# Biostimulation of Resident Bacteria flora from Freshwater during Biodegradation of Organotin Compounds

**M. L. Ate**
Joseph Sarwuan Tarka University
**Tersagh Ichor** ( smart.ichor@uam.edu.ng )
Joseph Sarwuan Tarka University
**Esther E. Ebah**
Joseph Sarwuan Tarka University
**Q. Amua**
Joseph Sarwuan Tarka University

**Research Article**

**Keywords:** Organotin, Biostimulation, Biodegradation, Tributyltin

**Posted Date:** January 4th, 2023

**DOI:** https://doi.org/10.21203/rs.3.rs-2422330/v1

# Abstract

Water samples obtained from five (5) locations along River Benue were spiked with organotin (TBTCl) in a ratio of 3mM to 1 liter. Two nutrient amendments (NPK fertilizer and poultry dung) were added to stimulate bacterial activity as degradation set up of 3 treatments including a control was monitored for 56 days. Organotin degradation activity was measured by monitoring decrease in TBTCl using X-ray fluorescence spectrometer on day 0, 14, 28, 42 and 56. Test chemical concentrations as well as microbial counts were recorded accordingly. Bacteria which demonstrated consistent growth in the presence of test organotin after 56 days were screened for organotin resistance potential by culturing on MSA medium containing varying concentrations of test organotins; 5 mM, 7 mM, and 10 mM. Organotin utilizing bacteria from fresh water were molecularly characterized and sequenced using pacBio sequencing. Degradation experiment showed an initial increase in microbial growth with counts hitting peak values on the 28th day after which microbial counts continuously dropped until the 56th day. There was a corresponding reduction in the concentration of the test organotin from 350 mg/L on day 0 to an average of 66.78 mg/L on day 56 indicating an average of 81.67% reduction. Organotin degradation by bacteria was significant $p < 0.05$ (One Way Anova) and Pearson correlation analysis showed a significant relationship between decrease in test organotins and bacterial growth ($r = -215$; $p = 1.33$). Metagenomic results showed that 76.27% of bacteria capable of organotin utilization belong to the phylum *Proteobacteria. Azospira spp 36%* was identified as a potent *organotin degrader* while *commamonas spp* 6.7%, *Sedimentibaacter spp 3%, Clostridium bowmanii 2.66% and Serpens flexibilis 2.08%*, demonstrated only mild growth. Rate of degradation of test organotins was faster when stimulated with NPK fertilizer compared to poultry dung. Further research on large scale, on-site biostimulation strategies is highly advocated.


# Introduction

The use of organotins as pesticides and as antifouling agents in boat paints has made these compounds to be common contaminants of marine and freshwater ecosystems [1].

Most problems arising from organotin compounds in the natural environment have resulted from human sources; some organotins have been deliberately introduced, especially those used for agricultural purposes while others have appeared by unintentional introduction, as in discarded wastes. The discharge of inorganic substances which became substrates leading to biogenic organometals has been identified as an indirect anthropogenic source [1].

The toxicity of organotins to organisms belonging to the five taxonomic kingdoms have been reported including endocrine disruption, and mitochondria function impairment [2] [3] [4].

Organotin contamination being one of the most important ecotoxicological problems has attracted interest from many researchers. Findings show that microorganisms play significant role in the degradation of organotins in the marine environment which could become a breakthrough in the

remediation of organotin contaminated sites [3] [5]. The degradation of hydrocarbons by indigenous microorganisms is however a slow process that can take many years if not assisted, hence the addition of nutrients is therefore frequently undertaken to enhance the biodegradation process.

Although NPK fertilizer [5] and Chicken manure [6] have both been used as stimulants in remediation studies, and the findings suggest that the addition of nutrients to an organotin contaminated site may result in enhanced biodegradation when compared against un-mended controls, a comparative study is required to ascertain the best amendment for organotin degradation.

In addition, most researchers of organotin remediation studies have explored mainly the marine ecosystem [7] [6] as a result, literature on organotin degradation using isolates from fresh water ecosystems is rare. Flooding has made our rivers to have continuous contact with household paints, furniture and other materials which are usually painted with organotin formulations. There has also been an increase in the use of pesticides and herbicides on irrigation farms cultivated along river banks and hence eutrophication of our freshwater bodies. This has thus increased concentrations of organotins in our fresh water habitats.

A study of this nature targeted at identifying bacteria that can degrade organotins and identifying suitable stimulants that can aid the degradation process in order to address the recent issues surrounding our fresh water habitats is therefore necessary.

The objectives of the research were to:

1. Monitor growth of bacteria in the presence of TBTCl
2. Compare microbial utilization of organotins in the presence of two biostimulants (NPK fertilizer and poultry dung) to determine the best amendment for organotin degradation.
3. Identify bacteria capable of degrading organotins (TBTCl) from river Benue using conventional culture and molecular approaches

## Materials And Methods

# Sampling Procedures

Water samples were obtained from the surface (10–20 cm) from five locations using sterilized 1 litre capacity plastic bottles. All samples were collected and transported immediately to the laboratory for analysis. Standard procedures were employed to prevent contamination of samples.

# Enumeration of heterotrophic bacteria

The total heterotrophic bacterial (THB) count was determined using the spread plate method on nutrient agar using methods described by APHA [8]. Ten-fold serial dilutions of samples were prepared using one milliliter of water and nine (9) milliliters of physiological saline as diluents. Dilution $10^{-4}$ was used for

inoculation using the pour plate technique in duplicate and the colony forming units of heterotrophs were counted after incubation at 28$^{o}$C for 24hours.

# Bioremediation Setup

Water samples obtained from the 5 sampling points were mixed and separately transferred into bowls based on the three treatments to be used. Each treatment was set up in duplicate.

The bioremediation set up comprised of the following:

Treatment A→ Tributyltin chloride + NPK + water

Treatment B → Tributyltin chloride + Poultry dung + water

Treatment C (Control) → Tributyltin chloride + water

The ratio of the treatment to the water was 3.0mM to 1 Litre, adopted from the works of Ebah *et al.*, [5]. Treatment A received 20 g of NPK, treatment B received 20 g of poultry dung while treatment C which served as a control had no nutrient amendment. The treatments were mixed once weekly to ensure sufficient oxygen supply to microorganisms so as to encourage aerobic activity as suggested by Burton and Turner [9]. Sampling was done on day 0, 14, 28, 42 and 56 respectively.

# Determination of Viable Counts

Viable counts of organotin utilizing bacteria were determined using spread plate method on mineral salt agar (MSA), containing 3 mM TBTCl while the control was plated without TBTCl. Standard methods used here were similar to those described by Ebah *et al.*, [5]

## Screening Of Tbtcl Resistant Bacterial Isolates

Bacterial isolates which grew on MSA with 3.0mM TBTCl were subcultured continuously on MSA with varying concentrations of TBTCl 5 mM, 7 mM and 10 mM. Isolates showing varying range of tolerance to TBTCl (5 mM, and 7 mM) were selected for further characterization.

## Identification Of Tbtcl Resistant Bacterial Isolates

# Biochemical Identification

Tentative identification of TBTCl degrading bacteria was done using the following tests; Grams Reaction, Citrate utilization, Glucose, Indole test, Lactose, Mannitol, Sucrose, Motility, Urease test, Voges –Proskeur test, Oxidase test

# Molecular Analysis

Molecular analysis methodology was based on PCR and metagenomics analysis.

# DNA Extraction from water Samples

DNA extraction was done using the MoBio Powerwater DNA isolation Kit following the MoBio extraction, manufacturer's instructions

## Polymerase Chain Reaction Analysis

The PCR programme was as follows; denaturing step at 95˚C for 3 min, followed by 33 cycles of 30 sec at 95 ˚C for 30 sec at 55 ˚C and extension at 72 ˚C for 1 min, followed by a final extension at 72˚C for 7 min and held at 4˚C. Amplified DNA was presented for sequencing.

# DNA Sequencing

DNA Sequencing was performed by pacBio sequencing technique to determine the nucleotide sequence of all microorganisms present in the water sample. Sequencing of samples was based on the sequel system by pacBio (www.pab.com). Raw threads were processed through the SMRTlink (V9.0) Circular Consensus Sequences (CCS) algorithm to produce highly accurate reads (> Qv40). These highly accurate reads were processed through vsearch (http://github.com/torognes/vsearch) and taxonomic information was determined based on QIMME2

## Results

# Total heterotrophic bacterial count

Total heterotrophic bacterial count in water ranged between $3.7x10^5$cfu/ml and $5.25x10^5$ cfu/ml. Mean THB count across locations was $4.66x10^5$ ± 50384 cfu/ml.

In treatment A which had TBTCl in water treated with NPK, there was an initial increase in microbial growth with counts hitting peak values on the 28th day after which microbial counts continuously dropped until the 56th day. Test chemical was also reduced by 87.77% from 350 mg/L on day 0 to 42.8 mg/L on day 56. See Fig. 2

Treatment B which was amended with poultry dung recorded a lower percentage reduction in test chemical compared to treatment A. 350 mg/l on day 0 was reduced to 99.2 mg/l on day 56, indicating 71.66% reduction. See Fig. 3

Treatment C was neither supplemented with NPK nor poultry dung. The treatment recorded lowest microbial activity with maximum counts on day14 ($4.21x10^4$ cfu/ml). Organotin degradation was also comparatively minimal as initial test organotin concentration of 350 mg/L was only reduced by 63.34% to 128.3 mg/L. See Fig. 4

In all treatments, statistical results showed a significant impact of microbial growth on the test organotins.

After 56 days of degradation, microbes were sub-cultured on mineral salt agar incorporated with 5 mM, 7 mM and 10 mM concentration of organotin to test for microbial resistance and ability to utilize organotin.

As a general observation, bacterial population and resistance significantly reduced with increasing concentration of test organotin as out of the $52.3x10^2$ cfu/ml bacterial population which grew on MSA, only $1.2x10^1$ cfu/ml were able to grow on MSA + 7 mM and none grew on MSA + 10 mM in all the treatments. Results of analysis showed that test chemical had significant impact on the microbial growth $p = 0.04 (< 0.05)$. See Table 1

**Table 1: Total Viable counts of organotin resistant bacteria.**

| Treatment | MSA only | MSA + 5 mM | MSA + 7 mM | MSA + 10mM |
|---|---|---|---|---|
| A | $52.3x10^2$ | $14.6x10^1$ | $1.2x10^1$ | Nil |
| B | $48.1x10^2$ | $16.7x10^1$ | $1.2x10^1$ | Nil |
| C | $33.6x10^2$ | $10.8x10^1$ | $1.0x10^1$ | Nil |

Metagenomic analysis of extracted DNA showed that of the total DNA screened for organotin resistant gene, 76.27% of TBTCl degraders belong to the phylum Proteobacteria. The largest genus associated with TBTCl degradation was found to be *Azospira* (36.4%). Others were *Clostridium 5.69%, Comamonas 6.76%, Sedimentibacter 2.66% and Serpens 2.08% Pseudomonas 2%.* The dominant bacteria species reported for TBTCl degradation was *Azospira spp 36%, while Clostridium bowmanii 2.66%, Serpens flexibilis 2.08%, Sedimentibacter 3% and Comamonas spp 7% demonstrated mild growth.* 40% of bacteria species shown to have TBTCl utilization potential had no resemblance with bacteria spp in the NCBI taxonomic database hence were tagged unknown. See Figs. 5

# Discussion

Microbial population in the river Benue is considerably high. Statistically, total heterotrophic bacteria count varied significantly ($p < 0.05$) at various locations with counts ranging from $3.6x10^5$ cfu/ml to $5.25x10^5$ cfu/ml across the sampling locations. Run off of organic fertilizers applied to support irrigation farming from the banks into the river, and the disposal of cattle and human waste are assumed to have contributed to the high microbial counts in these locations

Bacterial growth pattern observed was typical of a batch or closed system. Organotin concentration consistently decreased with each passing day while microbial abundance initially increased

spontaneously at the initial stages of the experiment, reaching peaks and then taking a downward turn as counts gradually declined until the end of the 56 days.

Continuous growth of freshwater bacteria in the presence of test organotin during the 56 days of experiment is an indication that river Benue also harbors bacteria that are resistant to organotins.

Treatment E which was not amended with any supplement recorded the lowest rate of organotin degradation as only 63.34% of TBTCl was degraded. This agrees with previous findings that degradation is faster and more efficient with the addition of nutritional supplements. Previous findings [5] [7] all point to the fact that even though the environment can clean itself of TBT contamination using indigenous microbes, this process can be slow without intervention. Biostimulants are therefore catalysts of the bioremediation process

Degradation was observed to be faster in treatments amended with NPK as compared to the treatments which were amended with poultry dung. While 87.77% of organotin was degraded in treatment A (which composed of TBTCl in water amended with NPK), only 71.66% was degraded in treatment B which had the same composition but amended with poultry dung.

It has been reported that the addition of some nutrient amendments may stimulate degradation of carbon compounds other than the desired pollutant or stimulate the proliferation of non-hydrocarbon degrading microorganisms at the detriment of the hydrocarbon degrading microorganisms [10]. In addition, research has shown that the presence of an alternative and perhaps preferential carbon source which is easier to metabolize than the contaminant of interest may slow down or inhibit contaminant degradation [11]. Since the poultry dung so employed in this study contained rice husk (a cellulosic waste often applied as base on the floor to prevent direct passage of poultry dung on the floor), it is very likely that this may have made degradation rate of the test chemical to be slower when compared to the use of NPK as a stimulant.

When compared with Treatment C (Control) which had no nutrient amendment, this present research however, concluded that amendment with poultry dung obviously aided organotin degradation only that percentage of chemical degraded, was not at the same level with the treatments amended with NPK fertilizer after 56 days of degradation.

Bacteria species identified in this research to have organotin-degrading capabilities belong to just 6 known phyla from a total of 31 in the NCBI taxonomy database. *Proteobacteria* (76.27%) being the dominant phylum. This agrees with other findings [12], further establishing that members of this phylum can be potent tools for bioremediation studies.

The findings here also show that *Azospira spp*, demonstrated consistent and abundant growth in the presence of different concentrations of organotin. The genus *Pseudomonas* was again identified as an agent for TBTCl degradation agreeing with previous findings [5] [7]. *Sedimentibacter spp, Serpens*

*flexibilis* and *Clostridium bowmanii* however demonstrated only mild growth. 40% of TBTCl degraders have no resemblance in the NCBI database

# Conclusion

Heterotrophic count shows heavy contamination of the river Benue in Makurdi stations especially at the Wurukum and Wadata stations.

The presence of bacteria with demonstrable ability to degrade organotin in river Benue indicates possible contamination of freshwater habitats with organotin compounds.

Continuous reduction in concentration of test organotins indicates that organisms were not only capable of growing in the presence of test chemicals but also utilized them as nutrient source hence reducing their concentrations during the 56 days of experiment

Organotin degradation is faster when carried out in the presence of a suitable biostimulant. And for TBTCl degradation, microbial utilization of test organotin is faster when stimulated with NPK fertilizer than poultry dung.

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# Figures

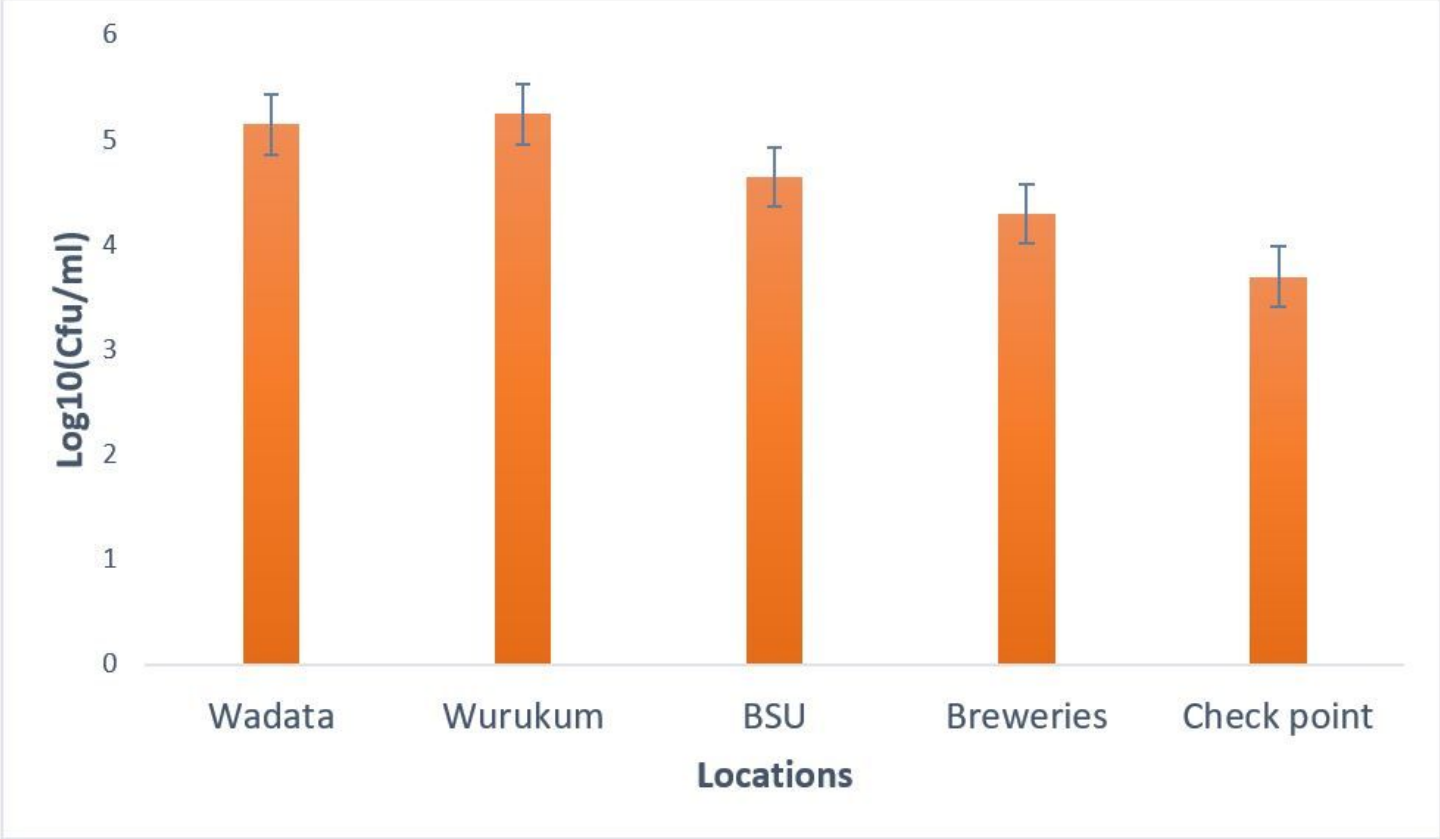


**Figure 1**

**THB count at various locations.**

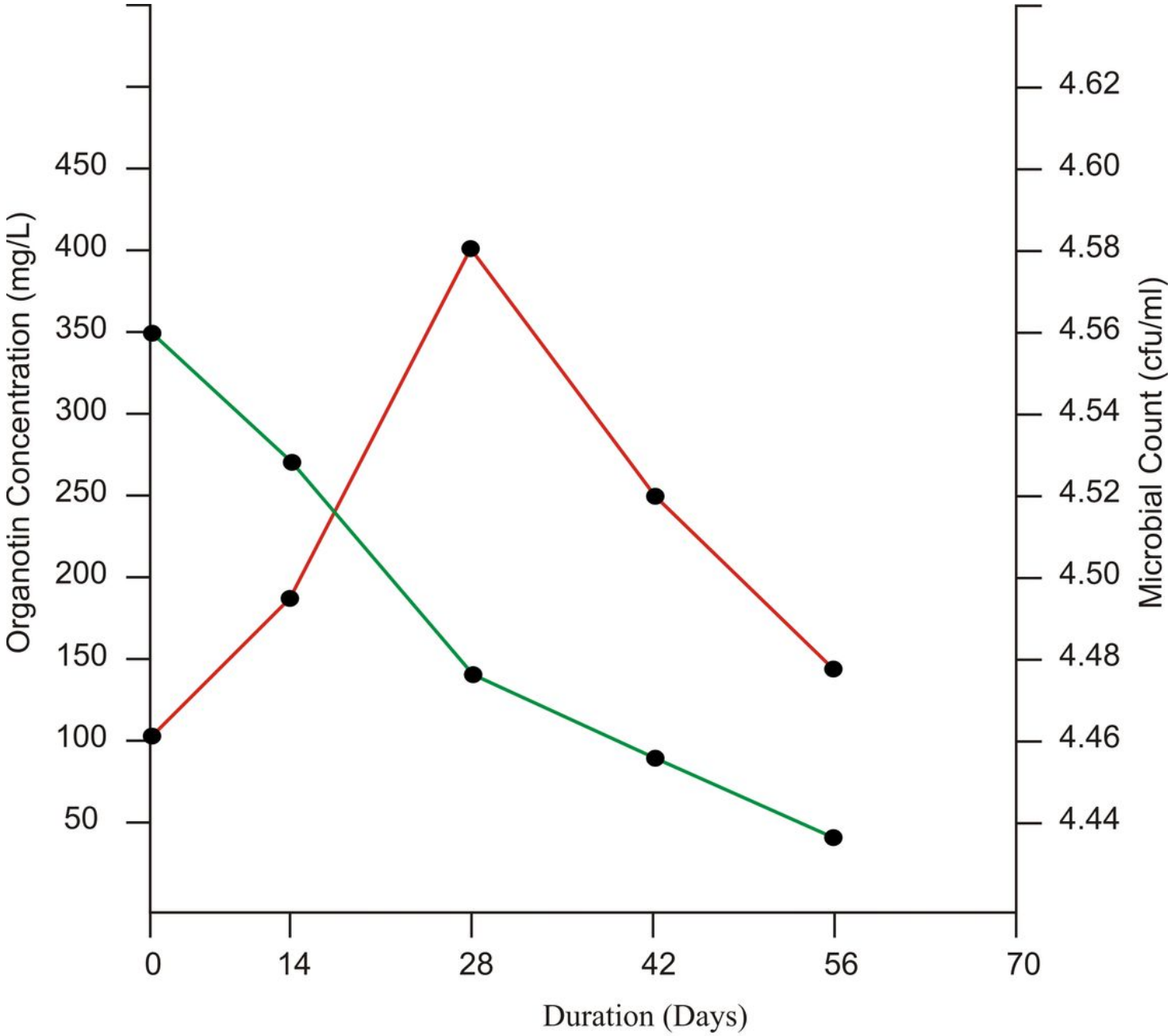


Figure 2: TBTCl in fresh water amended with NPK being utilized by Microbes

## Figure 2

See image above for figure legend.

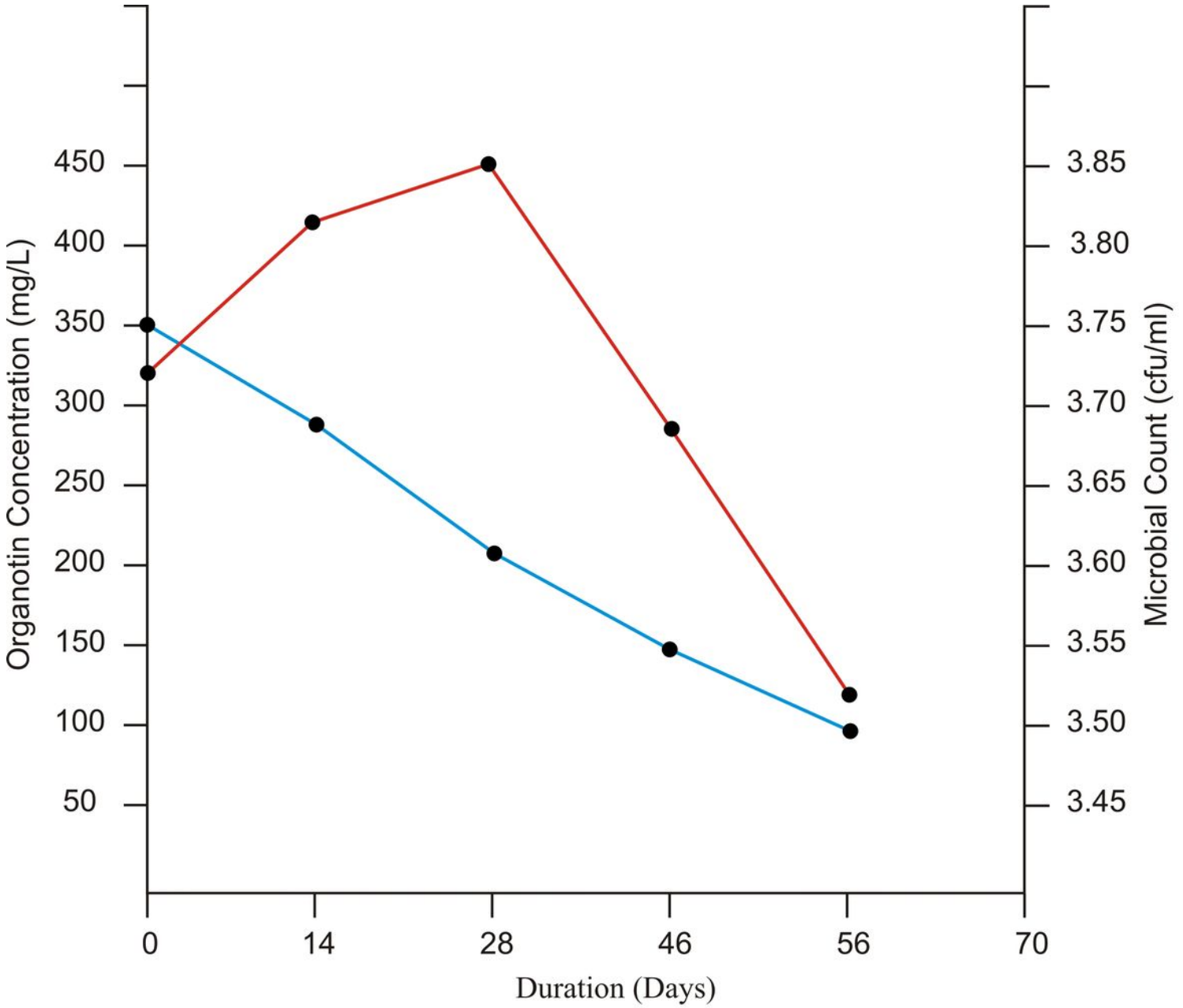


Figure 3: TBTCl in fresh water amended with Poulty dung being utilized by Microbes

## Figure 3

See image above for figure legend.

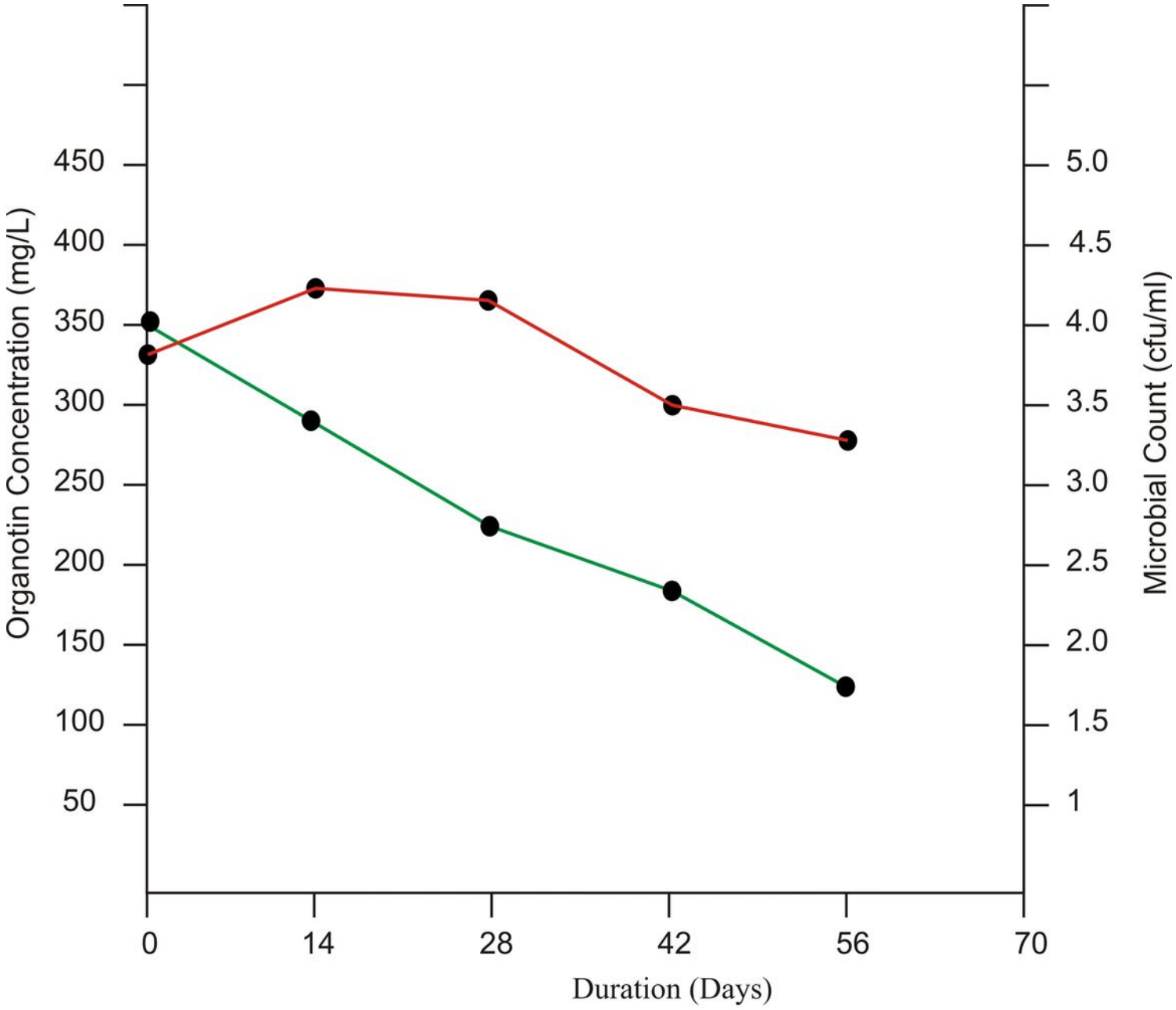


Figure 4: TBTCl in fresh water being utilized by Microbes (Control)

**Figure 4**

See image above for figure legend.

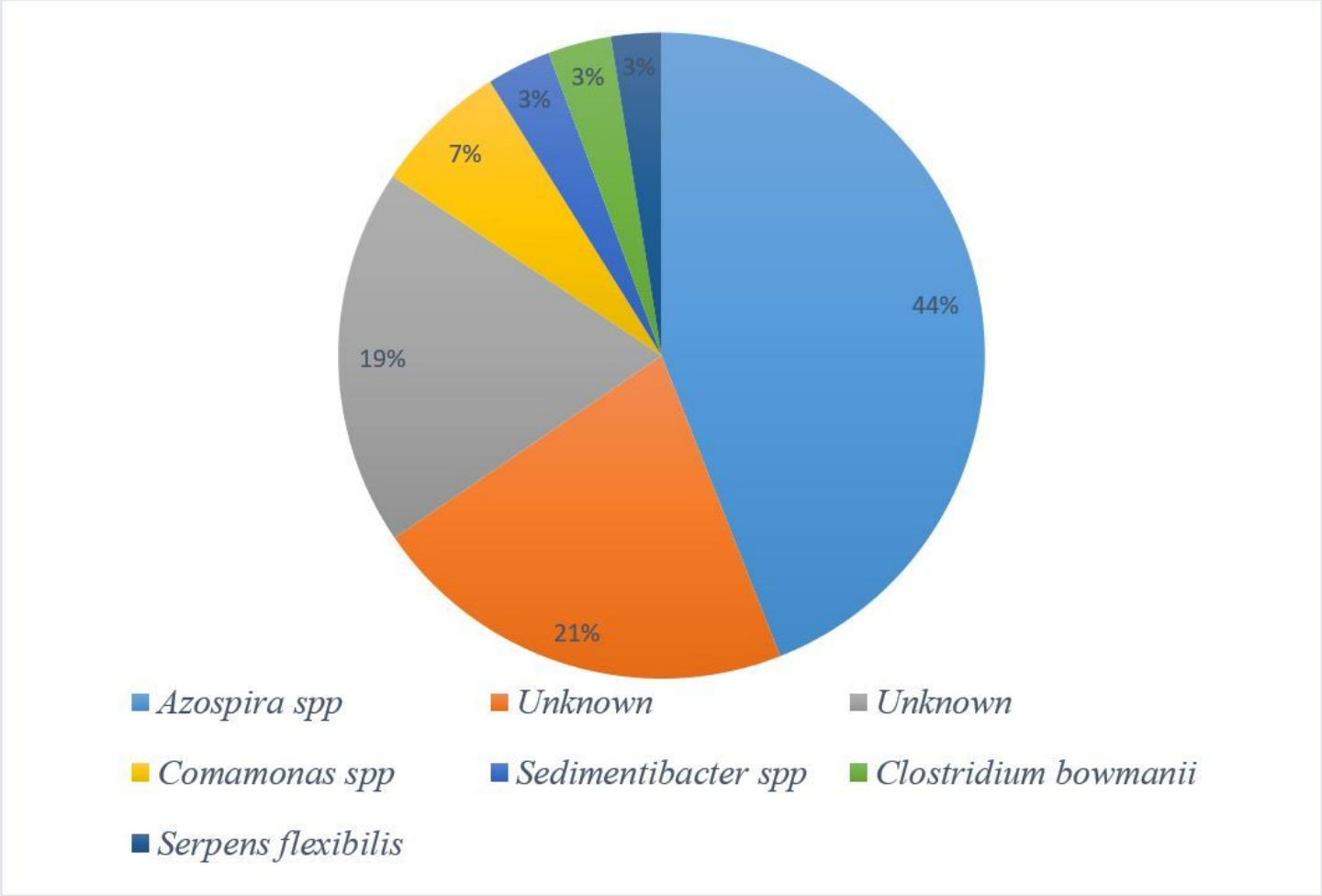


**Figure 5**

Top species classification of TBTCl degrading bacteria.