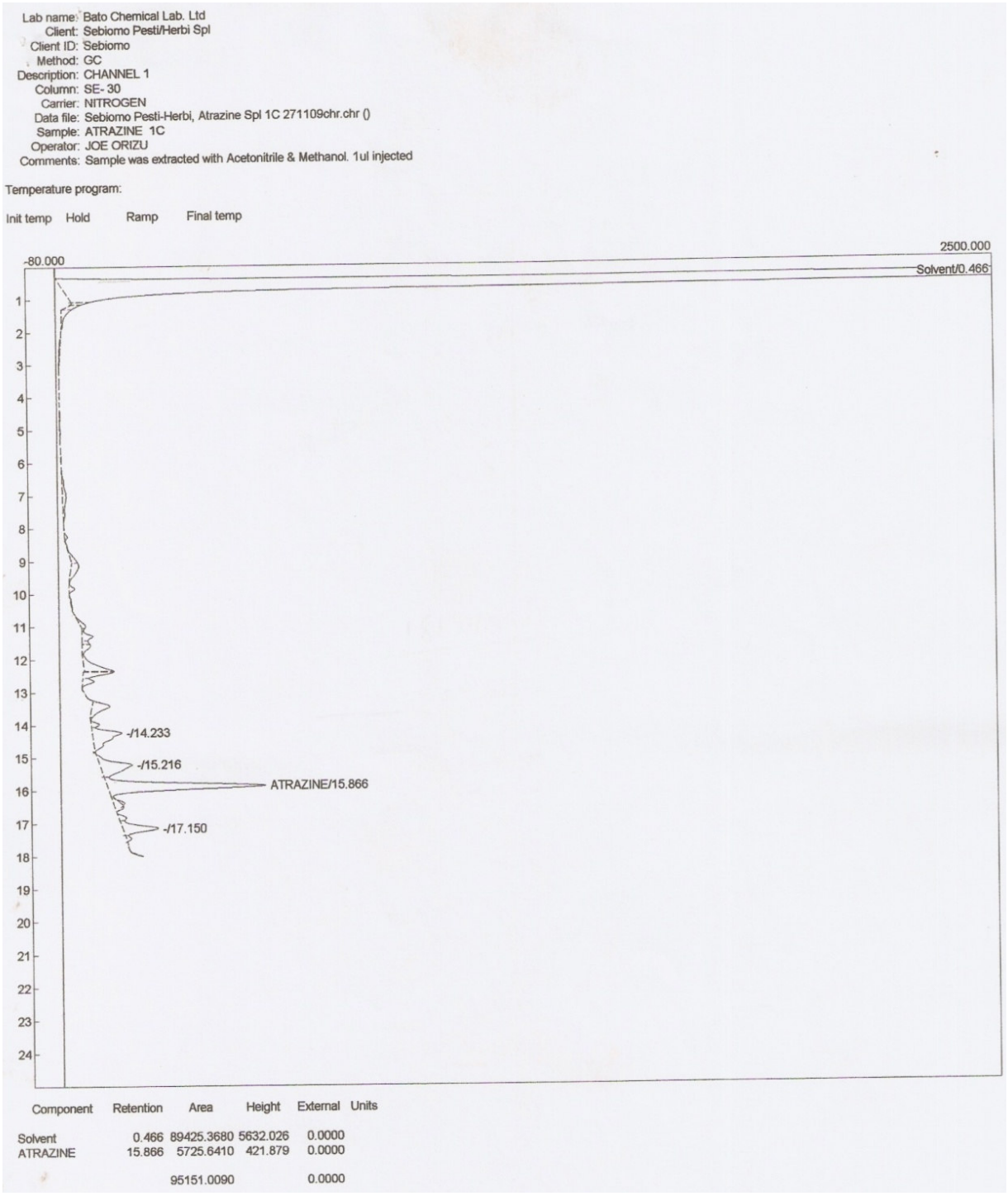

Figure 7. Degradation of atrazine after 2 weeks of treatment.

primextra detection peaked at retention time of 14.716 min. The area and height of primextra detection peaks were highest at the 2nd week (12822.7190 and 929.832) (Figure 10) of observation compared to the area and height of atrazine detection peaks (5725.641 and 421.879) (Figure 7). Atrazine recorded the lowest area and height detection peaks of 1088.1500 and 108.182 respectively at the 6th week of treatment (Figure 10) compared to primextra with area and height detection peaks of (5987.4940 and 755.354) respectively after the 6th week of treatment (Figure 12).

Figure 13 shows the concentration of residual atrazine and primextra from the second to the sixth week of treatment. The concentration of residual atrazine and

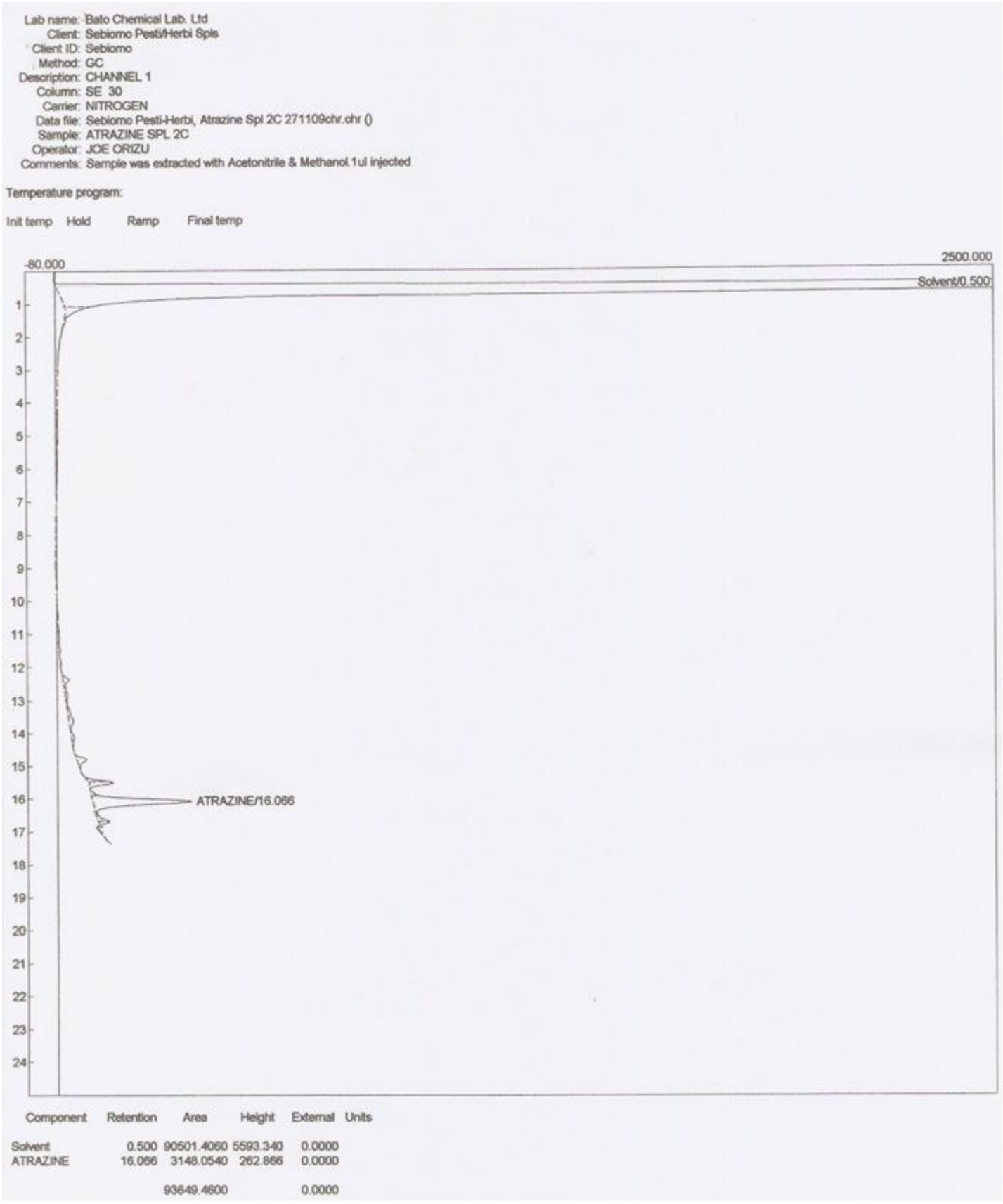

Figure 8. Degradation of atrazine after 4 weeks of treatment.

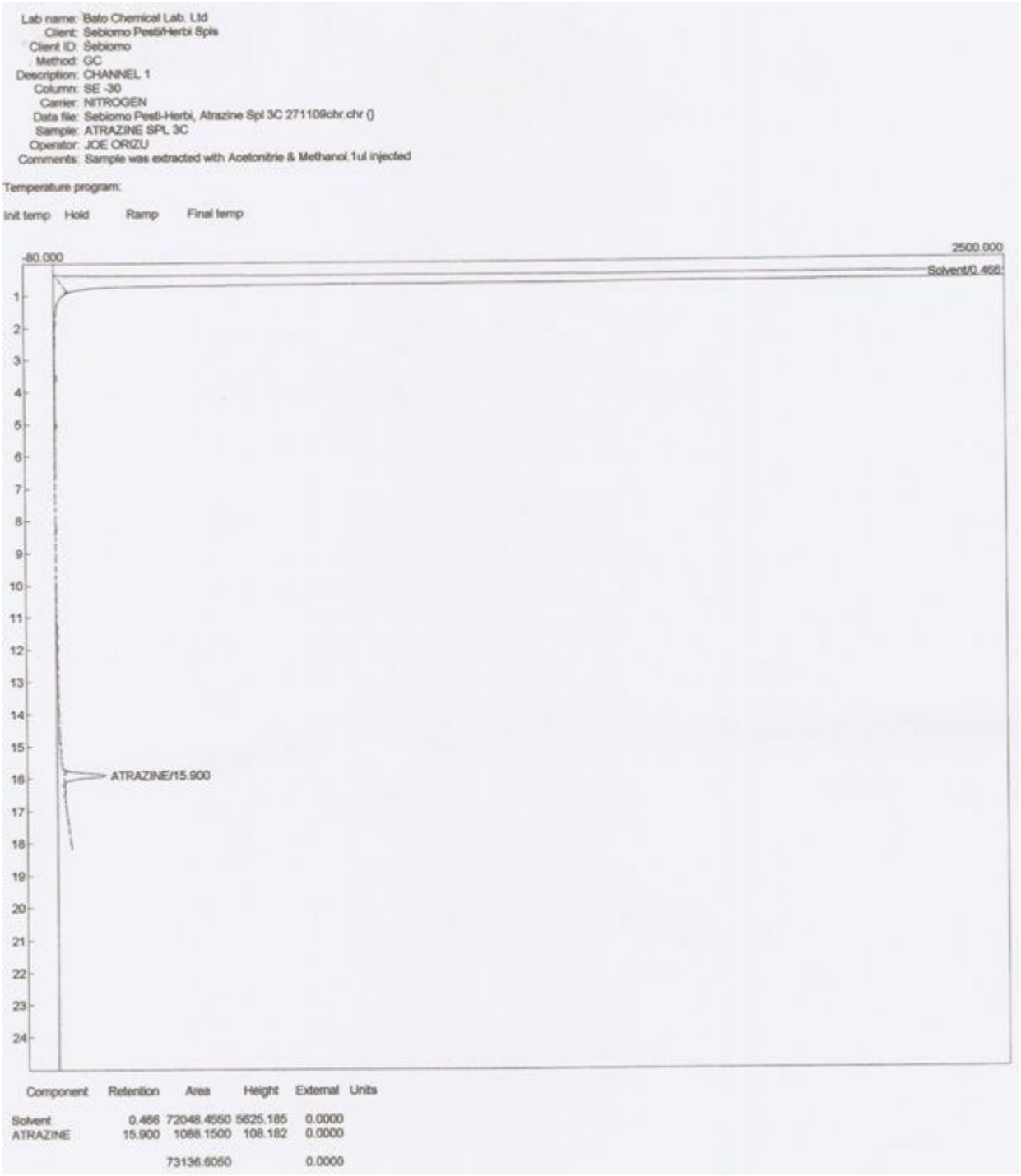

Figure 9. Degradation of atrazine after 6 weeks of treatment.

primextra recovered from soil samples decreased from the second to the sixth week of treatment signifying increased rate of both atrazine and primextra biodegradation. The concentration of residual primextra from 2nd to the 6th week of herbicide treatment was higher than that of paraquat (Figure 13) hence the soil retained much more of primextra than atrazine which was more easily degraded. The highest residual concentration of primextra of 88.53 mg/kg was recovered from primextra treated soils (after 2 weeks of treatment) compared to a residual concentration of 31.54 mg/kg of atrazine which was recovered from atrazine treated soils after 2 weeks of treatment. While the lowest residual concentration of 7.51 mg/kg of atrazine was recovered from atrazine

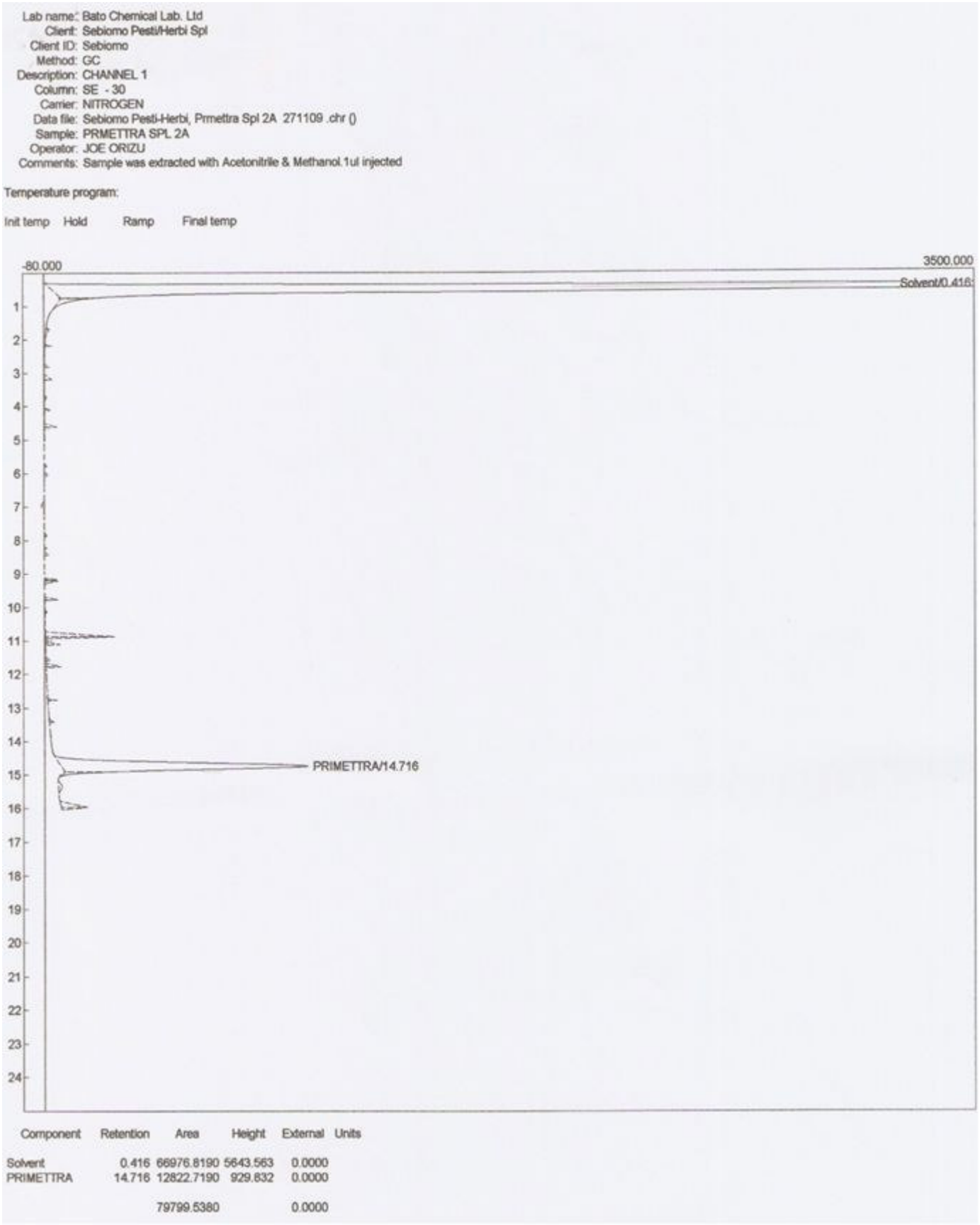

Figure 10. Degradation of primextra after 2 weeks of treatment.

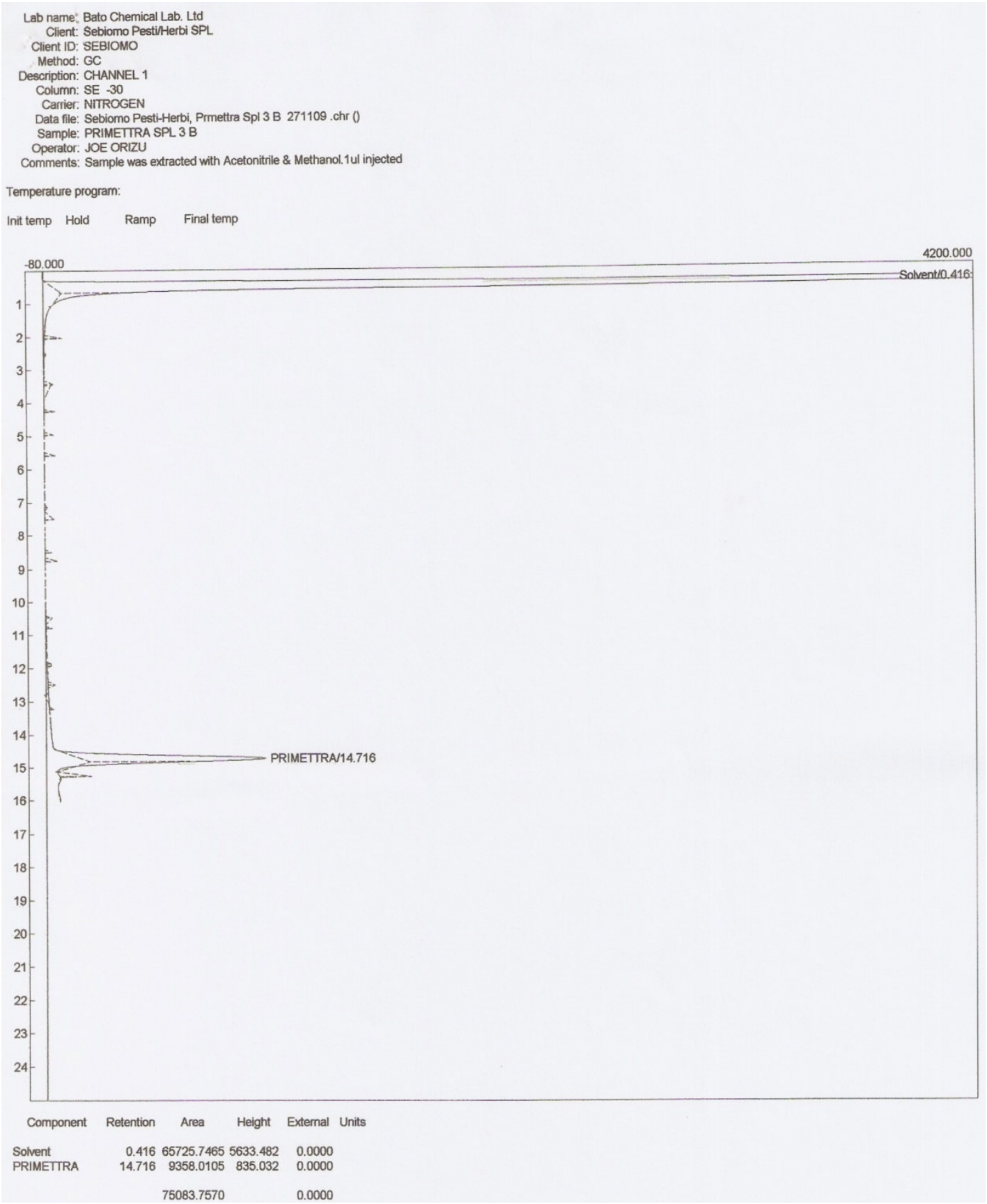

Figure 11. Degradation of primextra after 4 weeks of treatment.

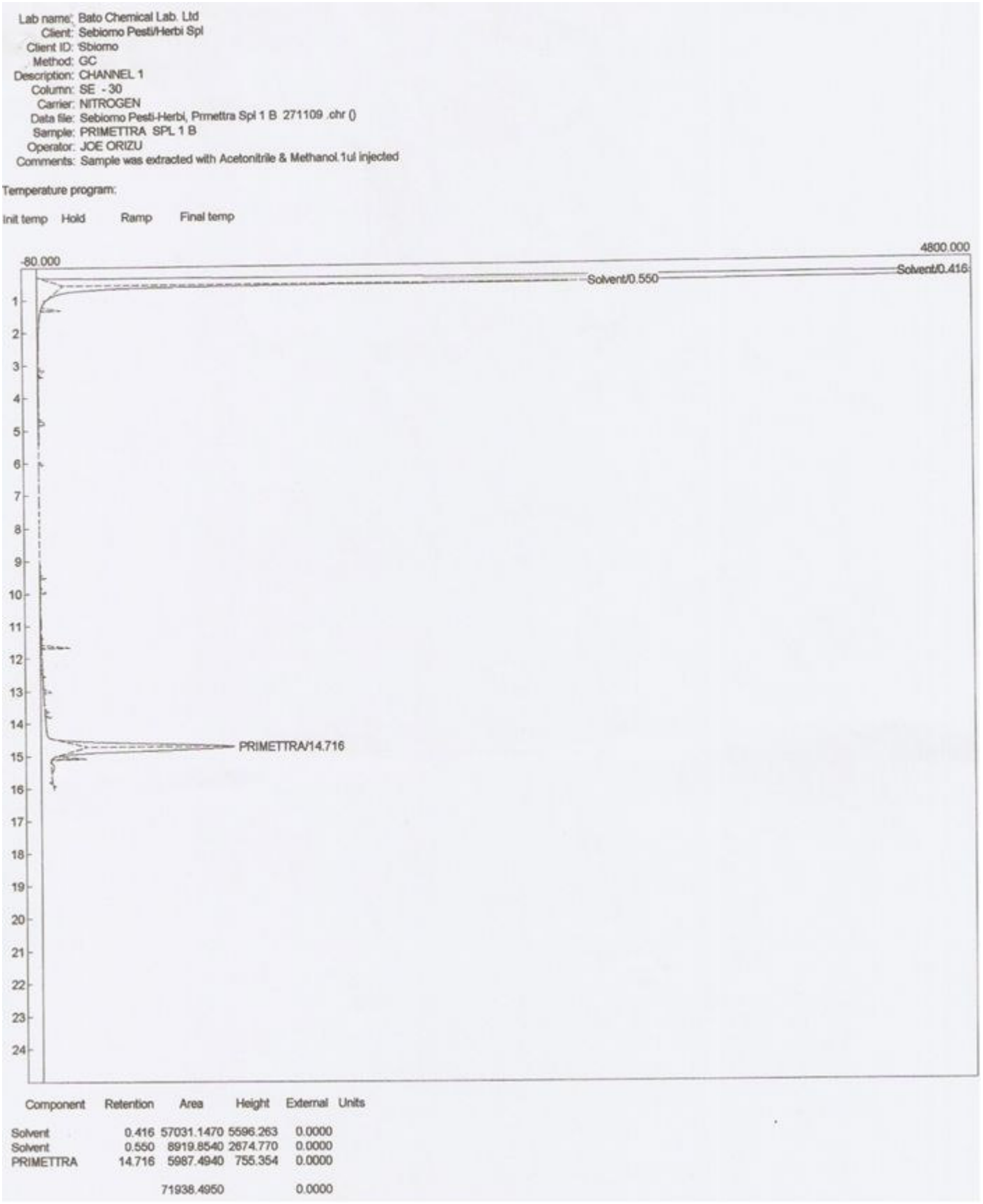

Figure 12. Degradation of primextra after 6 weeks of treatment.

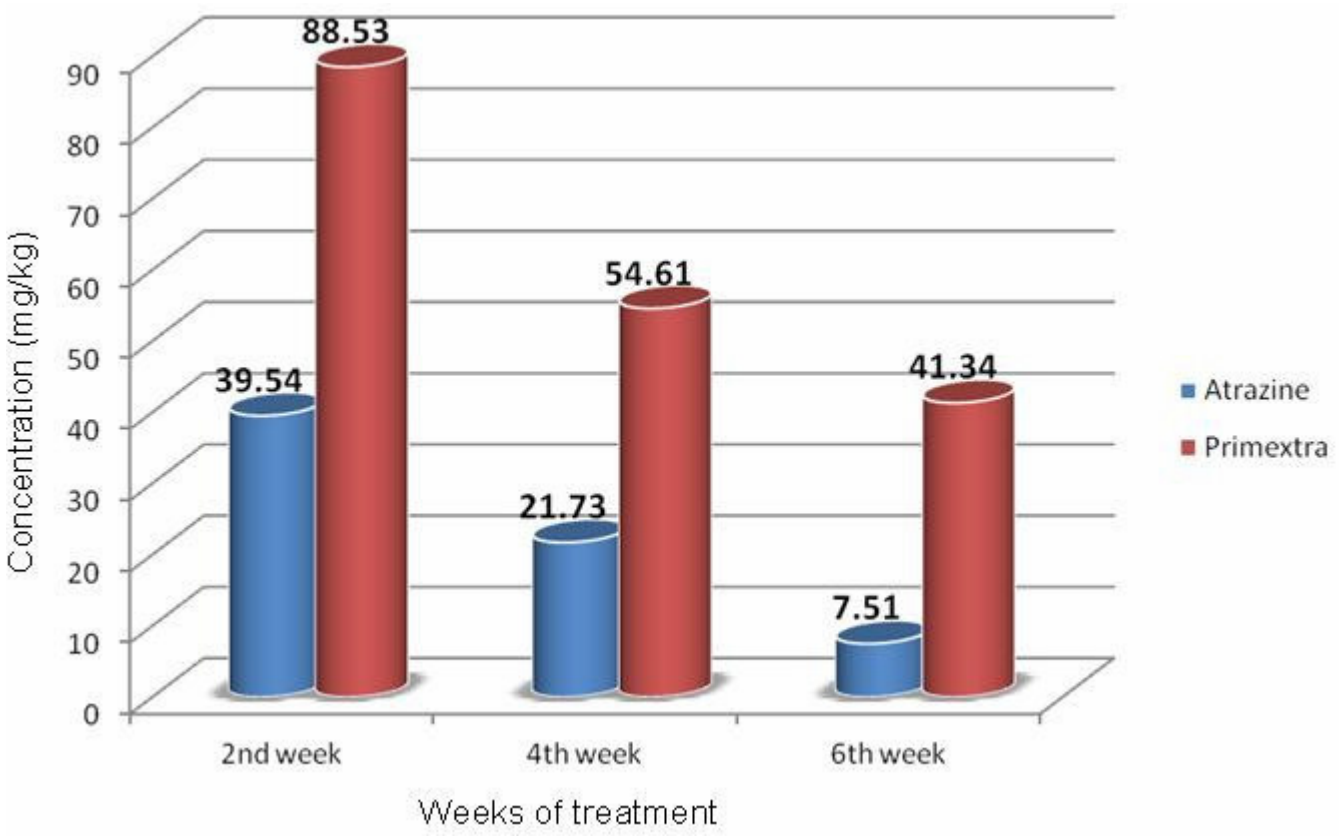

Figure 13. Concentration of residual atrazine and primextra.

treated soils compared to 41.34 mg/kg primextra obtained from primextra treated soils after the sixth week of treatment (Figure 13).

DISCUSSION AND CONCLUSION

Twenty-six bacterial isolates were initially obtained from soils treated with atrazine and primextra, on further experimenting to determine the herbicide utilisation patterns twelve bacterial isolates had the heaviest growth on minimal salt medium (thus producing the highest turbidity) which contained either atrazine or primextra as sole carbon source. The twelve bacterial isolates include *B. licheniformis, P. putida, B. subtilis, A. viscous, P. stutzeri*, *S. marcescens* (atrazine treated soil) and *B. megaterium, A. eutrophs, N. farcinica, B. subtilis, F. aquantile, A. bovis* (primextra treated soil). Ayansina and Oso (2006) isolated *Bacillus* sp and *Pseudomonas* sp from atrazine and primextra treated soils. In this study *Bacillus* sp. were found to be well distributed through soils treated with both atrazine and primextra.

The bacterial isolates used in the determination of the time utilisation of atrazine and primextra showed appreciable growth in culture medium containing either atrazine or primextra as carbon source. The differences observed in the growth of the isolates in the media are indications of the differences between the organisms in tolerating the herbicides. This study showed that the bacterial isolates grew maximally on atrazine and primextra especially *B. subtilis* which completely utilised atrazine and primextra in culture recording the highest viable counts and optical density. The growth profiles have shown that none of the bacterial isolates exhibited lag phase, because the microorganisms used in this study are indigenous to the soil from which they were obtained and consequently have adapted to the herbicides used in treatment. Martins et al. (2007) reported the ability of *Klebsiella pneumonia pneumonia* GC *s. B strain1*, *Pseudomonas alcaligenes*, *Enterobacter aerogenes* GC *s.A* and *K. pneumonia pneumonia* GC *s. B strain 2* to utilise metolachlor with *P. alcaligenes* demonstrating the fastest bacterial growth rate in a selective medium than in a rich culture medium.

The pH values produced by all the bacterial isolates in culture media significantly reduced from 0 h to the 30th day of incubation signifying the production of acidic metabolites. The reduction in pH of the culture fluids in the experimental flasks within the 30 day incubation period further confirmed chemical changes of the atrazine and primextra substrates which must have been precipitated by microbial enzymes. Similar observation

was reported by Atlas and Bartha (1972).

The gradual decrease in area and height of atrazine and primextra detection peaks which resulted in concomitant reduction in concentration of atrazine and primextra from the 2nd to the 6th week indicated increased biodegradation of the herbicides. The larger primextra area and height detection peaks shows the higher persistence of primextra in the soils compared to atrazine. Atrazine persistence in soil is characterised by a moderate, short half-life of 35 to 50 days (Topp, 2001), although faster atrazine mineralisation has been observed in soils which had been exposed to this herbicide as normal agricultural practice (Pussemier et al., 1997; Houot et al., 2000). Moreno et al. (2007) reported that after 16 days of incubation there was, at a maximum, 50% of the added atrazine remaining. Atrazine degradation in soil results from the activity of bacteria which are able to use the atrazine molecule as C or N source (Mandelbaum et al., 1993). Shaner and Henry (2007) reported that there was approximately a 3 to 5 fold difference between the rates of degradation in the rapid assay compared to field dissipation. According to Martins et al. (2007) three bacterial strains (*P. alcaligenes*, *E. aerogenes* GC *s.A* and *K. pneumonia pneumonia* GC *s. B strain 2*.) metabolized metolachlor from agricultural soil. One of them, another strain of *K. pneumonia pneumonia* GC *s. B strain 2*, isolated from conventional plantation soil was able to metabolize 99% of the herbicide. Metolachlor degradation rate yield was far superior to that shown by alachlor, another acetanilide compound (Ferrey et al., 1994).

The ability of bacterial isolates to utilise atrazine and primextra as carbon source has been demonstrated in this work. *B. subtillis* has shown the best ability to utilise atrazine and primextra respectively. Hence the bacterial isolates could be applied to remediate soils polluted with both atrazine and primextra. The progressive biodegradation of atrazine and primextra has also been elucidated in this work.

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