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Gas Chromatographic Approach to Evaluate the Efficacy of Organotin Degrading Microbes

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Abstract Tributyltin (TBT) is the most toxic chemical ever knowingly introduced into the marine environment, exerting lethal effects on a wide variety of marine organisms. Due to the impact of TBT a necessity exists for the screening of TBT resistant and degrading bacteria for the treatment of contaminated marine sediment. Therefore the approach taken for this study was to isolate and identify new TBT resistant and utilising microbes from marine sediments and soils. In addition to carry out and optimise batch TBT biodegradation assays to measure the TBT degradation rate and the production of the degradation products dibutyltin (DBT) and monobutyltin (MBT) over 21 days. Assays used to screen isolates identified six candidate strains which were characterised and utilised in biodegradation assays. Liquid, from biodegradation assays were analysed on gas chromatography mass spectrometry, which accurately measured and identified tributyltin and differentiated between TBT and the degradation products (DBT and MBT). Results showed a maximum decrease of ≥70% TBT in liquid samples recovered from batch assays and increases in the levels of DBT and MBT the least toxic of the compounds by 32% and 19% respectively, indicating the bacterial breakdown of TBT.

Keywords: *tributyltin, microorganisms, contaminated soils, marine sediments, bioremediation, characterisation, gas chromatography*

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1. Introduction

Tributyltin (TBT) is a synthetic organotin compound characterised by the presence of covalent bonds between three carbon (C) atoms and a tin (Sn) atom. Organotins have the general formula $(n\text{-}C_4H_9)_3Sn\text{-}X$, where X, is an anion normally a halogen ion such as Cl- or a group linked covalently through a heteroatom [1,2,3]. The number of Sn-C bonds and the length of the alkyl chain have an important impact on the physical and chemical properties of organotins. Generally, the toxicity of the organotin is influenced by the alkyl substitutes and the nature of X influences the relative solubility in water and non-polar solvents [2].

Organotins have a wide range of applications including: marine antifouling paints to prevent attachment of marine organisms; biocides in agriculture; stabiliser in plastic and as preservatives for wood, paper, leather and textiles [1,3-8]. The estimated global production of TBT was 50,000 tons per year which brought about the contamination of marine environments largely through leaching of TBT from antifouling paints [1,3,5]. This led to unexpected effects on non-target marine organisms such as oysters causing shell malformations (Arcachon Bay, France) and is associated with imposex in dog-whelk (*Nucella lapillus*) and the periwinkle (*Littorina littorea*) [2,7,8,9]. In addition, tributyltin is also recognised globally as an endocrine disruptor in mammals, high levels of TBT can affect the endocrine glands, upsetting the hormone levels in the pituitary, gonad and thyroid glands [1,6,8,10]. Due to the negative impact of TBT the International Maritime Organization (IMO) called for a global treaty banning the application of TBT bases paints in IMO member countries from the 1st January 2001 and a total prohibition by January 1st 2008 [1,3,8]. Unfortunately, impacted organisms have not recovered and levels in water still exceeds the maximum allowable environmental quality standard of 1.5 ngl^{-1} [11]. This is due to additional pathways such as, dumping of dredged marine and freshwater sediment, industrial discharge, cleaning activates in shipyards and the ongoing illegal use of TBT paints [2,3,6]. TBT bonds to suspended material and is deposited in benthic sediments because of its high specific gravity 1.2kg/l at 20℃ where it can last unaltered for decades, predominantly in anoxic conditions [1].

The most universally used instrument for the identification and determination of organotin compounds is gas chromatography (GC) due to its high resolution, separation ability and sensitivity of detectors available [7,10,12,13,14,15]. However, prior to GC analyses derivatisation is required in order to achieve more volatile compounds through several chemical reactions [7,13,15]. In brief this involves, alkylation using Grignard reagents, as a result of which the alkyl groups of alkylmagnesium chlorides are moved to the ionic organometal. Additionally by hydride generation, the organotin species are converted to a hydride form by sodium tetrahydroborate

($NaBH_4$,) [10,15]. It should be noted that Grignard reagents require extremely anhydrous conditions and non- protic solvents, [10,12,14] and severe interferences can be detected when hydridisation is applied to more complex matrices such as soil and sediment [7,10]. Therefore, ethylation by sodium tetraethylborate ($NaBEt_4$) is a preferred option. The chlorine atoms attached to the butyltin compounds are removed by the reagent and replaced with ethyl groups. Derivatisation can, either take place in an aqueous methanolic environment, or the reagent can be used directly in-situ for a simultaneous derivatisation and extraction, which reduces the number of analytical steps and potential errors [7,10,13].

Biological degradation has been suggested as the main pathway for the removal of TBT from the estuarine and marine sedimentary environment compared to physicochemical methods such as thermal treatment, steam striping and chemical oxidation [2,5,8,16]. Specifically bacterial isolates that are indigenous to the local environment are preferentially selected for bioremediation because of problematic interactions between the non-indigenous bacterial community and physical characteristics of the environmental location such as pH, dissolved oxygen and salinity [5,8]. The major benefits of biological degradation is its low cost compared to physicochemical approaches as it is non-invasive and the ecosystem will remain intact [2,5,8].

The aims of this paper may be summarised as follows: (1) to isolate and screen microorganisms capable of growth on TBT from sediment and soil from Haulbowline harbour and a boatyard on Dinish Island Co. Cork; (2) to carry out and optimise batch TBT biodegradation assays to measure the TBT degradation rate and the production of the degradation products dibutyltin (DBT) and monobutyltin (MBT) over 21 days. To this end an investigation of TBT and degrading compounds DBT and MBT from the biodegradation assays took place which required accurate sample preparation and chemical analyses consisting of derivatisation and liquid-liquid extraction followed by GC-MS analysis for separation, identification and quantification.

2. Materials and Methods

2.1. Chemicals and Reagents

All chemicals and reagents were purchased from Fisher Scientific Ireland unless otherwise stated. Tributyltin chloride (TBT 95%) dibutyltin dichloride (DBT 97%) and butyltin trichloride (MBT, 95%) were purchased from Sigma-Aldrich Ireland. Individual stock solutions of MBT, DBT, and TBT were prepared in methanol and were kept in the dark -4°C. Sodium tetraethylborate (NaBEt4 97%) was used as the derivatisation agent and prepared in tetrahydrofuran (HPLC Grade, Sci-Chem Ireland). Sodium acetate, acetic acid and sodium hydroxide was used to adjust the pH. 2, 2, 4-trimetylpentane (99% Sigma-Aldrich Ireland) was used for extraction.

2.2. Environmental Samples

Soil samples were collected from a traverse pit, located on Dinish Island, in Bear Heaven, approximately 2 km from Castletownbere, Co Cork. The site is the location of a syncrolift, which facilitates the removal of boats from the harbour for painting, shot blasting and general maintenance. Soil samples consisted of sandy fine to medium sub-angular gravel with finer materials in the upper surface including fine sand and a high content of paint flecks. Sediment samples were obtained from Haulbowline, an island 0.339 km2 in Cork harbour (latitude 51°50'43.8" north and longitude 8°16'35.8" west) where long term historical dumping of process slag from the steel works has resulted in environmental pollution. Sediment samples were obtained using an Ekman grab sampler using standard technique [17,18]. Sampling equipment was cleaned with water and rinsed with methanol between stations. Samples were stored on ice in quart glass jars.

2.3. Screening of TBT Resistant Bacterial Isolates

TBT-resistant bacteria from both soil and sediment samples were isolated via ten-fold serial dilution and spread onto nutrient agar (NA) plates. NA plates were prepared according to manufacturer's instruction and supplemented with 50 $mgl^{-1}$of TBTCl. NA plates without TBTCl served as controls. The temperature range for growth was determined by incubating cultures at 4, 12, 16, 20, 24, 28, 32 and 37℃ for 5 days. Resulting bacterial isolates resistant to 50 mgl^{-1} TBTCl were subcultured onto NA plates up to three times to ensure pure cultures were obtained, this was confirmed using Gram staining. Bacteria Isolated from the resistance screening were subsequently inoculated into minimum medium (MM) broths, as described by Abraham [19], containing 50 mgl^{-1} TBTCl. Isolates with the ability to grow in broths at this TBTCl concentration were successively transferred to broths of higher TBTCl in a stepwise manner through 100, 150, 200, 250, 500, 600, 800 and 1000 mgl^{-1}, after 72 hrs. All broths were incubated in an orbital shaker at 28℃, 90 rpm. Both solid and liquid media incubations were carried out in the dark to prevent photochemical degradation. Control samples included non-inoculated medium and inoculated medium with the same volume of methanol that was required to make up the various concentrations of TBT solutions. All incubations were performed in triplicate (n=3) for each experimental condition.

2.4. DNA Extraction, 16S rRNA Amplification and Phylogenetic Analysis

Total genomic DNA was extracted from pure culture isolate samples using Invitrogen™ genomic DNA extraction kit. Extracted DNA was visualised by UV excitation after electrophoresis in 1% agarose gels (w/v) 1× TAE (40 mM Tris-base, 1 mM EDTA, 1.14 mM glacial acetic acid; pH 8) gel containing 1 μg ml^{-1} GelRed™ (Bioscience) with Hyperladder IV (Bioline) as a molecular weight marker.

Bacterial 16S rRNA genes where amplified with the forward primer 27F (5'-GAGTTTGATCCTGGTCAG-3' [20]) and reverse primer 1329R (5'-ACGGGCGGTGTGTRC-3' [21]). All PCR reactions (50 μl) were carried out using the GoTaq™ G2 (Promega) kit and contained; 50mM Tris-

HCl (pH 9.0); 50mM NaCl; 5mM MgC_{l2}; 200µM each of dNTP (dATP, dGTP, dCTP, dTTP), 12.5 pmol of each primer, 200 ng template DNA and 1.25 U Taq DNA polymerase. A 'touchdown' PCR was used to specifically amplify bacterial 16S rRNA genes, with the following conditions: denaturation at 95℃ for 10 min, followed by 10 cycles of 94℃ for 60 s, annealing at 63℃ for 60 s and extension at 72℃ for 120s, where the annealing temperature was reduced by 1℃ for each cycle; this was followed by 20 cycles of denaturation at 94℃ for 60 s, annealing at 52℃ for 60 s and extension at 72℃ for 120 s, which were in turn followed by a 10-min final extension at 72℃. Negative controls containing no DNA were used, while *E.coli* DNA was used as a positive control. PCR products where visualised as described above. Sequencing of purified PCR products was performed using the cycle sequencing technology on ABI 3730XL sequencing machines (MWG BIOTECH, Milton Keynes, UK). The resultant sequence data were compared to nucleotide databases using basic local alignment search tools BLASTn and the Ribosomal Database Project II (RDP) (8.1) as previously described by Olsen [22] and Altschul [23]. Sequences were then aligned using ClustalX [24]. The phylogenetic inference package Geneious© (Biomatters, Ltd) was used for all phylogenetic analysis, using the Kimura-2 parameter correction [25]. The resulting partial 16S rRNA gene sequences generated by this study were deposited in the GenBank database under the accession numbers KX881904–KX881909.

2.5. Bioremediation Batch Assays

Bacterial strains C3, C6, C7, C18, C21 and C22 were inoculated using aseptic techniques into 10 ml of MM supplemented with 100 mgl^{-1} of TBT in triplicate (n=3). All broths were incubated in an orbital shaker at 28℃, 90 rpm for a period of 21 days in the dark to prevent photochemical degradation. Liquid samples were withdrawn aseptically on days 0, 14 and 21, followed by TBT extraction and derivatisation. Five point calibration curves were established for each analyte of interest using a 10, 20, 30, 40, and 50 mgl^{-1} mix of TBT, DBT and MBT prepared in methanol (99.7%). Quantitative and qualitative analysis was carried out on a Varian 450-GC, 220-MS to determine the presence of TBT intermediates MBT and DBT, in TBT supplemented broths which contained microbial growth, as described below. All sampling was carried out in triplicate (n=3).

2.6. Ethylation with $NaBEt_4$

Ethylation was carried out on 2 ml broth samples and standards as follows; 1 ml of acetate buffer (82 g/l sodium acetate in deionised H_2O, pH 4.7) followed by the addition of 1 ml of sodium tetraethylborate solution ranging from 1-5 % to investigate the optimal conditions (1 g of $NaBEt_4$ dissolved in 100 ml tetrahydrofuran) at 3 min intervals for 12 min, total addition 4 ml under continuous stirring [26]. After sample derivatisation pH was adjusted to 12.5 dropwise with 1 M sodium hydroxide solution to decompose boroxin side reactions, ethylated butyltin compounds were not affected [27]. Samples were then extracted with 5 ml of 2, 2, 4-trimetylpentane resulting in separation into two phases. The clear upper layer was retained for GC-MS analysis.

2.7. GC-MS Operating Conditions

Analysis of TBT, DBT, and MBT in liquid samples from bioremediation batch assays were performed in a Varian 450-GC, 220-MS system, with CombiPAL auto sampler (Varian Inc., Walnut Creek, CA). Separation was carried out on a capillary column coated with 5% biphenyl and 95% dimethylpolysiloxane (30 m length x 0.25 mm internal diameter x 0.25µm film thickness). The injector volume was 1 µL in a splitless injection mode and injector temperature was maintained at 280℃. Helium was employed as the carrier gas, at a flow rate of 1 ml min^{-1}. The temperature program was as follows: 50℃ (1 min) to 300℃ (4 min) at a rate of 10℃ min^{-1}. The MS-detector was operated in full scan mode in the range of 40-650m/z at a temperature of 280℃. Peak areas were used for quantitative calculation using the external standard method, peaks in the chromatograms were assigned to individual ethyl– derivatives of organotin compounds on the basis of retention time and identified using the comparison of their ion trap mass spectra with the standard MS spectra from the NIST library.

3. Results and Discussion

3.1. Bacterial Isolation and TBT Resistant Screening

Bacterial strains were isolated from soil and sediment samples collected from a traverser pit located on Dinish Island and Haulbowline, an Island in Cork harbour. It was decided to screen for tributyltin resistant and degrading bacteria at this site because of reported high concentration of TBT and other pollutants in the area. The sediment samples were taken near the coastline where industry contributed to heavy environmental pollution and the soil samples were taken from the boat yard where repairs and cleaning of marine craft took place. Fifty four bacterial strains were isolated from the soil and sediment samples sites.

The preliminary screening for TBT resistant isolate revealed 37 of the isolates were not resistant to TBT, while 17 bacterial isolates had the ability to grow on NA plates supplemented with TBT ranging from ≥50 mgl^{-1} to ≥1000 mgl^{-1}. From these 17 isolates, 7 were capable of growth on TBT concentrations ≥50 mgl^{-1}, 4 at concentrations ≥100 mgl^{-1}, and 6 at concentrations ≥1000 mgl^{-1}. These six isolates, C3, C6, C7, C18, C21 and C22, were the isolates used in the bioremediation assays and further characterised. Bacterial isolates C3, C7, C18 and C21 were isolated from several soil samples taken from the traverser pit and isolates C6 and C22 came from sediment samples taken from Cork harbour. Interestingly, the soil samples provided a higher percentage of resistant bacteria, possibly due to the present of TBT in paint flakes that bound to the soil particles due to cleaning activities.

3.2. Primary Screening for TBT Utilising Strains

Despite the severe impact of TBT on living organisms, several studies have reported the existence of TBT resistant bacteria isolated from various environments which included the following genera, *Klebsiella*, *Alcaligenes*, *Aeromonas*, *Enterobacter*, *Bacillus*, *Pseudomonas*, and *Citrobacter* [5,8,28-33]. Although it has been demonstrated that bacteria are able to degrade TBT into less toxic DBT and MBT and inorganic tin the removal efficiency and degradation rates vary from bacterial genera and also between identical species or strains [31,34].

Furthermore, there is no standard resistance level generically used to determine TBT resistance. According to Cooney TBT resistant bacteria are defined as being able to grow in the presence of an amount of TBT which kills 90%, or more of the cultivable population [31,35]. Conversely, Cooney also stated since several factors such as chemical, biological and physical conditions can influence TBT toxicity this definition should be adjusted to each individual situation [31,35]. To this end previous published work has defined TBT resistant bacteria, as bacteria able to cultivate in various media and various concentrations of TBT 8.4µm, 10µm, 50µM, 100µM, 2mM, 3mM, 4.5mM, 7mM, 10mM and 50 mgl^{-1} [5,18,28,30,31,33,34,36]. In fact several mechanisms were suggested for the survival and resistance of bacteria in the presence of TBT. In brief (1) efflux pump (efflux of TBT outside the isolated cell), (2) adsorption, (3) biosorption, (4) bioaccumulation, (5) detoxification (6) dealkylation and metabolic utilisation of TBT as sole carbon source [5,30,31,34].The mechanisms of interest in this study, is dealkylation of tributyltin ($C_{12}H_{27}Sn^{+}$) by enzyme activity to produce DBT ($C_8H_{18}Sn^{2+}$), MBT ($C_4H_9Sn^{3+}$) and eventually inorganic tin (Sn^{4+}), becoming increasingly less toxic in the process (Figure 1) [1,37].

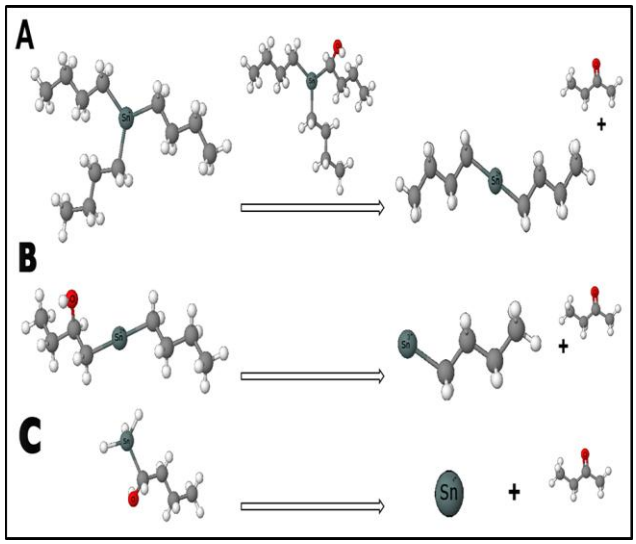

Figure 1. Degradation of TBT by sequential dealkylation. (A) TBT ($C_{12}H_{27}Sn^{+}$) forms β -hydroxybutyl-dibutyltin ($C_{12}H_{27}OSn^{+}$) through Dioxygenases (incorporating an oxygen atom into the substrate through enzyme activity), to DBT ($C_8H_{18}Sn^{2+}$) and methyl ethyl ketone. B, β – hydroxybutyl-butyltin ($C_8H_{18}OSn^{2+}$) degrades to MBT ($C_4H_9Sn^{3+}$) and methyl ethyl ketone. C, β-hydroxybutyl ($C_4H_{12}OSn^{3+}$) to tin (Sn^{4+}) and methyl ethyl ketone

As such, the 17 resistant isolates were investigated to determine their ability to utilise TBT as a carbon source. The TBT growth assays in MM broths indicated that the six isolates, that had the highest resistance levels as recorded in preliminary screening, C3, C6, C7, C18, C21 and C22, also had the ability to grow in TBT supplemented MM broths at concentrations ≥1000 mgl^{-1}. The control samples of MM broths and methanol did not support the growth of the isolates, suggesting that the isolates have the ability to utilise TBTCl as a sole carbon source. The 6 successful candidate isolates were selected for characterisation and TBT bioremediation batch assays.

3.3. Bacterial Characterisation

It has been reported that TBT is toxic to both gram negative and gram positive bacteria. However studies have revealed, that gram negative species are mainly responsible for TBT degradation in environmental samples [8,31]. Similarly, in this study, all 6 TBT degrading isolates were gram-negative with Blastn searches revealing these six belong to the Proteobacteria, a phylum which contains several genera noted for their bioremediation of environmentally polluting compounds, including polycyclic aromatic hydrocarbon, chlorinated-solvents, heavy metals and pesticides [38,39]. Isolates C6, C18, C21 and C3 are members of the class Beta-Proteobacteria, genus Achromobacter while Isolates C7 and C22 are members of the class Gamma -Proteobacteria, genus Enterobacteriales (Figure 2). In addition, a link between salt tolerance and TBT resistance has been previously shown increased concentrations of NaCl can reduce the toxicity of TBT due to the osmotic response of organisms or due to the Na+ and Cl– moieties [30]. However, salt tolerance tests conducted on the six isolates revealed the optimum growth ranges between 4–6 % NaCl (data not shown), slightly outside the definition of salt tolerant strains (6.5% NaCl).

3.4. Secondary Screening of Isolates by GC-MS

The bacterial isolates C3, C6, C7, C18, C21 and C22 that had the ability to utilise TBT as a sole carbon source were selected for secondary screening, to establish if the bacteria strains can degrade TBT through sequential dealkylation (Figure 1) were by TBT is degrade in to its less toxic compounds DBT and MBT over a period of 21 days.

The chemical analyses consisted of the following: extraction, derivatisation, clean up, separation, identification and quantification. The challenges posed and the main critical steps in organotin analysis are the extraction and derivatisation of MBT and DBT, since low yields can occur at this stage and lead to an underestimation of their content in the samples due to their more ionic nature. The gas-chromatographic conditions showed reproducible separation of the organotin compounds after derivatisation was optimised (optimum pH 4.7 and 2% NaBEt4). The compounds were extracted with iso-octane and injected into the GC in splitless mode to maximise sensitivity, the column temperature increased slowly to enable good separation of the eluting compounds [13].

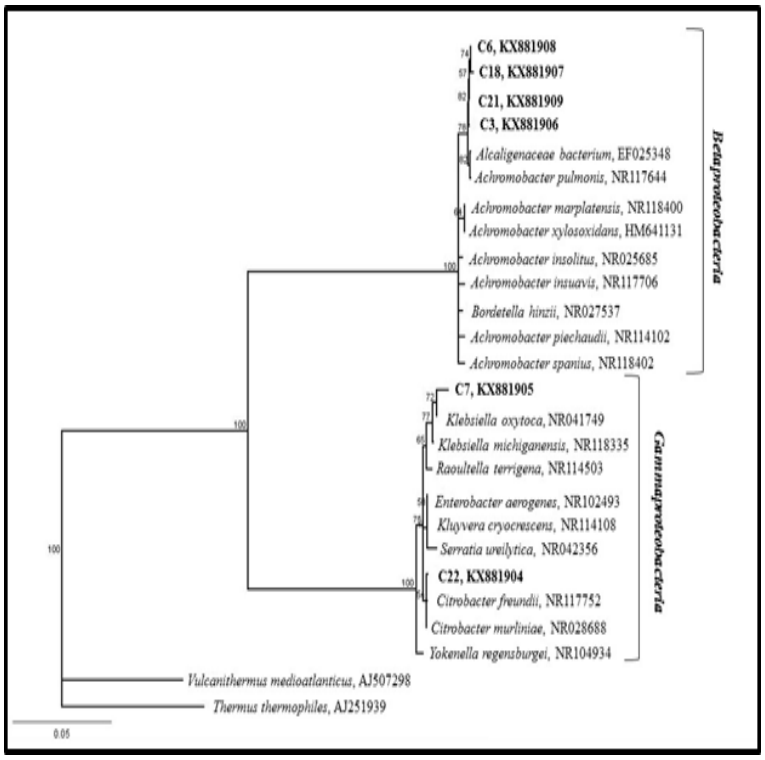

Figure 2. Phylogeny of bacterial sequences based on the Kimura-2 model and the neighbour-joining method of Saito and Nei (1987). Bootstrap replicates (out of a total of 100 replicate samplings) that supported the branching order are shown at relevant nodes. The scale bar represents 1 nucleotide substitution per 100 sequence positions

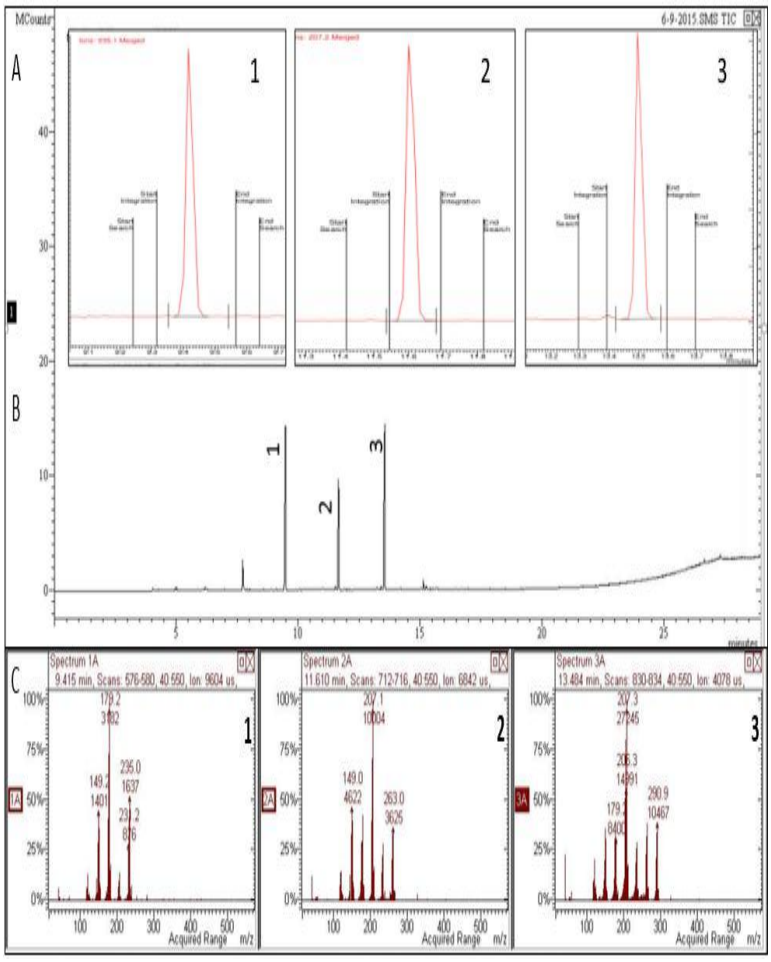

Figure 3. Identification of MBT (peak 1), DBT (peak 2) and TBT (peak 3) of known samples (**B**) chromatographic separation and (**C**) mass spectrum of each compound). Showing unique fragmentation pattern of eluted organotins (C 1-3) in order to identify according to its mass to charge ratio (m/z) and determine retention times

The compounds elute according to their boiling point, and the elution sequence can be predicted by calculating the total number of carbon atoms after derivatisation. The elution sequence of the organotin compounds are MBT (10 C atoms), DBT (12 C atoms) and TBT (14 C atoms). The ethylation by sodium tetraethylborate showed reasonable derivatisation effectiveness for organotin compounds and produced three distinct (ethyl –derivative) peaks, of TBT, DBT and MBT (Figure 3).

3.4.1. Qualitative Analysis of TBT and Degradation Products

An analytical procedure for separation and identification of organotin compounds was optimised on standard samples and then compared to liquid samples from bioremediation batch assays. Qualitative analysis was carried out comparing peak retention time (RT) of standard solutions against peak retention times of liquid broths (Table 1). In addition, a comparison was made of the ion trap mass spectra of the liquid broth samples against the standard ion trap mass spectra, from the reference library National Institute of Standards and Technology (NIST) Mass Spectrometry Library, (Edition 2008). A general guide from the NIST Standard Reference Database, 900 or greater is an excellent match; 800–900, a good match; 700–800, a fair match. Less than 600 is a very poor match [40]. Results show the characteristic ions for the three ethyl- derivatives and gives an average match factor of 840, 902 and 860 further confirming degradation of TBT by bacterial isolates tested (Table 1, Figure 4).

Table 1. Organotin species characteristics and associated retention times mean ± standard deviation (σ)

Peak	Cpd	Average match factor	Characteristic Ions *(m/z)*	Standards Retention time (min)	Samples Average Retention time (min)
1	MBT	860	179, 149, 235	9.434	9.422 ± σ 0.00027
2	DBT	902	149, 207, 263	11.614	11.60 ± σ 0.00030
3	TBT	840	177, 207, 235	13.497	13.515 ± σ 0.00024

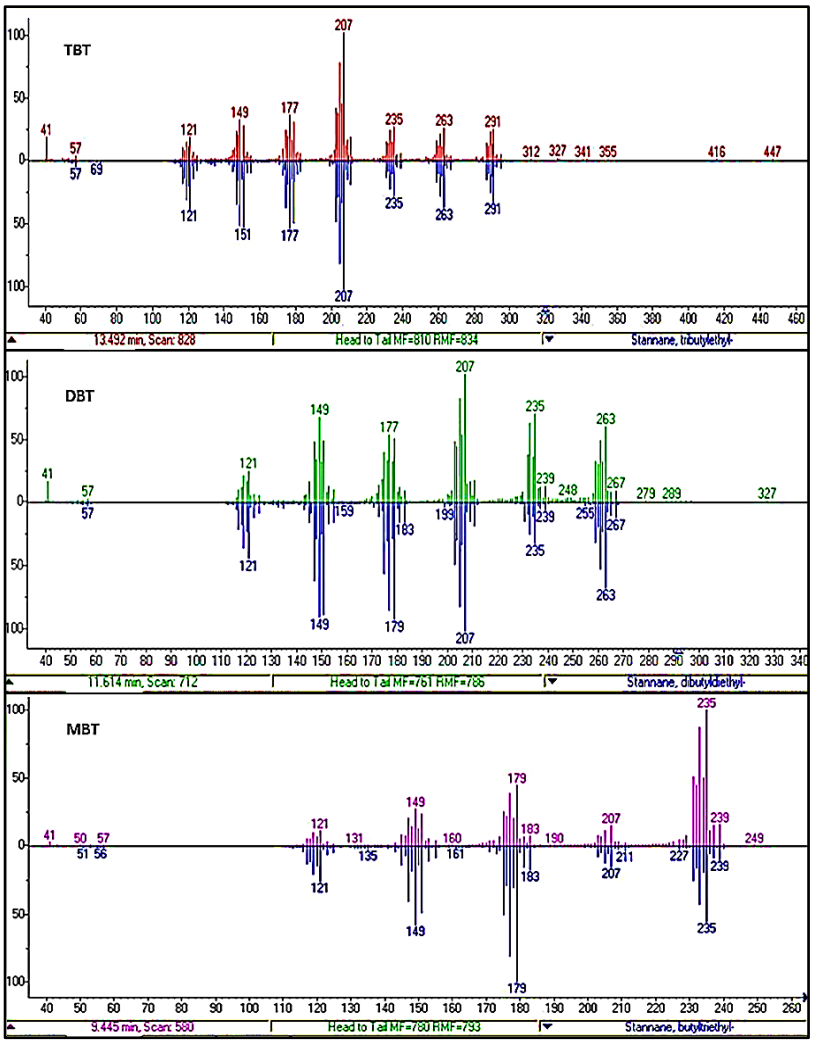

Figure 4. (Colour online) Mass Spectrums fragmentation patterns (finger prints) of ethyl–derivatives, TBT, DBT and MBT respectively from sample isolate C7 (KX881905) on day 21 accurately showing the mass measurement of the molecular ion (M+) peak for, TBT at m/z 291, DBT at m/z 263 and for MBT at m/z 235 and further the base peak at m/z 207 for TBT and DBT and m/z 235 for MBT which confirms all three identities based on its mass spectrometric properties. A comparison was also made of the fragmentation patterns found in sample isolate C7 (KX881905) against the underlying NIST mass spectrometry library spectrums which identified TBT and degrading products DBT and MBT with good to excellent match factors [40]. Additional from the mass spectrum the characteristic ions for TBT and degrading products DBT and MBT can be seen and are listed in Table 1. (y-axis, relative abundance, x-axis, m/z)

3.4.2. Quantitative Analysis

There are three basic quantitative analytical methods that can be used; the internal standard method, the external standard method and the normalisation method. These methods have various and different attributes. Quantitative analysis was carried out on the (ethyl –derivatives) of TBT, DBT and MBT measuring peak area (external standard method) from the 3 distinctive peak in the chromatographs. Five point calibration curves were generated using triplicate injections of 1µl of TBT, DBT and MBT standards which had good linearity with r2 values of 0.992245 for MBT, 0.991919 for DBT and 0.991232 for TBT. Additionally the following limits of quantification (LOQ) were obtained for TBT 0.9 mgl^{-1}, DBT, 1.3 mgl^{-1} and MBT 1.6 mgl^{-1}. Batch bioremediation assays containing bacterial isolates showed that enhanced TBT degradation in controlled conditions with a minimum reduction of ≥22% and a maximum reduction of ≥70% of total TBT concentration over 21 days. The bacterial isolates also exhibited a breakdown of TBT through sequential dealkylation showing an increased percentage of the degradation products. Results show a minimum and maximum DBT increase of ≥11% and ≥33%, respectively (Figure 5 and Figure 6). Furthermore, the bacterial isolates increased the levels of MBT the least toxic of the compounds by a minimum of ≥12% and maximum of ≥20%. In particular, the *Gamma-Proteobacteria*-like isolates, C7 (*Klebsiella*-like) and C22 (*Citrobacter*-like), showed the highest TBT removal rates (≥ 69%) over the 21 days (Figure 5 and Figure 6). Previously studies, have reported that both *Citrobacter braakii* [30] and *Klebsiella pneumoniae* strain SD9 [5] represent "high resistance TBT degraders". As such, isolates C7 (KX881905) and C22 (KX881904) degraded a 100 mgl^{-1} TBT spiked sample to 30 mgl^{-1} and showed to have a minimum and maximum efficiency degrading rate of ≥6% and ≥49 % compared to the other isolate in the group and therefore may represent two new previously unstudied high resistance TBT degrader from these environmental samples based upon TBT degradation ability.

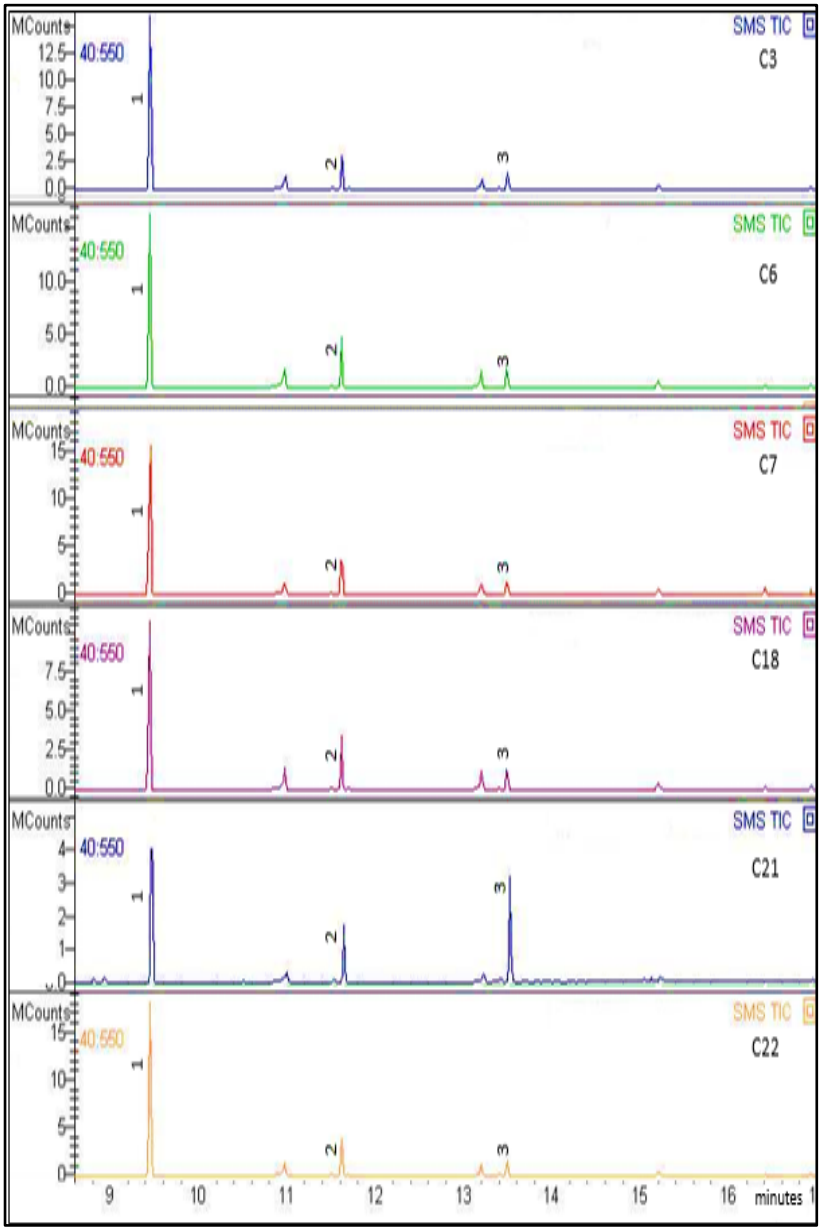

Figure 5. (Colour online) Secondary screening of isolates, from biodegradation assays showing chromatographic separation of organotin species and further demonstrating TBT (peak 3) degradation to DBT (peak 2) and MBT (peak 1) on day 21, detector response (y-axis) against retention time (x-axis)

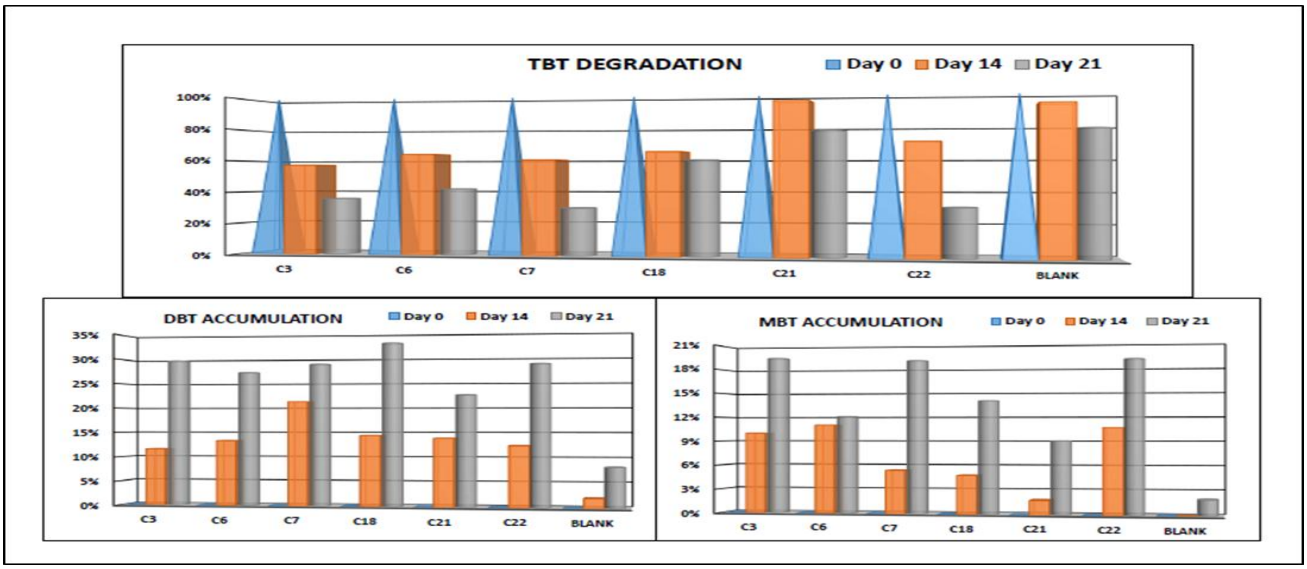

Figure 6. (Colour online) (Pyramid Day 0, Box Day 14, Cylinder Day 21) (Top), percentage TBT removal by isolates C3, C6, C7, C18, C21, and C22 over 21 days in cultivated MM broths (initial TBT concentration 100 mgl^{-1}), (Left) DBT (initial concentration 0 mgl^{-1}) accumulation over 21 days, (Right) MBT (initial concentration 0 mgl^{-1}) accumulation over 21 days

4. Conclusion

Over the last ten years it has been recommended that biological degradation is the key pathway for the removal of TBT from the environment. The present investigation demonstrates that bacterial isolates C3, C6, C7, C18, C21 and C22 selected from this study are resistant to high concentrations of TBT and can utilise TBT, as a sole carbon source. Quantitative and qualitative analysis of liquid broths containing the selected isolates and TBT took place over 21 days.

Chromatographic analysis further confirmed degradation of TBT by showing the presence of DBT and MBT peaks and by comparison of ion trap mass spectra's from the NIST library. In particular, isolates C7 (*Klebsiella*-like) and C22 (*Citrobacter*-like), showed the greatest potential for utilisation in a bioremediation trial of TBT contaminated sites. Nonetheless, for a real practical application of the isolates, the effects of other environmental factors such as aeration, temperature, pH, and indigenous bacterial community need to be investigated further. Arising from this a bench scale, remediation of spiked sediment is currently ongoing for further evaluation of these bacterial strains. Bacteria isolates have an important role in biogeochemical transformations of toxic chemicals acting as natural decontamination agents. To this end the identification of a microbial strain or group of strains that could reduce or eliminate the potential risk posed by TBT would be of great value to both the marine and waste industries.

Conflict of Interests

The authors declare they have no conflict of interests.

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