University of Strathclyde
Department of Civil Engineering

Enhancement of Tributyltin Biodegradation for Sustainable Remediation of the Environment

by

Arthit Sakultantimetha

A thesis presented in fulfilment of the requirements for the degree of Doctor of Philosophy

2010

Declaration of Author's Rights

This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

Signed: *Date:*

Acknowledgements

I wish to express my gratitude towards my supervisor, Dr. Helen Keenan for her kind assistance and suggestions. I also wish to express my appreciation to Dr. Tara Beattie for her valuable support and constructive criticism. I express my gratitude to Dr. Arinthip Thamchaipenet, Dr. Kannika Duangmal and Dr. Thomas Aspray for their support in experimental facilities. Special thanks to Laura McGregor for her generous time on the GC-MS and Olga Cavoura for her valuable support in this thesis.

I would like to acknowledge the Greensolutions (Scotland) Limited for their financial support on this project and British Waterways Scotland for the sediment samples used in the experiments. I also acknowledged the Department of Chemistry, Kasetsart University, Bangkok, Thailand and Department of Civil Engineering, University of Strathclyde, Glasgow, UK for research facilities.

I would like to dedicate my indebtedness to Dr. Sornnarin Bangkedphol for his immense assistance, patience, understanding, encouragement and attention throughout the duration of my study.

I wish to express my high appreciation and deepest gratitude to my parents, grandparents and Sornnarin's mother for inspiration, encouragement, and the financial assistance which made my graduate studies possible. This thesis could not be achieved without all of these supporters.

Arthit Sakultantimetha

October, 2010

Abstract

Tributyltin (TBT) is a compound released into aquatic environments through anthropogenic activity, which persists in the surrounding water and sediment. Generally, natural attenuation by intrinsic microorganisms can reduce levels of TBT, but the process is slow, and persistence of this xenobiotic substance can cause long-term negative impacts in the environment. This study experimentally examines the isolation of bioaugmentation species, the optimisation of biostimulation, and finally, employs techniques to enhance the removal of TBT from contaminated sediment.

Four strains of bacteria were successfully isolated from TBT-contaminated sediment collected from Bowling Basin in Glasgow, Scotland. This was achieved using a medium of glycerol containing tributyltin chloride (TBTCl) at a concentration of 130μM. Each isolated bacteria was then exposed to an initial concentration of 1μM TBTCl and levels of degradation were investigated. It was found that TBT was degraded at 3.9, 8.29, 11.43 and 16.87% by, respectively, *Alcaligenes faecalis*, *Citrobacter braakii*, *Pseudomonas fluorescens* and *Enterobacter cloacae*. This study has discovered two new species of TBT degrader, *C. braakii* and *E. cloacae*, which have never been previously studied or isolated through investigation of TBT degradation ability. Based on degradation efficiency and resistivity, *E. cloacae* were considered to be the best degrader amongst the isolates.

The capability of a sediment culture (SED) and *E. cloacae* to degrade TBT were optimised using biostimulation; respiration conditions, pH of the media and ambient temperature were studied for their effect on the efficiency of the treatment process. Aeration, which provided excess dissolved oxygen, extensively improved degradation, giving half-lives of 4.08 and 3.16 days for SED and *E. cloacae*, respectively. Variations in the pH of the media (pH 6, 7 and 7.5) moderately affected degradation rates, mainly at the early stages of log phase. Selection of an appropriate pH was found to alter K_d and K_{ow} values, which increased bioavailability of TBT, and also the characteristics of TBT. The shortest half-life for SED was reduced to

3.53 days at pH 7.5, while *E. cloacae* showed a reduction to 3.10 days at pH 7. Study of ambient temperature and degradation indicated that strong seasonal variations were likely. Half-lives were minimised at 37˚C for SED (3.22 days) and at 28˚C for *E. cloacae* (3.12 days). Reduction in half-life was shown between 10-28˚C, but was slightly reduced at the higher temperature. This suggests 28˚C would be an appropriate temperature control for industrial scale treatment.

The ability of individual organic nutrients and growth factors to enhance TBT degradation by SED and *E. cloacae* was also investigated. Most of the supplements supported bacterial growth and differing amounts of biomass were produced. Only supplements that produced a high biomass yield were selected for study on the activity required for TBT degradation. Negative or limited degradation was observed with some supplements, indicating that increased biomass did not necessarily promote degradation. Further, the absence of degradation by *E. cloacae* confirms that the supplement did not promote enzyme activity. Consequently, the addition of nutrients was expected to increase activity and increasing the degrader population was expected to boost the amount of enzymes to facilitate degradation. However, excess amounts of added nutrients showed an inhibitory effect and prolonged the degradation lag phase. Biostimulation therefore requires supplements at an appropriate concentration to increase the capability of TBT degraders.

Bioremediation of TBT-contaminated sediment was studied through investigation of enhancement and improved bioavailability. In TBT spiked sediment, the rate constant of TBT in the control sample (C), representing natural attenuation, was 0.0012 day^{-1}, indicating its persistence. In the stimulated sample (S) (pH 7.5, aerated and incubated at 28 °C), the rate constant was significantly increased to 0.0649 day^{-1}. Further stimulation by nutrient addition (SN) (succinate, glycerol and L-Arginine) and inoculation with *E. cloacae* (SI) (~10^7 viable cells/g of sediment) resulted in increasing of rate constant to 0.0776 day^{-1} and 0.0688, respectively. To improve bioavailability, addition of surfactant, adjustment of salinity and sonication were studied. The highest percentage solubilisation of TBT in water was obtained by adjusting salinity to 20 psu, which increased the solubility from 13.2% to 32.9%.

Degradation rate constant after bioavailability was improved were 0.1508, 0.1637 and 0.1887 day^{-1} for S, SN and SI, respectively. However, natural attenuation (C) was not enhanced. This suggests that in order to enhance TBT biodegradation it is necessary to provide suitable conditions. Further, improving bioavailability additionally increased the rate and amount of TBT degraded. Unfortunately, nutrient addition and inoculation of the degrader did not enhance degradation appreciably.

Publications

• From this thesis

A. Sakultantimetha, H.E. Keenan, T.K. Beattie, S. Bangkedphol, O. Cavoura. Bioremediation of Tributyltin Contaminated Sediment: Degradation Enhancement and Improvement of Bioavailability to Promote Treatment Processes. *Under submission*.

A. Sakultantimetha, H.E. Keenan, T.K. Beattie, S. Bangkedphol, O. Cavoura. Effects of Organic Nutrients and Growth factors on Biostimulation of Tributyltin Removal by Sediment Microorganisms and *Enterobacter cloacae*. *Applied Microbiology and Biotechnology* **2010**, DOI: 10.1007/s00253-010-3023-3.

A. Sakultantimetha, H. E. Keenan, T. K. Beattie, T. J. Aspray, S. Bangkedphol, A. Songsasen. Acceleration of Tributyltin Biodegradation by Sediment Microorganisms under Optimized Environmental Conditions. *International Biodeterioration & Biodegradation* **2010**, 64(6), 467-473.

A. Sakultantimetha, H. E. Keenan, M. Dyer, T. K. Beattie, S. Bangkedphol, A. Songsasen. Isolation of TBT-degrading Bacteria *Citrobacter braakii* and *Enterobacter cloacae* from Butyltin-Polluted Sediment. *Journal of ASTM International* **2009**, 6(6), 369-377.

Publications

• Related to this thesis

S. Bangkedphol, H. E. Keenan, C. M. Davidson, **A. Sakultantimetha**, A. Songsasen. Enhancement of Tributyltin Degradation under Natural Light by N-doped TiO_2 Photocatalyst, *Journal of Hazardous Materials* **2010,** 184(1-3), 533-537.

S. Bangkedphol, H. E. Keenan, C. Davidson, **A. Sakultantimetha**, A. Songsasen. The Partition Behavior of Tributyltin and Prediction of Environmental Fate, Persistence and Toxicity in Aquatic Environments, *Chemosphere* **2009**, 77(10), 1326-1332.

S. Bangkedphol, H. E. Keenan, C. Davidson, **A. Sakultantimetha**, M. Dyer, A. Songsasen. Development and Application of an Analytical Method for the Determination of Partition Coefficients of Tributyltin in the Forth and Clyde Canal, Glasgow, Scotland. *Journal of ASTM International* **2009**, 6(7), 3-19.

S. Bangkedphol, H. E. Keenan, C. Davidson, **A. Sakultantimetha**, A. Songsasen. Development of a Low Cost Method of Analysis for the Qualitative and Quantitative Analysis of Butyltins in Environmental Samples. *Journal of Environmental Science and Health, Part A* **2008**, 43(14), 1744-1751.

Table of Contents

List of Tables

List of Tables (Continued)

List of Figures

List of Figures (Continued)

List of Figures (Continued)

List of Figures (Continued)

List of Figures (Continued)

List of Figures (Continued)

Abbreviations

ACDP	Advisory Committee on Dangerous Pathogens
ANZECC	Australian and New Zealand Environment and Conservation Council
APHA	American Public Health Association
ARMCANZ	Agriculture and Resource Management Council of Australian and New Zealand
ASTM	American Society for Testing and Materials
BCF	Bioconcentration Factor
BOD	Biological Oxygen Demand
BPA	Baird Parker Agar
CFU	Colony Forming Units
CI	Confident Interval
CIRCA	Communication Information Resource Centre Administration
CMC	Critical Micelle Concentration
CSMC	Cockburn Sound Management Council
DBT	Dibutyltin
DBTCl	Dibutyltin Chloride
DEFRA	Department for Environment, Food and Rural Affairs
DFO	Fisheries and Oceans Canada
DNA	Deoxyribonucleic Acid
DoE	Department of Environment
EA	Environmental Agency
EC	European Community
EC_{50}	Effective Concentration
EPA	Environmental Protection Agency
EQS	Environmental Quality Standard
EU	European Union
FAP	Free Association Paint
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
FRTR	Federal Remediation Technologies Roundtable
GC	Gas Chromatography

Abbreviations (Continued)

HPLC	High Pressure Liquid Chromatography, High Performance Liquid Chromatography
IMO	International Maritime Organisation
IPCS	International Programme on Chemical Safety
LB	Luria-Bertini
LC_{50}	Lethal Concentration 50
LIST	Limnofix *In-situ* Sediment Treatment Technology
LOD	Limit of Detection
MBT	Monobutyltin
MBTCl	Monobutyltin Chloride
MC	Moisture Content
MCA	MacConkey Agar
MEPC	Marine Environment Protection Committee
MS	Mass Spectrophotometer
MSA	Mannitol Salt Agar
MSM	Mineral Salt Medium
OD	Optical Density
PAH	Polycyclic Aromatic Hydrocarbon
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
POP	Persistent Organic Pollutant
PPC	Pollution, Prevention and Control
PPT	Parts per Thousand
PSU	Practical Salinity Units
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
RNA	Ribonucleic Acid
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SEPA	Scottish Environment Protection Agency

Abbreviations (Continued)

SLS	Sodium Lauryl Sulphate
SPC	Self-Polishing Copolymers
SPM	Suspended Particulate Matter
TBT	Tributyltin
TBTB	Tributyltin Benzoate
TBTCl	Tributyltin Chloride
TBTF	Tributyltin Fluoride
TBTH	Tributyltin Hydride
TBTO	Tributyltin Oxide
TBTOAc	Tributyltin Acetate
TBTOH	Tributyltin Hydroxide
TE	Tris-EDTA
TeBT	Tetrabutyltin
TIC	Total Ion Chromatogram
TN	Total Nitrogen
TOC	Total Organic Carbon
TPH	Total Petroleum Hydrocarbon
UNEP	United Nations Environment Programme
USEPA	US Environmental Protection Agency
USGS	US Geological Survey
UV/Vis	Ultra Violet/Visible
ZMA	Zobell Marine Agar

Scope of thesis

The purpose of this study was to accelerate biodegradation of tributyltin (TBT). A shorter removal time would be expected to reduce the treatment costs and adverse effects that TBT poses to the environment. The main issue with TBT comes from use as a biocide in antifouling systems. The underestimated risk and extensive use have lead to high contamination in aquatic environments worldwide. The ban on TBT application was intended to control and reduce its impact on the environment; however, TBT persists and strongly accumulates in sediment. Degradation through natural attenuation is possible, but the process is slow. Therefore, “dig and dump” has been chosen as the main option to remediate contaminated sites to meet a regulation limit of contamination for land use. This method is not sustainable remediation since it merely transfers contamination from one site to another. The development of biodegradation is required for more sustainable management of environmental TBT contamination.

To achieve effective bioremediation in sediment, bioaugmentation and biostimulation were investigated to obtain appropriate techniques which, finally, can be applied to accelerate the degradation of TBT in sediment. For bioaugmentation, inoculation of cultured cells was performed to ensure the presence of a sufficient quantity of degraders in sediment. To do this, capable microorganisms with a high efficiency of TBT degradation and resistance were isolated from contaminated sediment. For biostimulation, suitable surrounding conditions were provided to support biodegradation. The respiration conditions, pH and temperature were examined to promise the conditions which gave the highest TBT-removal rate. In addition, organic nutrients, amino acids and vitamins were individually tested as supplements that promoted the activity of the enzymes required for degradation. Then, the stimulation conditions were controlled and the supplements were added into sediment to promote biodegradation. Besides demonstration of degradation enhancement in sediment by bioaugmentation and biostimulation, improvement of TBT bioavailability in sediment was also investigated. Since TBT strongly adsorbed on sediment is non-degradable, increased bioavailability promotes degradation.

CHAPTER 1

Introduction on Tributyltin

1.1 Physicochemical properties of tributyltin compounds

Tributyltin (TBT) is an organometallic compound. It is a member of the trialkyl organotin family. TBT (Bu_3Sn^+) has a central tin atom containing three butyl groups. It has covalent bonds between a tin atom and carbon atom of butyl groups. The structure of TBT is shown in Figure 1.1.

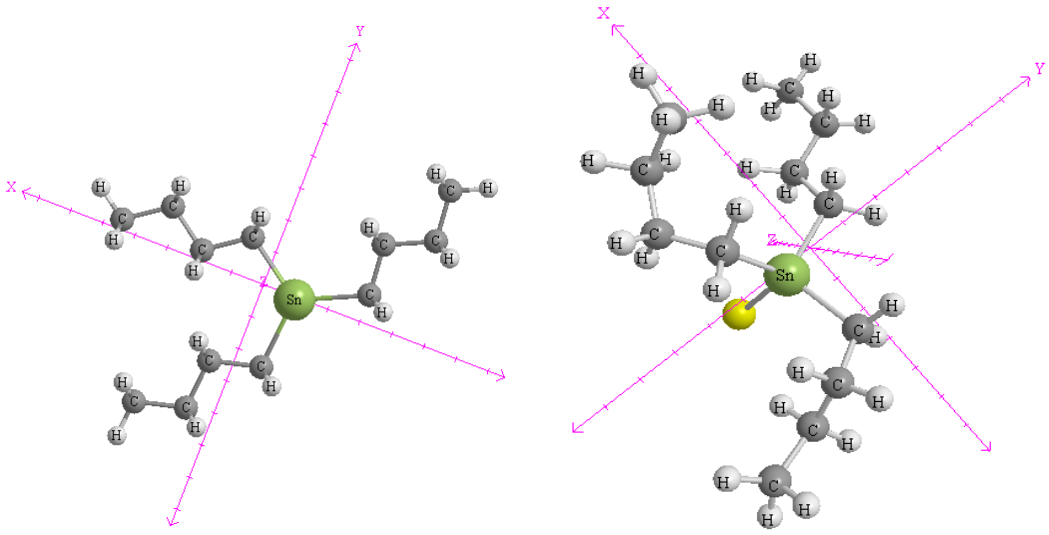


Figure 1.1 Molecular structure of TBT^+ (SP^2 hybridisation) and TBTX where X is anion atom (SP^3 hybridisation) (Bangkedphol, 2010).

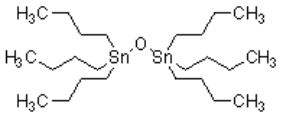
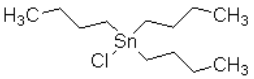
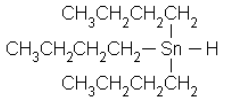
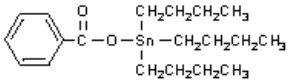
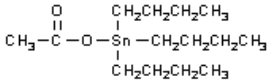
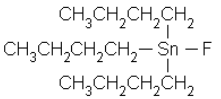
Butyltin compounds conform to the formula R_nSnX_{4-n}, in which R is a butyl group and X is an anionic species, such as hydride (TBTH), halide (TBTF, TBTCl), acetate (TBTOAc) or oxide (TBTO). TBT can be either liquid or solid at ambient temperature depending on the anion, and it has a characteristic odour. The nature of the anion influences the physicochemical properties, particularly the relative water solubility and the vapour pressure (Antizar-Ladislao, 2008). Depending on the property of the butyl-group, TBT is considered hydrophobic and, thus, has poor

water solubility (Oliveira and Santelli, 2010), but it is soluble in most organic solvents. The water solubility of TBT compounds is influenced by factors such as pH, temperature, redox potential, ionic strength and the concentration and composition of dissolved organic matter (Hall et al., 2000). The tin-anionic bond of TBT compounds can be dissociated in water, turning to positively charged cations as described by Equation 1.1.

$$Bu_3SnX + H_2O \rightarrow Bu_3Sn(H_2O)^+ + X^- \qquad \text{(Equation 1.1)}$$

TBT in marine water exists mainly as a mixture of the chloride, the hydroxide, the aqua complex, and the carbonate complex (Laughlin et al., 1986). In the environment, the behaviour and biocidal toxicity of TBT are attributed to the cation, but not to the associated anion (Antizar-Ladislao, 2008). An example of TBT compounds and their properties is shown in Table 1.1.

Table 1.1 The chemical identifier and physical properties of tributyltin compounds (Sigma-Aldrich, 2007).

TBT compounds	Oxide (TBTO)	Chloride (TBTCl)	Hydride (TBTH)	Benzoate (TBTB)	Acetate (TBTOAc)	Fluoride (TBTF)
Structure						
IUPAC	Bis(tributyltin) oxide	Tributylchlorotin	Tributyltin hydride	Benzyloxytributyltin	Acetoxytributyltin	Tributylfluorotin
Chemical Name	Tributyltin oxide	Tributyltin chloride	Tributyltin hydride	Tributyltin benzoate	Tributyltin acetate	Tributyltin fluoride
CAS Number	56-35-9	1461-22-9	688-73-3	4342-36-3	56-36-0	1983-10-04
Molecular Formula	$(C_4H_9)_3SnOSn(C_4H_9)_3$	$(C_4H_9)_3SnCl$	$(C_4H_9)_3SnH$	$C_6H_5CO_2Sn(C_4H_9)_3$	$CH_3\ CO_2Sn(C_4H_9)_3$	$(C_4H_9)_3SnF$
Molecular Weight	596.1 g/mol	325.51 g/mol	291.06 g/mol	411.17 g/mol	349.08 g/mol	309.04 g/mol
Boiling Point	180℃ 2 mmHg	171-173℃ 25 mmHg	80℃ 0.4 mmHg	135℃ 30 mmHg		> 350℃ extrapolate
Melting Point	< -45℃	-16℃		20℃	87℃	240℃
Flashing Point		>230℉	104℉	>230℉	>110℉	
Density	1.17 g/ml 25℃	1.2 g/ml 25℃	1.082 g/ml 25℃	1.193 g/ml 25℃		1.25 g/ml 20℃
Vapour Pressure	0.001 mmHg 25℃	< 0.01 mmHg 20℃		0.0002 mmHg 25℃		
Refractive Index	*n*20/D 1.486	*n*20/D 1.492	*n*20/D 1.473	*n*20/D 1.5182		

1.2 Production and application of tributyltin compounds

Organotin compounds are anthropogenic, and none of the compounds have a natural origin, except methyltin, produced by biomethylation of tin. Diethyltin-diiodide was the first organotin compound, synthesised in 1853 (Van der Kerk, 1975). Further investigation lead to the discovery of a vast number of organotin compounds, including compounds with effective biocidal properties in the 1950s. The tri-substituted organotin species, including TBT, were found to be the most powerful fungicides and bactericides in their family (Huang et al., 2004). TBT compounds were then produced and used worldwide as an important ingredient in antifouling paint, wood preservatives, fungicides and repellents. The compounds can be prepared via a two step reaction, in which tin halides are the initial substance reacted with suitable reagents to form tetra-alkyltin compounds. Routes of the first step synthesis can be by Grignard reaction, Wurtz reaction or the reaction with organo aluminium compounds (AlR_3). Tetrabutyltin, the product from the initial reaction, will then redistribute with tin tetrachloride in subsequent reactions to form less alkylated organotin chlorides (Hoch, 2001). The two step reactions are shown in Figure 1.2. Additionally, the reaction with organo aluminium compounds was the most competitive method and was largely operated for industrial production of organotin compounds.

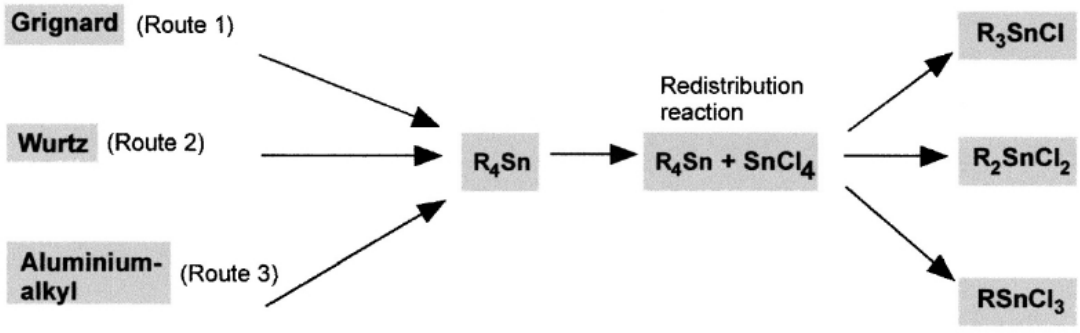


Figure 1.2 Two step preparation of organotin compounds (adapted from Hoch, 2001).

Extensive use of TBT worldwide started in the 1970s. The most important applications were as antifouling agents, and as pesticides in agriculture and wood preservation.

Antifouling paints: TBT-based paints were extremely effective as antifouling agents to prevent the settlement and growth of organisms on submerged structures like fishing nets, ship hulls and buoys. Fouling of ships via the attachment of marine organisms, such as gastropods and molluscs, results in roughness of the ships hull which increase drag and therefore fuel consumption (IMO, 2002). Fouling of a ship's hull is shown in Figure 1.3. The use of antifouling paints on hulls prevents organism attachment and therefore subsequent roughness; it also prevents the spread of alien species to international waters (Minchin et al., 2006). The protection from fouling provided by TBT based paints lasts more than 2 years. TBT-based paints are superior to copper-based paints as they do not promote bimetallic corrosion (Ebdoh et al., 1988). The primary antifouling mechanism is by dispersing the active ingredients (TBT) in a solid matrix, forming free association paints (FAP). The TBT leaches out of the painted surface preventing organisms settling on it. However, the release rate is uncontrolled, beginning with a very high rate that exponentially decreases over time. Further developments led to the use of superior paints containing copolymers. In these self-polishing copolymer paints (SPC), TBT is chemically bonded to a polymer; hydrolysis of the polymer by seawater results in the release of TBT. The surrounding water slowly erodes the coating and the antifoulant is always released at the same rate dependent on the property of polymer. This type of paint is more environmental friendly than FAP. The paints film surface is constantly renewed which reduces hull roughness (Omae, 2003). Antifouling paints were the most important source of TBT compounds in the environment. Major shipping routes, harbours and docks show very elevated TBT concentrations (Antizar-Ladislao, 2008).

Figure 1.3 Maintenance of ship and fouling on a ship hull.

Wood preservation: TBT oxide, TBT naphthenate and TBT phosphate were used to prevent the breakdown of cellulose in timber by insects, fungi and bacteria, whether by dipping, spraying, brushing and double vacuum impregnation. Application of the preservative to wood involves spraying processes in which TBT can be released into the environment due to leakage, accidental spills, effluents and air emission. Also TBT can be released via leaching from the treated wood (Hoch, 2001).

Pesticides: another extensive use of TBT compounds was as a biocide in industrial water systems, e.g. cooling tower and refrigerated water systems, wood pulp and

paper mill systems, and breweries. It was also used as a preservative to prevent the growth of mould and mildew, and as an antifungal agent in textiles (Antizar-Ladislao, 2008).

1.3 Fate and transportation of tributyltin

Environmental fate is the destiny of a chemical or biological pollutant after release into an environment. The environmental fate of chemicals describes the processes by which chemicals are transported and transformed in the environment. The processes include persistence in air, water, and soil; reactivity and degradation; migration; and bioaccumulation in organisms (Schnoor, 1996).

Large amounts of TBT, a Persistent Organic Pollutant (POP), have already been introduced into terrestrial and aquatic environments (UNEP, 2000). TBT compounds only enter the environment via industrial application, as the compounds have no natural source. The major sources of TBT that cause environmental impact are shipyard activities, e.g. painting of vessels and releasing of TBT from the painted vessels. TBT also runs-off agriculture areas into the local environment, or can be released from industrial manufacture facilities. Once in environment, the fate of TBT depends on its partitioning between media, the transport properties of the media, uptake/intake by organisms, and the transformation rate of TBT into other forms. The conceptual model displaying the pathway of distribution of TBT is shown in Figure 1.4.

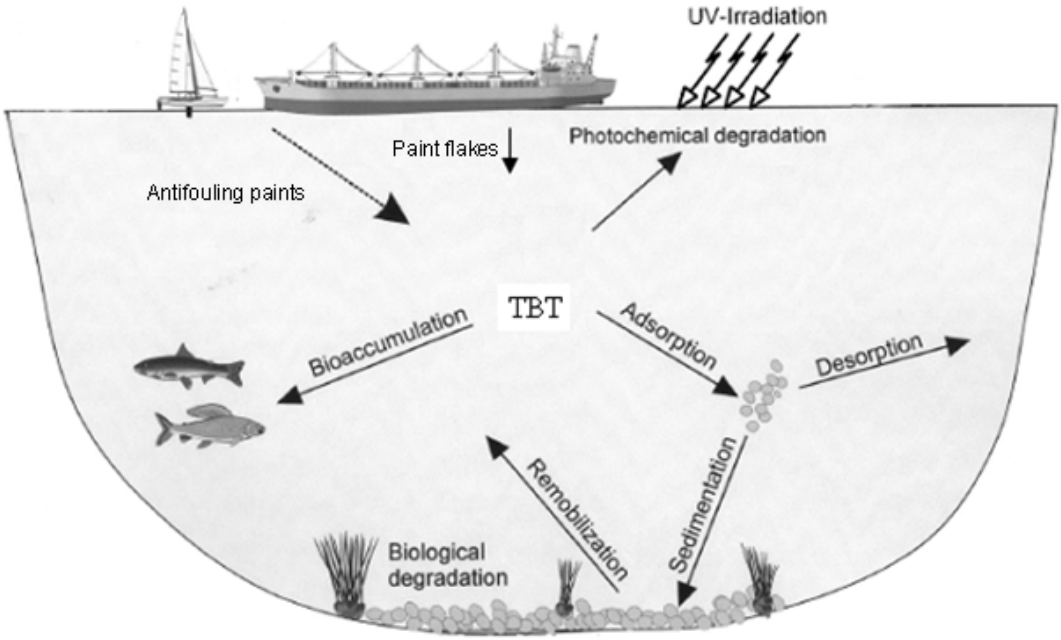


Figure 1.4 Distribution and fate of tributyltin into the aquatic environment (adapted from Hoch, 2001).

1.3.1 Adsorption, accumulation and transformation of tributyltin

TBT has low water solubility and a strong tendency to adhere to suspended material and sediment (Voulvoulis and Lester, 2006). TBT can adsorb to organic constituents of sediment e.g. organic components and sediment humus (carboxylic and phenolic ligands), and inorganic constituents e.g. sand, silt and clay (illite, kaolinite and montmorillonite) (Bangkedphol, 2010, Oliveira and Santelli, 2010). The adsorption mechanism of TBT is dependent on physiochemical parameters, including the mineralogical and chemical composition of the sorbent material and the content of organic matter (Hoch et al., 2003). The sorption of positively charged butyltins to sediments containing negatively charged surface sites (electrostatic bonding) is considered, together with the hydrophobic effect, as the most important explanation for the water to sediment fluxes of butyltin compounds. Van der Waals force is another minor force responsible for adsorption reactions of TBT (Tan, 1998).

TBT-adsorbed particles settle to the bottom of water bodies, minimising widespread surface water contamination. However, desorption of TBT from sediments is possible, thus, contaminated sediments are a continuing source of overlying water-column contamination (Landmeyer et al., 2004). Moreover, under inappropriate conditions for the degradation, TBT in the sediment can accumulate for up to ten years (Dowson et al., 1996). pH and salinity of the surrounding environment affect desorption of TBT from the sediment, and also modify the characteristics of TBT. Therefore, the pH and salinity reflect TBT partitioning in the environment (Langston and Pope, 1995). The partition between water and sediment is expressed in term of partition coefficient, K_d, or organic carbon partition coefficient, K_{oc}, when the proportion of organic carbon in the sediment is relevant to the amount of absorbed TBT (McKay, 2001). The distribution of TBT at the water-sediment interface is an important process for the transport and the fate of these compounds. The aqueous solubility affects leaching rates and the distribution ratio between water and sediment. Though the adsorption characteristics of TBT in several soils (clay, sand, topsoil and silt) have been examined and observed, no significant leaching of the compound occurred (Craig, 2003).

TBT adsorbs strongly to lipids in organisms, as a result of its low water solubility and lipophilic character, and causes bioaccumulation. Also, animal tissues are made up of proteins and lipids which contain nitrogen atoms. TBT shows a high affinity for substances containing nitrogen, oxygen, sulphur or phosphorus atoms because the tin easily forms a five- or six-coordination state with electronegative atoms (Omae, 2003). Therefore, biota will serve as a good accumulating matter for TBT. The partition between water and organism is expressed in term of bioconcentration factor (BCF) or octanol-water partition coefficient, K_{ow} (McKay, 2001). The accumulation of TBT in environmental media and organism's fatty tissue has become an important issue, particularly as it can be biomagnified in the food chain and impacts greatly upon the highest level predator-humans. Bioaccumulation of TBT can be very different from the theoretical calculations due to different excretion, uptake and degradation (detoxification) processes of the organisms and to the fat content of each organism (Gadd, 2000). Some organisms, including many fish

and crustaceans, are capable of enzymatically degrading butyltin compounds (Shimasaki et al., 2002). Other organisms, such as molluscs, lack these degradation mechanisms and are more vulnerable to butyltin compounds (Tang et al., 2010). The BCF is highly dependent on the bioavailability of TBT. Bioavailability expresses the level of the compound that is actually available to the biota (Salazar, 1986). The bioavailability of butyltins is lower in the presence of humic substances. Also, lower pH tends to decrease the bioavailability due to less hydroxide species (TBTOH), which are more easily taken up by organisms than the cationic species (TBT^{+}) (Looser et al., 1998).

Transformation of TBT in the environment can occur through biotic degradation, abiotic degradation and biomethylation (Oliveira and Santelli, 2010). Biotic degradation by animals or microorganisms is well known to be a major source of TBT removal in the environment (Liu et al., 2008). The enzymatic process can breakdown TBT via biotransformation or utilisation as a carbon source. The mechanism and pathway, influencing parameters, and microorganism activity will be discussed further in *Chapter 2*. Abiotic degradation of TBT to less toxic products is generally accelerated by sunlight via photo-degradation. However, photo-degradation is excluded in the areas where natural light cannot penetrate through the water column. Biomethylation, attachment or substitution of methyl groups, is a possible mechanism by bacterial enzymatic process (Vella and Adami, 2001). Also, humic substances or the presence of other methylated heavy metals, via a transmethylation reaction, may cause methylation of butyltins (Kuballa et al., 2004). These methylation processes are not only competing reaction pathways for the degradation of organotins, but they can also lead to fully substituted, volatile tin compounds with lower toxicity (Amouroux et al., 2000). Still, methylation does not seem to be a significant pathway for the transformation of butyltin compounds (Vella and Adami, 2001).

1.3.2 Distribution of tributyltin

After TBT is introduced into an ecosystem, it is transported in water, and adsorbs on to sediment and into the biota. The distribution is influenced by factors such as organic material (dissolved and suspended), pH, salinity, temperature and, species and density of organisms (Oliveira and Santelli, 2010). Since TBT has low volatility and a high boiling point, distribution of TBT into the atmosphere is negligible (USEPA, 2008b).

Aquatic systems: TBT compounds are generally present as TBT cation or TBT hydroxide species depending on the pH of the natural water (*see Section 3.3.2*). pKa is the dissociation constant where dissociation of TBT in water results in the presence of TBT^+ and TBTOH. At pH < pKa, TBT^+ is the major species, while TBTOH dominates when pH > pKa. Since pKa of TBT is in the range of 6.25-6.51 (Fent and Looser, 1995, Hunziker et al., 2001), at pH 8, the average pH of seawater, the major species of TBT are TBT hydroxide and TBT carbonate (Champ and Seligman, 1996). The levels of TBT in aquatic systems are greater in the regions with high levels of shipping, harbours and shipyards. The release of TBT from antifouling paint raises the TBT concentration in water, sediment and biota in seas, lakes or coastal areas. Beside antifouling paint, TBT has been detected in municipal wastewater and sewage sludge (Fent and Looser, 1995). The degree of tidal flushing and the turbidity of the water also influence TBT concentrations (Anderson et al., 2002).

Sediments: TBT is easily and progressively removed from water via adsorption onto sediment and suspended particulate matter (SPM) (Stasinakis and Thomaidis, 2010). TBT is retained under the sediment surface and persists within the sedimentary column over a long period of time (Quevauviller et al., 1994). TBT present on suspended matter or sediment is available for sediment-feeding organisms and leads to biomagnification. The re-suspension of particles and remobilisation of pollutants by travelling crafts, wave active and dredging can cause re-contamination of aquatic

systems (Miller and Boyle, 2003). Very fine sediments are relatively easy to re-suspend into the water column and represent a possible diffuse source of TBT.

Soils: TBT can contaminate land soil by dredging and landfilling of contaminated sediment, leaching of landfill and disposal of contaminated waste (Loch et al., 1990). Moreover, TBT may enter the ecosystem through atmospheric deposition by industrial application leading to the occurrence of TBT in rain and soil, and accumulation in the forest top soil (Huang et al., 2004).

Organisms: TBT can be uptaken/intaken by the wildlife living in a contaminated area, especially fish, molluscs and other water dwelling organisms. Furthermore, sediment dwelling organisms that are exposed to higher concentrations accumulated in sediment. TBT tends to concentrate within the liver, kidney and muscles of animals (Berge et al., 2004). Biomagnification in higher trophic predators can occurs through accumulation of TBT in aquatic organisms (Guruge et al., 1996). For humans, the exposure to TBT through the consumption of food is relatively low (Forsyth et al., 1994, Takahashi et al., 1999). The direct exposure to humans is often by workers who have contact with the substance.

1.3.3 TBT contamination of the environment

Environmental TBT contamination has been noted around the world, together with DBT and MBT as degradation products. The levels of TBT in water, sediment and biota are presented in Table 1.2. In all cases, TBT concentrations in sediment and biota were extensively higher than in water, even though TBT is initially released into the water column. Once TBT is released into water, it could be diluted by the current flow or tidal. However, the values in Table 1.2 still confirm large adsorption of TBT on sediment and accumulation in animals, which supports the biomagnification in food chains.

Table 1.2 Contamination of tributyltin in environmental media in many countries.

Location	Year	Levels of tributyltin			Reference
		Water (ng Sn/L)	Sediment (µg Sn/kg dry weight)	Biota (µg Sn/kg dry weight)	
Maizuru Bay, Japan	2007	1-2[a]	0.9-5[a]	2.4-4.1 (Mussel)[a,b]	Eguchi et al. (2010)
Sanricu Coast, Japan	2005		2–14,000	3–287 (Mussel)	Harino et al. (2007)[c]
North West Coast, Spain	2005		0.6–303	74–193 (Oyster)	Üveges et al. (2007)[c]
Coast, Thailand	2004		2-1,246[a]	4-45 (Bivalves) [a]	Harino et al. (2006)[d]
Southern Venice, Italy	2003	10–586	159–8,057		Berto et al. (2007)[c]
North Coast of Kyoto, Japan	2003	3.9–27	1.2–19	0.8–11 (Mussel)[b]	Ohji et al. (2007)[c]
Coast, Vietnam	2003		8.3–51	3.8–15 (Clam)	Nhan et al. (2005)[c]
West Coast, India	2001	123–345[a]	1.4–2,384[a]	124–261 (Oyster)[a]	Bhosle et al. (2004)[c]
Coast, Portugal	1999–2000		<3.8–12.4	<5.7–489 (Mussel)	Diez et al. (2005)[c]
North-Western Sicilian Coasts, Italy	1999–2000		3–27	<d.l.–91 (*H. trunculus*)	Chiavarini et al. (2003)[c]
Coast, Korea	1997–1998	<d.l.–4.5		16–1,610 (Bivalves) 7–323 (Starfish)	Shim et al. (2005)[c]
Coast, Malaysia	1997–1998		2.8–1,100[a,b]	2.4–190 (Fish)[a,b]	Sudaryanto et al. (2004)[c]
West and East Coast of Canada	1995	<d.l.–500	<d.l.–5,100	20–1,198 (Mussel)	Chau et al. (1997)[c]
Lake System Westeinder, The Netherlands	1993		6–520	180–2,500 (Mussel)[b] 7–63 (Fish)[b]	Stäb et al. (1996)[e]
Guadalete River, Spain	1992	9.6–116	26.5–601		Gomez-Ariza et al. (1995)[e]

[a] concentration of TBT instead of Sn; [b] wet weight; d.l. = detection limit;

presented by [c]Antizar-Ladislao, 2008; [d]Arai et al., 2009; [e]Hoch, 2001

1.4 Toxicity of tributyltin

The usage of TBT and its derivatives has drawn concern about the potential damage to the aquatic environment, especially from antifouling paints. Of particular significance is TBT, which is toxic to non-target organisms even at low concentrations due to the biocidal effectiveness (Hoch, 2001). The biological effects generally depend on the number of butyl groups bound to the tin ion; the tri-substituted form has proven to have the highest adverse effects per dose, with negligible or no effect from the anion species (Hoch, 2001). The World Health Organization has reported the lethal concentrations to aquatic species in the range of 0.04-16 μg/L for short-term exposures (World Health Organization, 1990).

TBT is an endocrine disruptor, which interferes with the endocrine system in many organisms, including humans (Cruz et al., 2010). TBT causes acute and chronic poisoning of sensitive aquatic life. It causes physical damage, impairs growth and development, it also has negative reproductive effects, teratogenic effects, immunological effects, carcinogenic effect and, the most worrying, causes imposex of molluscs, all of which can have drastic effects on population density (Antizar-Ladislao, 2008, Smith, 1981, United Nations, 2005). Other affected species include algae, plankton, molluscs and the larval stage and adult life of some fish. The rate of uptake and the rate of metabolic conversion/elimination varies from species to species, and results in different bioaccumulation ratios, and therefore different dose-related responses for each species (Meador and Rice, 2001), e.g. TBT is more toxic to Gram-positive bacteria than to Gram-negative bacteria (Lascourreges et al., 2000). Extended periods of exposure increase the susceptibility of organisms to TBT (Sousa et al., 2005). It has also been shown that embryonic larval stages of marine invertebrates are more sensitive than adults (Antizar-Ladislao, 2008). In addition, the effect of TBT is associated with environmental parameters, e.g. marine species are more susceptible to TBT than those freshwater (Leung et al, 2007). In some sensitive species, TBT can inhibit cytochrome P450, the enzyme system that is associated with degradation of TBT, resulting in higher accumulation and toxicity to the species than those without the inhibitory effect from TBT (Williams et al., 1998).

There is little known on the effects of TBT in humans, but disruption of lymphocytes in blood, which is the primary immune defence against tumours and virally infected cells, has been reported (Hoch, 2001). Generally, TBT causes skin, eye and mucous membrane irritation, and severe dermatitis after direct contact with liquid TBT chloride, resulting in symptoms of reddening, inflammation of the hair follicles, minute pustules and a faint erythema (Goh, 1985). Goh (1985) also reported that TBT causes nausea and vomiting after inhalation, and moreover extensively causes vesiculobullous lesions, erythema, and oedema. The effect of TBT on a variety of organisms is shown in Table 1.3, while Table 1.4 gives LC_{50} concentration of TBT for a variety of organisms; these tables indicate effects of TBT at concentrations of ng/L to µg/L.

Table 1.3 The effect of TBT on a variety of organisms at different concentrations and exposure time.

Specie	TBT concentration (exposure time)	Effect to organism	Reference
Macrobrachium rosenbergii (freshwater prawn)	3.12 mg/L (7 d)	delays embryonic development, reduces hatchability of eggs, reduces body growth and absence of eyestalk and internal organs	Revathi and Munuswamy (2010)
Fundulus heteroclitus (marine fish)	1 µg/L (life-cycle)	gonadal sex differentiation, spermatogenesis and reduces egg quality	Mochida et al. (2010)
Schmackeria poplesia (calanoid copepod)	10 ng TBTO/L (24 d)	reduces ovigerous females	Huang et al. (2010)
Sebastiscus marmoratus (mollusc)	100 ng/L (48 d)	induces masculinisation in female	Zhang et al. (2009)
Oncorhynchus tshawytscha (fish)	0.1-0.6 mg/L (24 h)	adverse effects on the function or development of fish immune systems	Misumi et al. (2009)
Mice	25-100 mg/kg	serious defects in testicular development and function of male mice	Kim et al. (2008)
Ruditapes philippinarum (clam)	0.6 µg/L (13 d)	prevents the development and survival of veliger larvae	Inoue et al. (2007)
Lymnaea stagnalis	1 µg/L	inhibits egg development, reduces survival hatchling and inhibits shell growth	Leung et al. (2007)
Chironomus riparius	2-200 µg Sn/L	generic adaptation and changes in reproductivity	Vogt et al. (2007)
Sillago japonica (fish)	200 mg TBTO/kg (30 d)	reduces quality of eggs, viable hatchability and total number of viable larvae	Shimasaki et al. (2006)
Tilapia nilotica (fish)	1 ng/L (15 d)	increases mortality, inhibits growth and inhibits ATPase activities	Zhang et al. (2006)
Mice	15 mg/L	affects the growth of preweanling mice and the receptors involved in memory and learning	Konno et al. (2005)
Pinctada fucata martensii (pearl oyster)	0.19 µg/L (7 d)	inhibits reproduction by decreasing embryo developmental success	Inoue et al. (2004)
Oncorhynchus mykiss (fish)	5µM (1 h)	strongly affects the mitochondrial activity in red blood cells	Tiano et al. (2003)
Paralichthys olivaceus (fish)	20 µg Sn/kg (10 d)	decreases hepatic cytochrome P450 content in liver	Shim et al. (2003)
Chlorella sp. and *Scenedesmus* sp. (microalgal)	0.1 mg/L (7 d)	reduces cell number and chlorophyll content	Tam et al. (2001)

Table 1.4 The lethal effect of TBT on a variety of organisms at different concentrations and exposure time (presented by Antizar-Ladislao, 2008).

Specie	Lethal concentration (exposure time)	Reference
Eurytemora affinis (zooplankton)	LC_{50}: 2.2 µg/L (48h), 0.6 µg/L (72 h)	Hall et al. (1988)
Phoxinus phoxinus (fish)	Complete mortality: 6.55-9.25 µg/L (96 h)	Fent (1991)
Sparus aurata (seabream)	LC_{50}: 28.3 µg/L (24 h) for fertilised eggs, 38.6 µg/L (24 h) for larvae	Dimitriou et al. (2003)
Tilapia species (fish)	LC_{50}: 3.8 µg/L (96 h) for adult life stages	Hongxia et al. (1998)
Palaemon serratus	LC_{50}: 22.30 µg/L (24 h), 17.52 µg/L (48 h)	Bellas et al. (2005a,b)
Thais clavigera (rock shell)	LC_{50}: 8.4 µg/L (24 h), 5.6 µg/L (48 h)	Horiguchi et al. (1998)
Haliotis discus (disk abalone)	LC_{50}: 5.4 µg/L (48 h)	Horiguchi et al. (1998)
Haliotis madaka (giant abalone)	LC_{50}: 3.9 µg/L (24 h), 1.2 µg/L (48 h)	Horiguchi et al. (1998)
Amphipod crustaceans	LC_{50}: 1.2-6.6 µg/L (48 h) for caprellids, 17.8-23.1 µg/L (48 h) for gammarids	Ohji et al. (2002)
Nitocra spinipes	LC_{50}: 13 µg/L (96 h)	Karlsson et al. (2006)
Tigriopus japonicus	LC_{50}: 0.149 µg/L	Kwok and Leung (2005)
Artemia salina	LC_{50}: 41.41 ng/L (24 h)	Panagoula et al. (2002)
Oryzias latipes	LC_{50}: 55 µg/L, 124 µg/L and 117 µg/L (96 h) for 0,3 and 5 day embryo, respectively	Bentivegna and Piatkowski (1998)

LC_{50}: lowest concentration to cause 50% lethality

1.5 Management and current standards for tributyltin compounds

1.5.1 Legislation and ban of tributyltin compounds

Since the 1980s, several national legislations have been implemented with the specific purpose of limiting the use of TBT. Due to the increasing knowledge of the environmental issues surrounding TBT in antifouling paints, France was the first country to ban TBT-based paints in 1982 (Miller and Boyle, 2003). To avoid distorted competition by national regulation, the European Community (EC) started regulating TBT and other organotin compounds in the region (Gipperth, 2009). EC legislation relevant for the control and reduction of inputs of TBT compounds to the aquatic environment include (EA, 2010, OSPAR Commission, 2006):

1) Directive 76/769/EEC relating to restrictions on the marketing and use of certain dangerous substances and preparations. However, this legislation has been amended many times, i.e. Directive 89/677/EEC and Directive 2002/62/EC and finally, in 2009, it was replaced by the REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) regulation EC 2006/1907, which came into force in June 2007.

2) Regulation (EC) No 782/2003 on the prohibition of organotin compounds on ships.

3) Directive 2000/60/EC establishing a framework for community action in the field of water policy (Water Framework Directive), Directive 76/464/EEC for aquatic environment, and Directive 2008/105/EC on environmental quality standard for surface waters.

Besides the European Commission, other regulations have also been adopted:

- In the US, the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) was adopted.
- In the Oceania/Australia region, the Protection of the Sea (Harmful Anti-fouling Systems) Act 2006 and Code of Practice for Antifouling & In-water Hull Cleaning and Maintenance.

As the problem of organotins is recognised globally, other related conventions have been established, i.e. Helsinki Convention (Helcom recommendation 20/4), OSPAR Convention and PARCOM recommendations 87/1 and 88/1. Finally, the Marine Environment Protection Committee (MEPC) established the International Convention on the Control of Harmful Anti-fouling Systems on Ships (the AFS Convention) for global agreement (Sonak et al., 2009).

After recognising the risks of TBT and other organotin compounds in the marine environment, the International Maritime Organization (IMO) began global regulation. In 1989, the IMO recognised the severe environmental effects of organotin compounds, which lead to adoption of the resolution by MEPC in 1990. The resolution recommended elimination of the use of antifouling paints containing TBT on non-aluminium hulled vessels of less than 25 metres in length or have a leaching rate of more than 4 µg TBT/day (IMO, 2002).

In November 1999, the IMO adopted a resolution for a global prohibition on application of organotin compounds. The resolution called for the control of antifouling system, and that all ships should not apply or re-apply organotins antifouling system as of 1 January 2003. Thereafter, all ships should either not bear the compounds on their hulls or external parts or surfaces, or should bear a coating that forms a barrier to prevent the compound leaching from the underlying non-compliant antifouling systems, as of 1 January 2008 (IMO, 2002). This regulation was also implemented into the EU by Regulation 782/2003 (EA, 2010).

In 2001, following the resolution adopted by the IMO, all members participating in the AFS Convention agreed to reduce or eliminate adverse effects on the marine environment and human health caused by antifouling systems. The agreement applies to all ships entitled to fly the flag of a member party or operate under the authority of a member party, and ships that enter the territory of the parties including ports, shipyards and offshore terminals. However, this agreement has an exception for ships only used on government non-commercial service (IMO, 2002).

In 1986, the UK government, Department of Environment (DoE), introduced regulations under Section 100 of the Control of Pollution Act 1974 to limit the concentration of TBT in paints and prevent retail sale of the most damaging TBT-based paints (Miller and Boyle, 2003). As of 1987, the UK banned the use of TBT paints on vessels less than 25 m in length and on all submerge equipment. Also all antifoulants had to be registered as pesticides, which require approval by a special committee for their sale and use (Yebra et al., 2004). TBT compounds are substances of potential environmental concern of national significance, signifying that their presence in the environment is of particular concern (EA, 2010). The UK also adopted the control of TBT compounds under the OSPAR and Helsinki Conventions - relating to protection of the marine environment, specifically that of the North Atlantic and Baltic seas. The Environmental Agency (EA) has produced a pollution reduction plan by complimenting restrictions on the supply and use of organotins in antifouling paints on vessels (and equipment intended to be fully or partially submerged) as contained in Annex 17 of the EU REACH Regulation. Implemented in the EU by Regulation 782/2003, the relevant domestic legislation for this agreement is covered by the Merchant Shipping (Anti-fouling Systems) Regulations 2009. All vessels entering UK waters/ports need to provide a certificate that declares either that the hull is free from TBT, or that the TBT anti-fouling system has been coated to prevent leaching into the aquatic environment (EA, 2010).

The releases of TBT compounds are controlled through the UK Pollution, Prevention and Control (PPC) Regulations, the Food and Environmental Protection Act (FEPA 1985), the Control of Pesticides Regulations (COPR 1986) and the UK

Surface Waters Regulations (SI 1997/2560). TBT compounds are included in the Surface Waters (Dangerous Substances) (Classification) (Scotland) Regulations 1998 (The National Archives, 1998). Implemented in Scotland by Scottish Environment Protection Agency (SEPA), the Scottish Pollutant Release Inventory (SPRI) reports the emission threshold as 0.005 kg/yr to water; 0.005 kg/yr to wastewater; 1.00 kg/yr to land (SEPA, 2010).

1.5.2 Environmental Quality Standard (EQS)

Environmental Quality Standard (EQS) values are maximum concentration limits for pollutants in ecosystems, i.e. soil, sediment, benthic organisms, water (whether surface/ground or marine/freshwater), and drinking water, etc. The values are established to protect environments, organisms or human uptake by consumption. Each EQS is specific to an individual substance and matrix of contamination.

The EQS values of TBT compounds (TBT-cation) are documented in the Directive 2008/105/EC. The quality standard is based on the annual average concentrations (AA-EQS), and is estimated from available information of the mollusc species *Necella lapillus* (dog whelk), which is the species most sensitive to TBT compounds. The quality standard based on the maximum allowable concentration (MAC-EQS), accounting for transient concentration peaks, is derived from the lowest acute toxicity test available on the most sensitive pelagic crustacean species to TBT compounds in both saltwater and freshwater, *Arcatia tonsa* (CIRCA, 2005). The AA-EQS in surface waters is 0.0002 μg/L, and the MAC-EQS in surface waters is 0.0015 μg/L (European Union, 2008). In addition, the Communication Information Resource Centre Administration (CIRCA) has developed TBT EQS values as shown in Table 1.5. However, only the EQS in waters have been adopted globally at the present.

Table 1.5 Specific TBT quality standards (CIRCA, 2005).

Protection objective	Quality standard
Pelagic community All types of surface water covered by the Water Framework Directive	0.0002 µg/L (corresponding conc. in suspended matter: 0.022 µg/kg dry wt)
Benthic community (freshwater and marine sediment)	0.0046 µg/kg wet wt 0.02 µg/kg dry wt
Predators (second poisoning)	230 µg/kg wet wt (corresponding conc. in water: 0.038 µg/L)
Food uptake by human	15.2 µg/kg seafood wet wt (corresponding conc. in water: 0.0025 µg/L)
Abstraction of water intended for human consumption (AWIHC)	< 1 µg/L
Water intended for human consumption (WIHC)	0.1 mg/L

US water quality standard values recommended by the EPA are 0.46 µg/L and 0.072 µg/L for acute and chronic toxicity, respectively in freshwater; 0.42 µg/L and 0.0074 µg/L for acute and chronic toxicity, respectively in marine water (USEPA, 2009). The Australian sediment quality guidelines for TBT, adopted by the Australian and New Zealand Environment and Conservation Council (ANZECC), and the Agriculture and Resource Management Council of Australian and New Zealand (ARMCANZ), are 5 µg/kg and 70 µg/kg for low and high trigger values (values which refer to the management response), respectively (ANZECC/ARMCANZ, 2000). Following PARCOM (Convention for the Prevention of Marine Pollution from Land-based Sources) recommendation 87/1, Denmark has adopted the quality standard for marine waters at 1 ng TBTO/L, Germany limits for suspended matter in fresh water, as well as in coastal water, is 0.025 mg/kg suspended particulate matter (OSPAR Commission, 2006). The UK has adopted the quality standards for marine waters at 2 ng/L, the Netherlands have adopted the limit values for marine water at 0.01 ng/L for the long term and 1 ng/L for the short term; for marine sediment at 0.007 µg/kg for the long term and 0.7 µg/kg for the short term (OSPAR Commission, 2006). EQS values specific to Scotland are recommended by

The Scottish Government, where TBT compounds in surface waters has a limit of 0.0002 μg/L (AA-EQS) and 0.0015 μg/L (MAC-EQS) (The Scottish Government, 2008).

1.6 Project aims and objectives

The aim of this research is to investigate enhancement techniques for bioremediation of TBT which can be practically applied to accelerate the treatment of TBT from contaminated sediment; the techniques include bioaugmentation and biostimulation. TBT-degraders were isolated for using as bioaugment species. Biostimulation was studied by optimising the suitable conditions and nutrient supplement for microorganism to promote degradation of TBT. The objectives of this research are:

1. Isolation and identification of sediment bacteria to obtain TBT-degraders.
2. Investigation of the degradation capability and resistivity of the isolates.
3. Optimisation of encouraging parameters e.g. respiration, pH and temperature to obtain suitable conditions, which accelerate TBT degradation.
4. Supplementation of nutrients and co-factors to encourage the enzymatic activity of TBT-degraders.
5. Employing optimised biostimulation and bioaugmentation by the isolate degrader to enhance degradation in contaminated sediment.
6. Improvement of bioavailability of TBT in sediment to increase the amount of degradable TBT.

CHAPTER 2

Isolation of Tributyltin-degrading Microorganisms and Biodegradation of the Compound

Objectives: To isolate potent single cultures that show efficiency in TBT degradation and are resistant to its toxic effects; also, to identify the isolated species that can be used as inocula for degradation enhancement by bioaugmentation in further studies, providing a rapid start for remediation treatment.

2.1 Background

Biodegradation is the process in which chemical substances are broken down to less complicated compounds by the enzymes produced by living organisms. Different species of microalgae, fungi and bacteria have been shown to be able to degrade TBT with varying degradation ability (Luan et al., 2006). Much of the evidence suggests that biotic degradation is the major pathway for removal of TBT in the aquatic and sediment environment (Tsang et al., 1999), therefore, these microorganisms are the key factor in biodegradation of TBT. The process involves progressive debutylation, the breakage of the tin-carbon bond to produce less toxic metabolites dibutyltin (DBT), monobutyltin (MBT) and mineralisation to inorganic tin as the final product. The anion bonded to the tin atom appears to have little toxicity towards microorganisms, however toxicity is greatly increased when tin is bonded to alkyl groups (Roy et al., 2004). Consequently, debutylation of TBT is one potential mechanism to reduce toxicity.

Even though the breakdown of TBT can take place via biotic and abiotic processes, natural abiotic degradation such as photolysis, chemical cleavage and thermal cleavage has negligible effect on the natural breakdown of TBT (Tsang et al., 1999). Consequently, only few non-biological reactions in nature lead to

breakdown of TBT comparable to that of biotic processes. Due to their ability to mineralise anthropogenic compounds, microorganisms play a large role in the processes taking place in soil, water and sediment and are frequently the sole means of degrading TBT (Alexander, 1999, Heroult et al., 2008, Liu et al., 2008).

2.1.1 Biodegradation pathway

TBT uptake by microorganisms involves biosorption and absorption (Tam et al., 2002). Despite the low water solubility of TBT, biosorption includes adsorption of TBT at the cell surface, following ion exchange process which is metabolism dependent. For absorption process, TBT is slowly absorbed through active uptake process which is also metabolism dependent. These processes lead to intracellular accumulation of TBT, mainly in the lipid-rich tissues or organelles (Swackhamer and Skoglund, 1993). The transport of TBT might be facilitated by some ion channels or carriers in the cell membrane, whether it is through facilitated transport or passive diffusion (Tsang et al., 1999). TBT exposed to microorganisms is instantly uptaken during the early stage of incubation; however, the uptake rate will decrease due to the efflux of the compound (Suzuki and Fukagawa, 1995).

The ability to adsorb and degrade TBT varies widely between species depending on their origin, shape, size, and cell wall composition (Tam et al., 2002). The adsorption of TBT will reach a maximum and degradation will then take place, reducing intracellular TBT (Taha et al., 2009). The accumulation induces metabolising enzymes in which TBT is degraded via a stepwise enzymatic debutylation mechanism inside the cells. With regard to adsorption, ligands in the cell wall can reduce the amount of accumulated TBT which would lead to lower degradation than in microorganisms without a cell wall (Taha et al., 2009). DBT, produced after intracellular degradation, can be gradually extruded from the cell and released into medium, despite being less lipophilic than TBT. It can also be further degraded to MBT which may be excreted into the medium or may accumulate inside the cells.

Such biological transformations which involve enzymes as catalysts often bring about extensive modification in the structure and toxicological properties of potential pollutants. Biodegradation of TBT proceeds via an oxidation reaction (β-hydroxylation) with the enzyme, dioxygenase (oxygen transferase) (Landmeyer et al., 2004, Lee et al., 1989). The TBT biodegradation pathway has been previously documented as shown in Figure 2.1. Butyltin compounds are oxidised by oxygenase enzymes and form β-hydroxybutyl-butyltins as intermediates. Next, the intermediates are catalysed and the intermediates are transformed into debutylated products and a by product, methyl ethyl ketone. These reactions change TBT to DBT, DBT to MBT and MBT to inorganic tin. However, for most of the microorganisms tested, MBT rather than elemental tin was the final metabolic product since the degradation rate of MBT was much slower than those of DBT and TBT (Tsang et al., 1999).

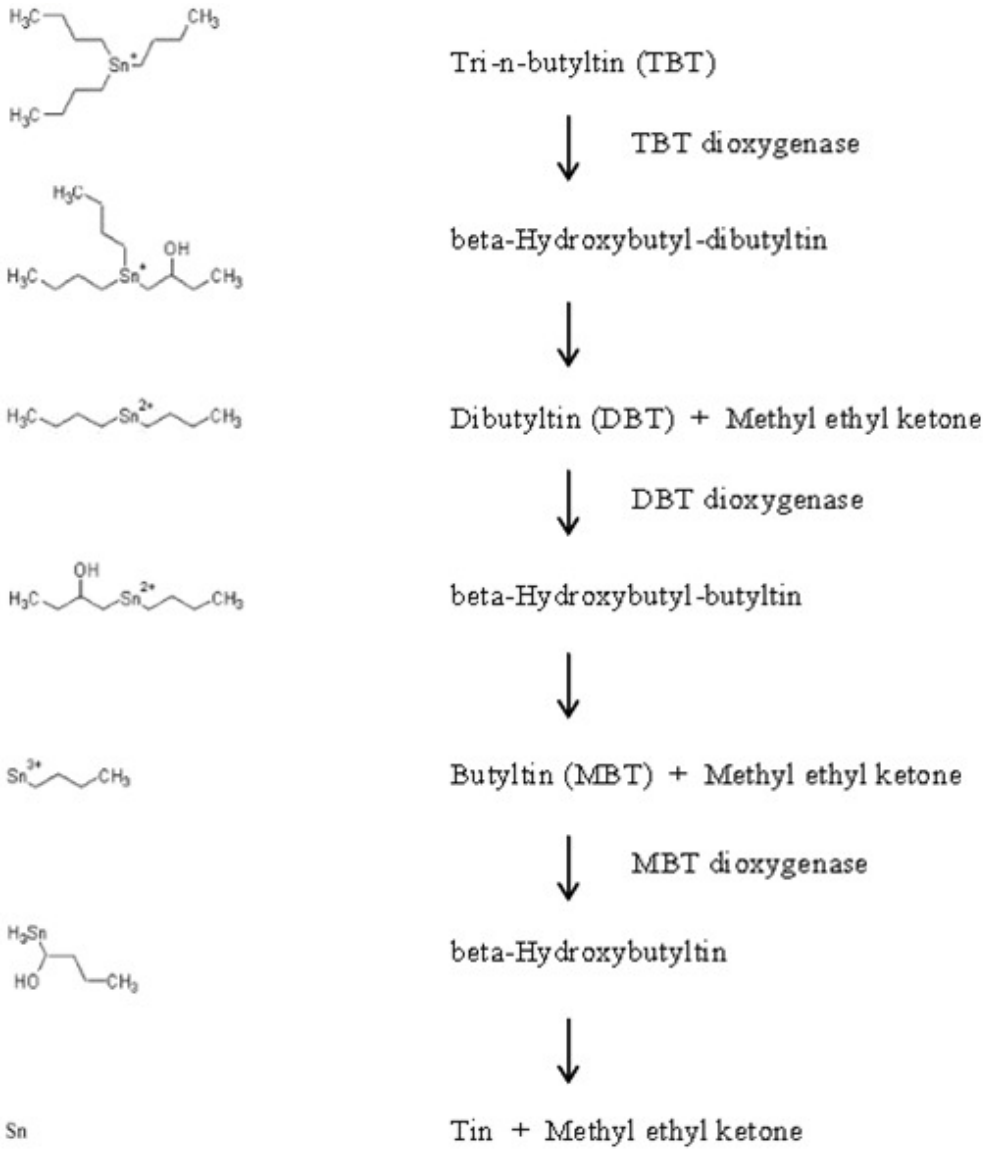


Figure 2.1 Degradation pathway of TBT via successive debutylation (Lee et al., 1989).

2.1.2 Cytochrome P450

Cytochrome P450 (also called CYP or P450) is an enzyme system that is responsible for the degradation of xenobiotic compound, a substance which is not normally produced or expected to be present in organisms. This enzyme has been identified from different lineages of life, including all animals, plants, fungi, and bacteria, and indicated by specific patterns of genes (Black, 1993). Cytochrome P450

forms part of multi-component electron transfer chains, which are significant oxidative enzymes of TBT. The oxidation reaction is an insertion of one oxygen atom into an organic substrate while the other oxygen atom is reduced to water as shown in Equations 2.1 & 2.2, where R is an alkyl group (Rein and Jung, 1993).

$$RH + O_2 + 2H^+ + 2e^- \rightarrow ROH + H_2O \qquad \text{(Equation 2.1)}$$

or

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+ \qquad \text{(Equation 2.2)}$$

The P450 catalytic cycle, shown in Figure 2.2, proceeds as follows (Guengerich, 1993): (1) the substrate binds to the active site of the enzyme, close to the heme group, opposite to the peptide chain; (2) the change in the electronic state of the active site favours the transfer of an electron via NADPH-dependent cytochrome P450 reductase, reducing ferric to ferrous; (3) molecular oxygen binds covalently to the ferrous heme iron, Fe^{2+}-O_2, and then undergoes slow conversion to a more stable Fe^{3+}-O_2^- (Archakov and Buchmanova, 1990); (4) a second electron is introduced into the cytochrome P450-dioxygen-substrate complex from the NADPH-dependent cytochrome P450 reductase or from the electron carrier cytochrome b_5, reducing the dioxygen to a negatively charged peroxo group, Fe^{3+}-O_2^{2-} (Egawa et al., 1991); (5) the peroxo group rapidly releases a water molecule; (6) P450 enzymes catalyse specific oxidative reactions or reductive reactions depending on the substrate; for TBT hydroxylation to occur (7), the product is released from the active site, and the enzyme returns to its original state.

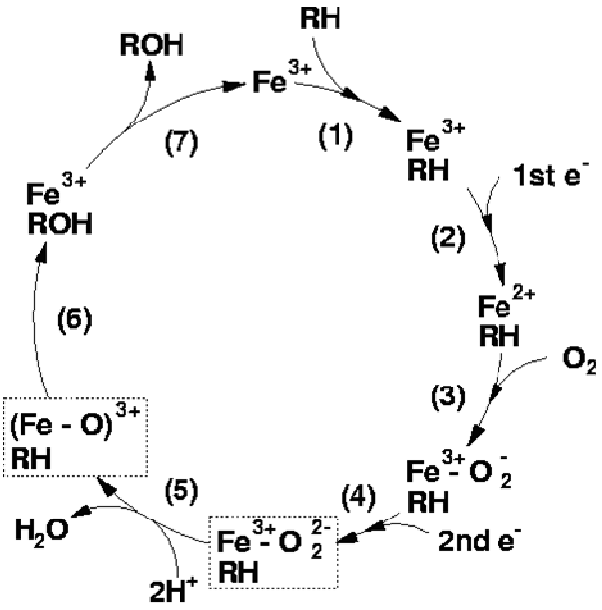


Figure 2.2 The catalytic cycle of cytochrome P450 (Segall, 1997).

Due to this electron transfer system, cytochrome P450 plays an important role in TBT degradation in organisms. Bernat and Długoński (2002) investigated the involvement of cytochrome P450, in a synthetic liquid medium, in the filamentous fungus *Cunninghamella elegans*, which is capable of TBT degradation. When cytochrome P450 inhibitors were introduced into the media, degradation activity disappeared. This finding confirms the participation of cytochrome P450 in the TBT degradation pathway (Bernat and Długoński, 2002). Further investigation with *Streptomyces* sp. by Bernat and Długoński (2009) confirms that the enzyme system also participated in DBT degradation.

2.1.3 Parameters influencing biodegradation

The effectiveness of biodegradation processes depends on the microbial populations which are available to destroy the target substances. At the same time, the growth and the physiological capabilities of these microorganisms depend on various factors (Atlas, 1991). With respect to TBT, the rate of biochemical transformation can vary greatly depending on the environmental conditions, the site

characteristics and the initial concentration of TBT (Stasinakis and Thomaidis, 2010). The major parameters that affect TBT biodegradation are:

Composition of the microbial community: heterotrophic bacteria must be present at high density. Biodegradation often requires the cooperation of more than a single species. Microorganisms should have an adaptation ability to resist high levels of TBT over the lethal or inhibitory concentration, and they should enzymatically degrade TBT, following effective induction of either existing catabolic genes or those horizontally transferred amongst the community (e.g., on plasmids).

Temperature: optimum temperature leads to the most rapid bacterial growth and highest removal efficiency (Krzywonos et al., 2008). The optimum temperature varies amongst bacteria in the community. The biodegradation rate is typically slower with decreasing temperature (FRTR, 2008). Therefore, biodegradation varies seasonally. In some environments, no TBT degradation is found in winter or with incubation at the *in-situ* temperature (5°C), but degradation is present in summer or with incubation at the *in-situ* temperature (28°C) (Olson and Brinckman, 1986).

pH: pH values of <5 or >10 are unfavourable for microbial activity because either proteins required for growth denature or the effectiveness of enzymes is decreased. Typically, optimal microbial activity occurs under neutral pH conditions in the range of 6 to 8 (Alexander, 1999). Nevertheless, the optimal pH is site specific since indigenous microorganisms will have adapted to the conditions in which they exist.

Electron acceptors: bacterial metabolic processes for energy production require a terminal electron acceptor to enzymatically oxidise carbon sources to carbon dioxide (USEPA, 2008a). Microorganisms through their cellular system collect the energy for their use (USGS, 2008). Therefore, aerobic bacteria require dissolved oxygen for TBT degradation (Bernat and Długoński, 2006). Other electron acceptors used are anaerobic, for example, nitrate or sulphate (Dou et al., 2008, Lu et al., 2008). In this case, facultative bacteria promote biodegradation since they can thrive under both aerobic and anaerobic conditions. The participation of oxygen in TBT degradation is

well documented as demonstrated previously (Lee et al., 1989). The anaerobic degradation of TBT by electron acceptors is considered to occur. Unfortunately, the specific mechanism of TBT anaerobic degradation has not been described. Due to the oxidative potential of electron acceptors, the butyl group might be completely mineralised to water and carbon dioxide resulting in DBT, MBT and tin formation (King et al., 1997).

Electron donors: organic compounds are generally utilised as electron donors and a source of carbon. Supplementation with other carbon sources may be used when there is a lack of nutrients present, resulting in limitation of cell growth.

Nutrients: microorganisms require inorganic nutrients such as nitrogen (N), phosphorus (P) and sulphur (S) to support cell growth and sustain biodegradation processes. The viable form of N and P are ammonium and orthophosphate, respectively (King et al., 1997). The presence of phosphorus has been related to the increasing of active TBT bioaccumulation (Singh and Joshi, 1991). Potassium has been reported to play a positive role in TBT biodegradation, and magnesium has been shown to support the growth and metabolism of microorganisms during TBT degradation (Bernat and Długoński, 2006).

Bioavailability: biodegradation is significantly affected if TBT is physically remote from potentially active microorganisms and well sorbed to matrices (Worsztynowicz et al., 2008). Since metabolism of TBT occurs inside cells, low bioavailability would reduce degradation yield and efficiency.

Level of metabolisable compound: the presence of contaminant at concentrations below the threshold can result in no biodegradation due to lacking of enzyme induction. On the other hand, very high concentrations of a contaminant can result in toxicity which inhibits cell multiplication and leads to a small density of capable bacteria. Hence, shock loading can cause a marked drop in remediation efficiency (Stasinakis and Thomaidis, 2010).

2.1.4 Utilisation of TBT and co-metabolism

Breakdown of contaminants can take place by growth-link biodegradation, where microbes utilise the contaminants as sources of carbon, energy, nitrogen, sulfur or other elements needed for multiplication. The contaminants are enzymatically transformed by heterotrophic microorganisms through characteristic metabolic pathways. For TBT, potential microbes utilise butyl groups from the compound as their growth substrate, i.e. source of carbon. In the situation that carbon content is limited, butyltin compounds become the sole source of carbon for survival. Stasinakis et al. (2005) investigated the degradation capacity of a culture obtained from activated sludge in synthetic wastewater without supplemental substrates, TBT was confirmed to be metabolised as the single source of carbon and energy. In addition, Cruz et al. (2007) demonstrated that the growth of *Aeromonas veronii* was increased in a minimal medium, containing all macro and micro nutrients, to which the sole carbon source was provided by TBT. Moreover, higher amounts of TBT added into a medium were found to improve numbers of the bacteria (Cruz et al., 2007).

Additionally, microorganisms can breakdown TBT as a co-metabolite (secondary compound). Co-metabolism is defined as a simultaneous metabolism of two compounds, in which degradation of the secondary compound depends on the presence of the primary compound. In the absence of a primary compound, the secondary compound is not degraded (Bernat and Długoński, 2006). This process is sometimes called biotransformation because metabolism of the second substance is a biological transformation that is not intended for growth, cell replication or energy support. Therefore, the breakdown of the co-substrate reflects the utilisation of the primary substrate for the growth of the microorganisms. The presence of glucose has previously been reported as primary compound for biodegradation of TBT (Bernat and Długoński, 2006).

2.1.5 Biodegradation at stages of bacterial growth

Since biodegradation is related to bacterial growth, the mechanism of growth and environmental conditions are associated with the chemical's breakdown. In autecological studies, almost all bacterial growth dynamics in batch culture exhibit four characteristic phases which are (1) lag phase; (2) exponential phase or log phase; (3) stationary phase; (4) death phase (Prescott et al., 2002).

Lag phase is the period after inoculation of cells into fresh culture medium. During the lag phase, the population remains temporarily unchanged. However, the cells are increasing in metabolic activity, growing in volume or mass and formation of proteins, RNA and other molecules.

Exponential phase (Log phase) is the period in which microorganisms are growing and dividing at the maximal rate possible given their genetic potential, the nature of the medium, and the conditions under which they are growing.

Stationary phase is the period in which microbes find a balance in terms of available nutrients, habitat and the concentration of their own waste products. At this equilibrium, microbial growth and death rates are similar which reflects the maximum rate of biodegradation.

Death phase is the period in which contaminants and nutrients diminish to a low level, there is a high accumulation of waste products and an overcrowding of the microbe population. The competition for available resources leads to an increase in death rate compared to cell division rate. The number of microbes declines. Degradation does not necessary stop, but significantly reduces.

The lag phase and the log phase are the key stages to the efficiency of bioremediation. The period of lag phase is also called the acclimation stage where no destruction of the pollutant takes place. This time interval is defined as the time between the introduction of the pollutant into an environment and the evidence of its

destruction (Suthersan, 1999). This stage is of considerable public health or ecological significance because the pollutant remains unaltered during this period (Alexander, 1999). It prolongs the period of exposure to the pollutant which raises the possibility of effects on and distribution in the environmental media it is in contact with.

The length of the lag phase is dependant on several factors. The density of the inocula, or the population of microorganisms acting on the contaminant, is relevant in this phase, because multiplication of the microbial cells leads to appreciable growth and loss of the chemical (Maier et al., 2009). Therefore, a small population extends the acclimation period. When transferred into a fresh medium (albeit the same or different) and with exposure to pollutants, the cells require a period of time to recover from the physical damage or shock of the transfer (Todar, 2008). High concentrations of pollutants also cause bacterial growth shock or kill the sensitive bacteria before tolerant bacteria recover (Kawai et al., 1998). Time is also required for the synthesis of essential coenzymes or division factors, and for synthesis of inducible enzymes that are necessary to metabolise substrates present in the medium (Stanier et al., 1970).

In addition, the type of pollutants, their concentration and the environmental conditions affect the duration of the acclimation period (Maier et al., 2009, Tarighian et al., 2001). Biodegradation is undetectable until potential species are present at a sufficient biomass to result in pollutant destruction. Because the biodegradation rate is usually determined from the amount of chemical loss, very high concentrations of the pollutant conceal trace levels of its reduction. The duration of acclimation is not only constant for a specific concentration, but varies from site to site. It is influenced by temperature, pH, aeration status, microbial community and, in some environments, nitrogen and phosphorus (Alexander, 1999). Another important factor is the presence of other toxic compounds, introduced initially or generated during biodegradation, which suppress the growth rate of degrading species and delay the acclimation time.

The growth of a single culture bacterium in a medium containing two carbon sources often does not show the same exponential growth phase as it does with an individual carbon source (Alexander, 1999). During reproduction, the substrate which permits faster growth is usually metabolised first, while the second substrate supports little or no growth. After depletion of the first substrate, the second substrate is used for growth. This biphasic growth and utilisation of two substrates in sequence is known as diauxie (Rajan, 2003). In some cases, organic compounds in an environment are used as the first substrate and a toxic contaminant is used as the second substrate. This utilisation of one substrate in preference to the target chemical tends to elongate acclimation (Östberg et al., 2007).

The exponential phase is the most influential period of growth and degradation (Maier et al., 2009). Although exponential growth is limited by depletion of nutrients and production of wastes in media, bacterial growth can be maintained by employing a continual stream of fresh medium or by serial transfer to maintain the level of essential nutrients and to dilute the amount of wastes (Thakur, 2006). As such, the rate of biodegradation of a second treatment batch could be the same as the final rate of first treatment. A greater rate on subsequent treatments can result from increases in numbers of degrading microorganisms after the first treatment or from skipping of the acclimation stage due to the community's active state. This is not always the case, however, since some microorganisms might lose their metabolic activity in the absence of the specific chemical during transfer, or there may be a reduction in number or biomass of the responsible microorganisms which results in a repetition of the acclimation period (Alexander, 1999). Therefore, cultivation of TBT-degraders in many studies acclimate the microbe in the employing medium containing TBT, which significantly enhances the degradation (Stasinakis et al., 2005).

2.1.6 Isolation and identification of TBT-degrading bacteria

In any environment, the community of microorganisms are site specific to the surrounding conditions in which they exist. Therefore, communities in different areas will exhibit dissimilar behaviour/response or capability towards the same thing. In addition, each individual microorganism has its own characteristic properties. Isolation of specific microorganisms is extensively done to obtain single strains which can be employed to achieve specific purposes. This technique has been used to isolate, for example, pollutant degraders (Dercová et al., 2008), probiotics to control infection (Aly et al., 2008), enzyme producers for fermentation (Cheng et al., 2008), disease inducers (Chang et al., 2008), etc. Not every species of microorganism in a community can be used in the treatment of TBT, which is the main objective of this work. Therefore, the isolation and study of single degrader strains were appropriate for investigation on degradation activity.

Generally, the isolation technique for TBT-degraders has been performed by addition of TBT into the sample or medium. This procedure directly screens the tolerate microorganisms which can degrade TBT and eliminates susceptible bacteria. To assure their degradation ability after screening, the isolates are streaked on mineral salt solid media containing TBT. A marine bacterium, *Vibrio* species has been isolated from an oil-field in India employing this technique, using Zobell Marine agar (ZMA) containing 20µM TBTCl (Murthy et al., 2007). Even though TBT can be degraded by freshwater and seawater bacteria, isolation of the degraders by acclimatisation of bacteria in TBT spiked samples is not always successful (Harino et al., 1997a).

To obtain only the degraders after screening, mineral salt medium (MSM) can be used to limit other carbon sources and force the bacteria to utilise TBT. Thus, non-degrading species and non-growth link degraders are removed. This technique was used to isolate TBT-degraders in surface water collected from an oil-field and a shipyard area in India. MSM agar containing 0.4mM TBTCl was used and

Pseudomonas species including *stutzeri*, *fluorescens* and *aeruginosa* were isolated (Roy et al., 2004).

In addition, microorganisms have been isolated from a potentially contaminated area without degrading assays. In order to acquire TBT-degrading cultures, they were tested individually for their degradation ability. Microorganisms in sediment and water collected from the north-west coast of Portugal have been isolated by Cruz et al. (2007) in non-selective medium without TBT. Following this, TBT degradation assays were carried out and *Aeromonas veronii*, a highly resistant TBT-degrader, was obtained (Cruz et al., 2007). Following this procedure, Bernat and Długoński (2009) isolated and found DBT-degraders from composting heaps, which were contaminated with pesticides and toxic metals.

In some studies, microorganisms, which were previously isolated for other purposes or obtained from culture banks, were used and found to be able to degrade TBT. Errécalde et al. (1995) reported the bacterial strain *Pseudomonas fluorescens* was a more potent strain for debutylation of TBT at ng/L level comparable to breakdown by several single strains of fungi, yeasts and bacteria. The bacterial strain *Pseudomonas diminuta* isolated from river water, previously used to degrade tributyl phosphate, was clearly shown the capability to degrade TBT (Kawai et al., 1998). Gram-negative bacteria *Alcaligenes faecalis* was able to degrade TBT via the debutylation process (Barug, 1981). A TBT-degrader, *Cunninghamella elegans*, a potent fungus known for its steroid hydroxylation, was previously used in polycyclic aromatic hydrocarbons (PAHs) degradation (Bernat and Długoński, 2002). The microalgae, *Chlorella vulgaris*, *Scenedesmus dimorphus* and *Chlorella miniata* were capable of degrading TBT at sublethal concentrations (Tam et al., 2002, Tsang et al., 1999).

All studies determined the degradation ability of their isolates under control conditions with different types of medium and different concentrations of TBT. Most of the isolate species were potent to degrade TBT, but might not completely perform debutylation. The pathway of stepwise debutylation of TBT is believed to involve a

cascade of enzymatic reactions, as mentioned in *Section 2.1.1*, involving different enzymes, which one isolate might not be able to produce (Tsang et al., 1999).

The basis of the identification technique is to identify genus and species of the microorganism. In general, the first step in the identification of a microorganism can be done through its morphology, Gram's staining, oxidase test, catalase test, oxygen utilisation and motility. This will categorise bacteria into groups, and then, more specific tests can be performed. The two methods that are widely acknowledged are the biochemical test following *Bergey's Manual of Systematic Bacteriology* (Krieg and Holt, 1984, Roy et al., 2004) and molecular biology by 16S-rRNA gene sequencing (Bosshard et al., 2006). To obtain the best confirmation, both methods are used in conjunction. Identification by biochemical tests has developed to reduce processing time, for example, through the use of the API system, a test kit which contains reagents ready for testing, or Vitek, a fully automated system for bacterial identification (bioMérieux).

2.1.7 Inhibition of bacterial growth from TBT exposure

TBT exerts risk through the interaction with membrane lipids (Cruz et al., 2010). The impact of TBT on microorganisms depends on the dose-response relationship and endpoint, i.e. the concentration which causes the study effect (USEPA, 1997). High concentrations of TBT are toxic to bacteria, while low concentrations might be insufficient for enzyme induction (de Lipthay et al., 2007). TBT can affect microorganisms by inhibiting the oxidative phosphorylation and mediation of anion exchange across membranes (Huang and Matzner, 2004). At high concentrations, TBT impacts on bacterial growth. The growth of the bacterial strain *Pseudomonas diminuta* was inhibited by the addition of TBT at higher than 20 mg Sn/L into estuarine seawater medium and consequently, TBT degradation was suppressed after a period of time (Kawai et al., 1998). In a microcosm, inhibition of growth was not found at a low concentration of TBT (10 µg/L) added into agar medium, but after increasing TBT concentration (100 µg/L), total inhibition of growth was detected (Suehiro et al., 2006). On the other hand, temporary inhibition

was found when colony forming units (CFU) of bacteria were decreased after exposure to TBT, but the population increased again after 2 days (Harino et al., 1997b). This suggests that the level of TBT introduced to bacteria caused toxicity shock, which temporarily inhibited their growth. In addition, higher TBT resistivity was observed in Gram negative than in Gram positive bacteria (Cruz et al., 2007).

Microorganisms can develop their resistivity since they can progressively grow at gradually intensifying levels of TBT, but die if exposed to a high initial level (2mM TBT) (Cruz et al., 2007). Exposure to a toxicant for some time can increase tolerance to that particular toxicant (Blanck and Dahl, 1996). It is postulated that the significant resistance to TBT is associated with the capacity to detoxify TBT (Bernat and Długoński, 2002).

Wuertz et al. (1991) demonstrated a bioassay of TBT in terms of the 50% effective concentration (EC_{50}) based on inhibition of colony formation. The EC_{50} for populations was higher at a TBT-polluted site than at a site without TBT, suggesting that exposure to TBT enhances the resistivity of the population to TBT in a contaminated environment. The EC_{50} for populations from TBT-contaminated freshwater sediments were nearly 30 times higher than those for populations from TBT-contaminated estuarine sediment, due to higher bioaccumulation occurring in the estuarine environment than in the freshwater environment. The toxicity of TBT in laboratory media was markedly influenced by the composition of the medium and whether it was liquid or solid (Wuertz et al., 1991).

Suzuki et al. (1992) demonstrated that TBT-tolerant bacteria accounted for 90% of the flora in natural seawater as observed when an amount of TBTCl was added. On addition of higher levels of TBTCl, the bacteria were found to be sensitive with the exception of *Vibrio* species. Total counts of viable bacteria did not decrease upon storage of the TBTCl-treated seawater, indicating that enrichment of tolerant strains took place.

The mechanisms, which have been proposed as being involved in promoting microorganism resistance to pollutants, are; (i) transformation to less toxic compounds; (ii) excretion from the cell, mediated by a multidrug efflux pump; (iii) degradation or metabolic utilisation; (iv) bioaccumulation without breakdown (Cruz et al., 2010). Microorganisms capable of performing any of these processes could reduce the toxicity of TBT and increase their resistivity. Degradation of TBT by *Dunaliella parva*, a microalga, resulted in its increased tolerance to TBT toxicity (Taha et al., 2009). It is possible that some microorganisms could reduce the toxicity by excreting a sorbent to the cellular surface to prevent the entry of TBT into their cells (Avery et al., 1993, Gadd et al., 1990).

2.2 Experimental

TBT can be broken down by debutylation to reduce its toxicity. Although the process can occur via physicochemical mechanisms including hydrolysis, photo-degradation and chemical cleavage (IPCS, 1990, Suehiro et al., 2006), biodegradation is an effective process which leads to more sustainable remediation. Biodegradation utilises microorganisms under the control of suitable parameters. The process can be used to reduce the level of TBT to a concentration that does not impact on living organisms and the environment, i.e. below the environmental quality standard.

Even though TBT can be naturally broken down in the environment by indigenous microbes, the process can be slow without intervention (Dowson et al., 1996). An understanding of the degradation process has led to an improvement in TBT remediation. This study was performed to isolate microorganisms that are resistant to the toxicity of TBT and also capable of degrading the compound. The identified isolates could then be used for degradation enhancement by bioaugmentation, which provides a quick start for remediation. In this study, bacteria were isolated from TBT-contaminated sediment in Glasgow, identified and investigated experimentally to determine their ability as TBT-degraders. Preliminary

information for further improvement of remediation using these isolated bacteria was also obtained.

2.2.1 Materials and equipments

All bacteriological nutrients and agars were obtained from Oxoid (Basingstoke, UK). Taq DNA polymerase, primers, dNTPs and ladder mix (GeneRuler) was obtained from Fermentas (Maryland, USA). Reagent for the oxidase test was obtained from BD (Maryland, USA). Gram's stain reagents were obtained from BDH (Poole, UK). Tris base, agarose, TAE buffer, bromothymol blue, ethidium bromide and sodium dodecyl sulphate for bacteria identification were obtained from Sigma-Aldrich (Steinheim, Germany).

All chemicals were used without additional purification. Tributyltin chloride (96% purity), dibutyltin chloride (96% purity), monobutyltin chloride (95% purity), tin (IV) chloride (99% purity), tetrabutyltin, tropolone (98% purity), sodium tetraethylborate ($NaBEt_4$), hydrochloric acid and acetic acid were obtained from Sigma-Aldrich (Steinheim, Germany). All solvents were HPLC grade obtained from Fisher Scientific (Loughborough, UK). All other chemicals used were of analytical grade obtained from BDH (Poole, UK). Helium gas and nitrogen gas were obtained from BOC (Manchester, UK).

The equipment and supplies used for this study included a Van Veen grab sampler (Petite Ponar, Wildco), filter paper no.54 (Whatman), shaking incubator (SI500, Stuart), orbital shaker (Stuart), autoclave (PriorClave, Tactrol), centrifuge (Micro Centaur, MSE), balance (EL104, Mettler Toledo), vortex (MaxiMatic, Jencons), PCR device (Tpersonal, Biometra), gel electrophoresis (MyRun, Cosmo Bio), gene purification kit (Geneaid), biochemical identification kit (API 20E, bioMérieux), gas chromatograph coupled with mass spectrometer (GC-MS) (Trace GC Ultra/DSQII, Thermo Scientific), chromatographic capillary column (DB5, 30 m x 0.25 mm i.d., 0.25 µm film thickness, Thermo) and UV/Vis spectrometer (Helios Zeta, Thermo).

2.2.2 Sample collection

The Forth and Clyde Canal was used as a main waterway between the east and the west coasts of Scotland and, as such, is a local contaminated site. The Bowling Basin (NS 450 735) on the canal was chosen as the sampling site, as it represents ongoing ship activity, shown in Figures 2.3 and 2.4. The sediment sample was collected in March 2007. Ten kilograms of surface layer sediment was taken using a Van Veen grab sampler at 15 cm in depth (Figure 2.5). The sediment was stored in sterile buckets. Samples were kept at 4°C in the dark immediately after transported to the laboratory. The study was performed within a few days after the sediment collection.

Figure 2.3 Picture showing Bowling Basin in the Forth and Clyde Canal.

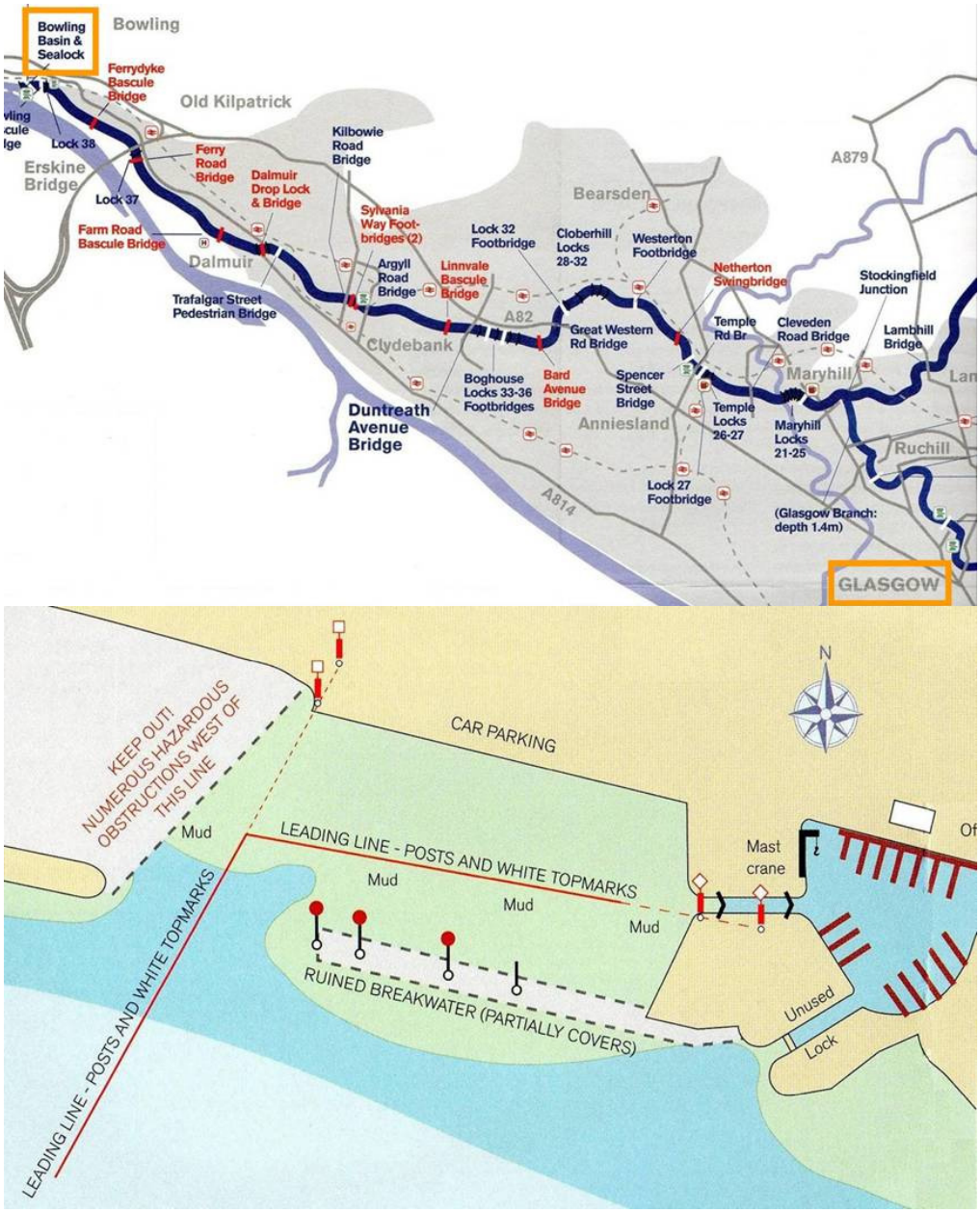


Figure 2.4 Map showing Bowling Basin in the Forth and Clyde Canal (adapted from British Waterways Scotland, 2008).

Figure 2.5 Sediment sample collection using a Van Veen grab sampler.

2.2.3 Isolation of TBT-degraders from sediment

The isolation of TBT-degrading bacteria was performed in two types of medium, 1) minimal medium (Visoottiviseth et al., 1995) and 2) glycerol medium (Inoue et al., 2000 with slight modification), both containing TBTCl (preparation of the media and employed concentrations of TBTCl were shown in *Appendix A*). Ten grams of sediment was suspended in 100 ml of each medium. Each suspension was filtered through a number 54 filter paper. A 5-ml aliquot of the filtered solution was inoculated into fresh medium of each type. The samples were incubated at 28°C on an orbital shaker at 150 rpm for 7 days. Subsequent to four inoculations and incubation under the same conditions, each culture solution was streaked on the solid medium containing the same amount of TBTCl in the screening medium. The streaked plates were incubated at 28°C for 24 h. Then, single colonies were picked up and re-streaked until pure cultures were obtained.

2.2.4 Identification of the isolated bacteria

Initially, simple identification techniques were carried out, e.g. Gram's staining, oxidase test and catalase test (Cappuccino and Sherman, 1998). Pure isolate cultures were differentiated on a non-selective medium (e.g. plate count agar, PCA), and selective media (e.g. mannitol salt agar, MSA), MacConkey agar (MCA) and Baird Parker agar (BPA). The plates were incubated at 28°C for 24 h, before observation and comparison. The isolates were kept as stocks at below 4ºC on slants made up from the same agar containing TBTCl.

Next, the bacterial isolates were subject to genotypic identification. 16S-rRNA gene sequencing was used. Complementary to this, biochemical tests in the form of API assays were used to phenotypically corroborate the identity of the new isolates that had never been identified as TBT-degraders.

The flow diagram of DNA extraction is shown in Figure 2.6 (Sambrook and Russel, 2001). For 16S-rRNA gene sequencing, all the isolates were cultured in Luria-Bertini (LB) liquid medium at 37°C, and shaking at 300 rpm for 24 h (Figure 2.6a). Chromosomal DNA was extracted from 1 ml of each culture suspension by centrifuging at 4,200 g for 2 min. The supernatant was discarded, and the cells were resuspended in 370 µl of Tris-EDTA (TE) buffer at pH 8.0, consisting of 10mM Tris.HCl and 1mM EDTA, with vortex (TE buffer was prepared with a stock solution of 1M Tris.HCl, mixture of Tris base 121.1 g and HCl 42 ml in 1 L of DI water, and 0.5M EDTA adjusted to pH 8.0 with NaOH). A 20 µl aliquot of 10% sodium dodecyl sulphate (SDS) was added and quickly inverted, then mixed thoroughly with 70 µl of 5M NaCl (Figure 2.6b). Afterwards, 200 µl of phenol:chloroform:isoamyl alcohol (25:24:1) were added, then vortexed and centrifuged at 16,800 g for 5 min (Figure 2.6c). The aqueous phase was transferred into a new tube, and then the volume was doubled with isopropanol. The tube was slowly inverted. After DNA stripes were visibly formed (Figure 2.6d), the sample was centrifuged at 16,800 g for 10 min and the solution was removed. The DNA was washed with 1 ml of 70% ethanol, followed by centrifugation at 16,800 g for 5 min. Alcohol was poured out and the

sample was left to dry at ambient conditions (Figure 2.6e). The DNA residue was reconstituted in 30-50 µl of TE buffer depending on the amount of the residue. The DNA were kept at 4°C if immediately used, or at -20°C for storage.

The quantity of extracted DNA was checked by gel electrophoresis technique. An agarose solution (2%) was prepared and left to set in a tray with a comb to make slots (Figure 2.6f). The gel was transferred into a gel electrophoresis device, and soaked with 0.5x TAE buffer. The mixture of 2 µl of DNA solution, 1 µl of 10x dye bromothymol blue and 3 µl of DI water was filled into the gel slots. DNA Ladder Mix (2 µl) was filled into a gel slot for sizing and approximate quantification (Figure 2.6g). The equipment was operated for 45 minutes at 100 volt. The DNA was dyed by soaking the gel in ethidium bromide solution for 10 minutes then rinsed with water for 5 minutes. The picture of DNA banding was captured under UV lamp (Figure 2.6h).

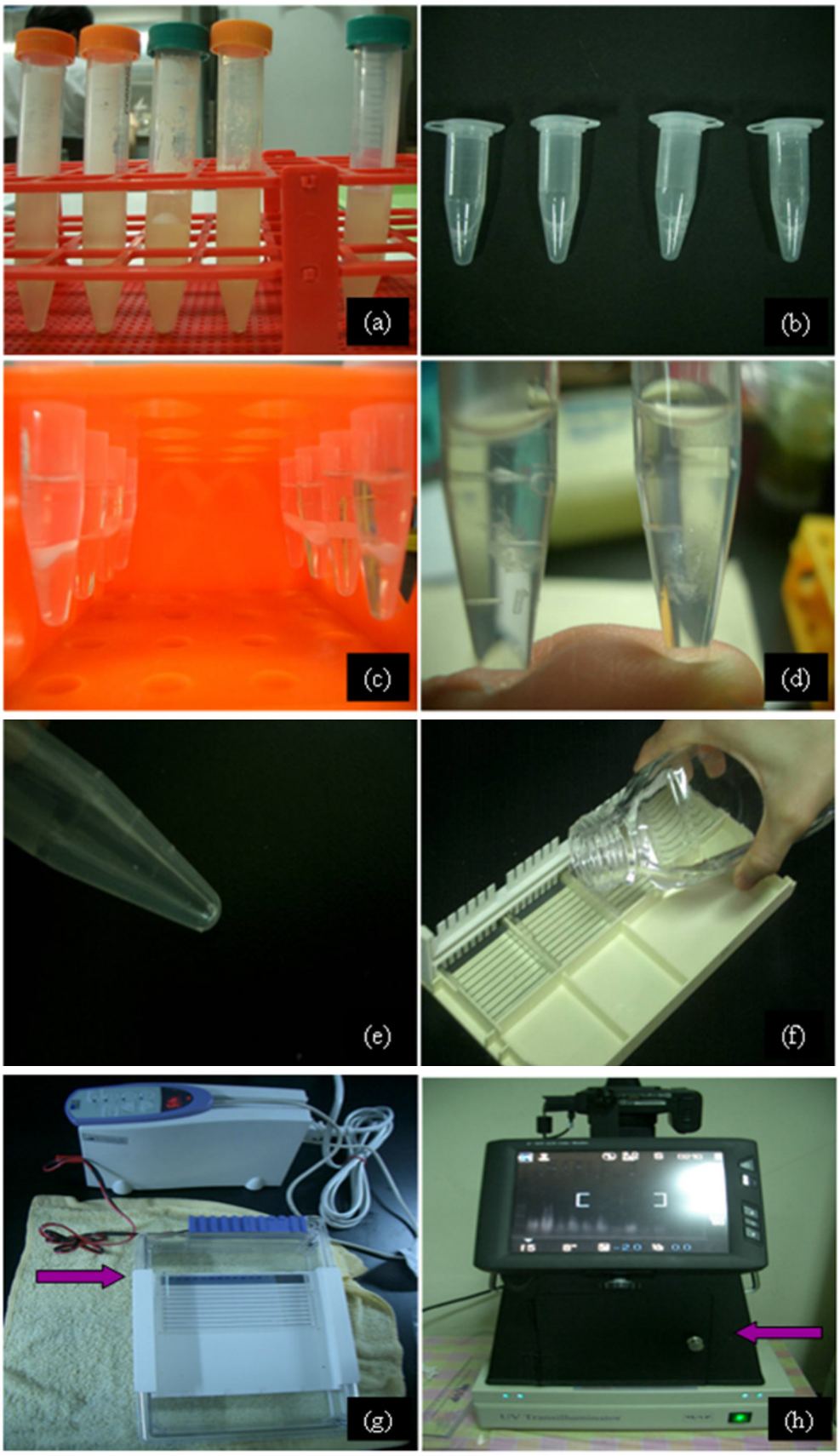


Figure 2.6 Flow diagrams of DNA extraction procedures.

Polymerase Chain Reaction (PCR) was performed by using two universal bacteria primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') where M is A or C, and 1525R (5'-AAGGAGGTGWTCCARCC-3') where W is A or T, and R is A or G. The solution for PCR reaction (50 µl) contained: 5 µl 10x buffer, 0.8 µl 10mM dNTPs, 1.5 µl 50mM $MgCl_2$, 0.625 µl 20mM of each primer, 0.25 µl 5U/µl Taq DNA polymerase and 2 µl DNA. The PCR conditions were as follows: initial denaturation at 95°C for 5 min; 30 cycles of denaturation (1 min; 95°C), primer annealing (1 min; 52°C) and primer extension (1 min; 72°C), with a final 10 min annealing step at 72°C. Once the DNA was successfully amplified, the PCR products were purified using a purification kit. The recovered PCR products were sent out to 1st base, Wardmedic, Malaysia, for sequencing using 27F primer. Nucleotide sequences were analysed using sequence alignment editor (Isis Pharmaceuticals) and the *BLASTn* program, which provided highly-probable identification of isolates based on the GenBank database at National Institute for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

The biochemical tests were employed using API 20E (bioMérieux), a standardised identification system for *Enterobacteriaceae*. The incubation box was prepared by distributing 5 ml of distilled water into honey combed wells of the tray to create a humid atmosphere, before an API 20E strip was placed in the incubation box. The inoculum was prepared by removing a single well isolated colony of pure culture and carefully emulsifying to achieve a homogeneous bacterial suspension in sterile saline water containing 0.85% NaCl. The bacterial suspension was transferred into the tubes of the API strip. Mineral oil was overlaid on some tubes to create anaerobiosis. The incubation box was closed and incubated at 37°C for 24 h. During incubation, metabolism produced colour changes that were either spontaneous or revealed by the addition of reagents. The reactions were read according to the reading table (*Appendix B*), and the identification was obtained by referring to the analytical profile index or using the identification software.

2.2.5 Determination of butyltin compounds

2.2.5.1 Analysis of butyltin compounds by GC-MS

Prior to the degradation study, effective determination for the presence and the concentration of butyltin compounds was developed. Butyltins were determined using GC-MS with the addition of tetrabutyltin as an internal standard. A working solution was prepared to indicate the retention time of each butyltin species. Stock solutions of TeBT, TBTCl, DBTCl, MBTCl and $SnCl_4$ were prepared separately to a concentration of 1,000 mg/L in hexane. Stock solutions were made up by adding 25.42 µl of TeBT standard solution, 21.7 µl of TBTCl standard solution, 26.04 mg of solid standard of DBTCl, 15.4 of µl MBTCl standard solution or 25.25 mg of solid standard of $SnCl_4$ to 25 ml volumetric flasks and made up to volume with hexane. The stock solutions were refrigerated.

The stock solutions of butyltins were mixed and diluted to give 10 mg/L in hexane, with 25 mg/L of the internal standard. Subsequently, a 0.5 ml aliquot of the mixed butyltins working solution was transferred into a vial. For the derivatisation step, ethylation of every tin species except tetrabutyltin was performed by addition of 1 ml of freshly prepared 0.3% $NaBEt_4$ and 5 ml of 0.1M acetate buffer: 1 L of the buffer was prepared by dissolving 13.6 g of sodium acetate in a beaker and adjusted the pH to 5 with glacial acetic (Ceulemans et al., 1998). The mixed solution was then manually shaken for 5 min, before the organic layer was collected and dried with anhydrous sodium sulphate (anh.Na_2SO_4), prior to GC-MS analysis.

The derivatised solution 1 µl was introduced into the GC-MS using helium as the carrier gas, with the flow rate of 1 ml/min. Injector and detector temperatures were held at 240°C and 200°C, respectively. The solvent delay was 4 min. The column oven temperature was programmed from an initial temperature of 100°C, held for 5 min, to a final temperature of 300°C at a rate of 15°C/min, and held for 5 min. Sample injection was split mode. The mass spectrometer was full scan (*m*/*z* 50-

400) in EI mode. The peak areas and mass spectra (Total Ion Chromatogram, TIC) were recorded.

To produce a calibration curve, working solutions of butyltins at different concentrations were prepared in hexane. TBT was spiked into samples, while DBT and MBT were formed from the degradation of TBT. Therefore, working solutions of TBT had higher concentrations than those of DBT and MBT. Stock standards of butyltins were mixed to make a calibration curve of TBT at 10, 20, 30, 40 and 50 mg/L, DBT and MBT at 2, 4, 6, 8 and 10 mg/L, with each solution containing 25 mg/L of TeBT. The solutions were derivatised as described above and analysed with GC-MS. The peak areas were plotted against working standard concentrations to form the calibration graph.

2.2.5.2 Extraction conditions and derivatisation procedures for butyltin compounds

The extraction and derivatisation steps used were a slight modification of those described by Bangkedphol et al. (2008) and Ceulemans et al. (1998). Briefly, NaCl was added into 50 ml of each sample at 0.02% w/v and adjusted to a pH ranging between 1.6 and 2 with 1M HCl. Each sample was extracted 3 times with 5 ml hexane/ethyl acetate (1:1) containing 0.05% tropolone on a shaker at 300 rpm for 30 min. The extracts were combined with 0.5 ml of internal standard (25 mg/L). Each sample was pre-concentrated under a gentle stream of nitrogen gas to obtain a 0.5 ml final volume, and then derivatised, dried and analysed with GC-MS as described in *Section 2.2.5.1*.

The methods by Bangkedphol et al. (2008) and Ceulemans et al. (1998) were developed for environmental samples. Therefore, optimisation of extraction conditions and derivatisation procedures for cell culture solution was required to validate the method, which performed in triplicate. Firstly, the quantity of derivatisation was checked to ensure the complete ethylation of butyltin compounds. The working solution at 50 mg/L of mixed butyltin compounds containing 25 mg/L

of internal standard was prepared. A 0.5 ml aliquot was derivatised in 5 ml of acetate buffer with 1, 2, 3 or 5 ml of 0.3% $NaBEt_4$.

Second, the effect of pre- and post-derivatisation was tested to obtain a better recovery. The glycerol medium employed in the experiment was used as a matrix. Stock standard 100 mg/L of mixed butyltins in methanol was spiked into the medium to a final concentration of 1µM (~0.3255 mg/L, initial concentration utilised in further experiments). For pre-derivatisation, a 50 ml of spiked sample was adjusted to pH 5 with 1M acetate buffer. After derivatised with 1 ml of 0.3% $NaBEt_4$, the sample was extracted and internal standard was added at 25 mg/L. Then, the sample was pre-concentrated, dried and analysed with GC-MS. The post-derivatisation procedures were done using the method previously mentioned based on Bangkedphol et al. (2008) and Ceulemans et al. (1998).

Third, the suitable extraction conditions obtained were tested with the medium with bacterial cells. The sediment microbes were isolated by mixing 10 g of sediment in 100 ml of glycerol medium. A 5 ml aliquot of supernatant was transferred to fresh medium and incubated at 28ºC for 7 days to reach maximum growth of the cells. The cell suspension was autoclaved to stop further activities follow by addition of butyltins standard, 100 mg/L in methanol, to give a final concentration of 1µM (0.3255 mg/L). The spiked sample was kept in the dark for 24 h to allow cellular absorption before analysis. The sample was extracted 3 times. The upper layers were collected and emulsion was removed using excessive amount of anh.Na_2SO_4. Finally, the sample was pre-concentrated, post-derivatised with 1 ml of the derivatising agent and analysed with GC-MS.

Fourth, the conditions were tested with different initial concentrations of butyltin compounds. Autoclaved culture solution was spiked at 0.05, 0.10, 0.20, 0.25 and 0.32 mg/L. The samples were extracted and analysed with GC-MS after sample preparation.

2.2.6 Degradation of TBT by single cultures in liquid medium

TBT-degradability of the isolates was screened in the glycerol medium (n=3) as previously prepared in *Section 2.2.3*. Each strain was pre-cultured in the medium without TBTCl at 28°C on an orbital shaker at 150 rpm for 24 h. Triplicates of each strain were contained in separate containers. The culture suspensions (2.5 ml) were inoculated into 50 ml fresh medium with 1µM TBTCl. Each sample was incubated under the same conditions and analysed for butyltins after 14 days. The extraction of each sample and their triplicate was performed in the same container without transfer. A control sample containing TBT without cells, allowing for photo/chemical degradation was prepared. Under these conditions, TBT-degraders were screened. Moreover, degradation of TBT-degrading bacteria was studied and continually analysed for TBT at 0, 1, 3, 7, 10 and 14 days of incubation.

2.2.7 Resistance of the isolates after TBT exposure in liquid medium

Growth curves of each isolate in the glycerol medium containing different concentrations of TBT were prepared to determine their TBT-resistivity. The isolated bacteria were cultured in the glycerol medium without TBTCl at 28°C on an orbital shaker at 150 rpm for 24 h. Five millilitres of inocula were transferred into 100 ml of fresh medium containing 0, 1, 10 and 130µM TBTCl, and incubated under the conditions. Optical density (OD) of the supernatants was subsequently determined using a UV/VIS spectrometer at 600 nm until the stationary phase of bacterial growth was reached (Cruz et al., 2007). The bacterial growth at various levels of TBTCl and of different strains was compared.

2.3 Results and discussion

2.3.1 Isolation and identification of TBT-degraders

The sediment from Bowling Basin was previously reported to be highly contaminated with butyltins which enormously exceed environmental quality standard shown in *Section 1.5.2* (Bangkedphol et al., 2008). In the canal water, 0.84, 1.13 and 2.16 µg/L of TBT, DBT and MBT were found, respectively. In sediment 153.44 µg/kg of TBT was measured. Also Bangkedphol et al. (2008) reported the high organic carbon content of Bowling Basin's sediment which leads to high accumulation of butyltins.

After the screening in minimal medium was completed, no turbidity was observed in the culture solution indicating no microbial growth. Spreading of culture solution on minimal agar containing TBT showed no microorganisms survived after the screening process, as shown in Figure 2.7a. Though not a single colony was formed refers that the sediment bacteria had no ability to debutylate TBT as sole carbon supply.

In glycerol medium, after incubation of the fourth transfer, the solution was highly turbid indicating the presence of microorganisms. Yet, it cannot be concluded that all the microorganisms present could degrade TBT. Due to TBT-tolerance microbes could remained in the presence of other carbon sources. As a result, checking the isolates capability for TBT degradation was compulsory. Figure 2.7b shows various types of bacteria on glycerol medium agar containing TBT.

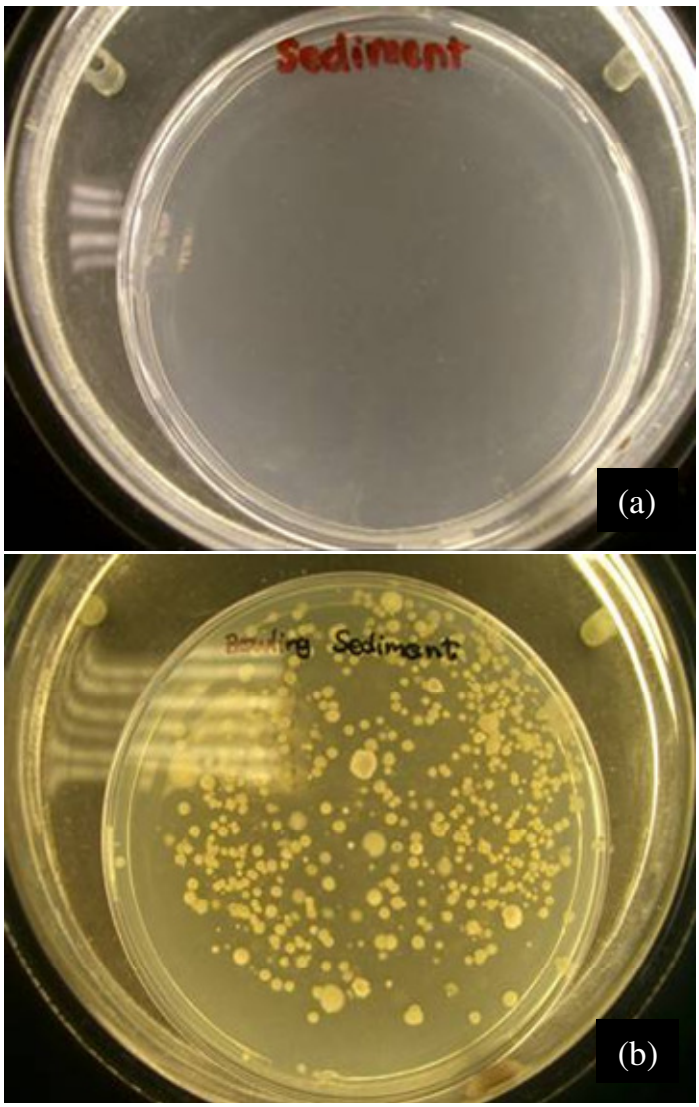


Figure 2.7 Screening of TBT-degraders from sediment bacterial cultures in (a) minimal medium with 8 mg/L TBTCl; (b) glycerol medium with 55 mg/L TBTCl.

After separation by morphology including shape, size and Gram's stain colour, 6 bacteria were isolated, purified and designated as B1, B2, B3-1, B3-2, B4 and B5. Figure 2.8 shows all the isolates are short rod Gram negative bacteria. Except B4, all the isolates showed negative oxidase test and positive catalase test. B4 is the only isolate that showed a positive reaction for both tests.

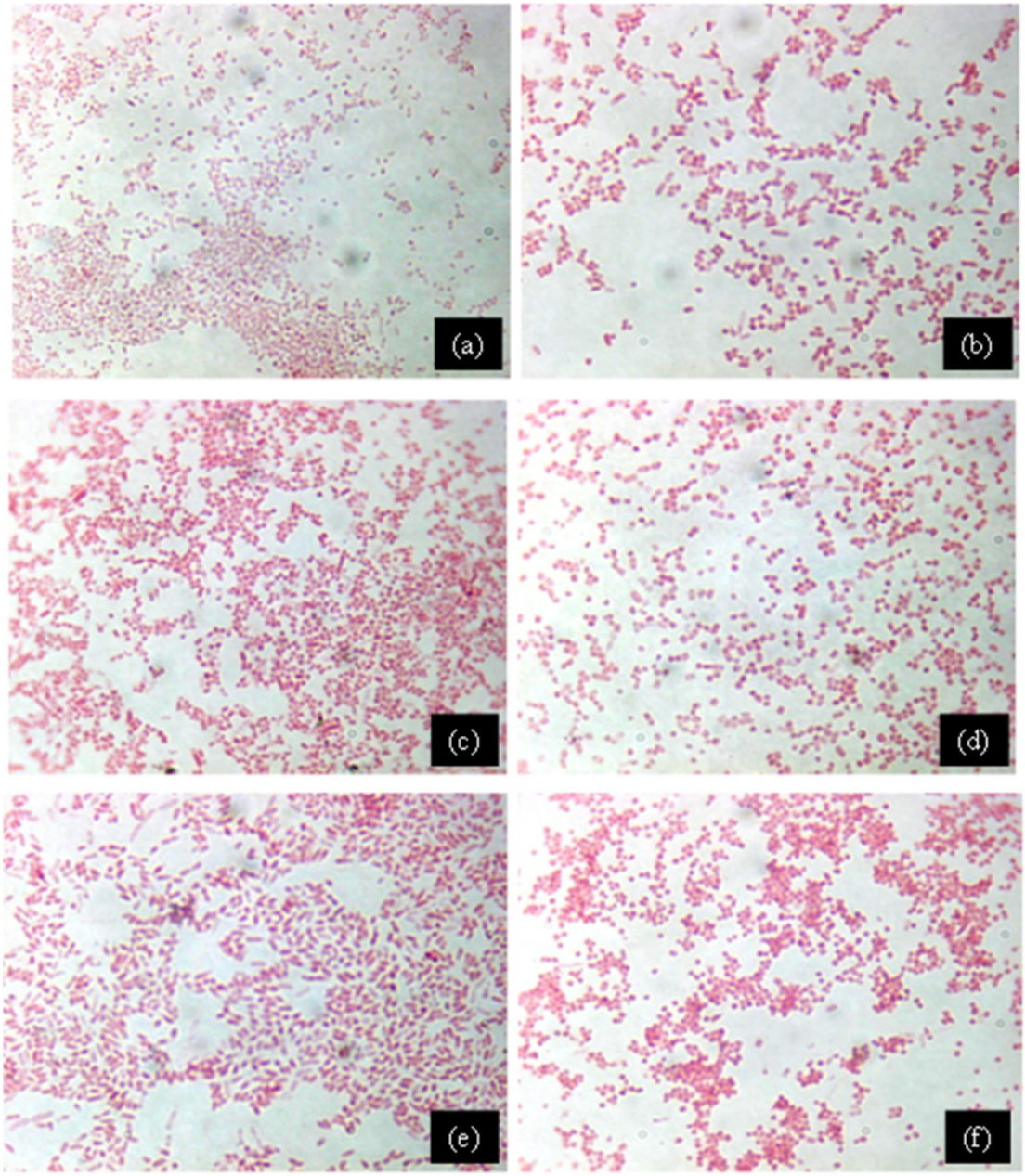


Figure 2.8 Microscopic photos of the isolated bacteria under oil immersion magnification at 1000X (a) B1; (b) B2; (c) B3-1; (d) B3-2; (e) B4; and (f) B5.

Subsequently, the isolates were transferred onto a non-selective agar (PCA) and three selective agars (MSA, MCA and BPA) which allow them to grow and show their character on each medium differently. Since the isolates are unknown, the three distinguish selective media were selected for preliminary separation of the isolates. The result is shown in Figure 2.9.

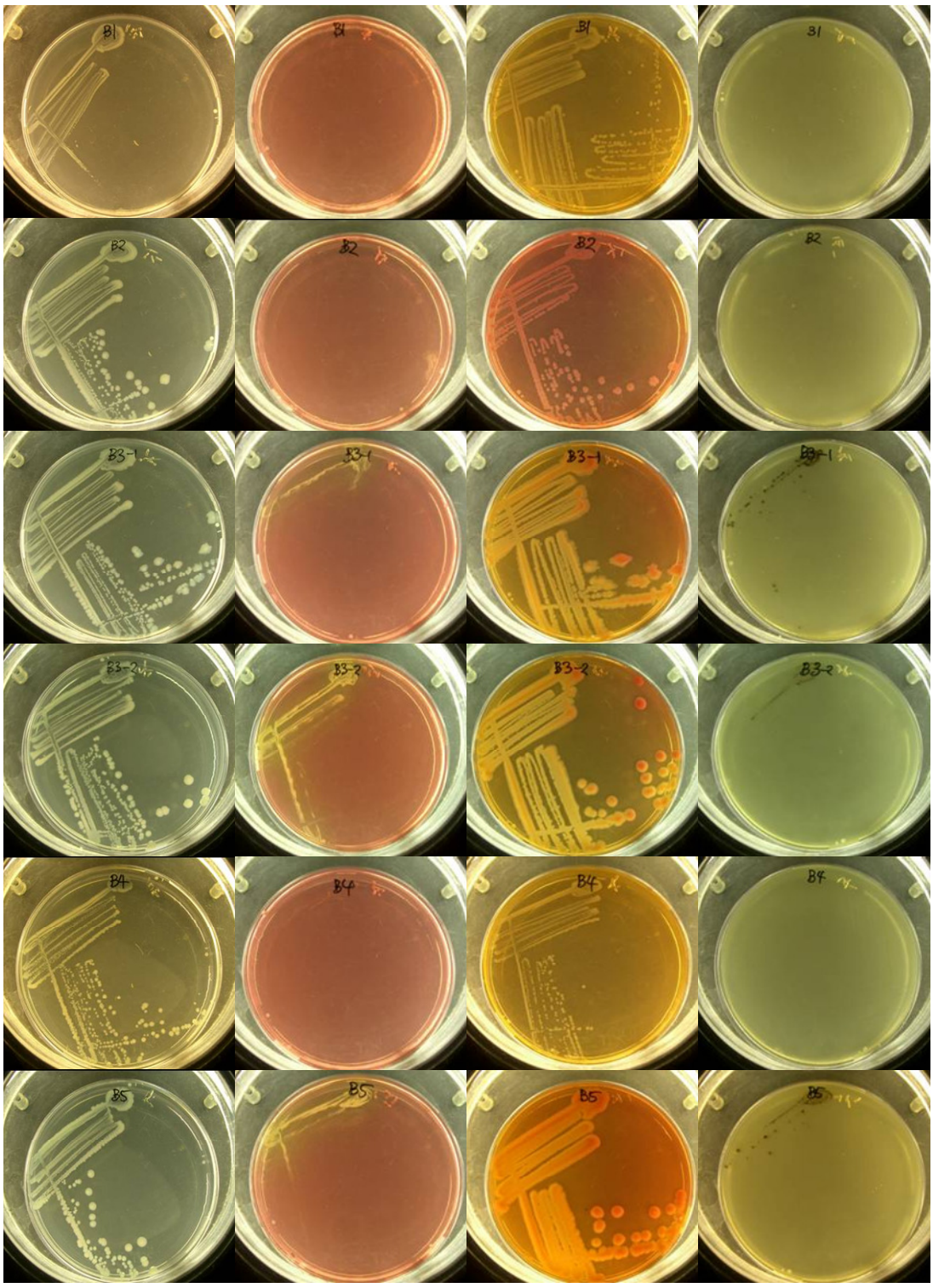


Figure 2.9 Growth of the 6 isolated sediment bacteria B1, B2, B3-1, B3-2, B4 and B5 on PCA, MSA, MCA and BPA, in the order from left to right.

All the isolates grew on PCA, a non-selective medium. On MSA, B3-1, B3-2 and B5 struggled to grow, while the growth of B1, B2 and B4 was not observed. This type of agar contains high level of NaCl which can inhibit growth of some bacteria. Due to the differential property of the agar by containing phenol red, mannitol fermentation by B3-1, B3-2 and B5 produces acid which changes the agar's red colour to yellow. MCA was used to distinguish the isolates as lactose fermenters (B2, B3-1, B3-2 and B5) produce acid and changes the colour of the pH indicator, neutral red, in the agar into deep red, and also produce pink colonies. On the other hand, non-lactose fermenters (B1 and B4) use peptone instead and release ammonia resulting in yellow colour of agar, and colourless colonies. On BPA, black, shiny, convex colonies of B3-1, B3-2 and B5 were present.

From the differentiating procedures, the isolates were distinguished as shown in Figure 2.10. Only B3-1, B3-2 and B5 were not able to be separated from each other.

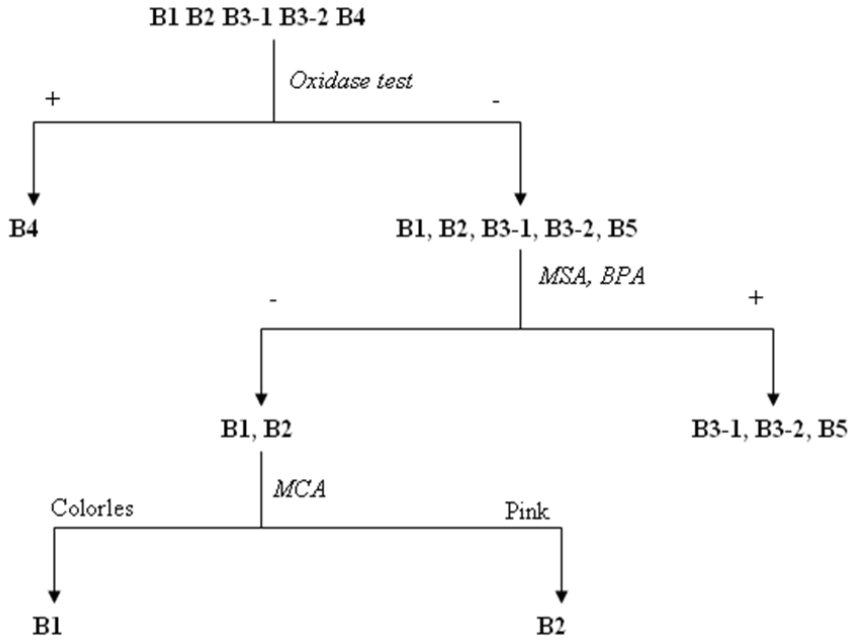


Figure 2.10 Differentiation of the isolates by biochemical tests and selective agars.

For chromosomal DNA extraction, the bands of genomic DNA were shown in Figure 2.11. High concentrations of DNA occurred at the top of the gel with a

smear of RNA at the bottom. Figure 2.11 also shows that the isolates B3-1, B3-2 and B5 contained plasmid DNA, the band in the middle of the gel. The extracted DNA was diluted as appropriate prior to PCR. The PCR products are shown in Figure 2.12.

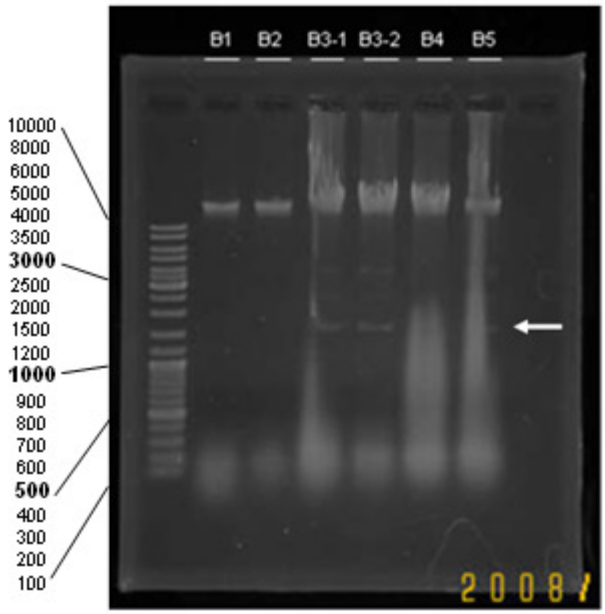


Figure 2.11 Extracted DNA of the 6 isolates on 2% agarose gel, the arrow points to evidence of plasmid DNA.

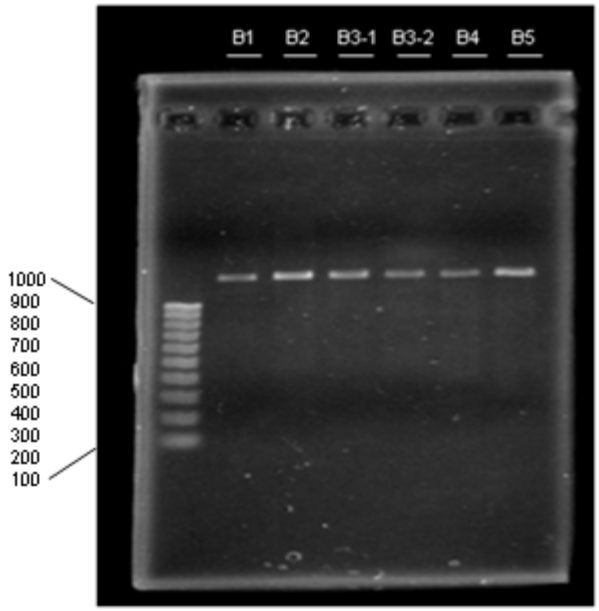


Figure 2.12 PCR products of the 6 isolates on 2% agarose gel using 27F and 1525R universal primers.

After purification, the PCR products were sequenced by 1st base, Wardmedic, Malaysia (results are shown in *Appendix C*). Comparison of the sequence in GenBank was confirmed 4 different species as shown in Table 2.1. The 16S rRNA alignment with BLAST 2 SEQUENCES program indicates the same strain among B3-1, B3-2 and B5.

Table 2.1 Comparison of the isolates based on partial sequencing of the 16S gene with the most closely related species from the GenBank database.

Strains	Genus/species	Accession No.	% Identity
B1	*Alcaligenes faecalis*	AY866407.2	99
B2	*Citrobacter braakii*	AF025368.1	99
B3-1	*Enterobacter cloacae*	EU733519.1	99
B3-2	*Enterobacter cloacae*	EU733519.1	99
B4	*Pseudomonas fluorescens*	EU586046.1	98
B5	*Enterobacter cloacae*	EU733519.1	99

Moreover, *Alcaligenes faecalis* (Barug, 1981) and *Pseudomonas fluorescens* (Errécalde et al., 1995, Roy et al., 2004) had previously been found to be TBT-degraders. *Enterobacter cloacae* have never been studied with TBT, but it was previously found to degrade pentaerythritol tetranitrate (Binks et al., 1996) and 2,4,6-trinitrotoluent (French et al., 1998).

The isolates, *A. faecalis* and *E. cloacae* are classified as class 2 biological agent by the Advisory Committee on Dangerous Pathogens (ACDP), as they have the potential to cause disease, but *C. braakii* and *P. fluorescens* are not include in the list (ACDP, 2004). It should be noted that these bacteria were isolated from natural environmental sample, and none of the species isolated have been associated with serious human disease.

The two new TBT-degrader strains, B2 and B3-1 (referred to as B3) were also identified with API 20E biochemical test kit as shown in Figure 2.13. After the interpretation as shown in Table 2.2, it was confirmed that B2 was *C. braakii*, while B3 was *E. cloacae* at 99.8% and 95.1% confident interval, respectively.

Figure 2.13 API 20E strips tested for B2 (above) and B3 (below).

Table 2.2 Biochemical characteristics of isolated TBT-degraders obtained from sediment sample using API 20E.

Characteristics	B2	B3
β-galactosidase production	+	+
Arginine dihydrolase production	+	+
Lysine decarboxylase production	-	-
Ornithine decarboxylase production	+	+
Citrate utilisation	+	+
H_2S production	+	-
Urease production	-	-
Tryptophane deaminase production	-	-
Indole production of tryptophane	-	-
Acetoin production	-	+
Hydrolysis of gelatin	-	-
Fermentation or oxidation of:		
- Glucose	+	+
- Mannitol	+	+
- Inositol	-	-
- Sorbitol	+	+
- Rhamnose	+	+
- Sucrose	-	+
- Melibiose	+	+
- Amygdalin	+	+
- Arabinose	+	+

Remark: + = Positive reaction; - = Negative reaction

2.3.2 Analysis of butyltin compounds

The analysis of organotin compounds with the GC-MS gives high sensitivity. However, analysis with GC requires derivatisation to form volatile compounds (Bowles et al., 2003). Ethylation, addition of ethyl- groups into the compounds with $NaBEt_4$ was carried out in buffer pH 5 to increase volatility of butyltins (Schubert et al., 2000). The reaction and the derivatised products are shown in Figure 2.14.

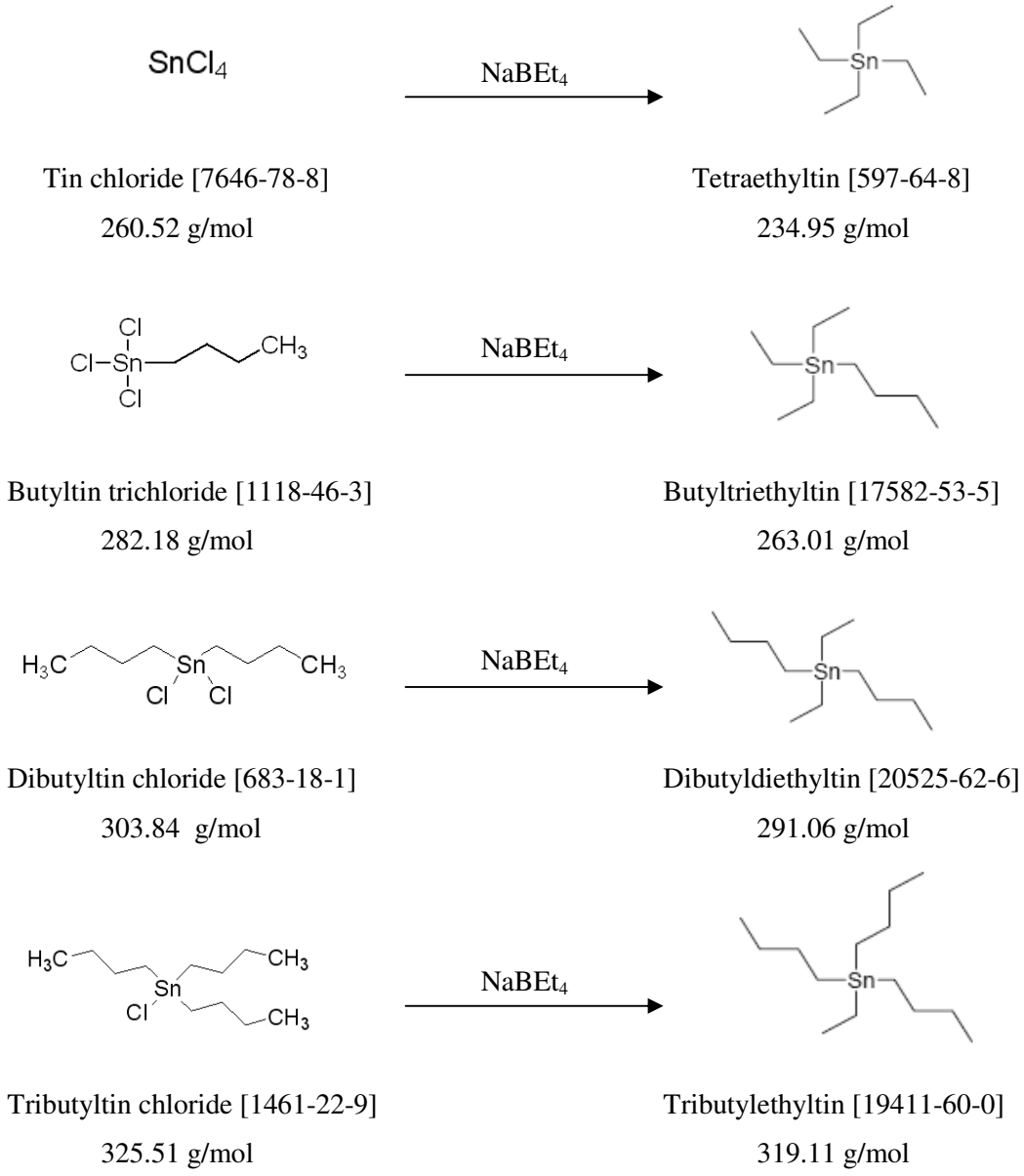


Figure 2.14 Ethylation of organic and inorganic tin with sodium tetraethylborate.

The chromatogram of the 10 mg/L working standard shown in Figure 2.15 indicates the retention time of derivatised tin species start from tetraethyltin at 4.21 min, butyltriethyltin at 7.42 min, dibutyldiethyltin at 9.57 min, tributylethyltin at 11.10, and finally tetrabutyltin at 12.29 min. The order of retention times was as expected from the separation in DB5 column where non-polar alkyl groups were retained by the stationary phase. As a result, tetraethyl-tin which contains 4 ethyl groups (C_2H_5) was the first compound detected, while tetrabutyltin with 4 butyl groups (C_4H_9) was the last.

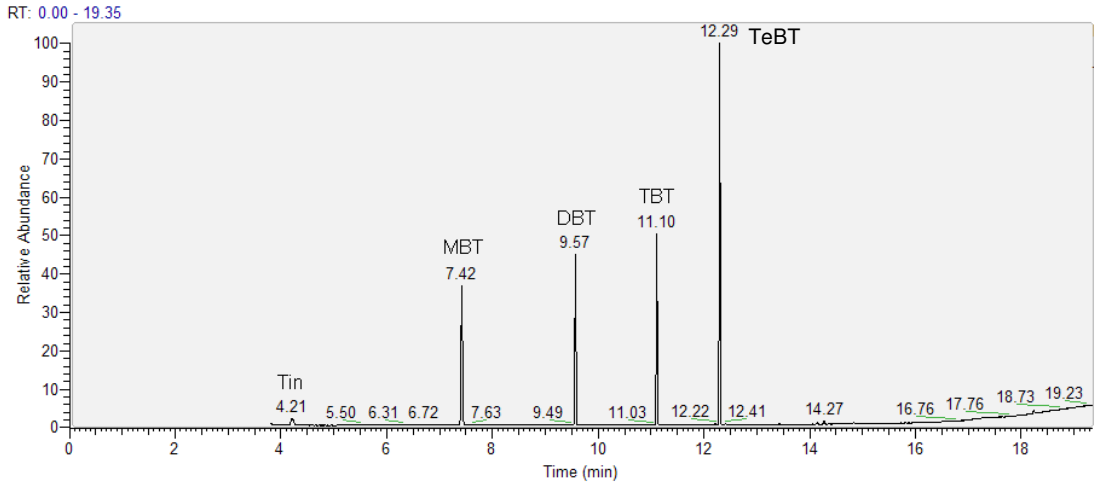
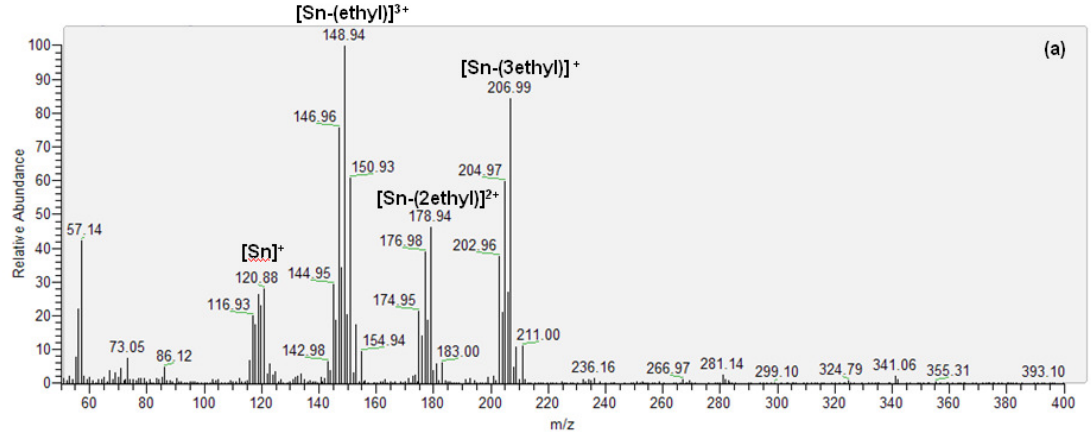
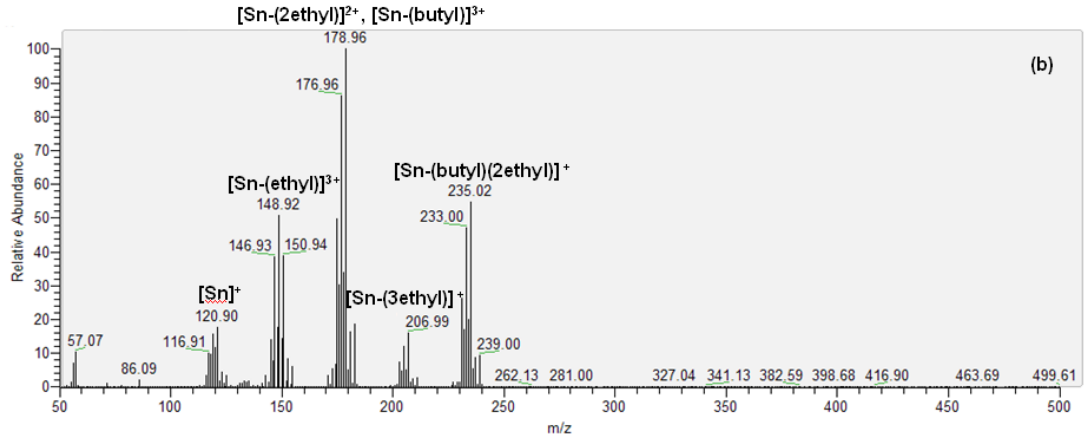
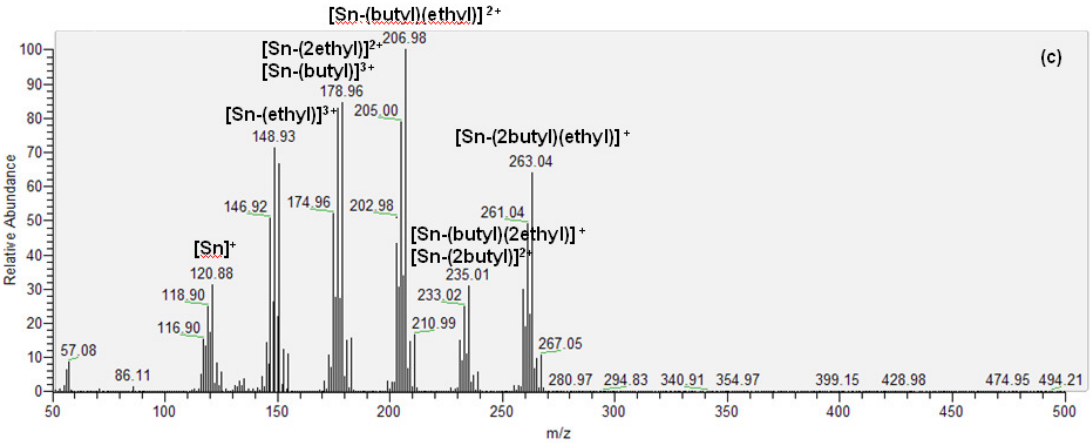


Figure 2.15 Chromatogram of tetrabutyltin (internal standard) and ethylated tri-, di-, mono-butyltin and tin analysed by GC-MS.

The parent molecular masses of tetraethyltin, butyltriethyltin, dibutyldiethyltin, tributylethyltin and tetrabutyltin are 234.95, 263.01, 291.06, 319.11 and 347.16 m/z, respectively. The ionisation of the compounds alternatively removed alkyl groups from tin atom. The specific ionisation series of molecular mass were shown in Figure 2.16.


[Sn-(ethyl)]3+
148.94
[Sn-(3ethyl)]+
206.99
146.96
150.93
204.97
[Sn-(2ethyl)]2+
178.94
57.14
[Sn]+
176.98
202.96
120.88
144.95
116.93
174.95
73.05
86.12
142.98
154.94
183.00
211.00
236.16
266.97
281.14
299.10
324.79
341.06
355.31
393.10
(a)
Relative Abundance
m/z


[Sn-(2ethyl)]2+, [Sn-(butyl)]3+
178.96
176.96
[Sn-(butyl)(2ethyl)]+
235.02
[Sn-(ethyl)]3+
148.92
233.00
146.93
150.94
[Sn]+
120.90
[Sn-(3ethyl)]+
206.99
57.07
116.91
239.00
86.09
262.13
281.00
327.04
341.13
382.59
398.68
416.90
463.69
499.61
(b)
Relative Abundance
m/z


[Sn-(butyl)(ethyl)]2+
[Sn-(2ethyl)]2+
206.98
[Sn-(butyl)]3+
178.96
205.00
[Sn-(ethyl)]3+
148.93
[Sn-(2butyl)(ethyl)]+
263.04
146.92
174.96
202.98
261.04
[Sn-(butyl)(2ethyl)]+
[Sn-(2butyl)]2+
[Sn]+
120.88
235.01
118.90
233.02
116.90
210.99
57.08
267.05
86.11
280.97
294.83
340.91
354.97
399.15
428.98
474.95
494.21
(c)
Relative Abundance
m/z

Continued

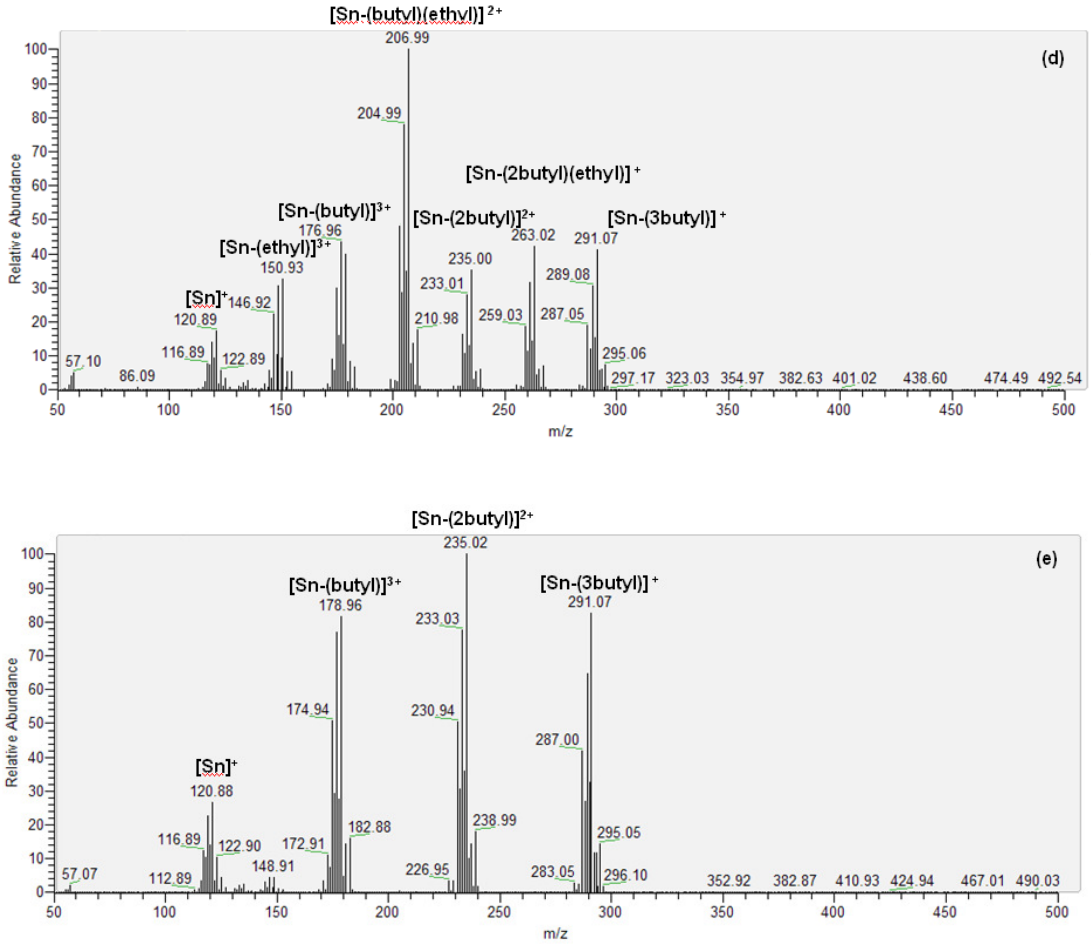


Figure 2.16 Mass spectra of (a) ethylated tin; (b) ethylated monobutyltin; (c) ethylated dibutyltin; (d) ethylated tributyltin; and (e) tetrabutyltin, analysed by GC-MS.

From the set of working solutions, calibration graphs, the linear relationships and correlation coefficients can be produced as shown in Figure 2.17. The limit of detection (LOD) was 1.99, 0.55 and 0.89 mg/L for TBT, DBT and MBT, respectively (relevant calculation of LOD is shown in *Appendix D*).

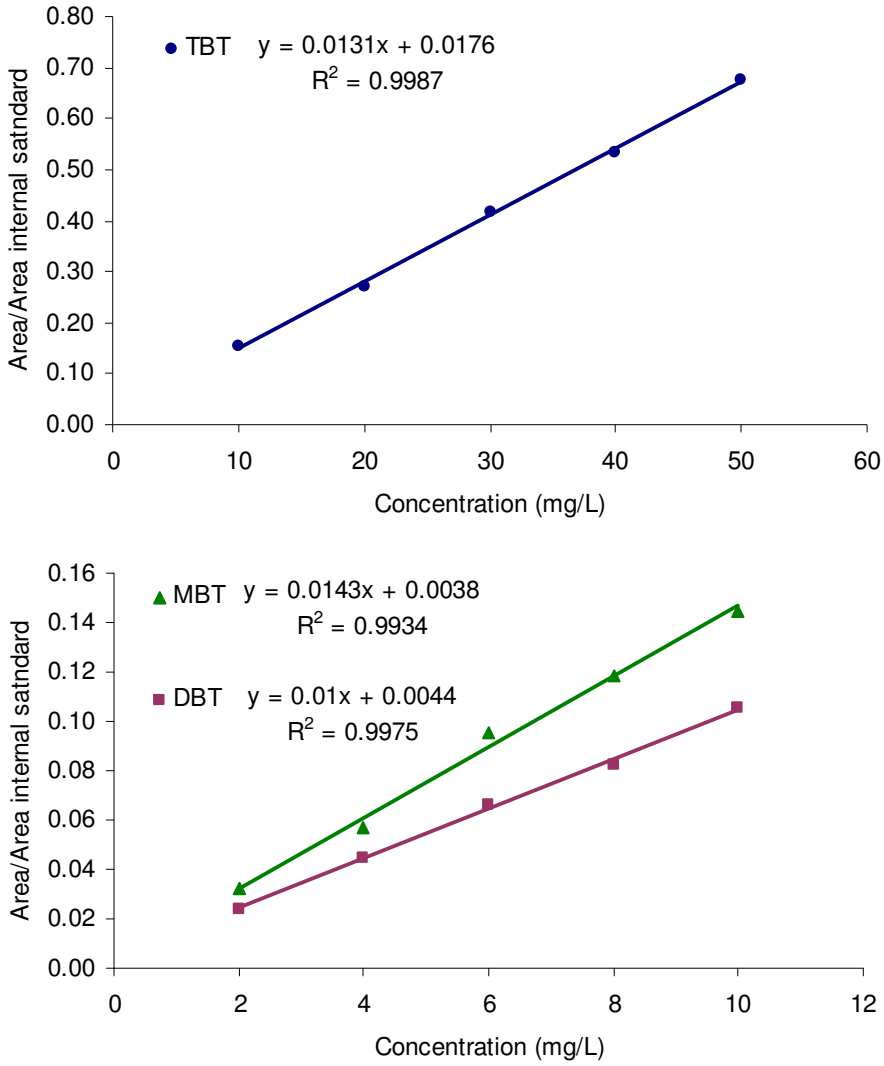


Figure 2.17 Calibration curves, linear relationships and correlation coefficients of butyltin compounds analysed by GC-MS.

2.3.3 Extraction of butyltin compounds in liquid media

The suitable volume of derivatising agent was checked to ensure the complete ethylation but not excessively added because the chemical is expensive. Figure 2.18 shows the percentage recovery of butyltin compounds where MBT was varied from 102.01 to 115.83 %, DBT was varied from 98.00 to 100.61 % and TBT was varied from 96.98 to 100.34 %. The results indicate that 1 ml of $NaBEt_4$ gave complete derivatisation of butyltins.

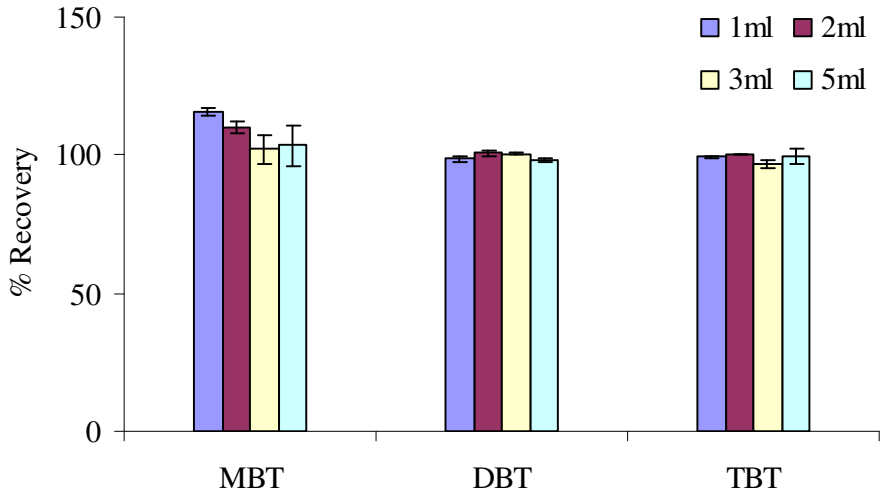


Figure 2.18 Recovery of butyltin compounds from liquid media using different amounts of derivatising agent (n=3, SD error bar).

Order of sample preparation was tested between derivatisation and extraction for improvement of the recovery. Derivatisation before extraction was intended to reduce polarity of butyltins instead of using tropolone, which tropolone is generally used to facilitate extraction efficiency (Bangkedphol et al., 2008). However, the matrix in the medium might lead to incomplete ethylation. Also, derivatisation increased volatility of butyltin compounds where they might disappear during the pre-concentration step. These effects severely reduced the recovery of MBT as shown in Figure 2.19. Pre-derivatisation was obviously unsuitable. On the other hand, extraction of butyltin compounds with tropolone encouraged the phase transfer of butyltin compounds, avoided volatisation during pre-concentration and allowed good ethylation. From the result, post-derivatisation was utilised.

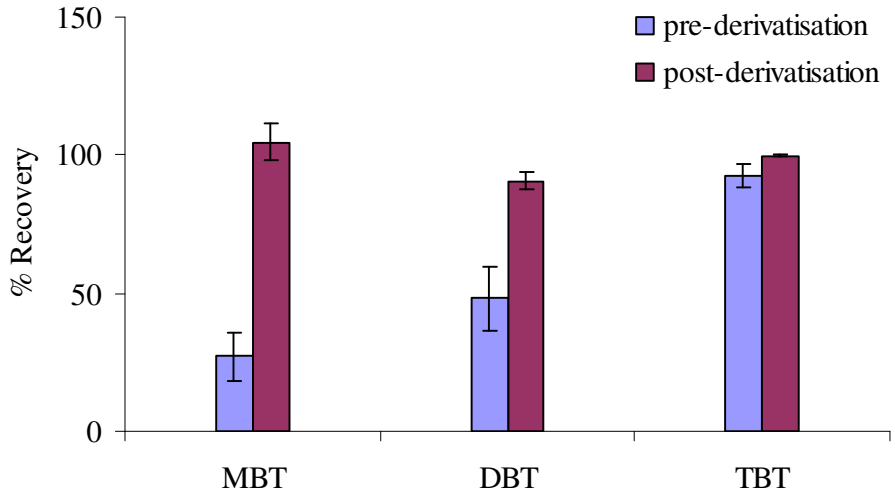


Figure 2.19 Recovery of butyltin compounds from liquid media derivatised before and after extraction (n=3, SD error bar).

The extraction conditions optimised previously were subsequently tested with the liquid medium containing bacterial cells. Butyltins partitioning property encourages adsorption onto suspended materials and sediment, and also bioaccumulation (Hoch, 2001, Voulvoulis and Lester, 2006). Besides, TBT degradation occurs inside bacterial cells and its metabolites are produced before being partially excreted into the medium (Tsang et al., 1999). Therefore, bacterial cells are a matrix that decreases extraction efficiency. The recovery in Figure 2.20 demonstrates a small reduction in medium containing cells. Additionally, when bacterial cells were present, emulsion between organic solution and medium was formed. To eliminate emulsion, anh.Na_2SO_4 was added to absorb water, which then set the organic layer free.

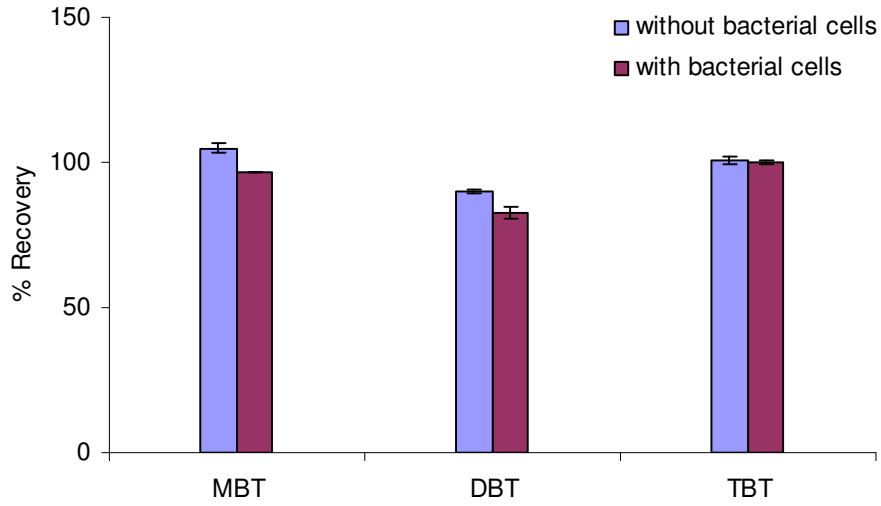


Figure 2.20 Recovery of butyltin compounds extracted from liquid media with and without bacterial cells (n=3, SD error bar).

Figure 2.21 shows recovery of butyltin compounds at different concentrations from optimised extraction. Due to degradation, the concentrations of butyltin compounds were continually changed. TBT was reduced after a period of time and resulted in increasing levels of DBT and MBT. Also during the production of DBT the substance was broken down to MBT. From this reason, the extraction procedures must be practicable for the concentrations that would occur in the experiment. The results show small variation of recovery of butyltin compounds. However, the low deviation of the triplicate samples ensured feasibility of the optimised method. This study confirms the validity of the extraction and derivatisation conditions. Therefore, these conditions were used for further studies.

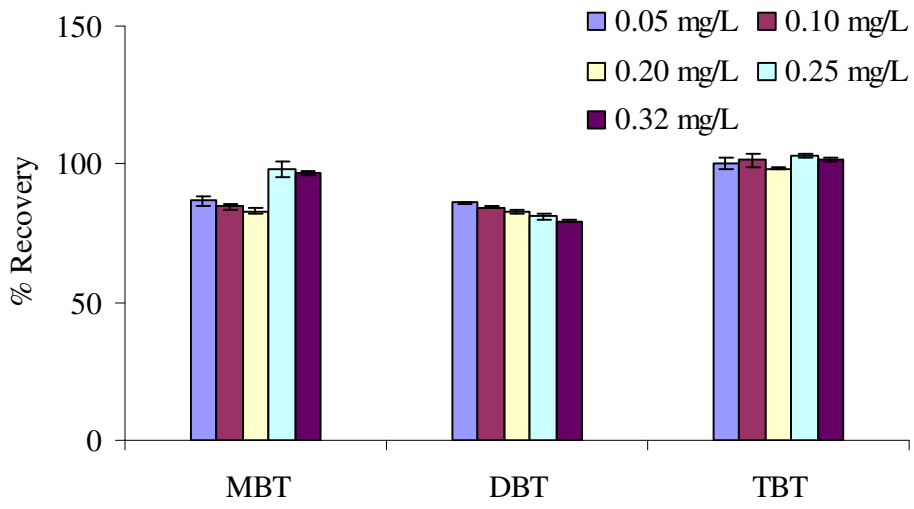


Figure 2.21 Recovery of butyltin compounds from liquid media at different initial concentrations extracted from medium containing bacterial cells (n=3, SD error bar).

2.3.4 Degradation of TBT by the isolated cultures

After 14 days incubation, bacterial activity was expected to be minimised due to depletion of nutrients and increasing of waste. Moreover, degradation activity in synthetic medium solution has been shown to occur significantly in the first week (Bernat and Długoński, 2002, Bernat and Długoński, 2006, Kawai et al., 1998, Tsang et al., 1999). Following the analysis of butyltins, the degradation in glycerol medium is shown in Figure 2.22. The amount of TBT, DBT and MBT measured from the samples of *A. faecalis* were 0.3129, 0.0059 and 0.0086 mg/L; *C. braakii* were 0.2985, 0.0098 and 0.0086 mg/L; *E. cloacae* were 0.2706, 0.0324 and 0.0124 mg/L; *P. fluorescens* were 0.2883, 0.0249 and 0.0102 mg/L, respectively. The concentration of butyltins was examined with respect to the LOD demonstrated in *Section 2.3.2*. All bacteria acquired from the sediment showed the potential to degrade TBT under co-metabolism conditions. So, there was the possibility of debutylation of TBT in Bowling Basin. From the majority of TBT reduction and production of its metabolites, the comparable activity of pure cultures was *E. cloacae* > *P. fluorescens* > *C. braakii* > *A. faecalis*.

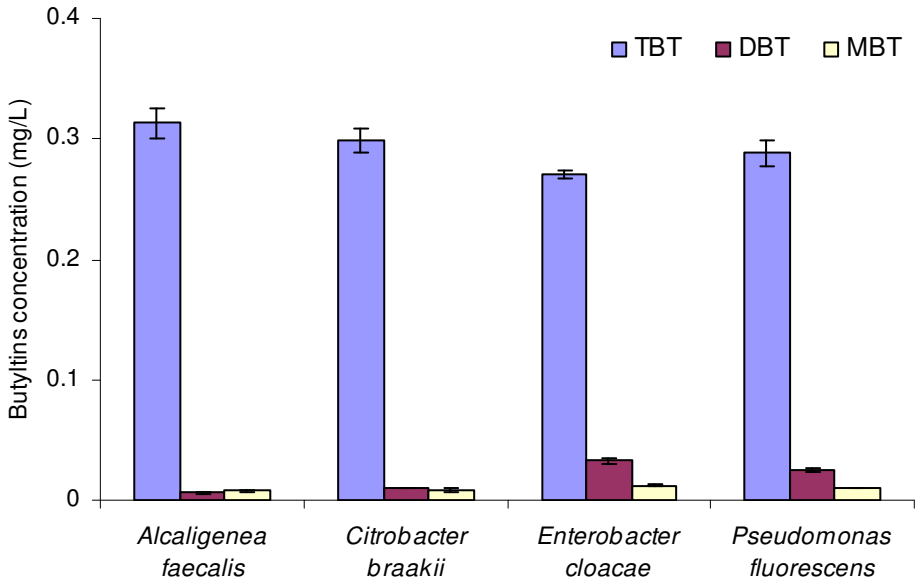


Figure 2.22 The concentration of butyltins after biodegradation of TBT by the isolated sediment bacteria for 14 days in glycerol medium, at initial concentration of 0.3255 mg/L (n=3, SD error bar).

Additional kinetic study of degradation was completed for all TBT-degraders. Figure 2.23 shows degradation during 14 days of incubation. The results confirm the efficiency of *E. cloacae* > *P. fluorescens* > *C. braakii* > *A. faecalis*.

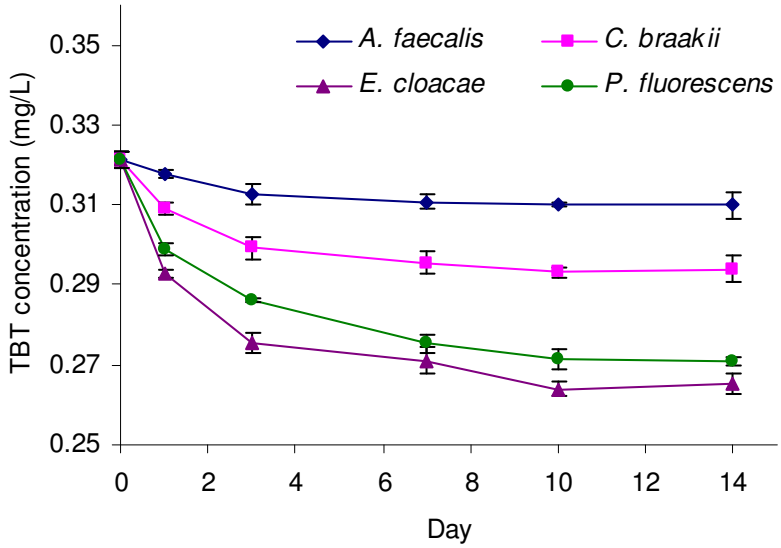
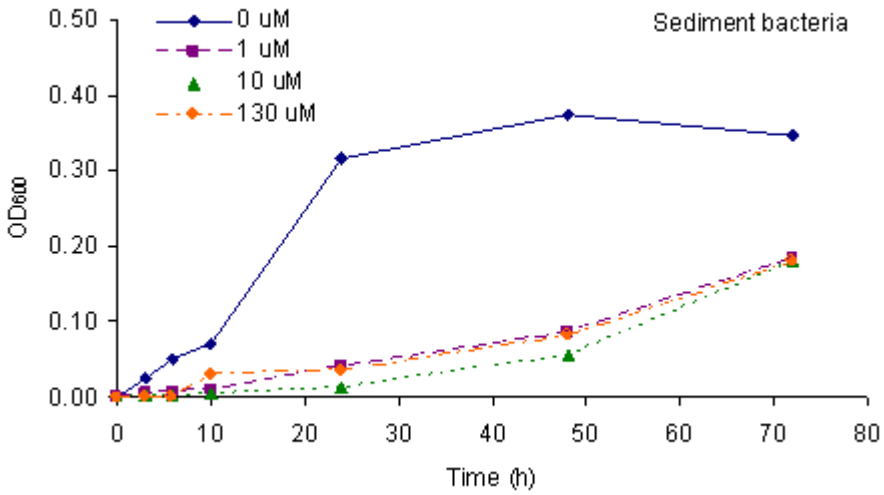
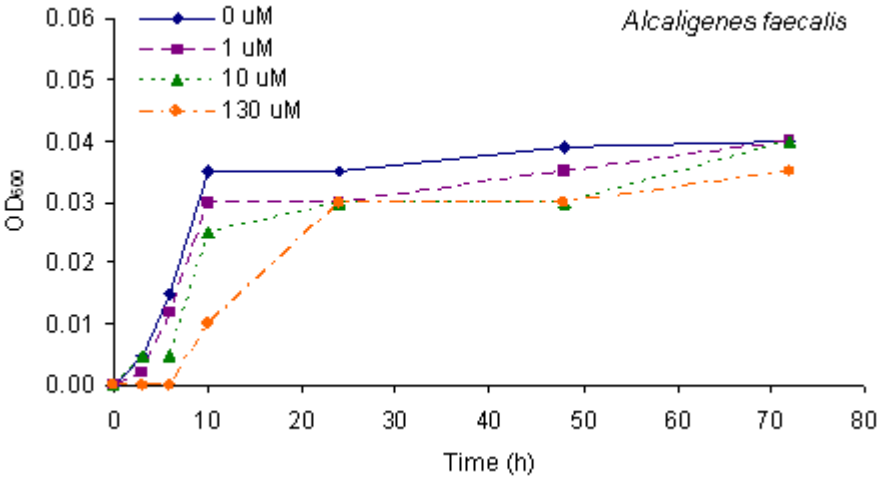


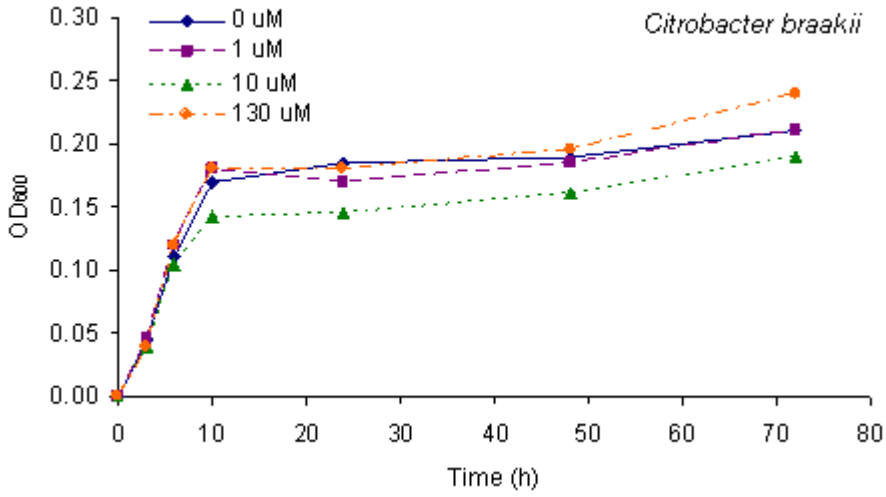
Figure 2.23 Degradation of TBT by isolated bacteria during 14 days of incubation (n=3, SD error bar).

2.3.5 Resistance of the isolated cultures

The observation of bacterial growth in different levels of TBT is shown in Figure 2.24. The growth was observed in every strain without total inhibition. The results show that TBT-resistant bacteria were presented in TBT-polluted sediment. However, not every strain was resistant to the toxicity. The sediment sample was an example, as most of the microbes died or shocked instantly when exposed to any level of TBT. As a result, bacterial growth was severely dropped. After a period of time, the number of surviving microbes was multiplied and they became the majority population. *A. faecalis* and *P. fluorescens* were considerably inhibited by TBT. Their growth was reduced upon increasing TBT concentration, as higher concentration of TBT gave lower turbidity during exponential growth compared to the control. For *C. braakii*, TBT barely affected its growth when compare with the control sample without TBT. In contrast, the addition of TBT promoted the growth of *E. cloacae*. Only an overload amount of TBT (130μM) shocked the *E. cloacae* before it recovered.


Sediment bacteria
0 uM
1 uM
10 uM
130 uM
OD600
Time (h)
0.00
0.10
0.20
0.30
0.40
0.50
0
10
20
30
40
50
60
70
80


Alcaligenes faecalis
0 uM
1 uM
10 uM
130 uM
OD600
Time (h)
0.00
0.01
0.02
0.03
0.04
0.05
0.06
0
10
20
30
40
50
60
70
80


Citrobacter braakii
0 uM
1 uM
10 uM
130 uM
OD600
Time (h)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0
10
20
30
40
50
60
70
80

Continued

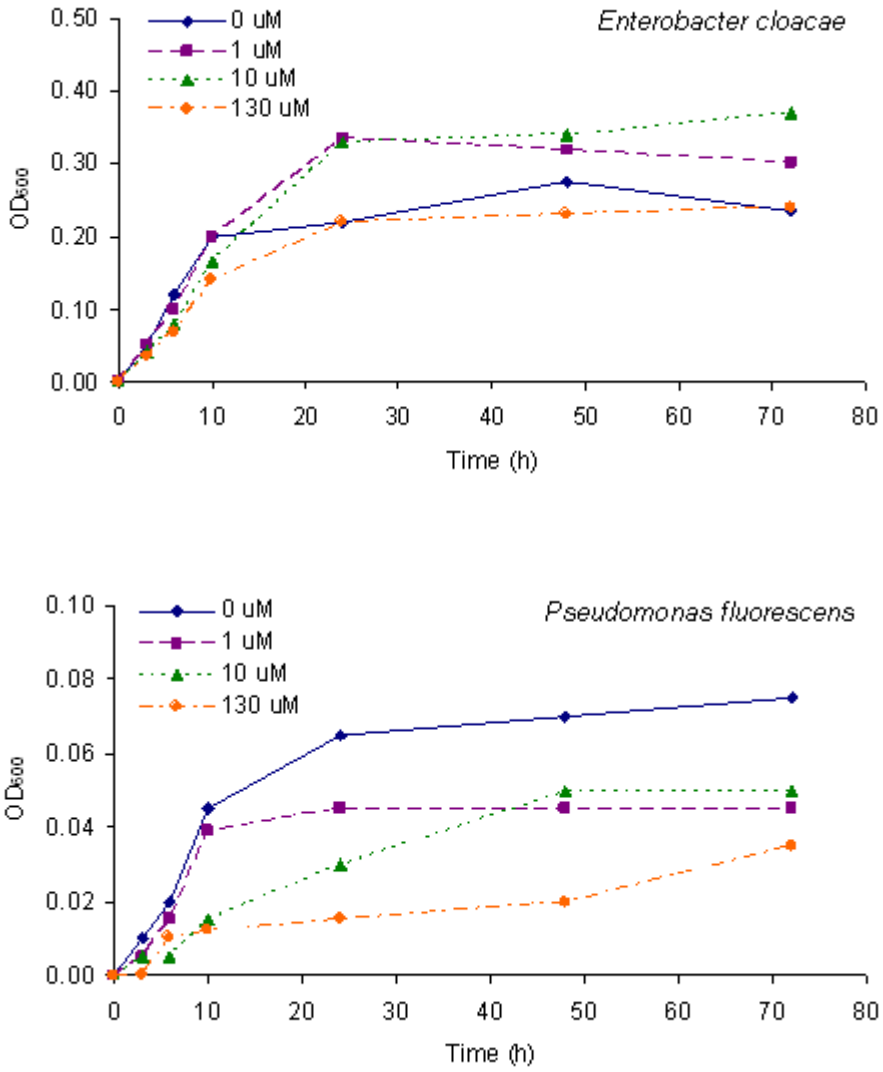


Figure 2.24 Effect of TBT on bacterial growth studied in glycerol medium containing different levels of TBT, incubated at 28ºC and analysed with UV/Vis spectrometer at OD_{600}.

From the results, it can be assumed that the resistivity to TBT was *E. cloacae* > *C. braakii* > *A. faecalis* > *P. fluorescens*. The degradation activity and its ability to resist TBT toxicity were considered together. In this study, *Enterobacter cloacae* (B3) isolated from Bowling Basin's sediment was the best TBT-degrader and most preferred for further studies.

2.4 Summary

TBT compounds released into the aquatic environment are generally degraded by bacteria in water and sediment. The isolation of TBT-degraders from TBT polluted sediment leads to the identification of potential species. Under co-metabolism conditions, four strains of TBT-degraders, *A. faecalis*, *C. braakii*, *E. cloacae* and *P. fluorescens* were successfully isolated, using glycerol medium containing TBTCl at 130µM (55 mg/L), from contaminated sediment collected from Bowling Basin in Glasgow. However, the sediment microorganisms were unable to utilise TBT without the presence of other carbon sources.

Degradation of an initial concentration of 1µM TBTCl (0.3255 mg/L) was investigated after 14 days, with the isolated bacteria. It was found that TBT was degraded at 3.9, 8.29, 16.87 and 11.43% by *A. faecalis*, *C. braakii*, *E. cloacae* and *P. fluorescens*, respectively. Based on degradation capability of the isolates, *E. cloacae* were considered to be the best degrader amongst the isolates. Effects of TBT on bacterial growth indicated that resistivity of *E. cloacae* > *C. braakii* > *A. faecalis* > *P. fluorescens*. The isolates were found to be tolerant towards TBT at concentrations significantly higher than the concentration of TBT measured at the basin, suggesting a low effect of TBT on the growth and activity of the bacteria. To meet the objective of this study, *E. cloacae* was the preferred isolate to be used as an inoculum for degradation enhancement (*Chapters 3 & 4*), because of its potential to degrade TBT and resistivity to the toxicity.

Moreover, amongst the four isolates, this study has discovered two species of high resistance TBT-degraders e.g. *C. braakii* and *E. cloacae* which have never been previously studied or isolated based upon TBT degradation ability.

CHAPTER 3

Stimulation of Tributyltin Biodegradation by Sediment Microorganisms under Optimised Environmental Conditions and Nutrient Supplementation

Objectives: To study biostimulation techniques by adjustment of environmental conditions and nutrient supplementation for better degradation capability. Optimising conditions involves controlling the surrounding environment to enhance TBT bioremediation. The organic nutrients and growth factors which promote dioxygenase activity can then be selected as amendments to support the enzymatic degradation process.

3.1 Background

Biotic degradation has been shown to be the major pathway for removal of TBT contamination in water and sediment (Tsang et al., 1999). However as TBT is a persistent pollutant, under unfavourable conditions for microorganisms, accumulation in the environment can occur and poses a risk to organisms exposed to TBT (Dowson et al., 1993). Natural attenuation of contaminants can be accelerated by biostimulation and/or bioaugmentation (Sarkar et al., 2005). Biostimulation is the process of promoting biodegradation through modification of the environment to encourage existing competent bacteria, while bioaugmentation involves direct addition of capable microorganisms to accelerate degradation (DFO, 2010). Bioaugmentation will be discussed in *Chapter 4*. This chapter will concentrate on biostimulation through the controlling of surrounding conditions and addition of various forms of nutrients. A hospitable environment and essential resources will support bacterial activity in which better degradation capability can be obtained. The parameters which influence biodegradation have been explained previously in *Section 2.1.3*.

Atmospheric conditions present in an environment will influence growth and activity of microorganisms depending on their characters e.g. aerobe, anaerobe or facultative anaerobe. Biodegradation can occur either aerobically or anaerobically (Dowson et al., 1996). Under anaerobic conditions, degradation results from anaerobes and facultative anaerobes using nitrate or sulphate as electron acceptors. In the environment with mixture of electron acceptors, the order of utilisation is typically dissolved O_2 (E_0=0.82V), NO_3^- (E_0=0.36, 0.52 or 0.74V), Mn(IV)-solids (E_0=0.52V), Fe(III)-solids (E_0=-0.05V) and dissolved SO_4^{2-} (E_0=-0.22V) (Eweis, 1998, USGS, 2009). Aerobic conditions are known to support TBT degradation better than anaerobic conditions (IPCS, 1990), since degradation under anaerobic is minimal (Voulvoulis and Lester, 2006). Dissolved oxygen has been confirmed to be an essential factor that promotes TBT degradation (Bernat and Długoński, 2006). Dissolved oxygen can be provided via aeration (Dowson et al., 1996), or the addition of oxygen release compounds (Hicks, 1999). As a result of aerobic stimulation, a dynamic mixture of aerobic and anaerobic degradation processes is represented in the environment (Landmeyer et al., 2004). A pathway of aerobic degradation has been described in *Section 2.1.1*, where dioxygenase enzymes oxidise butyltins using molecular oxygen. Even though degradation can occur under anaerobic conditions, no literature could be found demonstrating an anaerobic pathway.

Many studies on TBT biodegradation adjust the surrounding pH to neutral which is appropriate for more efficient culture growth (Luan et al., 2006). Extreme pH is generally unfavourable for microbial activity, as proteins for growth may be denatured or the effectiveness of enzymes decreased. Typically, optimal microbial activity takes place under neutral pH conditions, in the range of 6 to 8 (Alexander, 1999). Nevertheless, the optimal pH can be site specific as the indigenous microbes will have adapted to the conditions they inhabit.

The optimum temperature, which can lead to the highest TBT removal efficiency, varies for specific microorganisms and microbial communities. Enzyme-catalysed reactions of bacteria are temperature dependent (Prescott et al., 2002). Consequently, the surrounding temperature will directly affect the degradation

capability. Temperature can be adjusted to optimise the growth of cultures, or remain at room temperature. However, unpredictable ambient temperature is always experienced in natural attenuation due to seasonal variation (Olson and Brinckman, 1986).

Biostimulation of TBT-degraders also occurs via the addition of nutrients and organic compounds such as nutrient broth, or succinate and glycerol (Kawai et al., 1998, Roy and Cabral, 2002). Nutrients are an essential requirement for bacterial growth and to carry out various activities. Macro-elements, especially C, N and P, are required by all microorganisms and their availability can influence bacterial biodegradation (Atagana et al., 2003, Sarkar et al., 2005). Stimulation occurs by increasing biomass through consumption of prefer supplements (Roy and Cabral, 2002, Östberg et al., 2007, Radwan et al., 2000, Sarkar et al., 2005), follow by degradation of contaminants, leading to extension of the lag phase. To obtain initial degradation enhancement, amino acids and vitamins, which have been shown to promote initial stimulation of degradation, can be supplemented (Östberg et al., 2007, Radwan and Al-Muteirie, 2001).

Depending on the degrader, the addition of organic and inorganic nutrients for degradation enhancement has been associated with the type and concentration of both the xenobiotic and the supplement. The addition can result in enhanced degradation, where the lag phase is prolonged and major acceleration in degradation occurs after the lag period, or enhanced degradation where the lag phase is shortened and accelerated degradation is found immediately. However, unsuccessful enhancement is also possible where no stimulation takes place regardless of increased microbial biomass (de Lipthay et al., 2007).

From the literature, only nutrient broth (complex nutrient) and a mixture of succinate and glycerol (S/G) was studied and found to stimulate TBT degradation (Kawai et al., 1998, Roy and Cabral, 2002).

3.2 Experimental

With regard to slow natural attenuation, this chapter was intended to accelerate biodegradation. The findings would increase knowledge on biostimulation techniques for TBT degradation since information is still limit at present. This study investigated the capability of sediment microorganisms (SED), which representing a community with mixed potent and impotent degraders, and a previous isolate, *Enterobacter cloacae* (B3), which represents a single culture of degrader with confirmed effectiveness in relation to TBT degradation.

This study examined TBT degradation when atmospheric conditions, pH and temperature were varied to obtain better degradation capability. Although these effects can be expected to encourage natural attenuation, they have never been studied or optimised together to approach the best conditions which enhance TBT removal. From diverse techniques that can be utilised for treatment of contamination in an environment, this study will indicate appropriate options, which are more specific for TBT removal based on the experimental half-life.

This study also investigated enhancement through addition of supplements, including organic nutrients and growth factors. Due to the different enhancement efficiency and inhibition effect from supplements, this study will provide information on the suitability of supplements to accelerate TBT degradation based on the amount of degraded TBT. Even though materials obtained from industrial processes can be used for complex nutrients, this study was designed based on chemical aspects to investigate stimulation potential of individual nutrients, surfactants, amino acids and vitamins specifically for TBT mineralisation.

The optimised conditions and suitable supplements obtained from this study could be used to stimulate biodegradation in contaminated sediment.

3.2.1 Materials and equipments

The reagents and equipments used were as described in *Section 2.2.1*. In addition, amino acids, vitamins, Triton X-100, sodium lauryl sulphate (SLS) and sodium succinate were obtained from Sigma Aldrich (Steinheim, Germany). Potassium nitrate (KNO_3) and other carbon supplements were obtained from BDH (Poole, UK). The additional equipments used for this study included 0.2 μm air filters (Sartorius), 0.2 μm liquid filters (Nalgene), VoluPAC tubes (Sartorius), an air pump (ACO-9610, Hailea) and a total organic carbon (TOC) analyser (Teledyne Tekmar).

3.2.2 Bacterial cultures

A sediment culture (SED) was prepared by re-suspending 10 g of sediment slurry into a 100 ml aliquot of sterile DI water. The sediment was allowed to settle for 10 min, after which the supernatant was filtered and transferred into a glycerol medium as prepared in *Section 2.2.3*. The sediment culture was then incubated at 28˚C on an orbital shaker at 150 rpm, for 24 h to obtain approximately 10^8 CFU/ml. The resultant culture solution was then used to inoculate samples. *Enterobacter cloacae* (B3), the TBT-degrader isolated in *Chapter 2*, were studied for degradation by a single species. This degrader has already been deposited for public access as *Enterobacter cloacae* strain TISTR1971 (Sakultantimetha et al., 2009). Culture conditions were identical to those for SED.

3.2.3 Effect of respiration conditions on TBT-removal

Anaerobic respiration requires an alternative electron acceptor to oxygen to stimulate the biodegradation of TBT, and nitrate was selected for this purpose as it is the most prefer electron acceptor to facilitate anaerobic degradation. The appropriate concentration of nitrate was determined prior to use to prevent inhibition of microbial growth. To do this, nitrate was screened under aerobic conditions in glycerol medium. A stock solution of nitrate, 100 g NO_3^-/L, was prepared by

dissolving 16.30 g of KNO_3 into 100 ml of glycerol medium. The stock solution was subsequently diluted to make up test solutions at 0, 0.1, 0.5, 1.0 and 5.0 g NO_3^-/L. Then, 2.5 ml of SED or B3 culture was added to 50 ml of each test solutions. Test solutions were incubated at 28°C on an orbital shaker at 150 rpm for 7 days. Aerobic plate counts using PCA were prepared for each test solution to determine the number of CFU, which was then plotted against nitrate concentrations. Inhibitory effect of nitrate was insignificant at 0.1 g NO_3^-/L, therefore the concentration was used.

For the degradation study, test solutions were made up in sterile bottles by adding 2.5 ml of SED or B3 culture to 50 ml of TBT glycerol media which had been spiked with 100-µg/L TBTCl. As shown in Figures 3.1 and 3.2, the test solutions were exposed to three respiration conditions, prepared as follows:

1) unenhanced aerobic conditions with limited dissolved oxygen, i.e. no additional oxygen was added to the media;
2) anaerobic conditions, where the medium was bubbled with oxygen-free nitrogen gas for 6 h, then KNO_3 was added to give an initial concentration of 0.1 g NO_3^-/L. The inoculation was performed in an anaerobic box, shown in Figure 3.3. The head space of anaerobic samples was filled with nitrogen gas, and each container was capped and sealed;
3) enhanced aerobic conditions with aeration, i.e. the media was aerated for 6 h to allow for saturation with dissolved oxygen at the beginning of the experiment and was aerated throughout the incubation period.

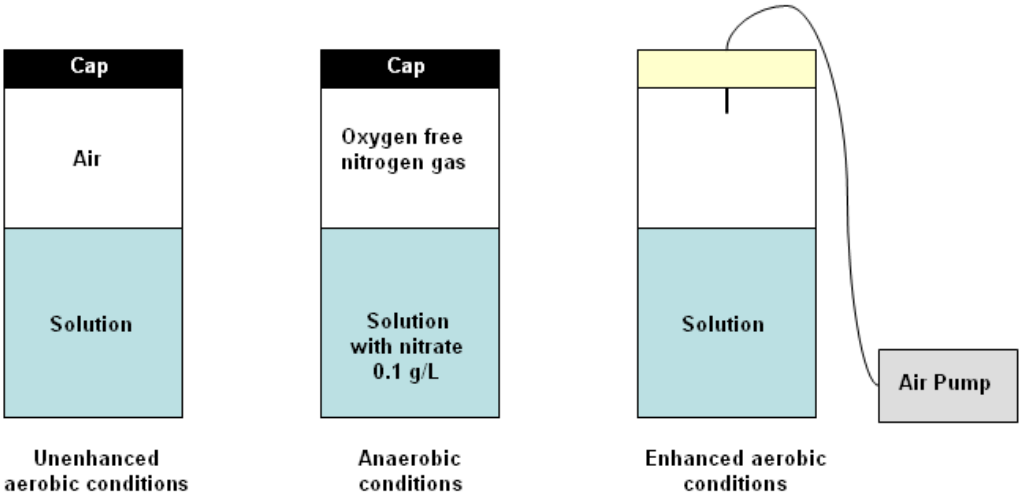


Figure 3.1 Sample solution set up for TBT degradation study under different respiration conditions.

Under enhanced aerobic conditions, test bottles were aerated at a constant flow rate with an air pump fitted with a 0.2 µm pore size air filter. All bottles were incubated at 28°C, on an orbital shaker at 150 rpm. All degradation studies were carried out in triplicate (n=3), which were prepared in separate bottles. A control sample of each respiration conditions was also prepared without inoculation. The starting point was counted as day zero of incubation. The test solutions were analysed immediately for actual initial concentration of TBT. Then solutions were analysed subsequently after 1, 3, 5 and 7 days of incubation to observe degradation capability. The analysis of butyltin compounds was performed as described in *Section 2.2.5*.

Figure 3.2 Set up of TBT degradation for unenhanced aerobic degradation and anaerobic degradation (above), and enhanced aerobic degradation (below).

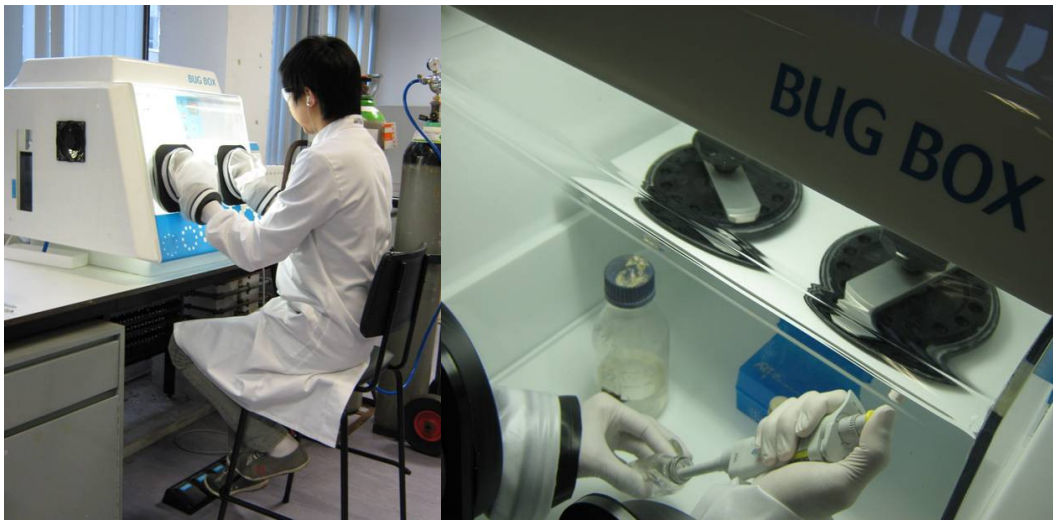


Figure 3.3 Inoculation of cultures to made up the anaerobic samples in an anaerobic box.

To obtain degradation rate constants (k) of TBT-degrading bacteria, degradation in the glycerol medium was repeated, but the sample was analysed for TBT at 0, 1, 3, 7, 10 and 14 days of incubation. Biotic degradation of TBT in batch reactors follows first order kinetics (Stasinakis et al., 2005) based on the assumption that the degradation rate is depends on the concentration of one reactant, TBT. The k was evaluated by the following equations:

$$C_t = C_0 e^{-kt} \qquad \text{(Equation 3.1)}$$

$$k = \ln(C_0/C_t)/t \qquad \text{(Equation 3.2)}$$

$$t_{1/2} = \ln 2/k \qquad \text{(Equation 3.3)}$$

where: C_t = total TBT concentration in the reactor at time t, µg/L;

C_0 = total TBT concentration in the reactor at time t = 0, µg/L;

k = degradation rate, day^{-1}; t = time, day; $t_{1/2}$ = half-life, day.

From the equations, a linear relationship was produced to evaluate the rate constants and half-lives where the slope is k. Finally, the potential for TBT degradation by the bacterial isolates was compared in term of the first-order rate (when possible). The bacterium that showed the highest TBT degradation rate was then preferred and was utilised as an augment species in further studies.

3.2.4 Effect of pH of media on TBT-removal

To establish the appropriate pH range to test TBT degradation, the effect of pH on growth of both cultures, SED and B3, was screened in a glycerol medium at pH 4, 5, 6, 7, 8, 9 and 10 containing 100 µg/L TBTCl. Exposure to TBT during the screening step was to obtain growth of actual sediment organisms that would be present during the degradation study. For each pH tested, the medium was adjusted by adding 5M HCl or 5M NaOH, and then sterilised by passing through a 0.2 µm pore size filter; autoclaving was not chosen for sterilisation since it can alter the pH of medium. Test solutions were prepared by adding 5 ml of either culture solution to 100 ml of test solution. Test solutions were incubated at 28°C, on an orbital shaker at

150 rpm. During 3 days of incubation, samples were collected from each test solution to determine the OD_{600} using a UV/Vis spectrometer.

From the screening step, pH 6, 7 and 7.5 were selected for the degradation study based on abundance of bacterial growth. For each pH to be tested, the medium was adjusted to the pH, sterilised and spiked with 100 μg/L TBTCl. SED and B3 were then inoculated. All degradation studies were carried out in triplicate (n=3), which were prepared in bottles separately. A control sample of each pH was also prepared without inoculation. The degradation of TBT was determined in aerated glycerol medium at each selected pH over 7 days using the culture conditions described above.

3.2.5 Effect of surrounding temperature on TBT-removal

Incubation temperatures of 4, 10, 20, 28, 37 and 44ºC were screened for the growth of SED and B3 in a glycerol medium, pH 7.5, containing 100 μg/L of TBTCl. Test solutions were prepared by adding 5 ml aliquots of inoculum to 100 ml TBTCl glycerol media. The test solutions were then incubated at 4, 10, 20, 28, 37 and 44ºC on an orbital shaker at 150 rpm. OD_{600} of the resultant growth medium was determined during 7 days.

After initial growth screening, incubation temperatures of 10, 20, 28 and 37ºC were selected to determine the effect on degradation in aerated TBTCl glycerol medium based on abundance of bacterial growth. The test solutions were prepared by inoculating SED or B3 culture into aerated glycerol medium at pH 7.5 containing 100 μg/L of TBTCl. All degradation studies were carried out in triplicate (n=3), which were prepared in separate bottles. A control sample of each temperature was also prepared without inoculation. The test solutions were incubated at the temperatures with shaking at 150 rpm. The initial TBT concentration was analysed immediately and samples were subsequently collected and analysed for butyltin compounds at 1, 3, 5 and 7 days of incubation.

3.2.6 Effect of nutrient supplement on TBT-removal

3.2.6.1 Screening of nutrient supplement

Potential biostimulation supplements were screened in relation to their effect on biomass production. The supplements were added to give an equivalent amount of carbon as provided by the glycerol medium (0.49 g C/L). In addition, a TOC analyser was used to determine the quantity of carbon in the long chain surfactant (Triton X-100) and polysaccharide (starch), due to their molecular weight being unspecified. Media were prepared as for the glycerol medium, but different carbon sources were added instead of glycerol. The list of studied compounds and their weight per litre is shown in Table 3.1 (relevant calculation in *Appendix E*). A carbon control sample was prepared without an addition of glycerol into the medium. All media were prepared and subsequently sterilised by passing through 0.2 μm filters (Nalgene).

Table 3.1 Carbon sources for biomass determination.

Type of supplement	Carbon supplement	Molecular structure	Molecular weight (g/mol)	Weight/volume per litre
Surfactant	Triton X-100	$C_{14}H_{22}O(C_2H_4O)_n$	-	0.715 ml
	Tween 80 (d 1.06 g/ml)	$C_{64}H_{124}O_{26}$	1310	0.783 ml
	Lauryl sulphate (SLS)	$NaC_{12}H_{25}SO_4$	288.38	0.980 g
Organic salt	Salicylate	$NaC_7H_5O_3$	160.11	0.933 g
	Acetate	$NaC_2H_3O_2$	82.03	1.673 g
	Citrate	$Na_3C_6H_5O_7.2H_2O$	294.10	2.000 g
	Succinate	$Na_2C_4H_4O_4$	270.00	2.754 g
	Tartrate	$NaC_4H_4O_6.2H_2O$	230.08	2.347 g
	Succinate/glycerol (7:6)			1.483g/0.462ml
Carbohydrate and sugar	Starch	$(C_6H_{10}O_5)_n$	-	0.916 g
	Glucose	$C_6H_{12}O_6.2H_2O$	198.17	1.347 g
	Sucrose	$C_{12}H_{22}O_{11}$	342.3	1.164 g
	Xylose	$C_5H_{10}O_5$	150.13	1.225 g
	Glycerol (d 1.26 g/ml)	$C_3H_5(OH)_3$	92.09	1.000 ml

Stimulation during lag time by amino acids and vitamins was studied in glycerol media. Amino acids and vitamins were added at 1mM and 1µM, respectively. The glycerol medium was made up without yeast extract, and each amino acid or vitamin was added individually. The list of studied compounds and their weight per litre is shown in Table 3.2. A growth factor control sample was prepared without the addition of yeast extract into the medium. All media were prepared and subsequently sterilised by passing through 0.2 µm filters.

Table 3.2 Amino acids and vitamins for biomass determination (Östberg et al., 2007).

Type of supplement	Growth factor supplement	Molecular structure	Molecular weight (g/mol)	Weight per litre
Amino acid	L-Valine	$C_5H_{11}NO_2$	117.15	0.117 g
	L-Arginine	$C_6H_{14}N_4O_2$	174.20	0.174 g
	L-Threonine	$C_4H_9NO_3$	119.12	0.119 g
	L-Glutamic acid	$C_5H_9NO_4$	147.13	0.147 g
	L-Methionine	$C_5H_{11}NO_2S$	149.20	0.149 g
	L-Tyrosine	$C_9H_{11}NO_3$	181.20	0.181 g
	L-Serine	$C_3H_7NO_3$	105.09	0.105 g
	L-Aspartic acid	$C_4H_7NO_4$	133.10	0.133 g
Vitamin	Thiamine (B1)	$C_{12}H_{17}N_4OS^+Cl^-.HCl$	337.27	0.337 mg
	D-Biotin (B7)	$C_{10}H_{16}N_2O_3S$	244.31	0.224 mg
	Cyanocobalamin(B12)	$C_{63}H_{88}CoN_{14}O_{14}P$	1355.38	1.355 mg
	Pantothenic acid (B5)	$C_9H_{17}NO_5.1/2Ca$	238.27	0.238 mg

To allow the microbes to become acclimatised to the different media, SED and B3 were inoculated into 10 ml of each medium and incubated for 24 h at 28ºC on an orbital shaker at 150 rpm. After incubation, 0.5 ml aliquots of each resultant cell culture was inoculated into appropriate fresh media and incubated for 7 days under the same conditions. Biomass of the resultant cell suspensions was determined using VoluPAC tubes. Briefly, 1 ml of each vortexed cell suspension was transferred into a VoluPAC tube and centrifuged at 2,500 g for 1 min as per the manufacturer's recommendation (Sartorius, 2010). Figure 3.4 shows cells suspension in a VoluPAC tube on the left and centrifuged cells in a VoluPAC tube on the right.

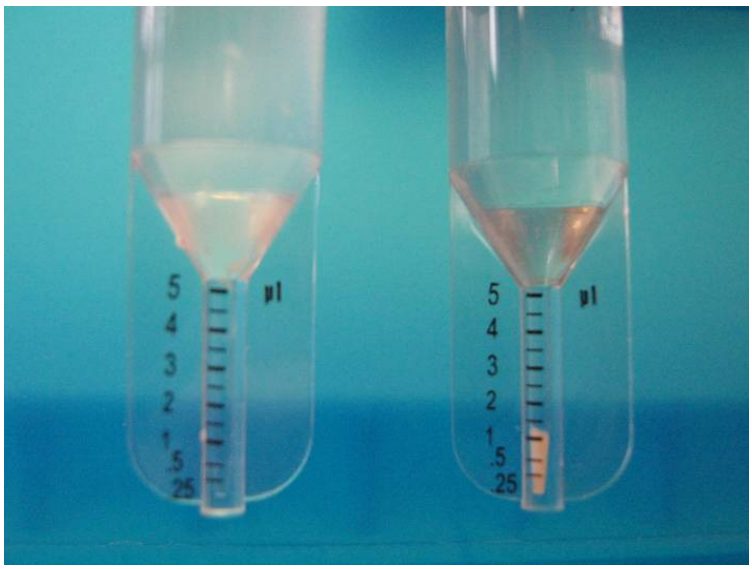


Figure 3.4 Biomass determinations using VoluPAC tubes.

Biomass was expressed as packed cell volume (PCV) and was read in the unit of µl from the graduated volume markers at the conical end of each tube. Percentage of PCV was calculated as follows in Equation 3.4 (Sartorius, 2010):

$$\%PCV = (PCV/\text{volume of cell suspension}) \times 100 \quad \text{(Equation 3.4)}$$

3.2.6.2 Biostimulation by nutrient supplement

The media which gave the highest biomass production during screening were selected to study TBT degradation. The nutrients were tested and their removal efficiency compared. The effect of nutrient concentration was also studied using the media prepared as described for the screening procedure with different amount of supplements at 0.1x, 1x and 10x strength media. For SED, starch, Tween 80, succinate/glycerol, L-Arginine, L-Threonine and D-Biotin were selected. Also biodegradation by SED was studied in the growth factor control. For B3, xylose, Tween 80, succinate and L-Serine were selected. Each medium was prepared with aerated DI water and adjusted to pH 7.5. A 2.5-ml aliquot of inoculates were transferred into 50 ml medium containing 100 µg/L TBTCl. Samples were incubated at 28ºC, shaking at 150 rpm with aeration for 7 days before analysis for butyltins. Triplicate samples were prepared in separate bottles.

3.3 Results and discussion

3.3.1 Degradation of TBT under different respiration conditions

To provide anaerobic conditions, the presence of oxygen molecules had to be prohibited, but without the electron acceptor, bacterial growth would be severely repressed. Nitrate was to be used as a supplement electron acceptor, however before this could happen screening was carried out to determine the appropriate nitrate concentration. Figure 3.5 shows how nitrate concentration affected the growth of SED and B3. Colonies were tolerant to low concentrations of nitrate (0.1 g NO_3^-/L); however, increasing nitrate to 0.5 g NO_3^-/L repressed growth of B3. Consequently, the initial concentration of nitrate used for anaerobic conditions did not exceed 0.1 g NO_3^-/L.

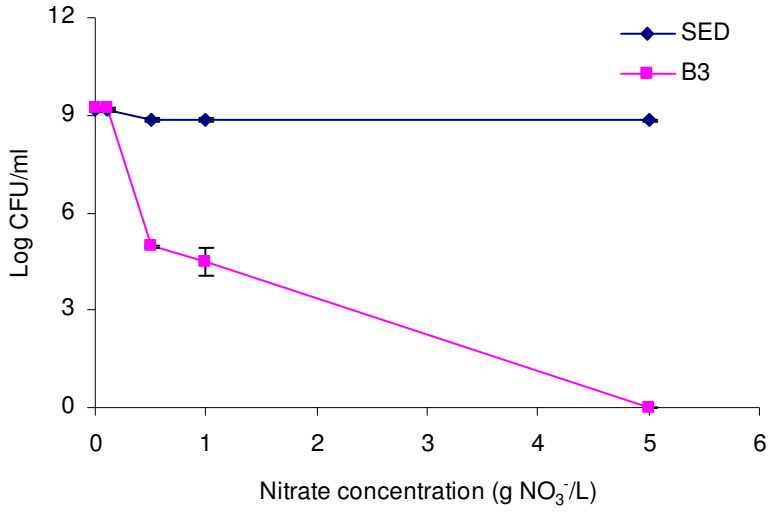


Figure 3.5 Log colony forming units of SED and B3 after exposure to various nitrate concentrations after 7 days in glycerol medium incubation at 28ºC, 150 rpm (n=2).

The degradation of TBT by SED and B3 under different respiratory conditions is shown in Figure 3.6. From the results, it can be seen that enhanced aerobic conditions were preferred for degradation of TBT by both SED and B3. This

finding is in line with Brandsch et al. (2001), who demonstrated preference for aerobic degradation.

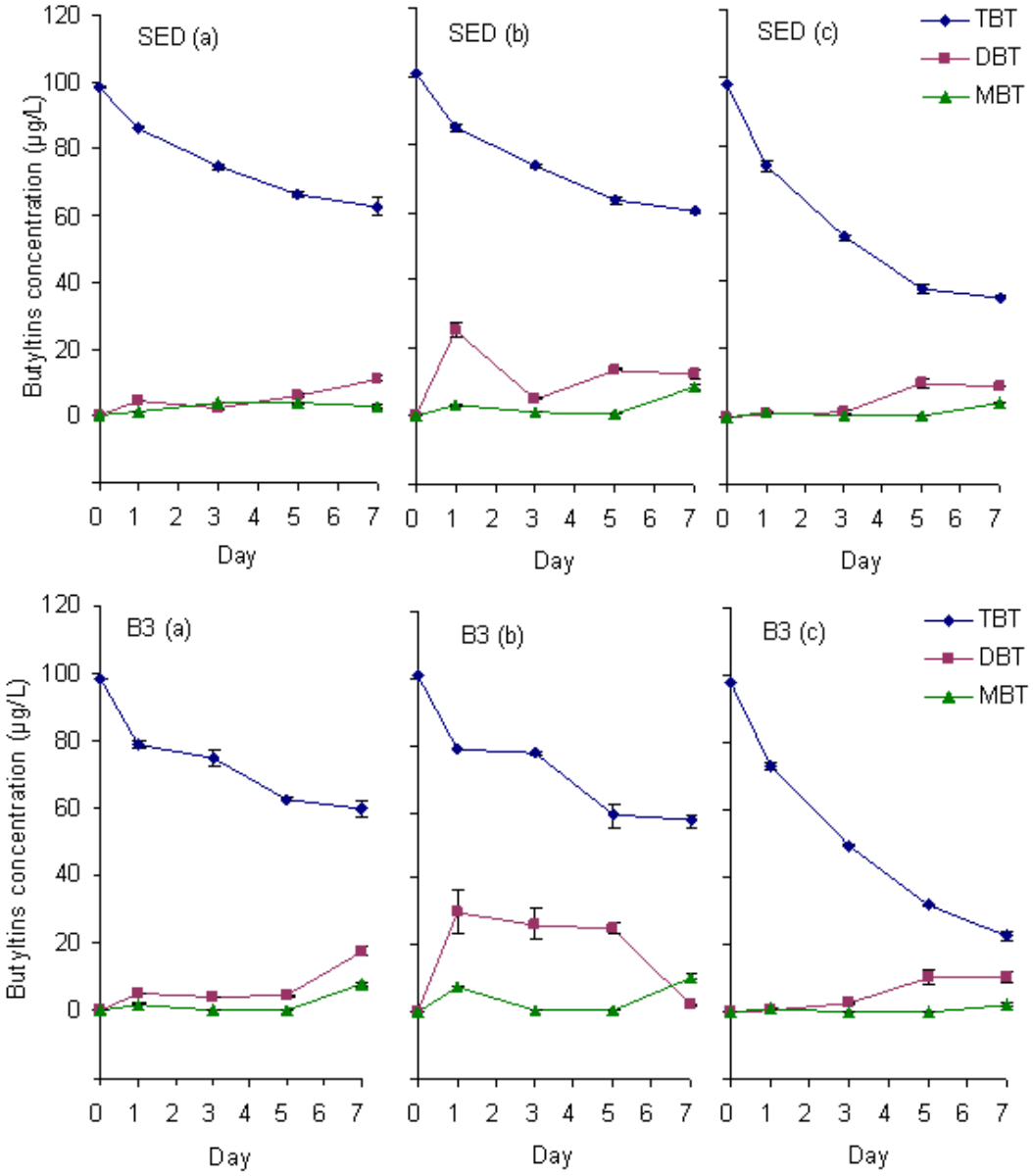


Figure 3.6 Degradation of TBT by SED and B3, and formation of DBT and MBT under (a) unenhanced aerobic; (b) anaerobic; and (c) enhanced aerobic conditions during 7 days of incubation in glycerol medium containing 100 µg/L TBTCl (n=3, SD error bar).

Figure 3.6 shows that adequate oxygen extensively enhanced degradation as determined by the final concentration of TBT. However, aeration alone did not prolong bacterial activity, which could be limited by the lack of essential nutrients, and affected by the accumulation of waste excreted by bacterial metabolism in batch reactor (Klein, 1996). In the aquatic environment, with circulating resources, enhanced aerobic conditions could extend the degradation until completion.

The degradation rate constants (k) were evaluated following the first order kinetics after 7 days of incubation (k_{7d}) by plotting ln C vs t, shown in Figure 3.7. Degradation rate constants were obtained from the slope from which the half-lives of TBT were then calculated. The efficiency of respiration conditions (Table 3.3) was determined by comparing the rate constants. The ratios of k_{7d} constants of unenhanced aerobic: anaerobic: enhanced aerobic conditions was 1: 1.16: 2.33 for SED, and 1: 1.11: 2.70 for B3. The k_{7d} and half-lives infer that the degradation ability under anaerobic conditions were slightly better than unenhanced aerobic conditions with limited oxygen. Providing adequate oxygen for the bacteria considerably reduced the half-life of TBT.

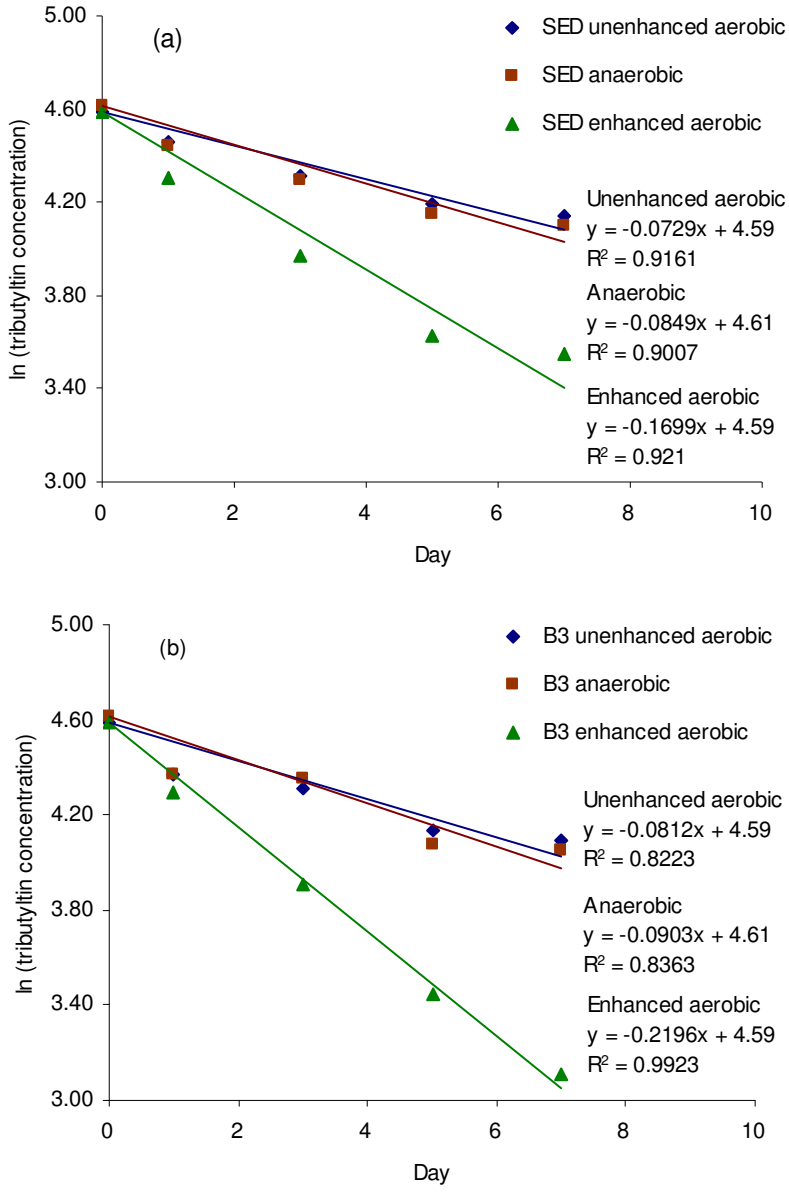


Figure 3.7 Linearisation of TBT degradation data by (a) SED; and (b) B3 under different respiration conditions for the calculation of rate constants and half-lives.

Table 3.3 Degradation rate constants at 7 days and half-lives of TBT by SED and B3 under aerobic and anaerobic conditions.

Respiration Conditions	SED		B3	
	k_{7d} (day^{-1})	$t_{1/2}$ (day)	k_{7d} (day^{-1})	$t_{1/2}$ (day)
Unenhanced aerobic	0.07	9.51	0.08	8.54
Anaerobic	0.08	8.16	0.09	7.68
Enhanced aerobic	0.17	4.08	0.22	3.16

Anaerobic conditions resulted in less effective degradation than enhanced aerobic conditions, yet B3 is a facultative anaerobe, which can live under both conditions, but still aerobic conditions were greater at facilitating TBT degradation. Under anaerobic conditions, nitrate is the most preferred electron acceptor as discussed in *Section 3.1* in these experiments; however, nitrate is likely to have been depleted in many sites including Bowling Basin since evidence of sulphate-reduction existed with the presence of hydrogen sulphide odours.

3.3.2 The effect of pH on TBT degradation

The pH value of a solution is an important parameter affecting oxidative degradation of pollutants (Li et al., 2009). Figure 3.8 shows the inhibitory effect of pH 10, most likely due to cell disruption under high alkaline conditions. Under mild acidic and alkaline conditions (pH 6 and pH 8), the growth of SED was noticeably suppressed when compared with neutral pH, and slightly reduced under stronger conditions (pH 5 and pH 9). The basic conditions at pH 8 were optimum for B3. Unexpectedly, B3 was tolerance of strong acidic pH 4 and pH 5, which gave sustained growth. The growth at pH 6 and pH 7 was lower than pH 4 and pH 5; however, this may have been due to biofilm suspension which was visible in the culture media, and can lead to lower OD readings. The results indicate that the majority of sediment microorganisms and *E. cloacae* are neutrophiles. The growth curves are shown in *Appendix F*.

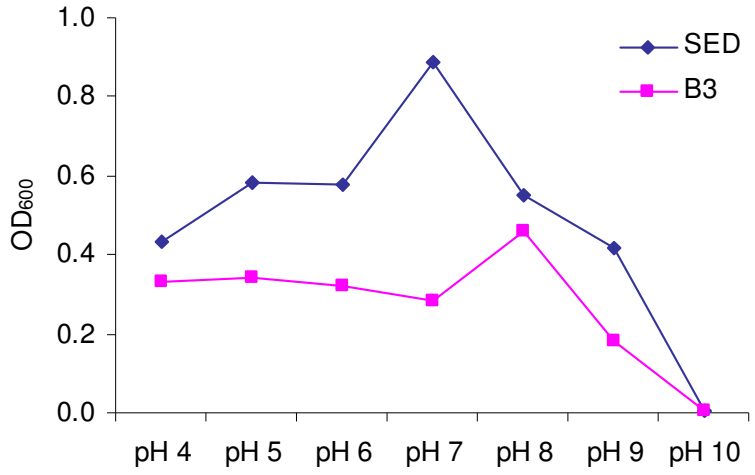


Figure 3.8 Bacterial growths of SED and B3 determined by UV/Vis spectrometer at 600 nm after 3 days of incubation in glycerol medium at different pH.

The pH range of this study was primarily chosen to encourage intrinsic microorganisms, rather than B3, as the sediment culture reflects the actual environment, rather than the single species. The initial range selected for the study of degradation capability was pH 6-8. Unfortunately, at pH 8 hydrolysis of magnesium to magnesium hydroxide occurred within the glycerol media, as indicated by a white precipitation. This resulted in a reduction of the pH from loss of hydroxyl groups in the system; nevertheless, magnesium was preferred for this study as it was recognised to support TBT degradation (Bernat and Długoński, 2006). Also at high alkaline pH, availability of phosphate can severely reduce. The pH under investigation therefore ranged from 6 to 7.5.

Figure 3.9 shows the degradation capability at different pH. The overall degradation capability was very similar in terms of the final concentration of TBT. However, the results can be differentiated by the stepwise degradation during exponential growth phase, i.e. during 0 to 3 days of incubation. It can be seen that alkaline pH results in better degradation from the first day for both SED and B3, while a major reduction at pH 6 was observed after 3 days.

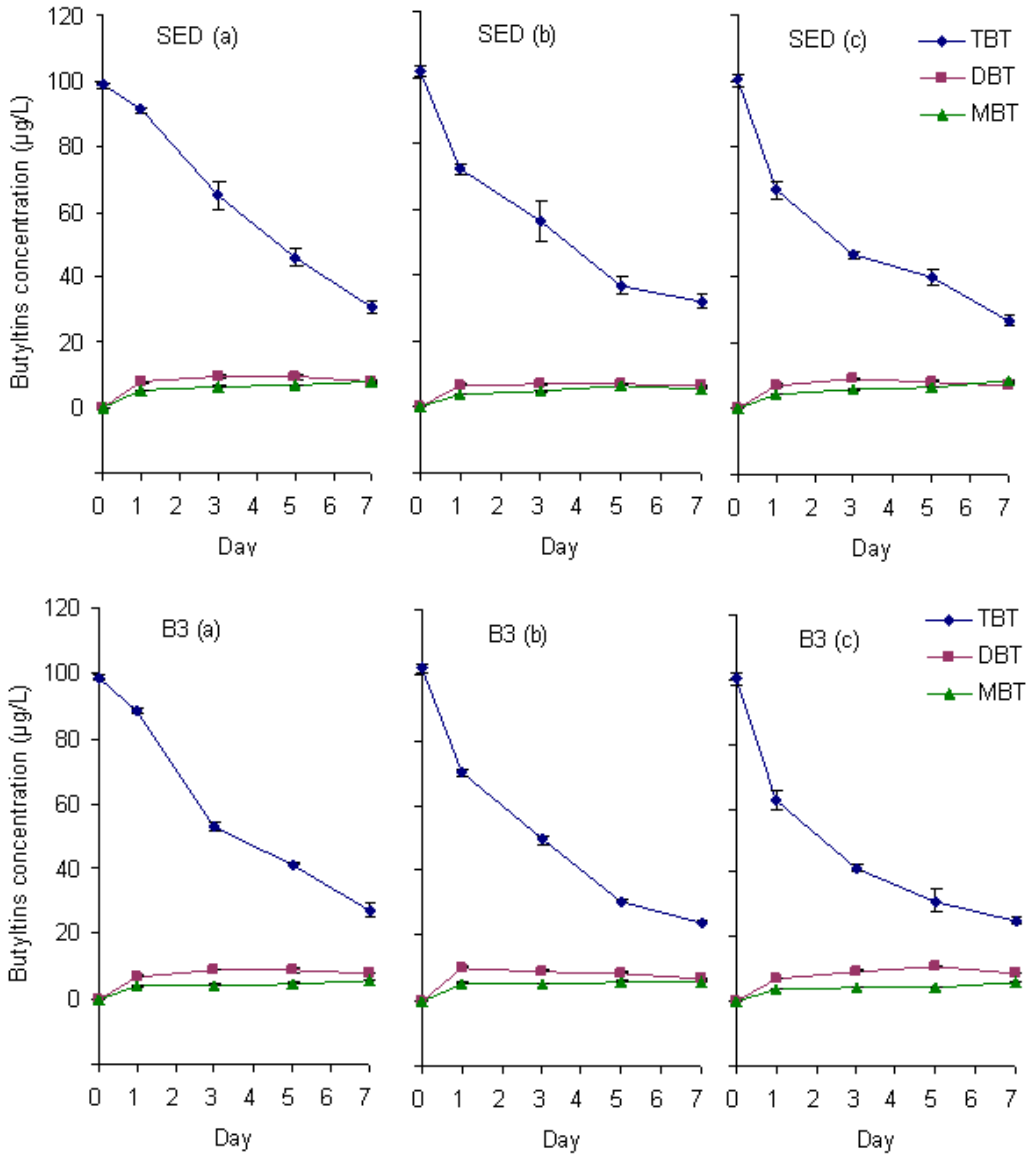


Figure 3.9 Degradation of TBT by SED and B3, and formation of DBT and MBT in culture media at (a) pH 6; (b) pH 7; and (c) pH 7.5 during 7 days of incubation in glycerol medium containing 100 µg/L TBTCl (n=3, SD error bar).

From Figure 3.10, the k_{7d} were evaluated following first order kinetics, and the half-lives of TBT were calculated as shown in Table 3.4. The effect of the surrounding pH was determined by comparing the k_{7d} constants, where ratios were in order of pH 6: 7: 7.5. The ratios of k_{7d} were 1: 1.16: 1.25 and 1: 1.22: 1.22 for SED and B3, respectively. The half-lives indicate greater degradation at neutral and alkaline pH, rather than acidic pH, and B3 was a better degrader than SED in

synthetic media. It can be concluded that pH slightly altered degradation during log phase. This was regarded as minor effect when compared with respiration conditions.

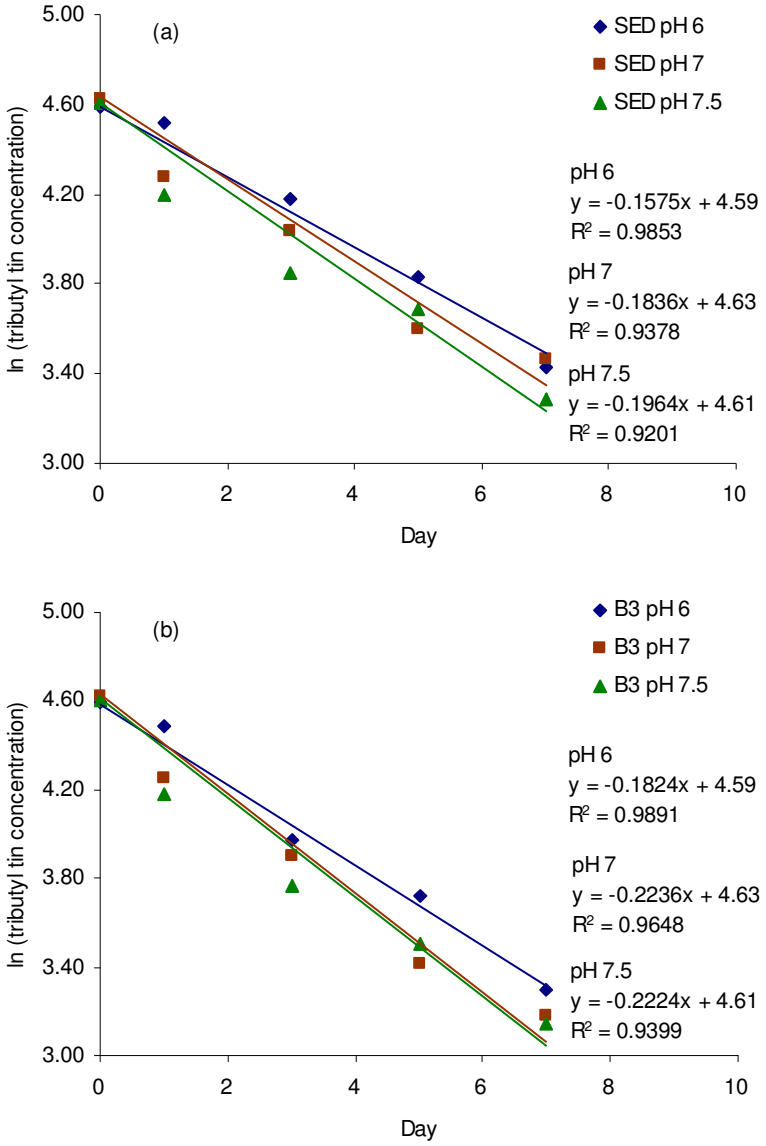


Figure 3.10 Linearisation of TBT degradation data by (a) SED; and (b) B3 under different pH for the calculation of rate constants and half-lives.

Table 3.4 Degradation rate constants at 7 days and half-lives of TBT by SED and B3 at pH 6, 7 and 7.5.

pH	SED		B3	
	k_{7d} (day^{-1})	$t_{1/2}$ (day)	k_{7d} (day^{-1})	$t_{1/2}$ (day)
6	0.16	4.40	0.18	3.80
7	0.18	3.78	0.22	3.10
7.5	0.20	3.53	0.22	3.12

Surrounding pH directly affects TBT speciation because the pK_a of TBT is in the region of 6.25 to 6.51 (Hunziker et al., 2001, Fent and Looser, 1995), therefore a pH value over or below pK_a will present different speciation of TBT. At pH lower than pK_a, TBT stays mainly in the cationic form (TBT^+), but the hydroxide form (TBTOH), so called the neutral form, is mostly presented at alkaline pH. However, at pH around the pK_a and at neutral, both forms are presented together (Hoch, 2001). The speciation of TBT is shown in Equation 3.5.

$$TBT^+ + nH_2O \leftrightarrow TBTOH + H^+ \qquad \text{(Equation 3.5)}$$

Degradation of TBT requires transfer of the compound into bacterial cells before the enzymatic activity is carried out. Although live and dead cells can readily adsorb TBT rapidly after exposure (Tam et al., 2002), pH still affects uptake capability. The forms of TBT, as shown in Equation 3.5, influence the partition of TBT from media onto the bacterial cell wall or into the internal cell of microorganisms. Octanol-water partition coefficient (K_{ow}) is one of the most important factors influencing TBT bioavailability. K_{ow} represents the distribution of a compound between octanol and water, where octanol represents the lipids in living organisms. The log K_{ow} of TBT is constant at pH 2-3 and begins to increase quickly at pH 4-7 and approach maximum constant log K_{ow} at pH 7-9 (Hunziker et al., 2001). This indicates better TBT uptake by bacteria when TBT is in neutral form rather than cationic form. It can be explained by lipophilicity that the neutral form of TBT is more non-polar than the cationic form, and is well adsorbed by non-polar lipid based

on “like dissolve like” theory (Corwin, 2005). Hunziker et al. (2001) also demonstrated that the K_{ow} of TBT^+ is 200, and TBTOH is 12,300.

From this explanation, the initial surrounding pH can dominate TBT degradation during the potential adsorption stage in terms of bioavailability. As a result, at pH 7.5, which has the highest K_{ow}, significant TBT reduction was shown after the first day of incubation. At pH 6, where K_{ow} is exponentially altered, removal was then found largely after 3 days.

Considering the effects of pH on bacterial cells, K_{ow} does not entirely reflect biosorption. Actual biosorption tends to increase with rising pH, but in contrast to K_{ow}, it can decrease at high basic pH (Avery et al., 1993, Gadd et al., 1990). At low pH (pH 4.5), biosorption is small because of minimal K_{ow} in the first instance, and competition with H^+ in the second. At increasing pH, TBTOH can greatly biosorbed in lipid (pH 6.5), but it is decreases at higher pH (pH 8.5) due to reduction of biological activity. Also proteins are denatured at intense alkaline pH (Avery et al., 1993).

To optimise the pH for bioremediation of TBT in sediment, it is essential to take sediment properties into account. Adjusting the initial pH, undoubtedly changes the characteristics of sediments, as demonstrated and explained earlier by Bangkedphol et al. (2009). These authors specified the log sediment-water partition coefficient (log K_d), which indicates the highest log K_d at approximately pH 6.5 to 7, but lower log K_d at pH <5 and >7. Approaching practical bioremediation in sediment, pH that shows the highest K_d should be avoided, since it clearly reduces bioavailability. Another reason to support neutral and alkaline pH to facilitate TBT degradation is the toxicity at low pH which can depress bacterial activity (Gadd, 2000). Extreme pH might also reduce availability of nutrients from reaction with hydronium ion and hydrolysis of minerals.

In summary, pH 7.5 was the most preferred in this study for the following reasons: (i) best degradation rate, (ii) highest K_{ow} and (iii) lowest K_d. However, it

may be impractical to control one optimum pH at all times because bacterial metabolism can change the surrounding pH along with the remediation. However, the initial pH adjustment is expected to increase degradation at the start of the process as determined by this experiment.

3.3.3 The effect of temperature on TBT degradation

The temperature of bacteria varies with that of external temperature. At low temperature, enzyme-catalysed reactions can be slowed down resulting in lower growth rate, but will increases with rising temperature until reaching the optimum (Prescott et al., 2002). For these reasons, growth at a wide range of temperatures was studied to obtain the optimum temperature and, therefore, the highest growth rate, which should accordingly show the best activity with respect to TBT degradation.

Figure 3.11 demonstrates that most of the microorganisms in the sediment and B3 are mesophiles. It should also be noted that growth of B3 was virtually inhibited at 44˚C. It can be seen from Figure 3.11 that the outstanding temperatures for SED and B3 were 20 and 28˚C. Additionally, there was also growth at 10˚C for SED, and at 37˚C for B3. Therefore, the effect of temperature on TBT degradation was delineated at 10, 20, 28 and 37˚C. The growth curves are shown in *Appendix F*.

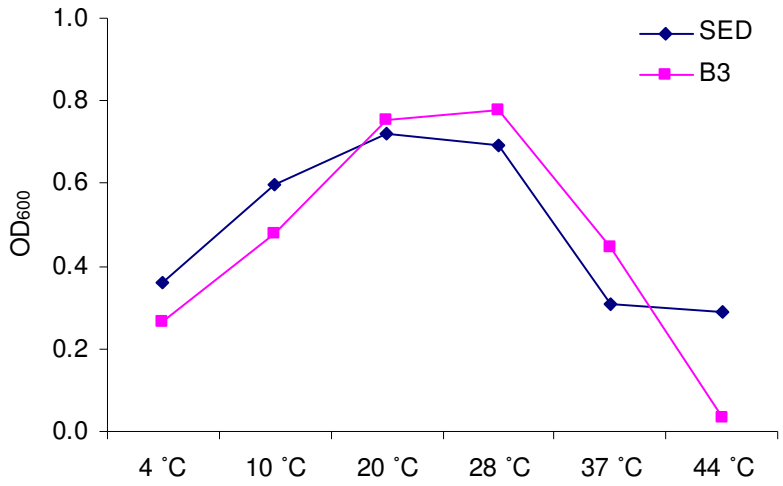


Figure 3.11 Bacterial growths of SED and B3 determined by UV/Vis spectrometer at 600 nm after 7 days of incubation in glycerol medium at varying temperatures.

As temperature is well associated with bacterial activity, poor degradation was expected at low temperature, and better removal when the temperature was increased, as suggested by Brandsch et al. (2001). These authors reported enhanced degradation of TBT by increasing the temperature from 5 to 55˚C. The results of the current study support this finding, with higher temperature significantly promoting SED activity on TBT, as shown in Figure 3.12.

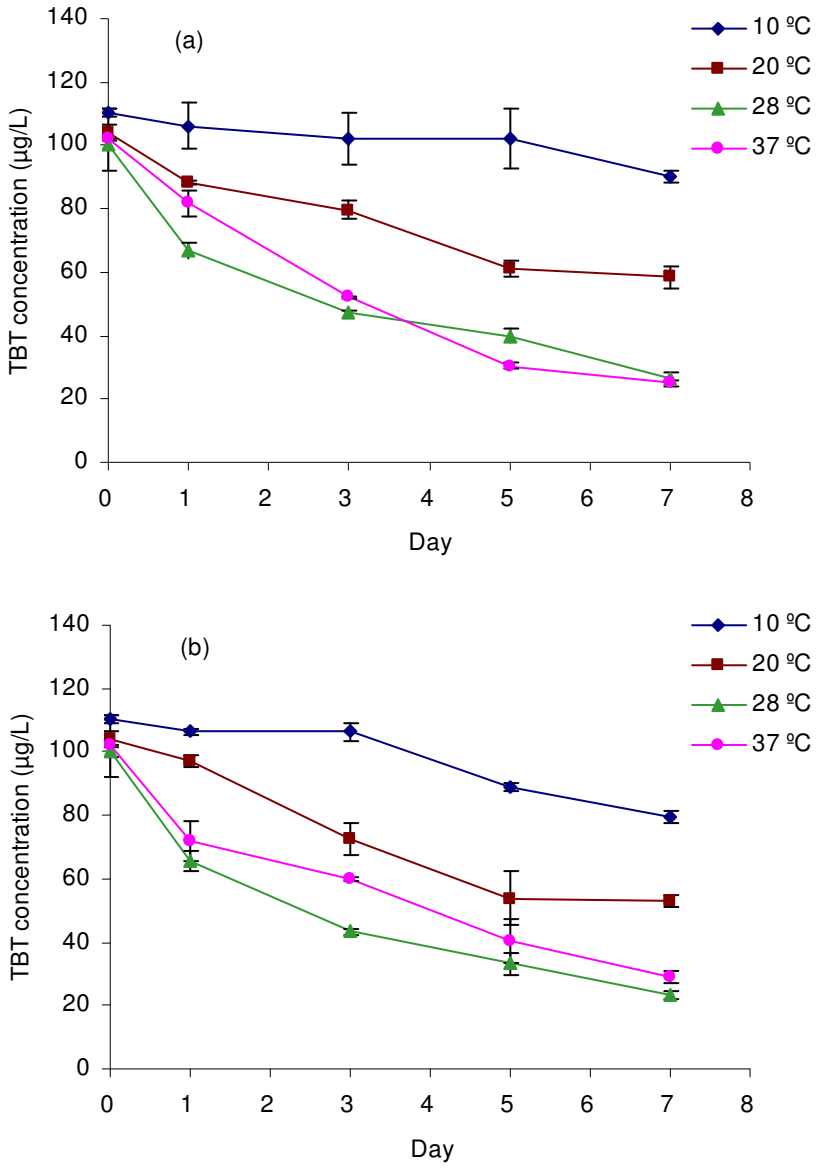


Figure 3.12 Degradation of TBT in glycerol media at different temperatures over a 7 days incubation period in glycerol medium containing 100 µg/L TBTCl (a) SED; and (b) B3 (n=3, SD error bar).

The k_{7d} were evaluated and half-lives of TBT were calculated, as shown in Figure 3.13 and Table 3.5. The enhancement due to temperature was compared in term of degradation rate constant ratio in the order of 10: 20: 28: 37˚C. The ratios of k_{7d} were 1: 3.67: 7.87: 8.66 and 1: 2.60: 5.27: 4.33 for SED and B3, respectively.

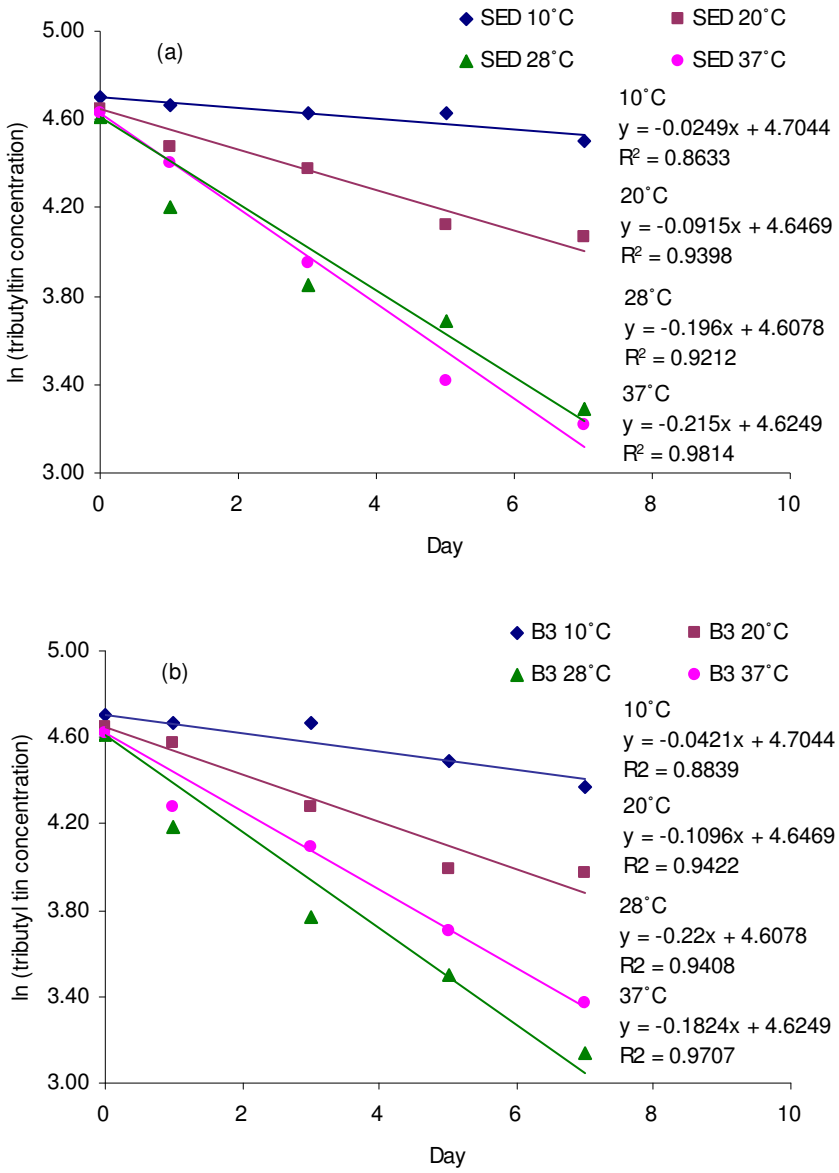


Figure 3.13 Linearisation of TBT degradation data by (a) SED; and (b) B3 under different temperatures for the calculation of rate constants and half-lives.

Table 3.5 Degradation rate constants at 7 days and half-lives of TBT by SED and B3 at 10, 20, 28 and 37˚C.

Temperature	SED		B3	
(˚C)	k_{7d} (day^{-1})	$t_{1/2}$ (day)	k_{7d} (day^{-1})	$t_{1/2}$ (day)
10	0.02	27.84	0.04	16.46
20	0.09	7.58	0.11	6.32
28	0.20	3.54	0.22	3.12
37	0.22	3.22	0.18	3.80

From the degradation results, the preferred temperature for TBT degradation was 28˚C. Adjusting the temperature to 37˚C did not improve half-life, but would boost the remediation cost. In addition, higher temperatures can amplify TBT solubility. Aqueous solubility of TBT will double when the temperature changes from 10 to 25˚C (Inaba et al., 1995). However, adsorption of TBT on sediment will also develop at higher temperatures. The trend of log K_d is continually increasing from 10 to 50˚C (Bangkedphol et al., 2009). Therefore, microorganisms have to compete with sediment adsorption to make contact with TBT for further reactions.

To achieve the most efficient degradation of TBT a compromise has to be reached between increasing the system temperature to improve the degradation ability of sediment culture, the operational cost for heating the system, and the effect on sediment adsorption. A temperature at 28˚C was found to be the best temperature from this study.

3.3.4 The effect of nutrient supplement on TBT degradation

Contaminant degradation mainly takes place during the exponential phase of bacterial growth, when nutrients are utilised for bacterial multiplication. It therefore seemed logical to assume that a greater population of microorganisms would result in increased biodegradation of contaminants (Atagana et al., 2003, Radwan et al., 2000). Screening of suitable carbon sources for biostimulation was therefore based on their potential to promote biomass production. TBT degradation was associated

with biomass because successive biostimulation was observed when the population of contaminant-utilising bacteria was increased (Bernat and Długoński, 2006, Roy and Cabral, 2002). The carbon supplements were added at the same concentration of carbon provided by glycerol because elemental carbon is utilised for bacterial growth and measured in biomass production. Therefore, comparatively all sources were equal in terms of carbon content.

The % packed cell volume (PCV) of each medium was shown in Figure 3.14. As expected no biomass was produced in the absence of a carbon source, demonstrating the essential nature of carbon for microbial reproduction. With respect to the surfactants, Tween 80 supported biomass production for both SED (0.08%) and B3 (0.05%); however minimal bacterial replication occurred in SLS and Triton X-100. Tween 80 is non-toxic to soil microorganisms and inert to soil matrix (Jayashree and Vasudevan, 2007); therefore, only Tween 80 was used as a carbon source to represent surfactants. Among organic salts, the addition of S/G resulted in the largest biomass for SED (0.20% PCV), while the addition of sodium succinate produced the largest B3 biomass (0.25% PCV). Even when the quantity of carbon was equal, biomass is still differed, this suggests that microorganisms utilise nutrients by selection which may be the results of their metabolism or properties of the nutrient (Barr, 1978, Roberts et al., 1996).

For carbohydrate and sugars (Figure 3.14), glycerol gave 0.12% PCV for both SED and B3. A basic carbon source like glucose is easily utilised by microorganisms (Bernat and Długoński, 2006, Östberg et al., 2007). For this reason, carbon sources with more complex structure i.e. sucrose (disaccharide of glucose and fructose), starch (polysaccharide consisting of a large number of glucose) and xylose (monosaccharide with 5 carbons structure) were studied in comparison with glucose to investigate the effect of chemical structure's complexity on bacterial utilisation. The highest % PCV of SED (0.25% PCV) and B3 (0.28% PCV) was obtained from starch and xylose, respectively. It appears that the complexity of the saccharide structure had no effect on bacteria metabolism. This result indicates that bacterial growth was dependent on the enzyme production for each type of nutrient.

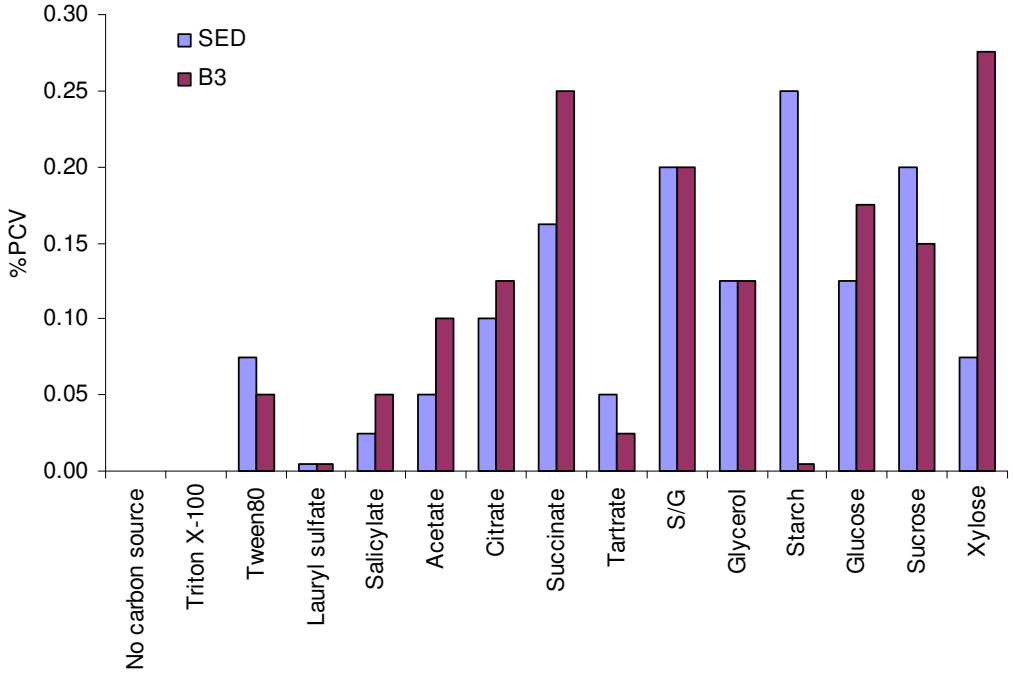


Figure 3.14 % PCV of SED and B3 in medium with different carbon sources after 7 days of incubation at 28ºC, shaking at 150 rpm (n=2).

The addition of an easily accessible carbon source might promote biodegradation, but the lag phase can be prolonged because microorganisms use the nutrients as the primary source for carbon and energy (Östberg et al., 2007). On the other hand, Östberg et al. (2007) demonstrated initial stimulation by amino acids and vitamins to shorten lag time of degradation. Since amino acids and vitamins were expected to be used as substrates/coenzymes in metabolic pathways or enzyme production of microorganisms rather than source of carbon and nitrogen, they were added in the same molar concentration.

Yeast extract in the medium evenly promoted biomass yield as shown in Figure 3.15, but provided unspecified growth factors. Accordingly, it was replaced by individual amino acids and vitamins to specify the influencing compounds. L-Serine was the best supplement for B3 (0.15% PCV), and it was previously reported to encourage growth of *Enterobacter cloacae* as a growth factor or as a reduced carbon source (Roberts et al., 1996). For SED, both L-Arginine and L-Threonine

likewise supported biomass production (0.12% PCV). The results imply that amino acids and vitamins were utilised as precursors, since the total amounts of carbon and nitrogen of each amino acid and vitamin were not related to biomass production. As a result, specific growth factors can be added to encourage TBT degradation.

For B3, Figure 3.15 shows the essential nature of amino acids as minimal biomass was observed when amino acids were absent. Even vitamins were believed to assist bacterial growth; however, it was shown in B3 that vitamins alone could not support the degrader in the absence of amino acids. In contrast, SED was able to cope with the lack of amino acids and, moreover, grew without any growth factors. Among the vitamins, D-Biotin (vitamin B7) gave the highest % PCV at 0.12 % for SED. Surprisingly, without growth factors, the % PCV of SED went up to 0.18%. The diverse species in sediment might produce and share various types of nutrients, including amino acids amongst the community, which would not occur in the sample with a single culture.

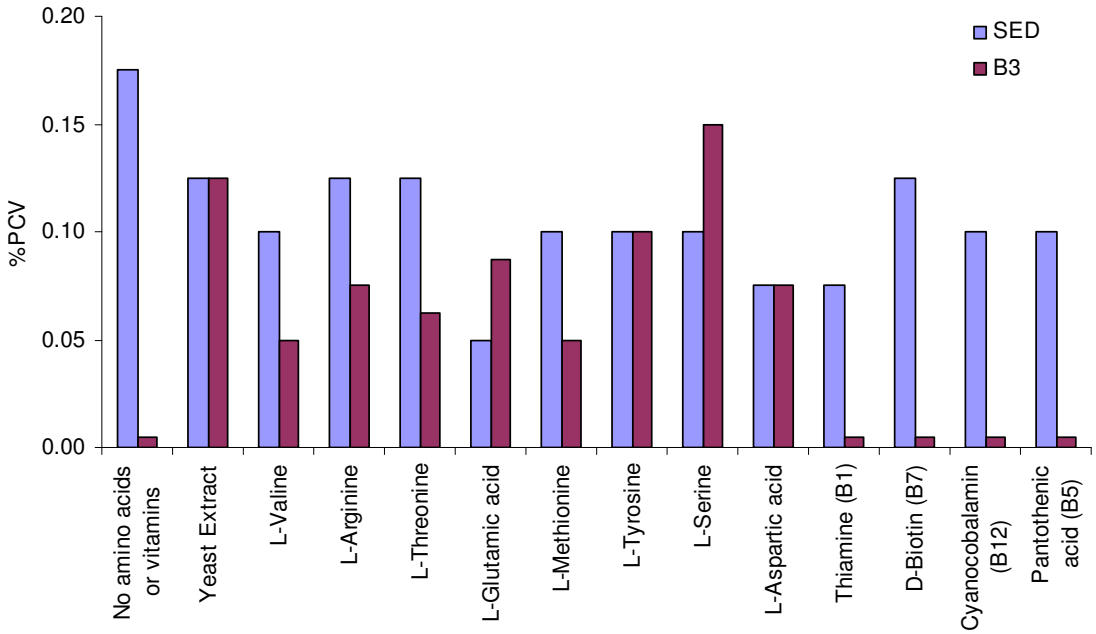


Figure 3.15 % PCV of SED and B3 in glycerol medium with different amino acids or vitamins after 7 days of incubation at 28ºC, shaking at 150 rpm (n=2).

From the screening step (Figures 3.14 and 3.15), the nutrients identified for biostimulation of degradation in the SED were Tween 80, S/G, starch, L-Arginine, L-Threonine, D-Biotin and growth factor control. Supplements for B3 were Tween 80, sodium succinate, xylose and L-Serine.

The type of nutrient and concentration present led to differing levels of TBT degradation. Figures 3.16 and 3.17 indicate that nutrients which enhanced biomass production do not necessarily promote initial TBT degradation, as some selected supplements showed no reduction of TBT after 7 days of incubation. TBT could be degraded as a secondary source when microorganisms were attracted by the easily accessible supplement (Stasinakis and Thomaidis, 2010). Accordingly, the stimulation was delayed and will occur after the primary source is depleted. However, this study intended to investigate immediate stimulation to reduce treatment time, which was only exhibited by some supplements.

Figure 3.16 shows the levels of TBT remaining after 7 days incubation with SED. Surfactants are more versatile when compared with other types of nutrients, since they supply both carbon and energy, as well as increasing the solubility of contaminants (Obbard et al., 2004). In surfactant-mediated bioremediation, the surfactant can improve the solubilisation of pollutants from soil, which in turn improves their bioavailability (Li and Chen, 2009) and biodegradation (Jayashree and Vasudevan, 2007). Surprisingly, the addition of the surfactant Tween 80 totally inhibited initial TBT degradation by SED. Tween 80 was previously reported to enhance endosulfan biodegradation by causing direct uptake of the xenobiotic compound along with the micelles (Jayashree and Vasudevan, 2007). Unfortunately, TBT was not degraded in the medium with Tween 80, even at a low concentration level (0.1x). The results indicate that the surfactant was not suitable for primary acceleration of TBT mineralisation; however it could still be used to increase TBT bioavailability in sediment. Degradation in starch medium was found to be about 25% at 0.1x supplement concentration, but no degradation occurred at higher concentrations, indicating that an excess amount of starch led to inhibition of initial TBT degradation by the microbes.

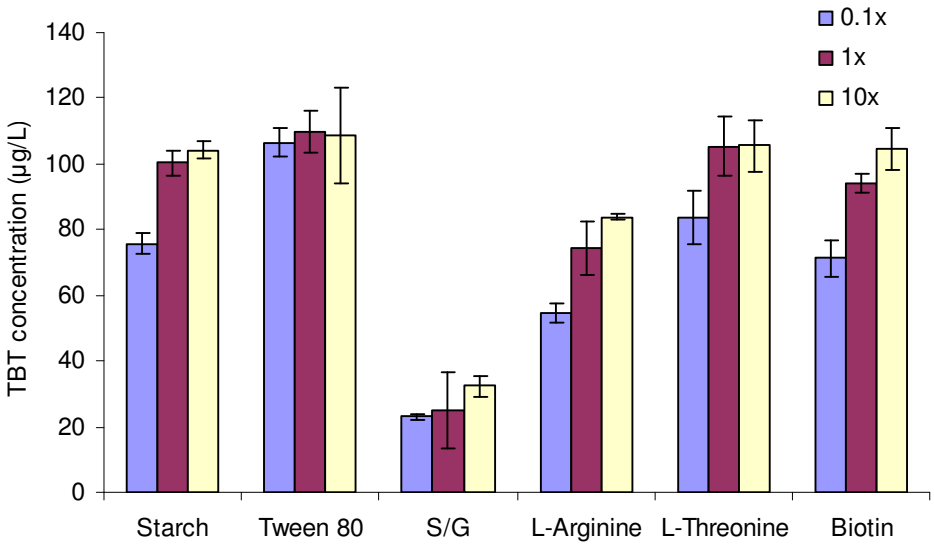


Figure 3.16 TBT concentrations after 7 days degradation by SED in liquid media with different concentrations of nutrient supplement (0.1x, 1x and 10x), at the initial TBT concentration of 100 µg/L (n=3, SD error bar).

As shown in Figure 3.16, the addition of S/G resulted in degradation of TBT by approximately 77 to 68% from 0.1x to 10x supplement concentrations. The mixture of S/G has been documented to enhance TBT degradation (Roy and Cabral, 2002). However, this study shows that excess amounts of the supplement may in fact inhibit the quantity of the TBT degraded. Succinate is part of the citric acid cycle; therefore, it is superior in providing energy for microbes when participating in the reactions in the cycle. From the experiment, S/G was the best to support initial TBT degradation. Even though Roy and Cabral (2002) stated that the stimulation effect was caused by the increased population, this study suggests that is not always the case. The nutrient did enhance the capability of microbes to degrade TBT, but increased bacterial growth was not always associated with improved degradation, i.e. starch produced the largest SED biomass, but did not result in the greatest level of initial TBT degradation.

Yeast extract, a complex nutrient for carbon source and growth factor, in the media was added as a source of amino acids and vitamins. Without the complex nutrient or growth factors, degradation of TBT by SED did not occur (not shown on Figure 3.16) indicating that growth factors were necessary for degradation. Amino

acids and vitamins can be produced by the SED for their growth, but in limited conditions SED were not able to debutylate TBT.

Amino acid addition provided energy and residues for enzyme production; also vitamins were added as co-factors. Degradation by the SED with L-Arginine varied according to the concentration of supplement, with the highest concentration showing the least degradation, 45% (0.1x), 26% (1x) and 16% (10x); L-Threonine was 16% (0.1x) and none for 1x and 10x; D-Biotin was 29% (0.1x), 6% (1x), none for 10x. Thus, it is shown in Figure 3.16 that excess amount of growth factors resulted in a reduction of degradation percentage.

The addition of amino acids can benefit auxotrophic bacteria that cannot synthesise certain specific amino acids by allowing them to participate in the degradation which results in biostimulation (Alexander, 1999). L-Arginine promoted TBT degradation by SED, suggesting that the sediment consortium cannot synthesise sufficient L-Arginine for enzyme production. For the same enzyme production and activity, individual microorganisms can be induced by diverse supplements, where each supplement contains different induction capability (Tokiwa and Calabia, 2006). From the experiment, L-Arginine is a good enzyme inducer to increase the capability of microorganisms in sediment consortium for TBT degradation.

For B3, Figure 3.17 shows xylose was a good supplement and degradation was observed at 36% (0.1x), 35% (1x) and 21% (10x). Tween 80 at a 0.1x supplement concentration was able to support degradation, reducing TBT by 8%, but no degradation was observed at 1x and 10x supplement concentrations. Succinate yielded degradation of 49% (0.1x), 75% (1x) and 77% (10x). Among the three carbon supplements, succinate was the most efficient because no inhibitory effect was observed at higher concentrations. Degradation was between 3% and 4% in the medium with L-Serine. L-Serine was inferior when compared with yeast extract. This infers that L-Serine was not associated with the degrading enzyme and is not suitable as a supplement for TBT treatment. Though the nutrient promoted growth of the single potential degrader (B3), again increased biomass was not always linked to

improved degradation ability. This indicates the importance of nutrients to increase dioxygenase activity of degraders rather than biomass. In addition, this study demonstrated co-metabolism degradation of TBT.

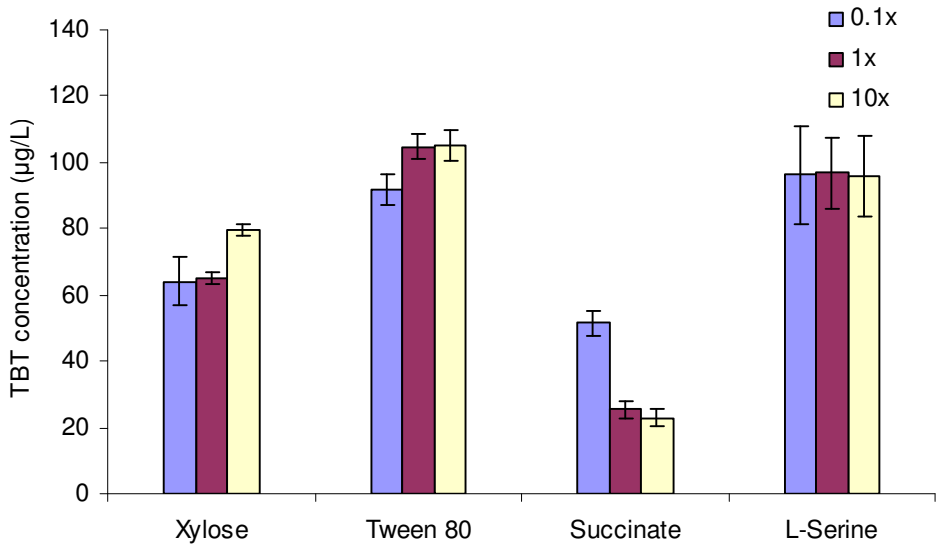


Figure 3.17 TBT concentrations after 7 days degradation by B3 in liquid media with different concentrations of nutrient supplement (0.1x, 1x and 10x), at the initial TBT concentration of 100 µg/L (n=3, SD error bar).

From the results, the supplementation might cause i) induction of degradation; ii) formation of intermediates; iii) presence of analogues and precursors; iv) biomass production to enhance numbers and degradation, which aids biodegradation. Given that addition of nutrients, organic materials or individual chemicals can enhance degradation, stimulation will shows in terms of shortened lag time or accelerating exponential phase from enzyme induction and production. Appropriate amount and type of nutrient can lead to completion of TBT removal (Kawai et al., 1998). It was also hypothesised that biodegradation capability can be encouraged by the addition of relevant pathway intermediates (Singleton et al., 2008), such as the use of succinate and L-Arginine in this study. Moreover, L-Arginine added in this study may be a precursor which facilitates degradation enzyme production. However, nutrients and amino acids added can inhibit degradation, as they can serve as a source of carbon and compete with xenobiotic compounds (Swindoll et al., 1988). Östberg et al. (2007) demonstrated that

degradation slowed down after the addition of lactate, but gave a stimulatory effect towards the end of incubation period (120 days). This shows the inhibitory effect of easily accessible carbon sources to initial TBT degradation.

Although delay of TBT degradation resulted from the competition between TBT and supplements, the lack of degradation may be due to the added nutrients promoting the growth of the consortium except degraders. From this reason, lower biomass could show better degradation than that higher biomass (Moorman et al., 2001). Consequently, the most appropriate carbon source for biostimulation of degradation might not necessarily show the highest growth rate where the enzyme enhancing nutrients are preferred. In addition, the inhibitory effect of supplement concentration was clearly seen. This suggests an amendment procedure where carbon sources with inhibitory effects should be continually added at limited concentrations. Slow-release nutrient sources such as biosolids were previously used to stimulate biodegradation, which can minimise the contamination from excess nutrients into the ecosystem (Sarkar et al., 2005).

Initial stimulatory effect of amino acids by shortening lag time has been evident. In loamy sand a significant positive effect was shown by amino acids, but not carbon sources (Östberg et al., 2007). However, amino acids can serve as reduced carbon sources which, from this study, show inhibitory effects at excess concentrations. In addition, amino acids that show the best support for bacterial growth were not necessary to promote enzyme activity. This finding was in agreement with Jarerat et al. (2004) who demonstrated the supporting property of amino acids on bacterial growth without promoting enzyme activity or, conversely, increasing enzyme production but with very little cell growth.

3.4 Summary

The capability of a sediment culture (SED) and bacterial isolate *E. cloacae* (B3) to degrade TBT were studied by biostimulation; respiration conditions, pH of the media, ambient temperature and nutrient supplement, were all optimised.

Three respiration conditions were investigated; aerobic with/without aeration and anaerobic with 0.1 g NO_3^-/L. Among the three conditions, aerobic with aeration, which provided sufficient dissolved oxygen, extensively improved the degradation. Variations in the pH of the media moderately affected the degradation rate, mainly at the early stage of log phase. The shortest half-life was observed at pH 7.5 and pH 7 for SED and B3, respectively. Appropriate pH also increased bioavailability, which can be explained by K_d and K_{ow} values. The study of ambient temperature indicates strong uncertainty of degradation from seasonal variation. The half-lives were minimised at 37˚C for SED and at 28˚C for B3. Investigation of these factors for the optimum conditions led to modification of the environment, the consequence of which was more efficient biodegradation of TBT. The suitable conditions obtained from this study, aerobic conditions, at pH 7.5 and incubated at 28°C, were then applied to sediment to investigate the efficiency of TBT remediation from contaminated sediment (*Chapter 4*).

The ability of organic nutrients (surfactants, organic salts and saccharides) and growth factors (amino acids and vitamins) were investigated individually to enhance TBT biodegradation. Most supplements supported bacterial growth at different amounts of biomass produced. Only the supplements that produced high biomass yield were selected to support dioxygenase activity. However, increasing the biomass did not necessarily promote degradation. Appropriate supplements were able to encourage the production of enzymes for metabolism of TBT. These supplements were shown to stimulate TBT degradation, by increasing bacterial population concomitant with degrading capability. At different amounts of supplements, most nutrients and amino acids had an inhibitory effect at increasing concentrations. Excess amount of the nutrients added can inhibit the initial degradation of TBT. Therefore, TBT biostimulation requires supplements that increase the capability of TBT-degraders at an appropriate amount. This study has demonstrated that S/G and succinate were the most suitable supplements for SED and B3, respectively. From this study, enhance supplements which are S/G (0.74 g/kg of sodium succinate, 0.23 ml/kg of glycerol) and L-Arginine (0.087 g/kg) were selected as degradation enhancement in sediment (*Chapter 4*).

CHAPTER 4

Bioremediation of Tributyltin Contaminated Sediment: Degradation Enhancement and Improvement of Bioavailability

Objectives: To enhance TBT bioremediation in sediment by inoculation of the degrader cells, controlling stimulation conditions and addition of supporting nutrients (investigated in *Chapters 2 & 3*). Also, to improve TBT bioavailability which increases microorganism uptake, by addition of surfactant, adjusting of salinity and sonication. After enhancement, a shorter bioremediation time and higher amount of degraded TBT is expected.

4.1 Background

4.1.1 Remediation of contaminated sediment

Due to the severe toxicity of TBT, degradation of the compound is a solution to reduce the risks. Remediation can be defined as any process to recover the contaminated environment to its original or nontoxic state. Remediation can be engaged for specific contaminants or a general approach taken, such as the reduction of BOD and odour from organic rich sediment (Chan et al., 2002). Remediation technologies can be generally classified as *in-situ* or *ex-situ*. *In-situ* bioremediation is a removal of contamination on site, while *ex-situ* involves treatment elsewhere. Possible sediment remediation options are shown in Table 4.1.

Table 4.1 Overview of the remediation options for contaminated sediment (Lens et al., 2005, Rulkens, 2005, USEPA, 2005).

Remediation method	Treatment technologies
In-situ	**Natural attenuation**: involves the natural biodegradation of pollutants without external intervention. **Aerobic and anaerobic treatment**: addition of electron acceptors or electron donors to provide suitable conditions for biotic processes. **Chemical oxidation**: involves the oxidation reactions such as Fenton reagents (a solution of hydrogen peroxide and an iron catalyst), permanganate or ozone. **Sediment capping**: emplacement of materials, such as clean sand/gravel, geotextiles and liners, to physically isolate the contaminated sediment and prevent migration.
Ex-situ	**Dredging and excavation**: relocation of the contaminated sediment for disposal or treatment, or reuse as construction materials after stabilisation. **Aerobic composting**: treatment system in which partially dewatered sediment is intensively mixed with bulky natural organic materials and placed in an aerated stockpile. Mechanical aeration of the pile is performed to allow aerobic biodegradation. **Landfarming**: treatment in which partially dewatered sediment is spread out in a thin layer to allow aerobic biodegradation on the top layer. **Bioslurry systems**: a system consisting of a reactor in which the sediment slurry is mixed with electron acceptors or electron donors and conditions are controlled in order to stimulate the growth of microorganisms that can degrade the contaminants (biostimulation). These microorganisms are already present in the sediment or can be added during the process (bioaugmentation). **Anaerobic treatment**: microbiological breakdown of biodegradable material without oxygen such as anaerobic sewage sludge treatment.

4.1.2 Abiotic remediation of TBT in sediment and case study

4.1.2.1 Abiotic remediation of a contaminated harbour

Since validated methods for remediation of TBT-contaminated sediment are not yet established, the contaminated sediment is generally dammed up and isolated from the rest of the aquatic environment. A case study of TBT-removal in Vuosaari harbour, Helsinki, Finland demonstrates the management of a contaminated area for harbour structures (Piispanen, 2004). Vuosaari harbour had to be dredged because the shallow shipyard area results in a high tendency for sediment to migrate. The covering of the TBT-contaminated sediment would require large volumes of clean materials and was also not suitable for the project. Both surface and deeper sediments were found to contain very high concentrations of TBT, and, as such, sediment was not allowed to be dredged or transferred without permission. OSPAR Commission has issued guidelines for the disposal of dredged materials. The quality criteria for the materials are issued nationally as shown in Table 4.2.

Table 4.2 Action level concentrations (µg/kg dry weight) according to the quality criteria for TBT in dredged materials (OSPAR Commission, 2008).

Country	Action level1	Action level 2
Finland	3	200
Germany[a] (2001-2004)	20	600
(2005-2009)	20	300
(2010-)	20	60
The Netherlands[b]	0.000007	250
Ireland[c]	100	500
United Kingdom[d]	100	1,000
Denmark	7	200
Belgium	3	7

[a]concentrations (µg/kg) based on total sediment; [b]concentrations (µg Sn/kg) based on dry weight; [c]mixture of TBT and DBT; [d]mixture of TBT, DBT and MBT

Action level 1: environmental toxicity safe concentrations in dredged materials

Action level 2: concentrations based on technical and economic possibilities of reducing current TBT concentrations in dredged materials

To dredge the sediment, the process required a water permit and proper disposal of the TBT-contaminated sediment. After obtaining a permit, low contaminated sediment was dredged and disposed. Permission was granted for high contaminated sediment to be dredged, but it had to be separated into an excavated rock pool after dredging. The dredging of very highly contaminated sediment was prohibited to prevent distribution of the sediment. Without removal of the very highly contaminated sediment, new harbour structures could not be built. To solve the problem, Helsinki City Council firstly constructed permanent embankments to separate the contaminated area from the environment, and the embankments became part of the harbour structures. Then, sediment was dredged and only 5% of the dredged sediment escaped from the embankments. Finally, the sediment was stabilised by mixing with cement and other stabilisers, and used to replace blasted rock and sea sand by incorporating it into a five meter thick wall on a bearing fill layer serving as part of the structure. The volume of highly contaminated sediment dredged from the area was estimated to be approximately 300,000 m^3. The deposition and stabilisation of the sediment were protected by bottom and surface structures to avoid migration of the material in the aqueous system. Figure 4.1 shows the dredging in the construction area with embankments and curtains.

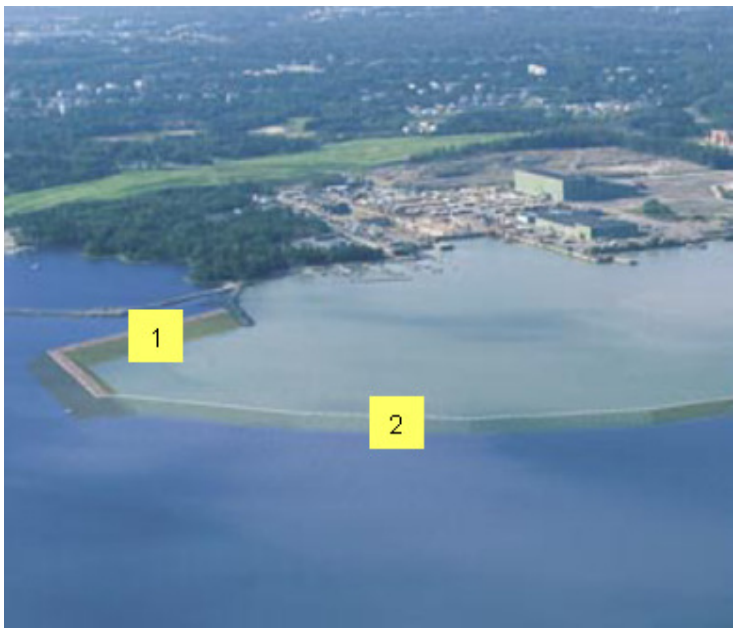


Figure 4.1 Birds eye view of the dredging area with (1) protective embankments and (2) temporary protective curtains (adapted from Piispanen, 2004).

4.1.2.2 Abiotic remediation of TBT contaminated sediment

The dredging of TBT-contaminated sediment from the site is an option to remediate the area, however, sediment is still contaminated and its transfer might cause distribution of TBT in terrestrial areas. The Department for Environment, Food and Rural Affairs (DEFRA) as the regulatory for sea disposal of dredged materials in England and Wales has funded a study on remediation technology for TBT (Reed et al., 2001). The study isolated the heavily contaminated components of the sediment to reduce the volume of grossly contaminated material. By employing froth flotation techniques, light fractions of sediment which had the highest contamination of TBT were separated. From the developing technique, disposal costs were reduced and easily managed.

The disposal of contaminated sediment still requires biodegradation to reduce the amount of TBT, or the contamination will remain in the containment without abiotic degradation. Although incineration of the sediment was assessed as a good TBT destruction method, temperatures of 1,000°C are necessary for TBT to be undetectable (Howard and Gkenakou, 2008). This technique is not likely to be commercially acceptable because of the operational costs. Steam stripping is another remediation technique introduced to separate TBT from matrices (Eschenbach et al., 2001). The technique can decontaminate different materials almost completely by using pressurised steam. The alteration of the physical-chemical conditions results in desorption and vaporisation of TBT. After gas-solid separation, TBT and water are condensed in a condensation chamber where liquid-liquid separation removes TBT from water. The contaminant discharge is then treated. A more recent technique introduced for TBT remediation is photo-degradation. Titanium dioxide and its doped species were used as catalysts which promoted the debutylation by natural light in this technique (Bangkedphol et al., 2010).

4.1.3 Bioremediation of TBT in sediment and case study

Despite many remediation techniques having been used and developed for removal of TBT, unfortunately, documentation on bioremediation case studies of TBT are very limited at present. A few scientific studies on TBT treatment in wastewater by aerobic and anaerobic activated sludge have been published (Stasinakis et al, 2005, Voulvoulis and Lester, 2006); however, the biotic removal of TBT in sediment was due to natural attenuation. Nevertheless, natural attenuation cannot appreciably reduce contamination unless enhancement is in place. With regard to bioremediation in sediment, only laboratory scale enhancement by aeration to provide aerobic conditions (Dowson et al., 1996) and optimisation of the surrounding temperature (Brandsch et al., 2001) have been acknowledged so far.

Biodegradation is a major process for TBT removal, particularly in sediment. This technique has more advantages over incineration and landfill disposal because biodegradation converts toxic compounds into less or non-toxic products, produces no waste, has lower cost of operation, reduces health and ecological effects, and can be performed *in-situ* without disturbing the environment (Sarkar et al., 2005).

4.1.3.1 Intrinsic bioremediation

Intrinsic bioremediation or natural attenuation is a general method of *in-situ* bioremediation. Natural attenuation is found in most contaminated areas with TBT metabolites, DBT and MBT, together with TBT being present (Landmeyer et al., 2004, Rodríguez et al., 2010). The method use indigenous microorganisms to degrade the contaminants of concern without human intervention. This method is only feasible when a capable microbial community, essential nutrients and appropriate conditions are present at the site. Although, in general, aerobic degradation has been proven to be the most effective in reducing contaminant levels, the *in-situ* bioremediation of contaminated aquatic sediment is likely to be anoxic, anaerobic, and co-metabolic modes which are well known to be slower than aerobic processes (Adventus, 2010). In other words, TBT will be degraded less by the

bacterial community in sediment as compared with water due to the reducing DO concentration with increasing depth (Dowson et al., 1996). Degradation of TBT within the sediment compartment is comparatively slow, with TBT half-lives in surficial sediments ranging from 360 to 775 days, but in anaerobic sediment, the half-life of TBT has been reported to be in the order of ten years (Dowson et al., 1996). The occurrence of high TBT levels in aquatic sediment could potentially serve as a long-term threat to aquatic inhabitants. For this reason, the persistence and the migration of a toxicant increase the toxicity to the organisms in contact with it. Therefore, appropriate stimulation and conditioning are needed to improve the treatment quality.

Landmeyer et al. (2004) working in South Carolina, USA, found that bed-sediment microbial community significantly reduced levels of TBT and increased the concentration of inorganic tin. These authors also found that TBT was degraded faster in organic rich sediment, which doubled (fieldwork) or tripled (laboratory) TBT degradation rates when compared to organic poor sediment. In organic rich sediment, TBT decreased from the system rapidly with a shorter acclimation period, but TBT was not completely removed, because (i) microbial oxidation was limited due to poor bioavailability resulting from absorption of TBT onto organic-rich sediments; (ii) the presence of anaerobic microenvironments that would result in essentially no biotransformation of TBT (Landmeyer et al., 2004).

Notable attenuation has been found with river (Suehiro et al., 2006) or estuarine sediment bacteria (Harino et al., 1997a, Mora et al., 1995), with no significant difference in TBT degradation rate, but in terrestrial soil, microorganisms have slow degradation leading to the accumulation of TBT on land (Huang and Matzner, 2004). The slower degradation rate in soil than in water indicates the greater stability resulting from absorption in soil.

4.1.3.2 Biostimulation

Biostimulation provides the environment and resources required by the native microbes to degrade target contaminants, hence reducing contaminant toxicity. The required substrates (electron donor, electron acceptor, specific carbon substrate, or nutrients) are added to stimulate the growth and enzyme production of a target consortium of bacteria. Generally, electron acceptors and nutrients are the two most critical components of any delivery system (Alexander, 1999). Sufficient amounts of appropriate nutrients can accelerate degradation by increasing the capability and biomass of degraders (Delgado-Moreno and Peña, 2009, Sarkar et al., 2005, Xia et al., 2006). Aerobic conditions are usually provided since biotic degradation of TBT is minimal in both laboratory and fieldwork under anaerobic conditions (Voulvoulis and Lester, 2006).

Chan et al. (2002) carried out work in the Shing Mun River, Hong Kong, which had odour problems results from large amount of sulphide and organic carbon in the sediment. The authors injected nitrate solution 15 cm beneath the sediment surface to oxidise sulphide to sulphate and breakdown organic materials to carbon dioxide. The acceleration of odour removal was accomplished to meet sediment-water quality and odour control in 4 years. In Hamilton Harbour, Canada, oil and PAHs were treated *in-situ* by injection of calcium nitrate and organic nutrients (Murphy et al., 1995). It was studied through bench, pilot and field scales without physical or biological limitations precluding matching the laboratory results in a real site treatment. The reduction of oil and PAHs confirmed the effectiveness of biostimulation.

4.1.3.3 Bioaugmentation

Bioaugmentation is the artificial introduction of microorganisms, which are capable of degrading target contaminants, when the native microbes are probably not able to achieve sufficient biodegradation. After the potent microorganisms are introduced, biostimulation can lead to faster and complete degradation of

contaminants (McKinsey et al., 2003). Nevertheless, the indigenous populations are usually acclimatised to the existing contamination and have developed to utilise or to transform the pollutants. In this case, bioaugmentation then accelerates degradation after inoculation of dense cells without delay (Lima et al., 2009). However, the effect of biotic and abiotic environments on microorganisms is critical in terms of survival, activity and migration of the inocula (El Fantroussi and Agathos, 2005). Therefore, augmentation with the indigenous microorganisms which contain the catabolic activity, especially specific strains, can lead to more effective treatment than the application of uncharacterised consortia (El Fantroussi and Agathos, 2005). Also, encapsulation techniques (Moslemy et al., 2003) and bioaugmentation with catabolic genes (Dejonghe et al., 2001) might be a solution to reduce the effect from the environment. From the literature review, no bioaugmentation for both *in-situ* and *ex-situ* TBT degradation in sediment has been studied at present. Most of the potential effective microbes from laboratory tests are single species; as a result they might not be competent or competitive in the real environment, which could result in a decline in numbers after introduction. Therefore, viability and potential to survive of the inocula in the environment is the key parameter for potential bioaugmentation.

4.1.4 Sediment characteristics and TBT bioremediation

In addition to microbial community and TBT characteristics, site characteristics are of particular importance to bioremediation. The factors influencing growth and degradation of the microorganisms have been discussed in *Section 2.1.3*, however additional factors are associated with biodegradation in sediment. In the real environment, other biodegradable materials, such as various organic and inorganic components present in the sediment, may be used by microorganisms in preference to TBT. Also, the presence of toxic substances, such as organic pollutants and metals in the sediment, can harm microorganisms, which could reduce the degradation capability (Scragg, 1999).

It has been reported that lower amount of TBT can be degraded in sediment compared to water (Brandsch et al., 2001). In activated sludge treatment of TBT in

wastewater, TBT can be completely adsorbed by the sludge in one day. The degree of adsorption is higher when the contact time is greater or the suspended solid concentration is increased (Stasinakis et al., 2005). Since degradation of TBT occurs inside microbial cells (Tsang et al., 1999), reduced bioavailability prevents microbes from approaching uptaking the molecule of TBT, prohibiting its breakdown.

After a pollutant released into an aquatic environment, it can partition into different media and become less available for microorganisms, as shown in Figure 4.2. Generally, bioavailability is the fraction of a pollutant that becomes available for activity in the target issue. Since bioavailable fraction, both readily and potentially available, of a pollutant might not bioreactive, then the term bioavailable used in this study only refers to biodegradable fraction.

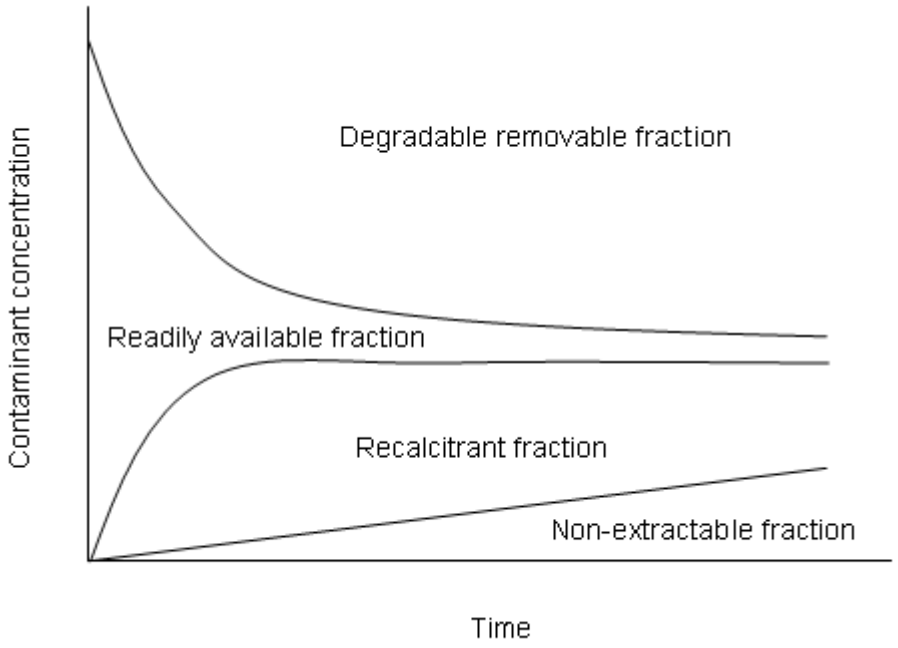


Figure 4.2 The influence of contact time on the extractability and bioavailability of a pollutant (adapted from Semple et al., 2003).

4.2 Experimental

Since TBT mainly accumulates in sediment after release in the aquatic environment, many studies to date monitor TBT contamination and natural attenuation in sediment at various sites globally. To accelerate degradation, many techniques can be applied. However, with regard to degradation enhancement, only aeration (Dowson et al., 1996) and temperature (Brandsch et al., 2001) have been documented for TBT in sediment. Therefore, this study aimed to develop the treatment of TBT contaminated sediment by providing aerobic conditions, suitable temperature and pH. Furthermore, biostimulation (nutrient supplement) and bioaugmentation (inoculation of *Enterobacter cloacae*) techniques were employed to support TBT natural attenuation. Since TBT is strongly adsorbed to sediment constituents, TBT uptake by microorganisms is hindered and remediation quality is negatively affected. This study also investigated the improvement of bioavailability by adding surfactant, adjusting salinity and sonication to obtain more efficient biodegradation in which larger amounts of TBT were removed in a shorter time.

4.2.1 Materials and equipments

The reagents and equipments were used similar to those noted in *Sections 2.2.1 & 3.2.1*. In addition, potassium chloride (KCl) was obtained from BDH (Steinheim, Germany). The additional equipments used for this study included pH meter (3520, Jenway), conductivity meter (PCM3, Jenway), oven (Gallenkamp), muffle furnace (Gallenkamp), incubator (Gallenkamp), ultrasonic bath (F5100b, Decon), centrifuge (ALC4218, Camlab), homogeniser (RZR 2051control, Heidolph) and total nitrogen (TN) analyser (Boat sample and Total nitrogen, Teledyne Tekmar). Compressed air (zero grade) and oxygen gas for TN analyser were obtained from BOC (Manchester, UK). Nitrate custom standard was obtained from Teledyne Tekmar.

4.2.2 Sediment sample and bacterial culture

The sediment sample was collected from Bowling Basin, Glasgow, UK (NS 450 735) in April 2009. Fifty kilograms of surface layer sediment was taken using a Van Veen grab sampler at 15 cm depth and was stored in sterile buckets. Sediment was kept at 4°C in the dark. To carry out the test, the sediment was passed through a 2 mm sieve and was homogenised using a stirrer at 1500 rpm for 30 min as shown in Figure 4.3. The sediment was tested for moisture content (MC) and TOC follows ASTM: D 2974-87 (ASTM, 2000), TN using TN analyser, pH and redox potential using a pH meter, and was analysed for butyltin compounds to assess existing contamination. The C to N ratio was adjusted to 10:1 by adding NH_4Cl into the sediment (Alexander, 1999). *Enterobacter cloacae* strain TISTR1971 was cultured in glycerol medium as previously described in *Section 2.2.3* to obtain approximately 10^8 CFU/ml for bioaugmentation.

Figure 4.3 Sediment sample preparation for TBT degradation study.

4.2.3 Optimisation of extraction and derivatisation conditions for butyltin compounds in sediment

The extraction and derivatisation steps of butyltin compounds in sediment were carried out as described previously by Bangkedphol et al. (2008) and Ceulemans et al. (1998), with slight modification. To each sample containing 10 g of sediment, 4 ml of DI water was added and pH was adjusted to between 1.6 and 2 with concentrated HCl. Extracting solvent, 10 ml hexane/ethyl acetate (1:1) containing 0.5% tropolone was added. The sample was then sonicated for 30 min in an ultrasonic bath at 40 kHz, with manually shaking every 10 min. Subsequently, the sample was centrifuged at 3,600 g for 5 min to separate sediment, water and organic solvent layers. A 5-ml aliquot of solvent was transferred and derivatised with 0.3% $NaBEt_4$ over 5 ml of acetate buffer, as previously described in *Section 2.2.5.1* for derivatisation of water sample extract. A cleanup step based on BS EN ISO 23161:2009 (British Standards, 2009) was incorporated for the sediment sample extract to reduce interference from sediment matrices, which would disturb the analysis and interfere with the GC-MS. The sediment extract was cleaned by passing through silica gel. The silica gel, bead size 63 to 200 µm, was pre-activated in a furnace at 440ºC for 24 h. Then, 0.3 g of silica gel was soaked in hexane and packed in a 1 ml size pipette tip. The derivatised sediment extract was passed through the column, followed by 10 ml of 50% acetone in hexane. In order to pre-concentrate the sample, an internal standard was added prior to evaporation under nitrogen gas to obtain 0.1 ml before analysis with GC-MS. Working solutions were prepared at 2, 4, 6, 8 and 10 mg/L of DBT and MBT, and 10, 20, 30, 40 and 50 mg/L of TBT with 25 mg/L of internal standard, TeBT.

The extraction procedures of sediment were optimised in triplicate to achieve optimum efficiency. First, the amount of derivatising agent was varied to find the appropriate amount to completely ethylate butyltin compounds. Sediment sample (10 g) spiked with mixed butyltin compounds at 100 µg/kg was studied. The sample was extracted, derivatised with 1, 5 and 10 ml of 3% $NaBEt_4$, followed by cleanup and

analysis. The results were compared regarding quantity of butyltin compounds extracted.

Secondly, the percentage of tropolone in the extracting solvent was increased to improve extraction of MBT and DBT. Following the pre-treatment method, spiked sediment sample was extracted with the solvent containing 0.5 and 1.0% tropolone and derivatised with 5 ml of 3% $NaBEt_4$.

Thirdly, replication of extraction was studied to improve the extraction of MBT and DBT. Following the pre-treatment method, the spiked sediment sample was single, double and triple extracted with 10 ml of the solvent containing 0.5% tropolone. For single extraction, 5 ml of extracting solvent was collected, while all organic layers were collected for double and triple extraction. The samples were derivatised with 5 ml of 3% $NaBEt_4$. To obtain the same pre-concentration factor, the final volume of the single extraction was 0.1 ml, while for double and triple extraction it was 0.2 ml.

Finally, without significant improvement from increasing amount of tropolone or extraction replication, the method was tested for the percentage recovery in sediment sample. The control sample of this experiment (sediment without spiking) was analysed in parallel with the spiked sample for background contamination of butyltin compounds. After subtracting the existing concentration, percent recovery was calculated based on the amount of butyltins added into the sediment.

4.2.4 Degradation of TBT in spiked sediment

The studied sediment was spiked with TBTCl at 100 µg/kg, by adding stock solution of TBTCl in methanol (>99%) into the sediment and mixed with homogeniser at 1500 rpm. To obtain a control sample for this experiment, 2 kg of spiked sediment was transferred into a container, the head space was filled with nitrogen gas and the sample was kept at ambient temperature in the dark. Three

enhanced samples were prepared by adjusting pH to 7.5, one of which was then biostimulated and one was bioaugmented. Biostimulation was carried out by nutrient addition with 1.48 g of sodium succinate, 0.46 ml of glycerol and 0.174 g of L-Arginine. For bioaugmentation inoculation, centrifuged cells of *E. cloacae* from 200 ml of the cell culture were added to the enhanced sample to obtain approximately 10^7 CFU per g of sediment. The samples were aerated and incubated at 28°C to start the degradation study. The sample set up is shown in Figures 4.4 and 4.5. Subsequently, the samples were homogenised at 1500 rpm and water was replenished before sediment was collected at 0, 3, 7, 14, 21 and 28 days. Determination of pH, redox potential, MC, TOC, TN and analysis of butyltin compounds were carried out in triplicate as described in *Sections 4.2.2 & 4.2.3*.

Figure 4.4 Bioreactors preparation for control (left) and stimulated sample (right).

Figure 4.5 Sediment samples set up for enhancement of TBT degradation.

4.2.5 Degradation of existing TBT contamination

Due to the reduced bioavailability with aged contamination, the potential to degrade existing TBT was tested in TBT non-spiked sediment. Two kilograms of sediment were transferred into two containers, one of which was used as a control sample and one as a stimulated sample. The control sample was flushed with nitrogen gas and left at ambient temperature in the dark, and the stimulated sample was adjusted to pH 7.5, aerated and incubated at 28°C. Water was replenished and sediment slurry was homogenised at 1500 rpm and withdrawn immediately from each sample after 0, 3, 7 and 14 days of incubation for determination of pH, redox potential, MC, TOC, TN and analysis of butyltin compounds in triplicate as described in *Sections 4.2.2 & 4.2.3*.

4.2.6 Improvement of bioavailability

Solubility and partitioning of TBT in the aqueous phase were improved by addition of surfactant, adjustment of salinity and sonication. The shake-flask method

used for the TBT desorption study by surfactant and salinity was slightly adapted from the method used by Bangkedphol et al. (2009).

Triplicate samples were prepared by transferring 100 ml of sediment slurry into a 250 ml conical flask, spiked with TBTCl at 100 µg/L, and filled with 100 ml of DI water. Non-ionic surfactant, Tween 80, was added to increase the solubility of TBT in water. Different amounts of Tween 80 were added at 0x, 0.1x, 1x, 10x and 100x critical micelle concentration (CMC). The CMC of Tween 80 is 15 mg/L which is equivalent to 11.45µM (Cheng and Wong, 2006). Therefore, 0, 1.41 µl/L, 14.10 µl/L, 0.14 ml/L and 1.41 ml/L of Tween 80 (density 1.064 g/ml) were added to obtain the samples at 0x, 0.1x, 1x, 10x and 100x CMC, respectively. The samples were shaken on an orbital shaker at 150 rpm, at 28ºC for 24 h. The samples were allowed to settle and 20 ml of supernatant was analysed for TBT. In addition, a control sample prepared by spiking 10 ml of sediment with TBTCl at 100 µg/L was analysed for TBT concentration.

The salinity of the system was adjusted to increase partitioning of TBT from sediment to water. The triplicate samples were prepared and appropriate amounts of KCl were added to obtain 0, 5, 10, 20, 25 and 30 psu, representing a range from freshwater to estuarine water. Salinity is a measure of the salt content of water, measured in parts per thousand (PPT) or more precisely, when calculated based on conductivity, Practical Salinity Units (PSU). The average salinity of different types of water was 0.5 psu for freshwater, 0.5-17 psu for brackish or estuarine water, and 32-37 psu for marine (APHA, 1999). The salinity was determined based on electrical conductivity measurement at 20ºC (APHA, 1999). The samples were shaken on an orbital shaker at 150 rpm, at 28ºC for 24 h and supernatants were analysed for TBT as was the TBT concentration in the control sample.

Sonication was performed to increase partitioning of TBT into water. The sediment samples were prepared in triplicate and sonicated in an ultrasonic bath (40 kHz) for 0, 5, 10, 20 or 30 min. Supernatants were withdrawn for TBT analysis without further shaking. The control amount of TBT was also determined.

4.2.7 Degradation of TBT with bioavailability improvement

Degradation of TBT in sediment spiked at 100 µg/kg was repeated, but with adjustment of sediment salinity to 20 psu. Sediment from the control and the three stimulated samples were withdrawn after 0, 3, 7 and 14 days of incubation for determination of pH, redox potential, MC, TOC, TN and analysis of butyltin compounds in triplicate as described in *Sections 4.2.2 & 4.2.3*.

After the first enhanced response, when no further increase in degradation occurs, re-enhancement was stimulated by repeating the process. For the enhanced sample, the pH was adjusted to 7.5 to provide favourable conditions. For the enhanced sample with nutrient supplement, addition of the nutrients was repeated. Finally, the augmented sample was re-inoculated with *E. cloacae*. The samples were incubated under the same conditions and samples were withdrawn at 21 and 28 days of incubation for determination.

4.2.8 Analysis of butyltin compounds

The analysis of butyltin compounds in sediment and water was performed as described after optimisation previously in *Sections 2.2.5 & 4.2.3*. Briefly, to each 10 g of sediment sample, 4 ml of DI water was added and pH was adjusted to between 1.6 and 2 with concentrated HCl. Extracting solvent, 10 ml hexane/ethyl acetate (1:1) containing 0.5% tropolone, was added. The samples were sonicated for 30 min in an ultrasonic bath (40 kHz). Subsequently, the samples were centrifuged at 3,600 g for 5 min and a 5-ml organic layer was collected. For the water samples, 20 ml of supernatants were adjusted to pH 2 and extracted three times with the solvent containing 0.05% tropolone on an orbital shaker for 30 min. The organic layers from triplicate extraction were collected and combined.

The extractant was derivatised in 5 ml of 0.3% $NaBEt_4$ prepared in acetate buffer pH 5, by manually shaking for 5 min. The extract was then cleaned by passing it through a silica gel column. In order to pre-concentrate the sample, an internal

standard was added prior to evaporate under nitrogen gas to obtain 0.1 ml, and analysed with GC-MS.

4.3 Results and discussion

4.3.1 Optimisation of extraction and derivatisation conditions for butyltin compounds in sediment

From the pre-treatment method, the amount of derivatising agent required was investigated, as shown in Figure 4.6. For the water sample, 1 ml of 0.3% $NaBEt_4$ allowed complete ethylation of butyltins. In the sediment sample, more derivatising agent was required, since matrices in sediment can obstruct the reaction or compete with butyltin compounds for the reaction. Therefore, 5 ml of the agent was utilised for further studies in sediment samples.

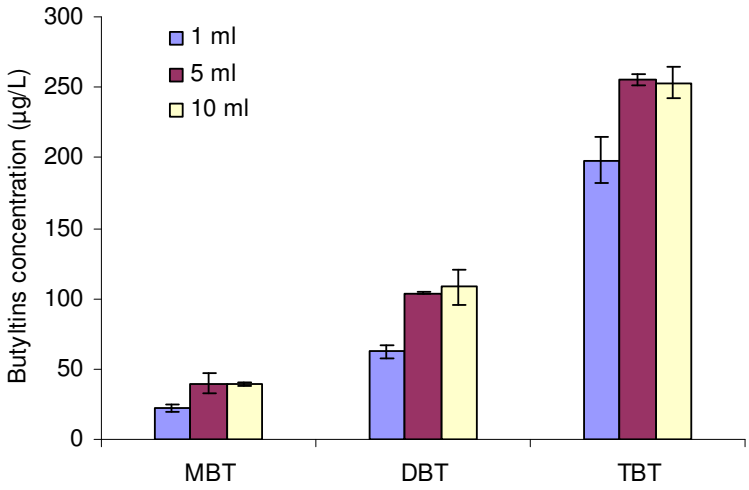


Figure 4.6 Concentration of butyltin compounds in spiked sediment sample, ethylated with different volume of derivatising agent (n=3, SD error bar).

Since the extraction of MBT and DBT was inefficient, tropolone, which can enhance extraction of MBT and DBT by complex formation with the substances, was increased. As shown in Figure 4.7, the increase in tropolone in the extracting solvent showed no significant improvement. This implies that tropolone at 0.5% was

sufficient to reach maximum extraction efficiency. This amount was already ten times higher than that used for water samples.

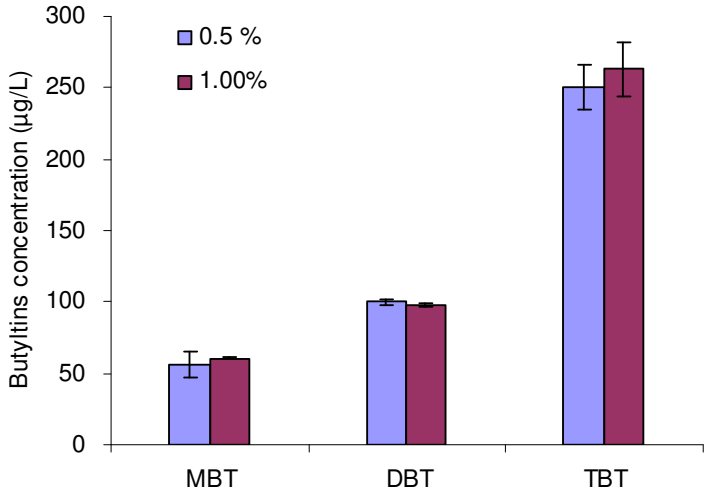


Figure 4.7 Concentration of butyltin compounds in spiked sediment sample, extracted with the extracting solvent containing different amounts of tropolone (n=3, SD error bar).

The replication of extraction was studied as it was expected that the extracted amount would increase with increased extractions. Figure 4.8 shows that extraction of DBT was slightly increased, however, no significant improvement was found for MBT and TBT. As a result, replication of extraction was unnecessary.

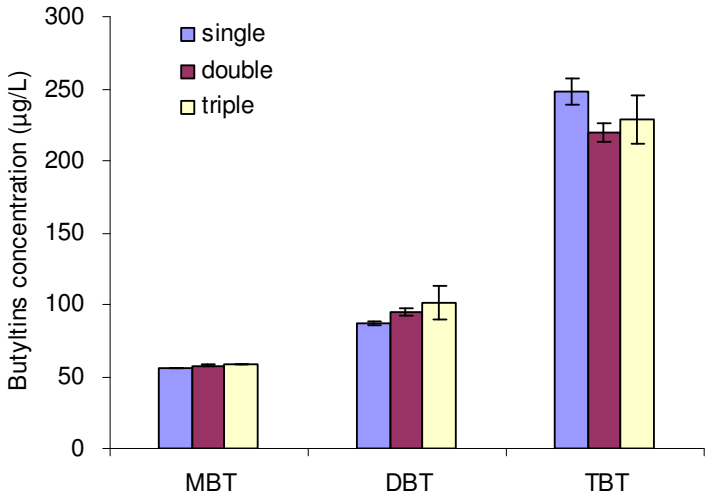


Figure 4.8 Concentration of butyltin compounds in spiked sediment sample, extracted with different replication (n=3, SD error bar).

Since there was no further improvement for extraction of MBT and DBT, the method was used without any modification. As illustrated in Figure 4.9, the sediment sample was contaminated with TBT and DBT at 0.07 and 0.14 mg/kg, respectively. After subtraction of background contamination, the % recovery of spiked butyltin compounds was found to be 46, 49 and 102% for MBT, DBT and TBT, respectively.

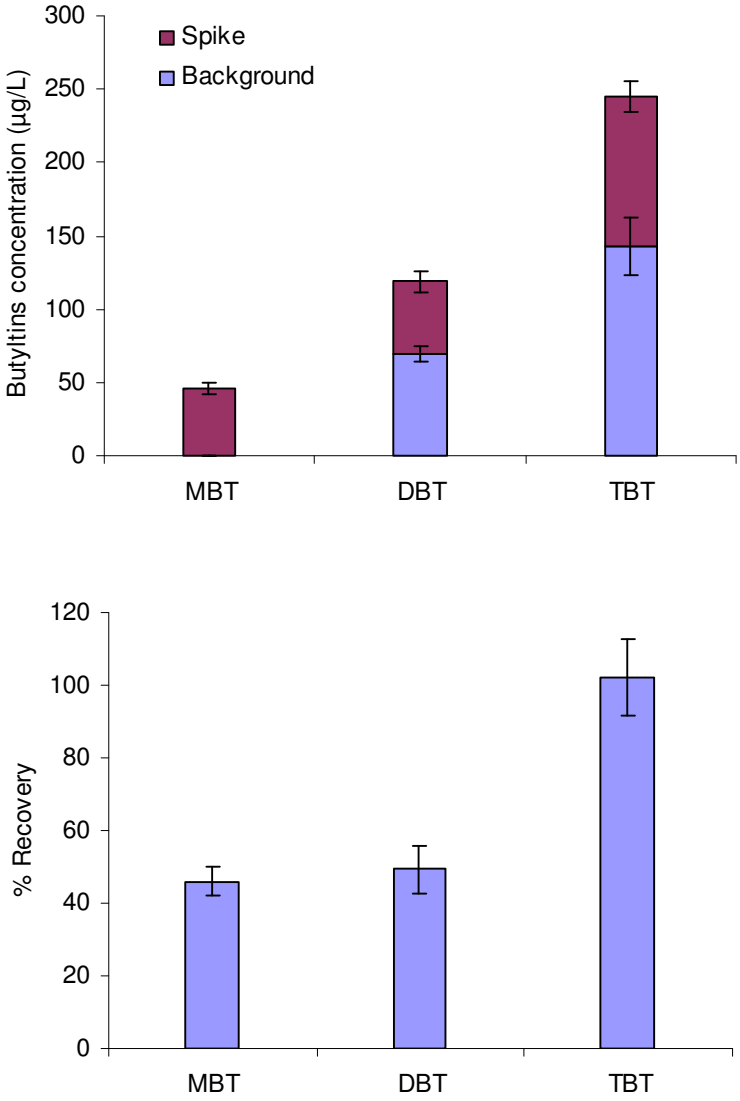


Figure 4.9 Concentration of background and spike butyltin compounds in sediment sample, and % recovery of spike butyltin compounds (n=3, SD error bar).

4.3.2 Degradation of spiked TBT in sediment

Since aeration caused dewatering of the sediment during the current study, water replenishment was required before the samples were withdrawn. Abiotic removal was insignificant because TBT is poorly volatile and well adsorbed onto the sediment surfaces (Voulvoulis and Lester, 2006). Additionally, Vouvoulis and Lester (2006) demonstrated that TBT is resistant to high temperatures (≤105°C). As a result, the reduction of TBT and the increase of its metabolites result from biotic processes.

The sampled sediment was found to be already contaminated with butyltin compounds. However, 100-µg/kg TBTCl was additionally spiked into the sediment as it assures bioavailability of TBT for microorganisms. The reduction of TBT shown in Figure 4.10 indicates the ability of the sediment microorganisms to degrade TBT. The control sample without stimulation represented natural attenuation under anaerobic conditions. Insignificant reduction of TBT was observed for the control sample which confirms long-term accumulation of TBT in the sediment. Application of biostimulation and bioaugmentation considerably promoted the degradation indicated by a decrease in TBT and an increase in DBT. However, the adjustment of environmental conditions was the most important factor in accelerating the degradation (Figure 4.10b). In addition, nutrient amendment encouraged degradation activity of the microorganisms (Figure 4.10c) and inoculation increased the number of potential microorganisms in the sediment (Figure 4.10d), and resulted in additional stimulation. The enhancement took place in the first week before the degradation slowed down again.

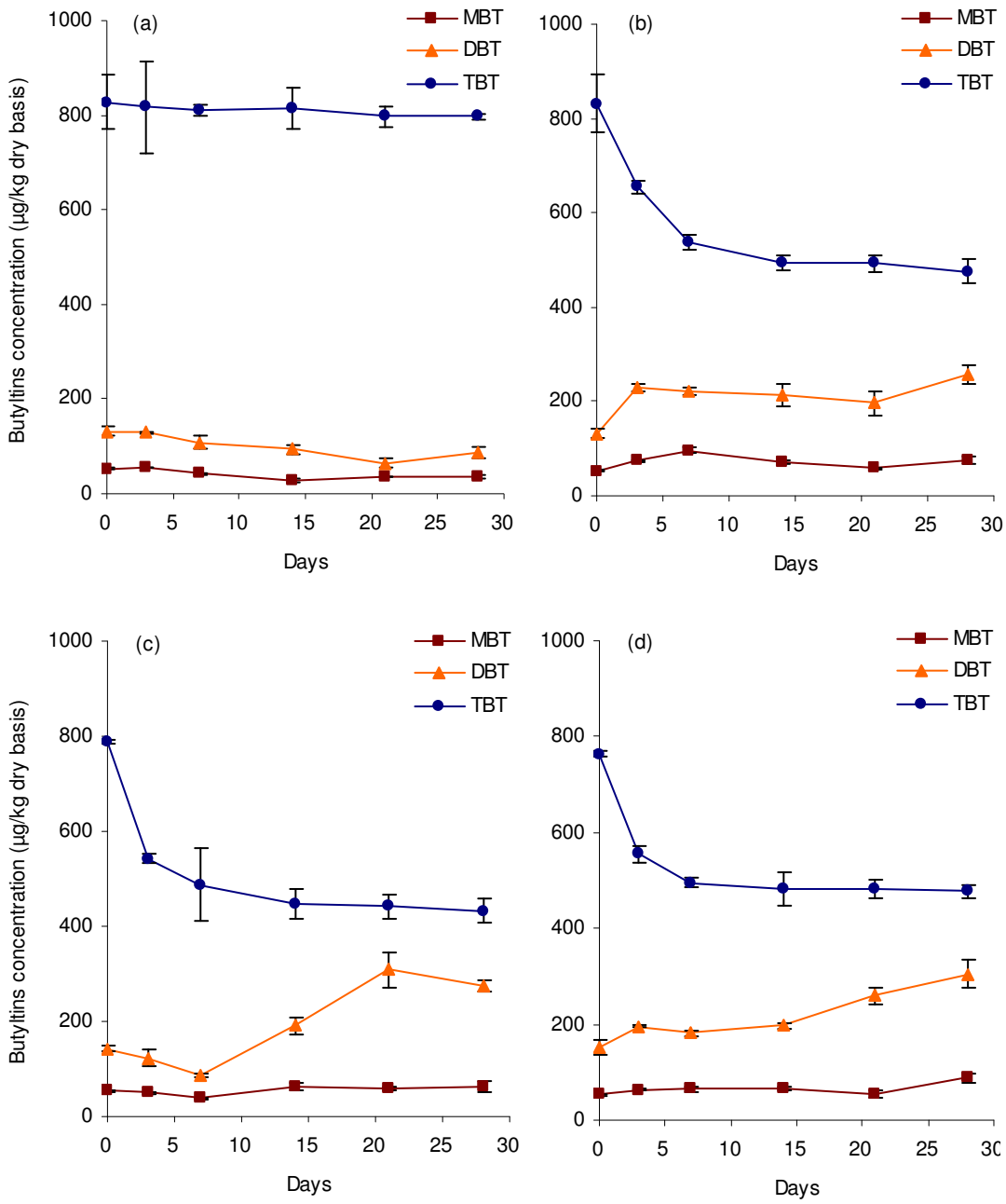


Figure 4.10 Degradation of TBT and formation of DBT and MBT in sediment during 28 days of incubation (a) control, C; (b) stimulation, S; (c) stimulation and nutrient addition, SN; (d) stimulation and inoculation of TBT-degrader, SI (n=3, SD error bar).

From Figure 4.11, comparison of % TBT degradation indicates that treatment of TBT contaminated sediment can be achieved solely by providing appropriate surrounding conditions. Nutrients can be supplemented for further stimulation;

however, TBT final concentrations were barely different after 7 days. Consequently, the addition of nutrients is not required with sufficient degradation time. Inoculation of the TBT-degrader also encouraged the degradation in the first 3 days, but the enhancement significantly reduced after this time. Therefore, inoculation was unnecessary and should be used only when the sediment community has no capability to degrade TBT. Additionally, the degradation enhancement was ineffective after one week. This result was in agreement with Delgado-Moreno and Peña (2009), and Sarkar et al. (2005), who studied biostimulation of organic pollutants by addition of organic and inorganic materials, respectively. In both cases, the addition initially enhanced degradation, but degradation slowed down and resulted in a similar rate to the control. It has also been observed that under appropriate conditions and capable indigenous microorganisms, the addition of inorganic material, co-substrates or surfactant might not appreciably enhance degradation (Lei et al., 2005).

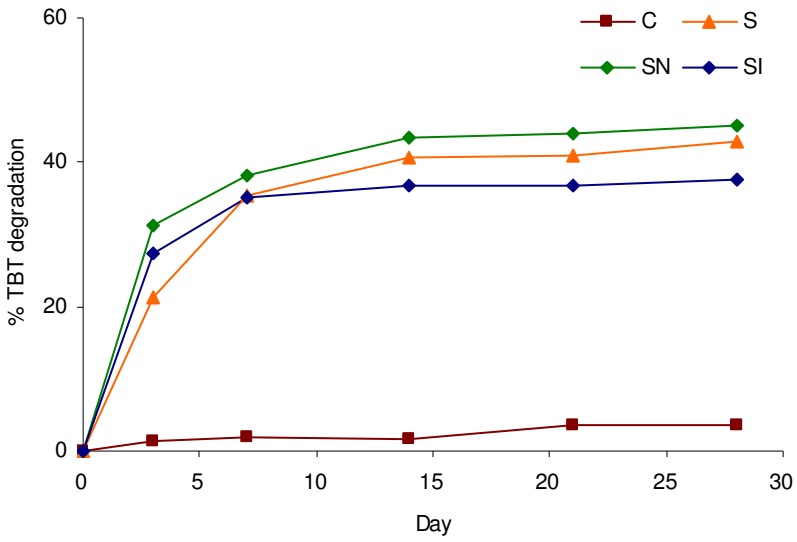


Figure 4.11 % TBT degradation of control (C); stimulation (S); stimulation and nutrient addition (SN); stimulation and inoculation of TBT-degrader (SI) during 28 days of incubation.

Rapid degradation of TBT was observed after the conditions were adjusted. Since the sediment was already contaminated with TBT on sampling, the intrinsic microorganisms were adapted and acclimatised before the experiment was

performed. It has been reported that acclimatisation increased the degradation rate 7.3 times when compared to non-acclimatised microbes (Stasinakis et al., 2005). The degradation rate of butyltin compounds in sediment is typically DBT>TBT>MBT, however, the order of degradation is altered to TBT>DBT>MBT after the degraders are acclimatised (Dowson et al., 1996, Voulvoulis and Lester, 2006). Since the sediment was previously contaminated, the result shows that TBT was degraded to DBT, and DBT had accumulated in the sediment. Reduction of DBT during the study period was observed only in the SN sample; although no obvious degradation of DBT and MBT was observed. This was because the rates of DBT and MBT degradation were slower than that of TBT.

Degradation rate constants were evaluated following first order kinetics as indicated in Figure 4.12. The degradation rate was considered as biphasic since stimulation accelerated the degradation in the first week, however after the first week the degradation rate dropped suddenly. Therefore the rate of stimulation was calculated separately rather than over the entire incubation period.

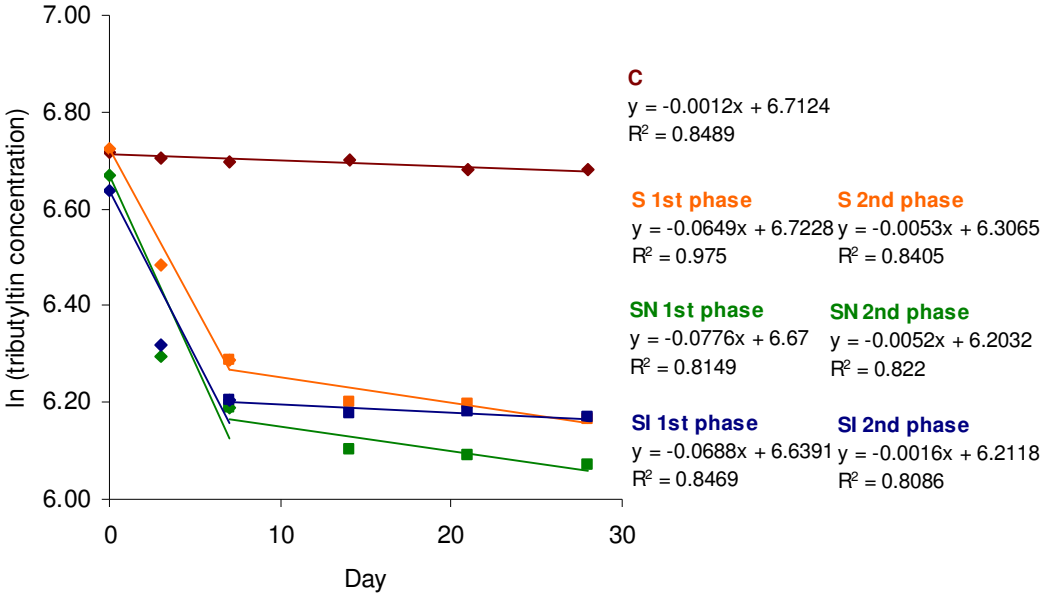


Figure 4.12 Biphasic linearisation of the degradation data of control (C); stimulation (S); stimulation and nutrient addition (SN); stimulation and inoculation of TBT-degrader (SI).

Degradation of hydrocarbons has previously been found to fit first-order; however, Sarkar et al. (2005) dissimilarly described hydrocarbons degradation as second-order, where the linear relationship obtained had a better correlation coefficient. For TBT, even though degradation in liquid media follows first-order kinetics (*Chapters 2 & 3*), first-order was not appropriate for sediment used in this study. This is because TBT in water is entirely available to bacteria and can be uptaken by bacterial cells quickly (Tsang et al., 1999), which does not apply in sediment. The fast and slow phases were previously described as rapid TBT degradation followed by slower rates for the remaining TBT (Dowson et al., 1996). As such, the biphasic degradation was applied to demonstrate the rate constants during enhancement as shown in Figure 4.12.

The low degradation rate of TBT in the control sample (0.0012 day^{-1}) was improved after enhancement during the first week (S = 0.0649 day^{-1}; SN = 0.0776 day^{-1}; Si = 0.0688 day^{-1}). Although further acceleration was insignificant after the first week, degradation rates were still higher than the control when the environmental conditions were continually controlled (S = 0.0053 day^{-1}; SN = 0.0052 day^{-1}; SI = 0.0016 day^{-1}). The rate constant of the SI sample at the second phase was the smallest when compared to the other stimulated samples. This might be due to the higher initial density of the bacterial cells which utilised more nutrients during the first week. After the decline of stimulation, lower nutrient supply remained in the sediment, resulting in lower activity of bacteria in the SI sample compared to those in the S and SN samples.

From Figure 4.13, it can be seen that the redox potential of the samples increased during the incubation period, while the pH of the samples became acidic. In the control sample, a steady redox potential and pH were observed. The changing redox potential was caused by aeration, which increases the oxidising agent in the system, and the change in pH was a consequence of bacterial metabolism. The C to N ratio was slightly varied but still close to 10:1.

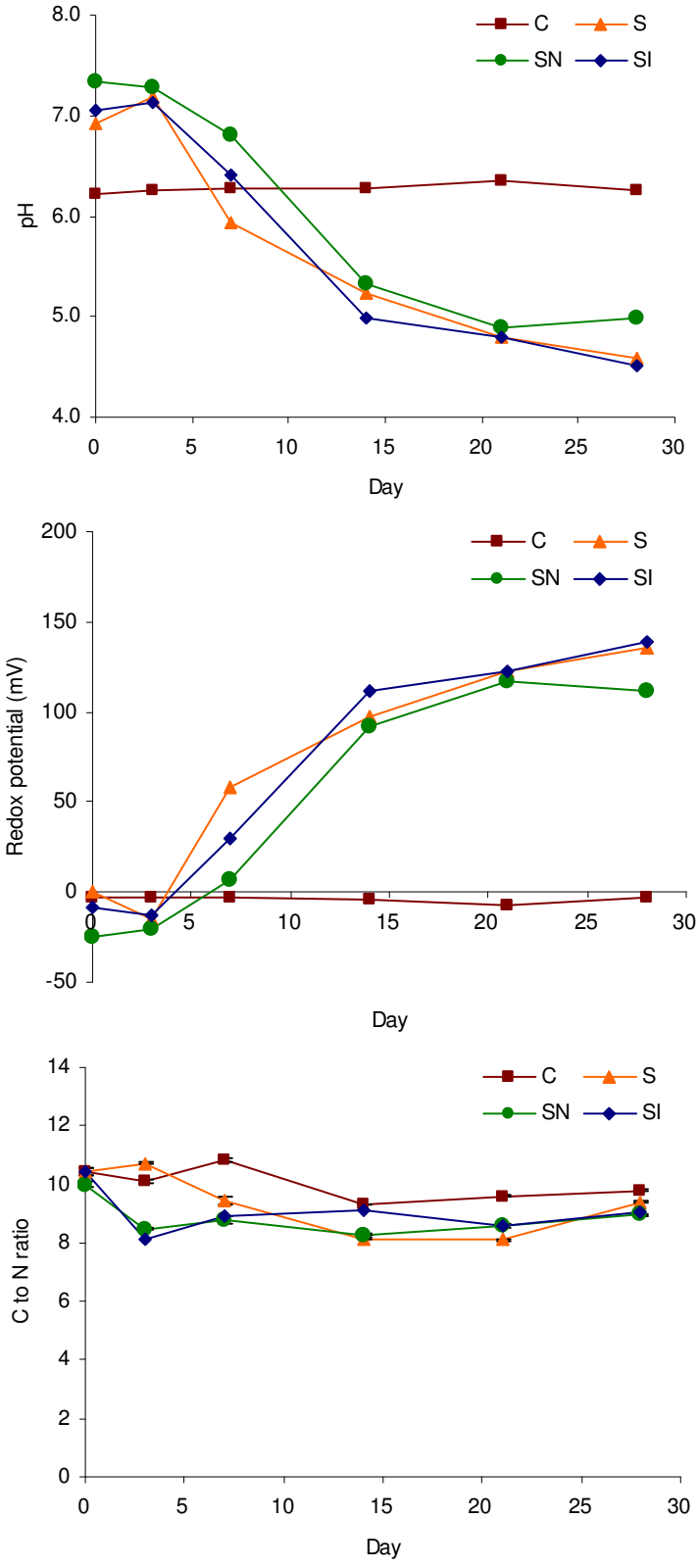


Figure 4.13 Sediment pH, redox potential and C to N ratio during the incubation period of control (C); stimulation (S); stimulation and nutrient addition (SN); stimulation and inoculation of TBT-degrader (SI).

The pH of the sediment reduced during incubation. This negatively affected degradation because lower pH decreases bioavailability by reducing the octanol-water coefficient (K_{ow}) as previously described in *Section 3.3.2*. Therefore, pH is one of the important parameters which can limit biodegradation. The reduction of pH, however commonly occurs during bacterial metabolism regardless of specific biodegradation (Atagana et al., 2003).

During incubation, the redox potential continually increased. A high and positive redox potential is present in a system that tends to acquire electrons and leads to oxidation in the system, whereas a negative and low redox potential leads to a reducing environment. The increase in redox potential in the sediment was a result of continuous aeration which added molecular oxygen, an oxidising agent, into the system. Since degradation of TBT occurs through stepwise oxidation by microorganisms (Lee et al., 1989), degradation of TBT is expected to be better at a high, positive redox potential. This assumption is supported by Rodríguez et al. (2010) who monitored contamination of butyltin in coastal sediment in Northern Spain. The degradation was observed by determining DBT and MBT in the sediment, where redox potential was determined in parallel to assess their relationship (Rodríguez et al., 2010). At the sampling locations with high redox potential, percent-butyltin degradation was higher than at those with low redox potential. Similarly, the increase in redox potential in the samples during incubation encouraged the degradation.

In many cases, nitrogen in sediment is limited rather than the carbon source. In this case, addition of inorganic materials to provide nitrogen can stimulate the growth of microorganisms and, moreover, biodegradation. Nitrogen can be added to achieve the C to N ratio of 10:1 as this is the optimum ratio generally used in bioremediation (Alexander, 1999, Atagana et al., 2003, Xia et al., 2006). The C to N ratio was slightly altered during incubation. Even though the forms of carbon and nitrogen can be changed through bacterial metabolism to less available forms, it was believed to cause only a small effect on the degradation and not to be the main parameter for the discontinuation of stimulation. Atagana et al. (2003) demonstrated

that the presence of nitrogen always enhanced biodegradation, but stimulation effectiveness was relatively poor at excess amounts of nitrogen.

4.3.3 Degradation of existing TBT contamination

Since TBT was spiked to approximately double the amount of TBT originally in the sample, and without ^{14}C isotope of TBT to confirm the degraded partition, the 40 % degradation obtained in the first experiment might come solely from TBT introduced through the spike, as it was more available for microorganisms than aged TBT in the sediment.

As indicated in Figure 4.14, existing TBT was biodegradable and stimulation promoted the degradation, however this was not as efficient as the degradation in the spiked sediment because of lower bioavailability. Degradation rates constant in the stimulation period (first week) were calculated. The *k* values were 0.0011 day^{-1} (R^2: 0.9971) and 0.0501 day^{-1} (R^2: 0.9399) for control and stimulated sample, respectively. Although degradation of aged TBT was evident, the rate constant was smaller than that of the spiked TBT. This confirms poorer bioavailability of aged TBT than spiked TBT. In addition, the same trends in redox potential, pH and C to N ratio were found.

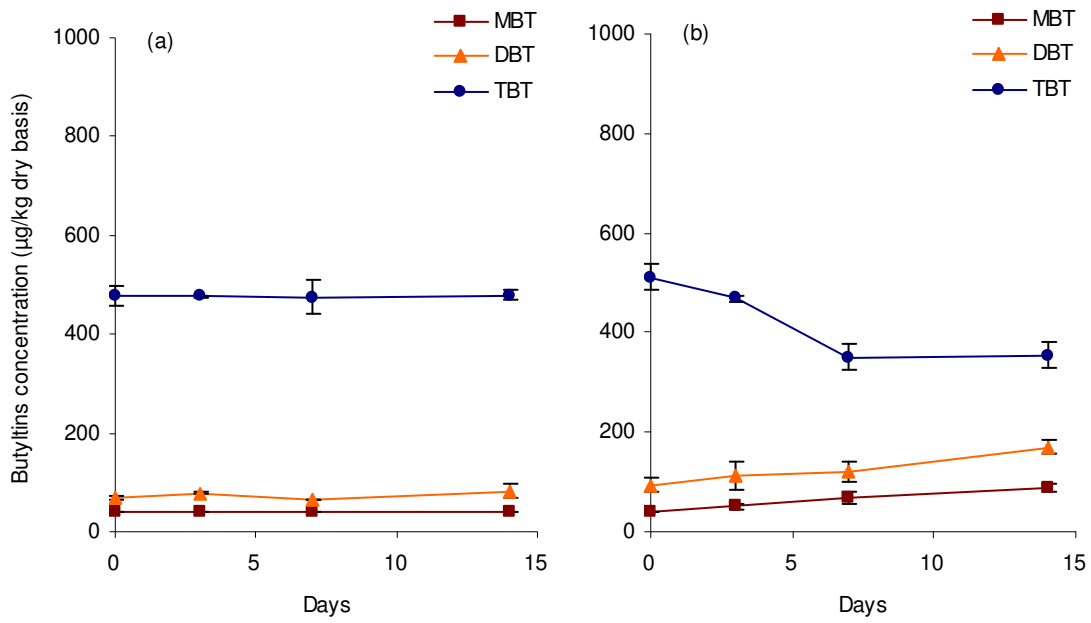


Figure 4.14 Degradation of existing TBT and formation of DBT and MBT in sediment during 14 days of incubation (a) control; (b) stimulation (n=3, SD error bar).

The quality criteria for dredging material in the UK are 100 µg/kg dry weight (action level 1) and 1,000 µg/kg dry weight (action level 2) of the mixture of TBT, DBT and MBT (OSPAR Commission, 2008). The existing butyltin contamination was below the action level 2 meaning the sediment can be dredged and disposed without further TBT removal. However, it can still pose risk to the environment because the concentration is over the action level 1. Enhancement of degradation reduced the amount of TBT which then reduced the toxicity. Even though the concentration of TBT was reduced, only a small reduction in the total concentration of butyltins mixture was occurred, as the final product of TBT is often not inorganic tin because MBT has slow degradation rate. To comply with the UK's regulation, extension of degradation time is required for dredged sediment with contamination over action level 2 to allow complete degradation of TBT to inorganic tin.

Aged contamination significantly reduces bioavailability of TBT as it can be adsorbed by organic (humus and organic substances) and inorganic (clay minerals)

components of sediment. Particularly, clay minerals which contain d-spacing such as montmorillonite (0.96-2.14 nm), illite (1.0 nm) and kaolinite (0.7 nm), allowing TBT to enter (Bangkedphol, 2010), but are too small for bacterial cells (≥0.2 μm) to access. Since aged contamination extends the time available for TBT to be absorbed by the clay minerals, a higher amount of TBT is unavailable for biodegradation. This is not always the case when TBT binds with organic matter via Van der Waals (TBTOH) and electrostatic forces (TBT^+) on the sediment surface since it can still be approached by degraders and can be degraded. Consequently this kind of adsorption is reversible. However, extended periods of contamination probably increase the binding strength between TBT and the matrix, both organic and inorganic, which then reduces extractability and decay rate (Huang and Matzner, 2004). This suggests that delayed treatment of the contamination will reduce efficiency of the treatment process. The effect of aged contamination has previously been examined in PAHs degradation where spike PAHs were well degraded, but all initial contamination still remained (Singleton et al., 2008). Despite this, the present experimental results proved the degradability of aged TBT.

4.3.4 Improvement of bioavailability

All the techniques were applied to improve percentage solubilisation of TBT in the aqueous phase which increased the bioavailability of TBT for biodegradation as shown in Figure 4.15. The solubilisation of TBT was 13.22 % without any improvement. Solubilisation was increased to the maximum at 25.92 % by addition of Tween 80 at 1x CMC. Solubilisation was reduced from this to 17.59 % by 100x CMC. This suggests the addition of Tween 80 at concentrations higher than CMC is disadvantageous. However, the presence of Tween 80 up to 100x CMC still enhanced the solubility compared to initial solubility.

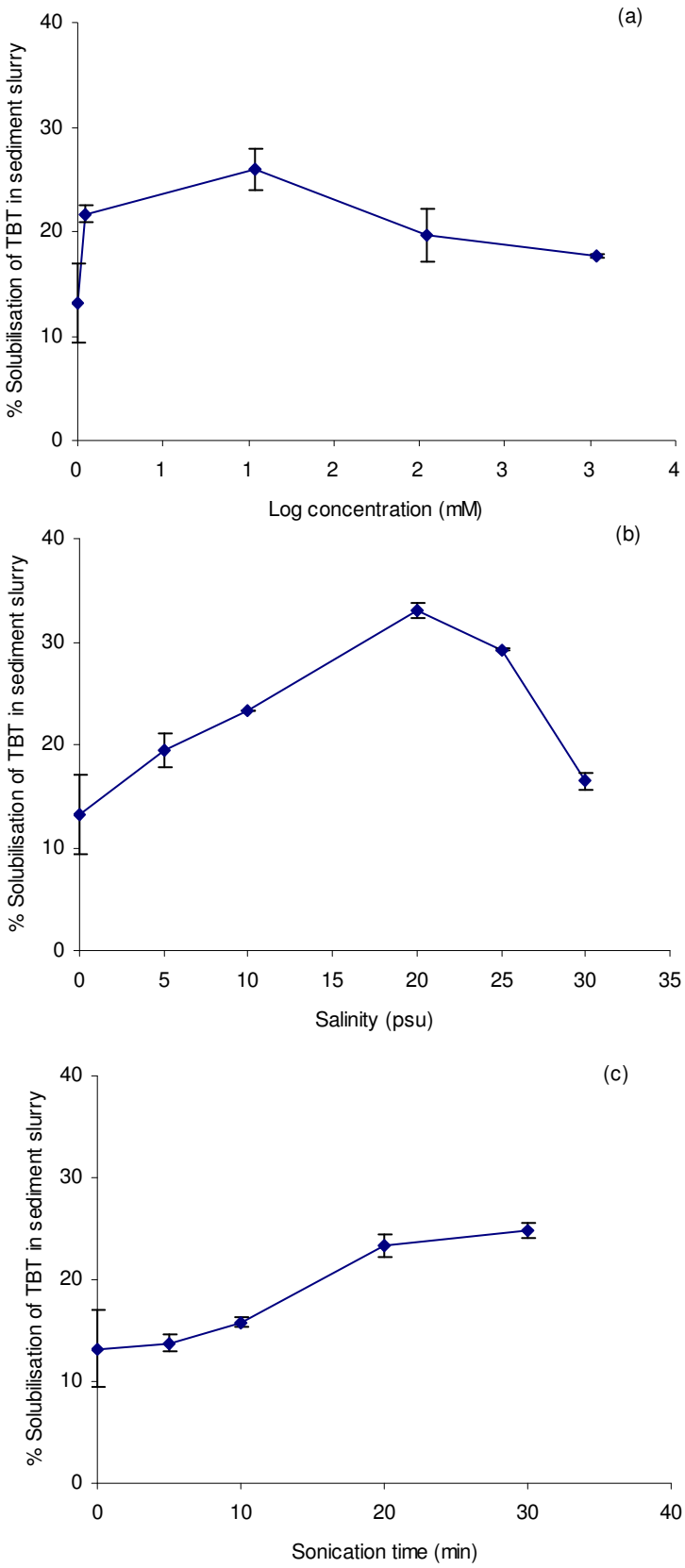


Figure 4.15 Improvement of TBT solubilisation in sediment slurry by (a) addition of surfactant; (b) adjustment of salinity; (c) sonication (n=3, SD error bar).

As represented in Figure 4.15, the salinity of the sediment was originally less than 2 psu. Adjustment of salinity to 20 psu continually increased the solubilisation from 13.22 to 32.94 %. Salinity higher than this, however, resulted in reduction of solubility to 19.24 and 16.46 % at 20 and 30 psu, respectively. With regard to sonication, more partitioning of TBT into water occurred when longer sonication times were applied. The solubilisation increased from 13.22 to 26.34 % when sonicated up to 30 min. Comparison among the techniques found that the highest solubilisation was achieved by adjusting salinity of the sediment to 20 psu.

Although aged contamination was degradable, bioavailability was still an issue. Therefore, improvement of TBT bioavailability was carried out. Surfactant has the ability to reduce the surface tension of water and allow hydrophobic compounds to dissolve. Addition of surfactant was expected to increase bioavailability and facilitate the uptake of contaminants into microbial cells along with micelle (Jayashree and Vasudevan, 2007). Tween 80, a non-toxic surfactant to soil and inert to soil matrix, was selected which has previously been used to enhance desorption and biodegradation for example in the degradation of chysene (Hadibarata and Tachibana, 2009), desorption of PAHs (Cheng and Wong, 2006), degradation of endosulfan (Jayashree and Vasudevan, 2007) and degradation of DDT (Karagunduz et al., 2007). TBT is an organometallic compound, which exhibits both hydrophobic and metal ions properties. Therefore, the interaction of both types of properties must be taken into account. In an environment with sediment and water, organic compounds are either adsorbed on sediment or dissolved in water. Surfactant added at a concentration lower than CMC can be adsorbed on sediment, consequently releasing organic compounds into water. Also, surfactants that stay in water can form hydrophobic interactions with organic compounds and increase the solubility of the compounds due to the amphiphile property of the surfactant. If the concentrations of surfactant are higher than CMC, micelles are formed and organic compounds will interact with the hydrophobic end in the nucleus of micelles (Karagunduz et al., 2007). With regard to metals, surfactant added below or above CMC can be adsorbed on sediment, but at different sorption sites, which possibly physically remove metals from sediment (Singh and Turner, 2009). There is no interaction between metals and

micelles except complexation. However, complexation of metals can only occur with anionic surfactants.

TBT can interact with surfactant as an organic compound and can also form complexes with surfactant as a metal (Louppis et al., 2010). Unfortunately, TBT was very well adsorbed on the sediment and micelles might not have been able to interact with the adsorbed TBT. Also no complexation took place because Tween 80 is a non-ionic surfactant. Therefore, the solubility of TBT was enhanced only by the replacement of the surfactant onto the sediment. On the other hand, micelles could increase the solubility of other organic compounds which then lessen water solubility and lead to resorption of TBT. Hence, % solubilisation at 10x and 100x was lower than those of 0.1x and 1x CMC.

Salinity initially increased the solubility. However, at salinity higher than 20 psu, solubility dropped (Figure 4.15b). This can be explained by two effects since TBT acts as both metal and organic. In freshwater, TBT acted as a metal ion and had an electrostatic interaction with the negative charge of humus in sediment. The solubility was then increased at higher salinity because K^+ from KCl competed with TBT to interact with sediment. This resulted in less available negative charged particulate matter releasing more TBT into the aqueous phase (Unger, 1988). At salinity higher than 20 psu, the hydrophobic character of TBT was more significant where lower solubility was typically caused by the salting out effect (Harris and Cleary, 1987).

Sonication is the use of ultrasound to agitate particles in a sample. Similar to the extraction procedures, sonication was used to increase desorption of TBT from sediment. However, desorption was low when compared to sonication in the presence of extracting solvent. This is because TBT in water was continuously transferred into the organic phase and allowed more TBT to partition into water. Without organic solvent, the dissolution of TBT was limited. The sonication time can be extended to increase % solubilisation, but it would harm microorganisms and reduce cell numbers (Joyce et al., 2003), which would affect degradation capability.

Additionally, extending sonication time can reduce solubility as reported in surfactant and salinity studies. The resorption of previously desorbed content is a result of the formation of smaller sediment particles which provide a larger surface area (Pumure et al., 2010). Therefore, amongst the studied techniques, salinity at 20 psu was the most effective in increasing bioavailability. Since estuary conditions aid TBT bioavailability, this suggests that *in-situ* remediation of contaminated estuarine sediment is expected to be more efficient than that in freshwater sediment.

4.3.5 Degradation of TBT with bioavailability improvement

It is clearly presented in Figure 4.16 that degradation enhancement was further promoted by adjusting salinity of the sediment to 20 psu. The degradation capability was in the order of SI>SN>S>C, as shown in Figure 4.16. Higher bioavailability did not encourage TBT degradation in the control sample. The degradation capability after bioavailability improvement indicates that stimulating conditions were the main factors in enhancing TBT degradation rate, as shown in the S sample. Nutrient addition and augmentation slightly increased degradation capability, where the efficiency of the SI sample with inoculation of the TBT-degrader became higher than that of the SN sample with nutrient addition after the improvement of bioavailability.

After 14 days, the accelerated degradation in the stimulated samples dropped, following which re-enhancement was again applied in the enhanced samples to indicate the associated factors. pH in sample S was adjusted to 7.5, nutrients were re-supplemented in sample SN, and sample SI was re-inoculated with *E. cloacae* cells. Re-enhancement did not show a further stimulation, except in the SI sample (Figure 4.17). This implies that the pH of the sample did not affect bacterial activity. Inoculation of the degrader failed to increase TBT degradation suggesting that microorganisms still thrived, but their TBT-degrading activity was decreased. Although a small improvement was observed after addition of the nutrients, it was insignificant and the degradation slowed down again after the first week of re-enhancement.

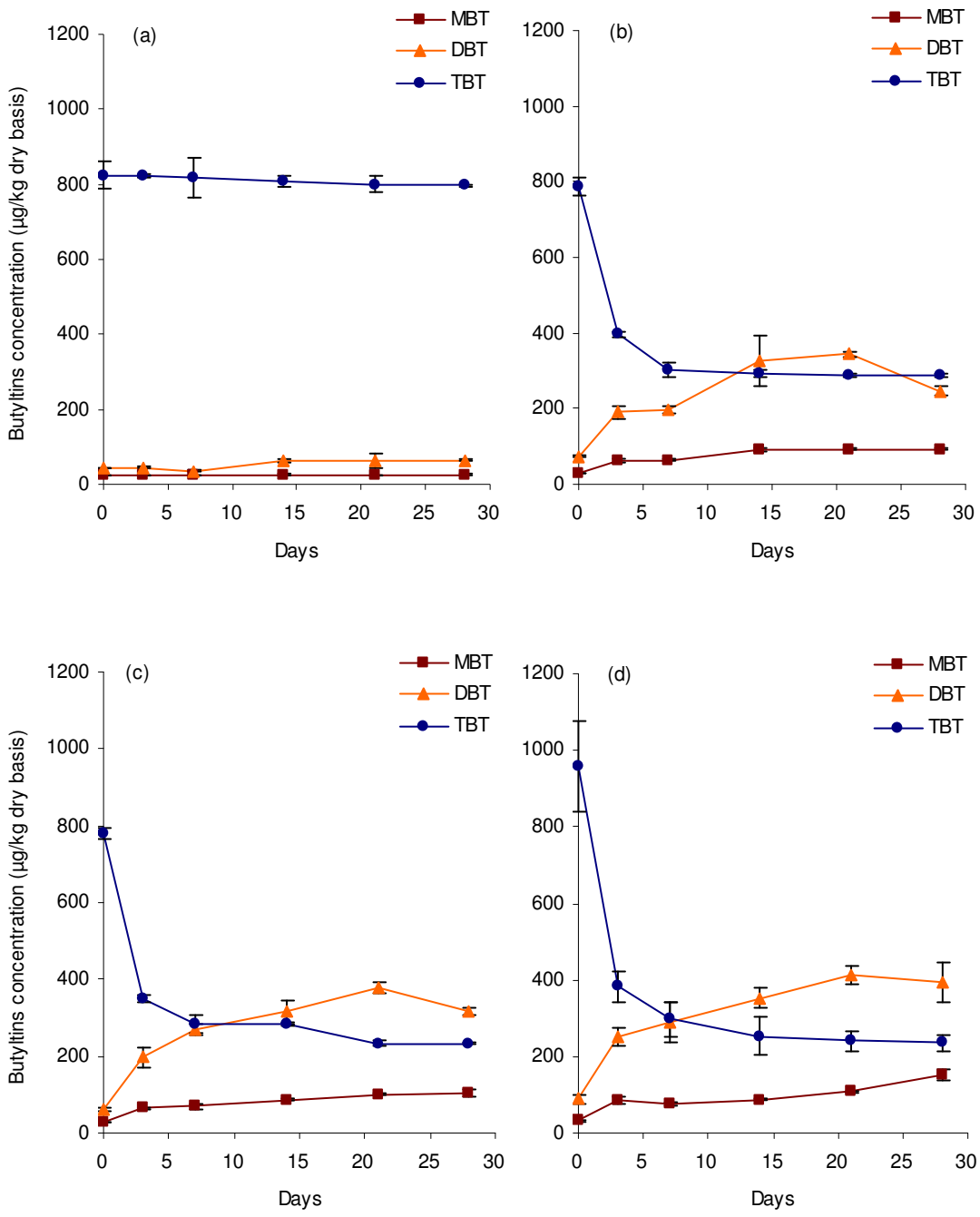


Figure 4.16 Degradation of TBT and formation of DBT and MBT in bioavailability-improved sediment with salinity at 20 psu during 28 days of incubation (a) control, C; (b) stimulation, S; (c) stimulation and nutrient addition, SN; (d) stimulation and inoculation of TBT-degrader, SI (n=3, SD error bar).

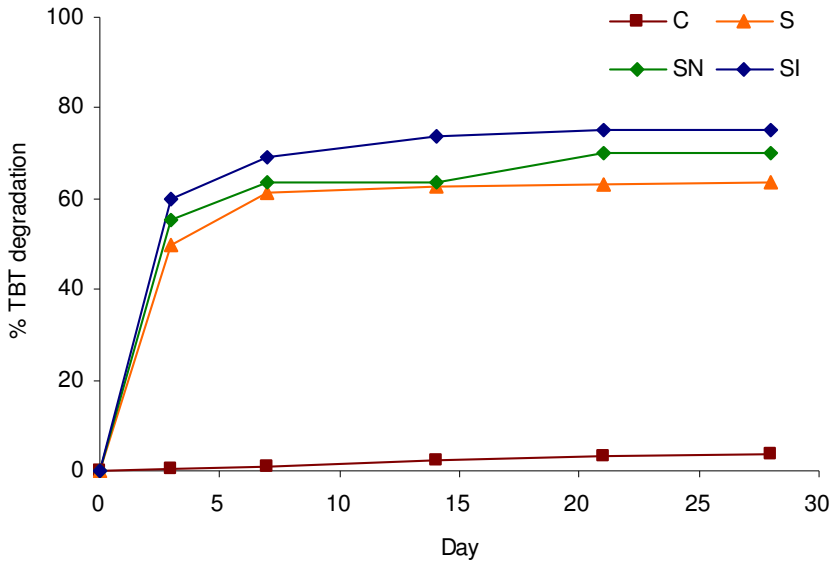


Figure 4.17 % TBT degradation of bioavailability improved sediment of control (C); stimulation (S); stimulation and nutrient addition (SN); stimulation and inoculation of TBT-degrader (SI) during 28 days of incubation.

Degradation rate constants in the stimulation period (first week) were evaluated following first order kinetics (Figure 4.18). The *k* values were 0.0013, 0.1508, 0.1637 and 0.1887 day^{-1} for C, S, SN and SI, respectively. The rate constants of the enhanced samples were approximately double when bioavailability was improved. Also the final concentrations of TBT were noticeably lower after the improvement. Without suitable conditions, the rate constants of the control with or without salinity adjustment were similar.

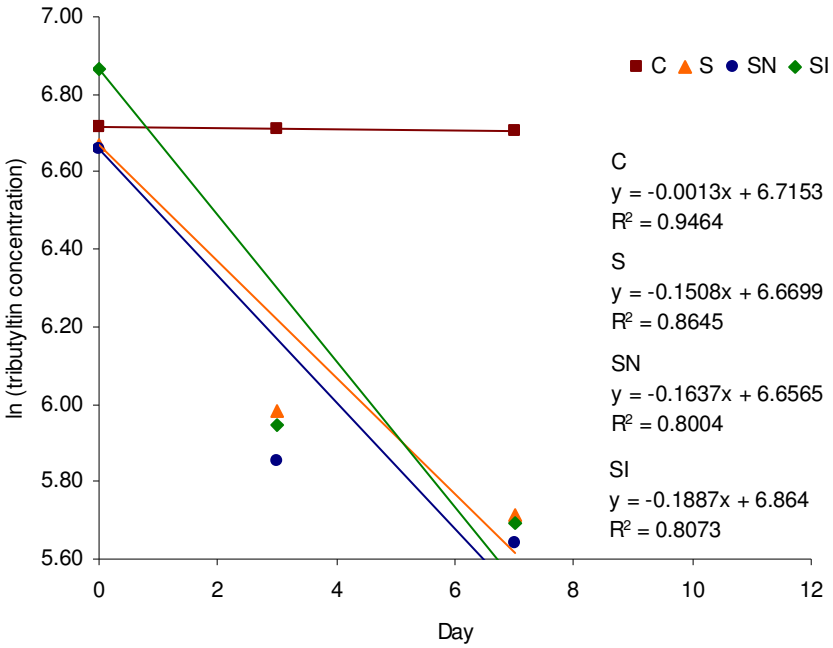


Figure 4.18 Linearisation of the degradation data in bioavailability improved sediment of control (C); stimulation (S); stimulation and nutrient addition (SN); stimulation and inoculation of TBT-degrader (SI).

After adjusting sediment salinity to 20 psu, degradation of TBT was significantly increased. This confirms that the adjustment improved partitioning of TBT, and made it available for microbes to uptake and to degrade. Also inoculation of the degrader showed better degradation than nutrient stimulation. This might be because the majority of TBT was well adsorbed on the sediment together with the indigenous microbes where addition of the nutrients increased their degradation capability. However, the augmented culture required some time to attach on the sediment, which hindered them in approaching TBT. By the time *E. cloacae* was established on the sediment, surrounding conditions might not have been suitable for the degradation. After salinity of the sediment was adjusted, a larger amount of TBT was initially partitioned into water, and was easily uptaken by the inoculants during enhancement. Moreover, the partitioning of TBT has an equilibrium point, a constant ratio of TBT in sediment and water (Bangkedphol, 2010, Landmeyer et al., 2004). Therefore, biosorption reduces the amount of TBT in water, and then induces additional partitioning of TBT from sediment into water. Also K_{ow} increases at higher salinity (Bangkedphol et al., 2009).

The cessation of the stimulation might have been a consequence of inappropriate sediment properties (e.g. pH), limited amount of nutrients, diminishing bacterial growth/activities, or declining TBT bioavailability. The re-enhancement confirms the problem of less available TBT since adjustment of pH, inoculation of the degrader or addition of the nutrients were insignificant in enhancing degradation. In a study of PAHs removal, the stimulation stopped even though the population of degraders still thrived (Lei et al., 2005). Also stimulation of TBT degradation in sewage sludge was unsuccessful even with increased surrounding temperature and aeration (Voulvoulis and Lester, 2006). The rapid decline in degradation after the first week, even when bioavailability was improved, might result from minimal desorption of TBT from sediment into water. In this case, it can, therefore, be deduced that bioavailability played the most important role amongst the parameters.

Similar trends of pH, redox potential and C to N ratio were observed. However, changes in the trends were presented after re-enhancement with an increase in pH and a reduction of redox potential in samples S and SN. After incubation, pH was reduced and redox potential was increased again as shown in Figure 4.19.

The findings from this study suggest that enhanced biodegradation is effective for remediation of TBT contaminated sediment, but a limited amount of TBT can be removed. From this study, it apparently does not result from the biotic activity but the limited bioavailability of TBT, since re-enhancement for microbial activity was not successful. Although the degradable fraction appeared to be depleted and the readily fraction was minimal, the recalcitrant and non-extractable fractions were still remained in the sediment without breakdown as previously demonstrated in Figure 4.2. Therefore, bioremediation could be performed to reduce an initial concentration of TBT, and then intensive techniques, such as chemical or thermal cleavage, could be used if further reduction is required.

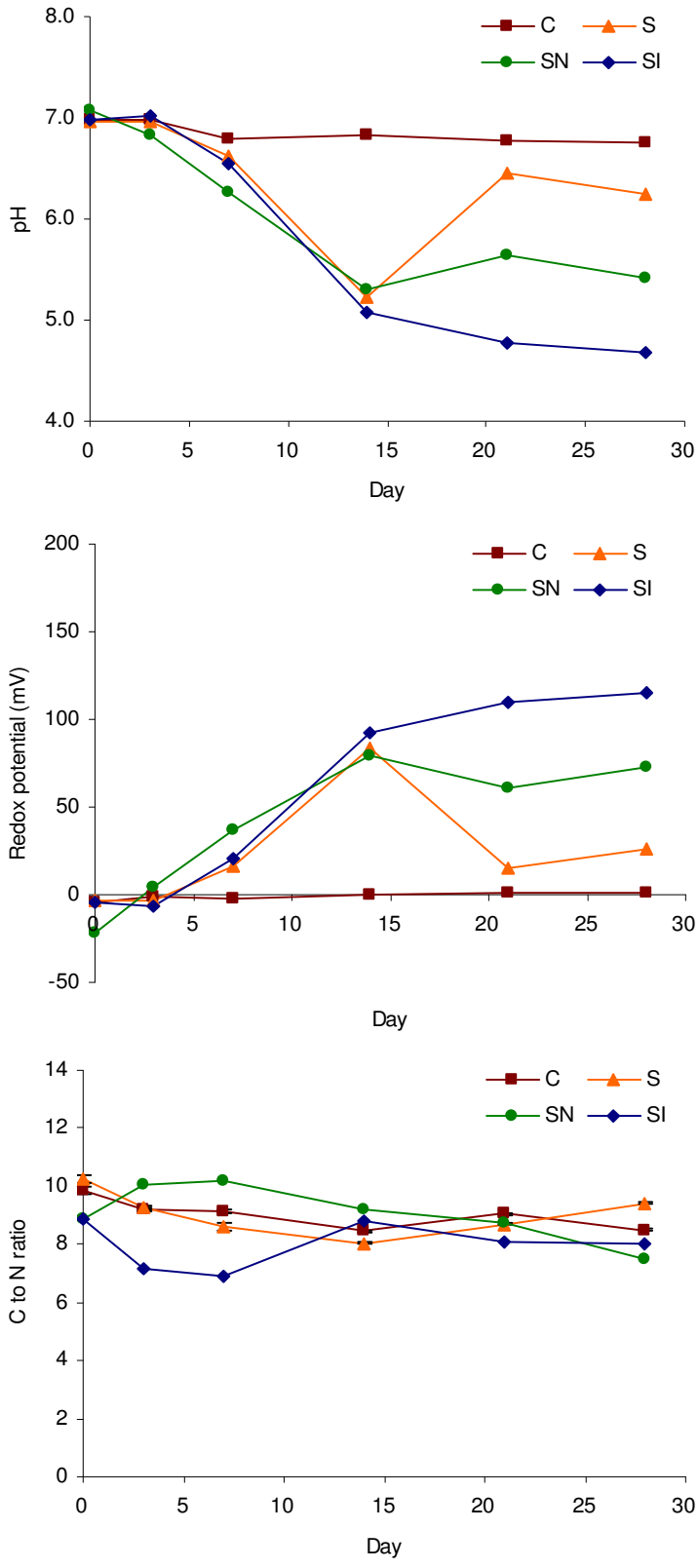


Figure 4.19 Bioavailability improved sediment pH, redox potential and C to N ratio during the incubation period of control (C); stimulation (S); stimulation and nutrient addition (SN); stimulation and inoculation of TBT-degrader (SI).

4.4 Summary

Since TBT accumulated in sediment, enhancement by the methods studied in *Chapters 2 & 3* were applied to increase the degradation rate. Natural attenuation of spiked TBT in sediment was accelerated and the rate constant was increased from 0.0012 day^{-1} to 0.0649 day^{-1} by providing suitable surrounding conditions, to 0.0776 day^{-1} by nutrient supplement with stimulating conditions, and to 0.0688 day^{-1} by inoculation of *E. cloacae* cells with stimulating conditions. This highlights the importance of the surrounding conditions on microbial activity. Nutrient addition and bioaugmentation slightly enhanced degradation, but inappreciably. With adequate remediation time, supporting conditions for capable microbes is the only thing required. Unfortunately, degradation enhancement dropped after one week of incubation.

The existing TBT (aged TBT) in sediment proved to be only partially bioavailable and biodegradable, it seems that long-term accumulation reduces degradation capability. Therefore, improvement of bioavailability was investigated by addition of surfactant, adjustment of salinity, and sonication. The adjustment of sediment salinity to 20 psu was found to be the best technique among the studied methods to increase TBT partitioning in water.

Degradation after improved bioavailability showed higher degraded concentration of TBT (approximately double from degradation without improvement of bioavailability), since microorganisms could uptake more TBT for intracellular metabolic processes. However, enhancement still dropped after one week. Re-enhancement by adjust the pH, addition of nutrients and re-inoculation was then carried out to indicate the cause of enhancement limitation. After re-enhancement, no significant degradation was observed. The finding indicates that even if TBT biodegradation was accelerated, TBT bioavailability was the most significant factor that limited degradation and preserved the amount of non-degradable TBT which would remain in the sediment.

CHAPTER 5

Conclusions and Recommendations for Future Works

5.1 Conclusions

The objectives of this study were achieved as described in the relevant chapters. For the isolation of TBT-degraders, *Alcaligenes faecalis*, *Citrobacter braakii*, *Enterobacter cloacae* and *Pseudomonas fluorescens* were successfully isolated under co-metabolism conditions from the contaminated sediment collected from Bowling Basin, Glasgow. Amongst the isolates *E. cloacae*, a new strain which has never been isolated and studied for TBT-degrading capability, had the highest capacity to degrade TBT and was highly resistant to the toxicity (*Chapter 2*). This strain was subsequently used as an inoculum.

Biostimulation studies were carried out (*Chapter 3*) and indicated that environmental conditions significantly influenced microbial activity and TBT characteristics, and appropriate supporting conditions extensively enhanced degradation. The optimised conditions were aerobic with a sufficient amount of dissolved oxygen, a pH of 7.5 and a temperature of 28˚C. The amendment of organic nutrients and growth factors were also achieved to biostimulate TBT degradation (*Chapter 3*). However, increasing the biomass did not necessarily promote degradation. The optimum amendment was that which increased degradation activity, in addition to increasing the degrader population to raise the amount of the enzyme. An inhibitory effect was shown when excess amounts of the nutrients were added.

Finally, the enhancement of bioremediation in sediment was achieved by bioaugmentation and biostimulation techniques, which increased degradation rates (*Chapter 4*). Results indicate that providing suitable environmental conditions considerably enhanced degradation. Further, stimulation by nutrient addition or

bioaugmentation with a proven degrader were not appreciably enhanced TBT degradation when competent microbes were presented and adequate remediation time was given. Limitation of bioavailability from long-term accumulation reduced the amount of degradable TBT. The bioavailability was improved by adjusting salinity to increase TBT partitioning in water.

Although bioremediation is preferred to treat contaminated sites and was proved in this study to be an effective strategy, it must be aware that bioremediation can be limited. The advantages and disadvantages of bioremediation are shown in Table 5.1.

Table 5.1 Overview on advantages and disadvantages of bioremediation strategies.

Advantages	Disadvantages
● Bioremediation is a natural process which relies on indigenous microbes that naturally occur in sediment and pose no threat to people at the site or in the community. ● No dangerous chemicals are used in bioremediation. ● The residues for the treatment are usually harmless products and include carbon dioxide, water, and cell biomass. ● If the right conditions exist or can be created at the site, sediment can be cleaned without excavation. This creates minimal disruption and disturbance to on-going site activities, decreases resuspension of contaminated sediment, does not produce waste products that must be disposed, and reduces worker exposure. ● Bioremediation does not require as much equipment or labour and is usually cheaper. ● Complete destruction by bioremediation is possible which eliminates the chance of future liability associated with treatment and disposal of contaminated sediment.	● Biological processes are often highly specific. Important site factors required for success include the presence of metabolically capable microbial populations, suitable environmental growth conditions, and appropriate levels of nutrients and contaminants. ● It may take anything from a few months to several years for microbes to process enough of the harmful chemicals to clean up the site. ● Low bioavailability of contaminant to microorganisms limits biodegradability. ● Uncertainty about the uniformity of treatment because bioremediation performance depends on several factors i.e. types and amounts of harmful chemicals present, size and depth of the polluted area, and type of sediment and the conditions present which are varying from site to site. ● Research is needed to develop and engineer bioremediation technologies that are appropriate for sites with complex mixtures of contaminants that are not evenly dispersed in the environment.

The bioremediation strategies include *in-situ* (monitoring natural attenuation, biosparging, bioventing and bioaugmentation), *ex-situ* (landfarming, composting and biopiles) and bioreactors (slurry reactors and aqueous reactors). Since specific investigation is required for each site to select an appropriate technique, it should be noted that there is difficulty to extrapolate from bench and pilot-scale studies to full-scale field operations.

This study was designed based on the feasibility of the laboratory, peer reviewed research and standard method for bioremediation, specifically for TBT. Excluding site characteristic information, this study was then contributed to *ex-situ* and bioreactors degradation. A local contaminated area was chosen as a study location. The sediment samples were collected on the surface as the active contaminated fraction rather than core sediment. Although aseptic conditions were controlled to prevent contamination of microorganisms, storage of sediment before studied might affect the microorganism's composition. This did not affect isolation of a competent TBT-degrader; however, it might affect degradation study in sediment. Therefore, the samples were collected and used without delay.

To isolate TBT-degraders, 28°C was utilised instead of ambient temperature at the site. Even the temperature might affect the screening of dominant species; the screening was expected to facilitate *ex-situ* remediation where temperature can be controlled for the highest bacterial growth and activity. To optimise supportive conditions for biodegradation, this study was performed in liquid media. As a result, the best conditions can be obtained without limitation of TBT availability, saturated conditions and co-contamination of other pollutants, which expected to promote unspecific sites. For study of TBT, all samples and their replicates were prepared in separate glass bottles, and the samples were given up for analysis. Withdrawing of the samples was avoided to prevent unreliable analysis of butyltin compounds because butyltins are well adsorb on glassware and bacterial cells which are not homogeneous in the solution.

Degradation in sediment was designed to perform in batch reactor with diffused aeration to increase DO and, in the same time, thoroughly mix the sediment. The study of bench-scale TBT degradation in sediment sample might have uncertainty when moved to pilot and full-scale remediation. Since laboratory results always higher than treatment in field site; however, the optimisation was expected to give the best performance possible for each site.

The finding from this study improves knowledge on the biodegradation of TBT, which is still limited at present. The achievement provides a step forward in the sustainable treatment of TBT-contaminated sediment. It does provide technical information on biodegradation for practitioners for better decision-making in selecting a suitable and feasible technique for their application. This study has shown the important of respiration conditions and temperature on biodegradation, which is necessary if biotic degradation of TBT is preferred. This study also amended the information on improvement of nutrient supplementation and leads to better nutrient selection by practitioners. Moreover, this study shown the effectiveness of biodegradation and make this technique became an option for treatment of TBT. On the other hand, this study also demonstrated the limitation of biodegradation by bioavailability which affects the remediation outcome.

5.2 Future works

Although the aim and objectives of this study were achieved, further interesting works can be performed to expand the knowledge of TBT biodegradation. These are suggested as follows:

- TBT-degraders were isolated for bioaugmentation; however, the potential of the bioaugmentation technique is generally dependent on the site characteristics. The population density might decline over time because of the unsuitable conditions for their growth, inhibition from other pollutants present at the site or unable to thrive with the indigenous microorganisms. Therefore, future work should study on genetic modification using the expressing gene which produces the degrading enzymes.

Consequently, inoculation of the gene instead of the cells would avoid inhibition of the augmented species by the surrounding conditions. The study of this technology can improve *ex-situ* bioremediation (i.e. bioreactor), since the release of genetically modified organisms/genes into the environment tend to be unfavourable.

● Using biostimulation, enhancing environmental conditions were proved to be to increase degradation rate, other factors influencing degradation e.g. C to N ratio, solid content in slurry, mixing rate and initial concentrations of TBT in the materials could be optimised in the future to approach better bioremediation efficiency.

● Since the study on nutrient supplement found that specific nutrients can support degradation capability, further work on the association of the essential nutrients to the TBT degradation metabolism of microorganisms would be worthwhile to give better understanding. This could lead to more efficient enhancement by nutrient addition.

● Limitation of bioavailability was proved to be the most important factor which reduces degradation capability. Future research for techniques which additionally improve bioavailability and, at the same time, mild for microorganisms is essential. As such, biodegradation can be performed solely to reduce most of the contamination instead of incorporation with physical-chemical degradation.

● The effectiveness of biodegradation presented from this study was in bench scale, therefore, the enhancement could be tested in both pilot scale and full scale remediation to ensure the practicability of the optimised method.

● Another suggested research area is a study on the relationship between the amount of degraded TBT and characteristics of the sediment, e.g. particle size, amount of organic content and ratio of clay minerals, which could indicate the causes of bioavailability limitation. This relationship will lead to better decision making on remediation techniques applied to contaminated sediment. For instance, the sediment

fractions with high contamination and low bioavailability can be separated for suitable abiotic degradation while the other fractions can be treated by biodegradation.

- Generally, bioremediation is associated with microbial population, environmental conditions and site characteristics. This thesis has mainly concentrated on microbial population and environmental conditions. Further study on site characteristics, e.g. contamination in sediment profile, current flow or tidal wave, salinity and temperature variation, and migration of the contaminated sediment, could progress the work on enhancement of *in-situ* bioremediation. Also, the effect of climate change should be considered in the study as it affects the site characteristics.

References

ACDP 2004. The approved list of biological agents. Norwich, UK.

ADVENTUS 2010. *In situ bioremediation/Groundwater remediation* [Online]. Available: http://www.adventusgroup.com/education/in-situ-bioremediation.shtml [Accessed 18 August 2010].

ALEXANDER, M. 1999. *Biodegradation and bioremediation,* London, Academic Press.

ALY, S. M., ABD EL RAHMAN, A. M., JOHN, G. & MOHAMED, M. F. 2008. Characterization of some bacteria isolated from *Oreochromis niloticus* and their potential use as probiotics. *Aquaculture,* 277, 1-6.

AMOUROUX, D., TESSIER, E. & DONNARD, O. F. X. 2000. Volatilization of organotin compounds from estuarine and coastal environments. *Environmental Science & Technology,* 34, 988-995.

ANDERSON, B. A., UNGER, M. A. & MOORE, K. A. 2002. Fate of tributyltin in a created tidal wetland. *Environmental Toxicology & Chemistry,* 21, 1176-1183.

ANTIZAR-LADISLAO, B. 2008. Environmental levels, toxicity and human exposure to tributyltin (TBT)-contaminated marine environment: A review. *Environment International,* 34, 292-308.

ANZECC/ARMCANZ 2000. Australian and New Zealand guidelines for fresh and marine water quality. Canberra: Australian and New Zealand Environment and Conservation Council/Agriculture and Resource Management Council of Australian and New Zealand.

APHA 1999. *Standards methods for the examination of water and waste water (APHA: 2520B),* Washington D.C., American Public Health Association, Water Environment Federation.

ARAI, T., HARINO, H., OHJI, M. & LANGSTON, W. J. 2009. *Ecotoxicology of antifouling biocides,* Tokyo, Springer.

ARCHAKOV, A. I. & BUCHMANOVA, G. I. 1990. *Cytochrome P-450 and active oxygen,* London, Taylor and Francis.

ASTM 2000. *Standard test methods for moisture, ash, and organic matter of peat and other organic soils (ASTM: D 2974-87)*, Philadelphia, ASTM international publisher.

ATAGANA, H. I., HAYNES, R. J. & WALLIS, F. M. 2003. Optimization of soil physical and chemical conditions for the bioremediation of creosote-contaminated soil. *Biodegradation,* 14, 297-307.

ATLAS, R. M. 1991. Microbial hydrocarbon degradation - Bioremediation of oil spills. *Journal of Chemical Technology & Biotechnology,* 52, 149-156.

AVERY, S. V., CODD, G. A. & GADD, G. M. 1993. Biosorption of tributyltin and other organotin compounds by cyanobacteria and microalgae. *Applied Microbiology and Biotechnology*, 39, 812-817.

BANGKEDPHOL, S. 2010. *Case study of sustainable managemnet of tributyltin contamination in the environment.* Doctor of Philosophy, University of Strathclyde.

BANGKEDPHOL, S., KEENAN, H. E., DAVIDSON, C. M., SAKULTANTIMETHA, A., SIRISAKSOONTORN, W. & SONGSASEN,

S. 2010. Enhancement of tributyltin degradation under natural light by N-doped TiO2 photocatalyst. *Journal of Hazardous Materials,* 184, 533-537.
BANGKEDPHOL, S., KEENAN, H. E., DAVIDSON, C., SAKULTANTIMETHA, A. & SONGSASEN, A. 2008. Development of a low-cost method of analysis for the qualitative and quantitative analysis of butyltins in environmental samples. *Journal of Environmental Science and Health Part A,* 43, 1744-1751.
BANGKEDPHOL, S., KEENAN, H. E., DAVIDSON, C., SAKULTANTIMETHA, A. & SONGSASEN, A. 2009. The partition behavior of tributyltin and prediction of environmental fate, persistence and toxicity in aquatic environments. *Chemosphere,* 77, 1326-1332.
BARR, J. G. 1978. Variation in metabolism of biochemical test substrates by *Klebsiella* species: An epidemiological tool. *Journal of Medical Microbiology,* 11, 501-511.
BARUG, D. 1981. Microbial-degradation of bis(tributyltin) oxide. *Chemosphere,* 10, 1145-1154.
BELLAS, J., BEIRAS, R., MARINO-BALSA, J. C. & FERNANDEZ, N. 2005a. Toxicity of organic compounds to marine invertebrate embryos and larvae: A comparison between the sea urchin embryogenesis bioassay and alternative test species. *Ecotoxicology* 14, 337-353.
BELLAS, J., HILVARSSON, A. & GRANMO, A. 2005b. Sublethal effects of a new antifouling candidate on lumpfish (*Cyclopterus lumpus* L.) and Atlantic cod (*Gadus morhua* L.) larvae. *Biofouling,* 21, 207-216.
BENTIVEGNA, C. & PIATKOWSKI, T. 1998. Effects of tributyltin on medaka (*Oryzias latipes*) embryos at different stages of development. *Aquatic Toxicology,* 44, 117-128.
BERGE, J. A., BREVIK, E. M., BJØRGE, A., FØLSVIK, N., GABRIELSEN, G. W. & WOLKERS, H. 2004. Organotins in marine mammals and seabirds from Norwegian territory. *Journal of Environmental Monitoring,* 6, 108-112.
BERNAT, P. & DŁUGOŃSKI, J. 2002. Degradation of tributyltin by the filamentous fungus *Cunninghamella elegans*, with involvement of cytochrome P-450. *Biotechnology Letters,* 24, 1971-1974.
BERNAT, P. & DŁUGOŃSKI, J. 2006. Acceleration of tributyltin chloride (TBT) degradation in liquid cultures of the filamentous fungus *Cunninghamella elegans*. *Chemosphere,* 62, 3-8.
BERNAT, P. & DŁUGOŃSKI, J. 2009. Isolation of *Streptomyces* sp. strain capable of butyltin compounds degradation with high efficiency. *Journal of Hazardous Materials,* 171, 660-4.
BERTO, D., GIANI, M., BOSCOLO, R., COVELLI, S., GIOVANARDI, O., MASSIRONI, M., GRASSIA, L. & CARDWELL, R. D. 2007. Organotins (TBT and DBT) in water, sediments, and gastropods of the southern Venice lagoon (Italy). *Marine Pollution Bulletin,* 55, 425-435.
BHOSLE, N. B., GARG, A., JADHAV, S., HARJEE, R., SAWANT, S. S., VENKAT, K. & ANIL, A. C. 2004. Butyltins in water, biofilm, animals and sediments of the west coast of India. *Chemosphere,* 57, 897-907.
BINKS, P. R., FRENCH, C. E., NICKLIN, S. & BRUCE, N. C. 1996. Degradation of pentaerythritol tetranitrate by *Enterobacter cloacae* PB2. *Applied Environmental Microbiology,* 62, 1214-1219.

BLACK, S. D. 1993. *Cytochrome P450 structure and function,* London, Springer-Verlag.
BLANCK, H. & DAHL, B. 1996. Pollution-induced community tolerance (PICT) in marine periphyton in a gradient of tributyltin (TBT) contamination. *Aquatic Toxicology,* 35, 59-77.
BOSSHARD, P. P., ZBINDEN, R., ABELS, S., BODDINGHAUS, B., ALTWEGG, M. & BOTTGER, E. C. 2006. 16S rRNA gene sequencing versus the API 20 NE system and the VITEK 2 ID-GNB card for identification of nonfermenting gram-negative bacteria in the clinical laboratory. *Journal of Clinical Microbiology,* 44, 1359-1366.
BOWLES, K. C., APTE, S. C. & HALES, L. T. 2003. Determination of butyltin species in natural waters using aqueous phase ethylation and off-line room temperature trapping. *Analytica Chimica Acta,* 477, 103-111.
BRANDSCH, R., NOWAK, K. E., BINDER, N. & JASTORFF, B. 2001. Investigations concerning the sustainability of remediation by land deposition of tributyltin contaminated harbour sediments. *Journal of Soils and Sediments,* 1, 234-236.
BRITISH STANDARDS 2009. *Soil quality - Determination of selected organotin compounds - Gas chromatographic method (ISO 23161:2009).*
BRITISH WATERWAYS SCOTLAND 2008. *Skiper's Guide*. Scotland.
CAPPUCCINO, J. & SHERMAN, N. 1998. *Microbiology: A laboratory manual,* California, Benjamin/Cummings Science Publishing.
CEULEMANS, M., SLAETS, S. & ADAMS, F. 1998. Speciation of organotin in environmental sediment samples. *Talanta,* 46, 395-405.
CHAMP, M. A. & SELIGMAN, P. F. 1996. *An introduction to organotin compounds and their use in antifouling coatings,* London, Chapman and Hall.
CHAN, K. H., TSUI, T. S. & FANG, Z. R. 2002. Long-term performance of sediment remediation in Shing Mun River, Hong Kong. Environmental Protection Department, The Government of Hongkong Special Administrative Region.
CHANG, S. C., KUNG, H. F., CHEN, H. C., LIN, C. S. & TSAI, Y. H. 2008. Determination of histamine and bacterial isolation in swordfish fillets (*Xiphias gladius*) implicated in a food borne poisoning. *Food Control,* 19, 16-21.
CHAU, Y. K., MAGUIRE, R. J., BROWN, M., YANG, F., BATCHELOR, S. P. & THOMPSON, J. A. J. 1997. Occurrence of butyltin compounds in mussels in Canada. *Applied Organometallic Chemistry,* 11, 903-12.
CHENG, K. Y. & WONG, J. W. C. 2006. Combined effect of nonionic surfactant Tween 80 and DOM on the behaviors of PAHs in soil-water system. *Chemosphere,* 62, 1907-1916.
CHENG, Q., BAI, X. & WANG, Y. 2008. Isolation and fermentation condition of milk-clotting enzyme producing strain from glutinous rice wine. *Sheng Wu Gong Cheng Xue Bao,* 24, 999-1003.
CHIAVARINI, S., MASSANISSO, P., NICOLAI, P., NOBILI, C. & MORABITO, R. 2003. Butyltins concentration levels and imposex occurrence in snails from the Sicilian coasts (Italy). *Chemosphere,* 50, 311-319.

CIRCA 2005. Environmental quality standard (EQS) substance data sheet: Tributyltin compounds (TBT-ion). *Common Implementation Strategy for the Water Framework Directive.* Brussels.
CORWIN, C. H. 2005. *Introductory chemistry: Concepts & connections*, New Jersey, Prentice Hall.
CRAIG, P. J. 2003. *Organometallic compounds in the environment: Principles and reactions,* New York, John Wiley & Sons.
CRUZ, A., CAETANO, T., SUZUKI, S. & MENDO, S. 2007. *Aeromonas veronii*, a tributyltin (TBT)-degrading bacterium isolated from an estuarine environment, Ria de Aveiro in Portugal. *Marine Environmental Research,* 64, 639-650.
CRUZ, A., HENRIQUES, I., CORREIA, A., SUZUKI, S. & MENDO, S. 2010. *Aeromonas molluscorum Av27: A potential natural tool for TBT decontamination*, Tokyo, Terrapub.
DE LIPTHAY, J. R. D., SØRENSEN, S. R. & AAMAND, J. 2007. Effect of herbicide concentration and organic and inorganic nutrient amendment on the mineralization of mecoprop, 2,4-D and 2,4,5-T in soil and aquifer samples. *Environmental Pollution,* 148, 83-93.
DEJONGHE, W., BOON, N., SEGHERS, D., TOP, E. M. & VERSTRAETE, W. 2001. Bioaugmentation of soils by increasing microbial richness: Missing links. *Environmental Microbiology,* 3, 649-657.
DELGADO-MORENO, L. & PEÑA, A. 2009. Compost and vermicompost of olive cake to bioremediate triazines-contaminated soil. *Science of the Total Environment,* 407, 1489-1495.
DERCOVÁ, K., ČIČMANOVÁ, J., LOVECKÁ, P., DEMNEROVÁ, K., MACKOVÁ, M., HUCKO, P. & KUŠNÍR, P. (2008) Isolation and identification of PCB-degrading microorganisms from contaminated sediments. *International Biodeterioration & Biodegradation,* 62, 219-225.
DFO 2010. *Glossary* [Online]. Fisheries and Oceans Canada. Available: http://www.dfo-mpo.gc.ca/Science/biotech/abgrds-srdbfa/gloss-eng.htm [Accessed 08 August 2010].
DIEZ, S., LACORTE, S., VIANA, P., BARCELO, D. & BAYONA, J. M. 2005. Survey of organotin compounds in rivers and coastal environments in Portugal 1999-2000. *Environmental Pollution,* 136, 525-536.
DIMITRIOU, P., CASTRITSI-CATHARIOS, J. & MILIOU, H. 2003. Acute toxicity effects of tributyltin chloride and triphenyltin chloride on gilthead seabream, *Sparus aurata* L., embryos. *Ecotoxicology and Environmental Safety,* 54, 30-35.
DOU, J., LIU, X., HU, Z. & DENG, D. 2008. Anaerobic BTEX biodegradation linked to nitrate and sulfate reduction. *Journal of Hazardous Materials,* 151, 720-729.
DOWSON, E. H., BUBB, J. M. & LESTER, J. N. 1993. Depositional profiles and relationships between organotin compounds in freshwater and estuarine sediment cores. *Environmental Monitoring and Assessment,* 28, 145-160.
DOWSON, P. H., BUBB, J. M. & LESTER, J. N. 1996. Persistence an degradation pathways of tributyltin in freshwater and estuarine sediments. *Estuarine Coastal and Shelf Science,* 42, 551-562.

EA 2010. *Tributyltin compounds: Pollution inventory substances* [Online]. Available: http://www.environment-agency.gov.uk/business/topics/pollution/39167.aspx [Accessed 09 July 2010].

EBDOH, L., HILL, S. & GRIEPINK, B. 1988. A programme to improve the quality of analytical results in the environmental monitoring of organotin compounds. *Environmental Technology,* 9, 965-968.

EGAWA, T., OGURA, T., MAKINO, R., ISHIMURA, Y. & KITAGAWA, T. 1991. Observation of the O-O stretching Raman band for cytochrome-P-450cam under catalytic conditions. *Journal of Biological Chemistry,* 266, 10246-10248.

EGUCHI, S., HARINO, H. & YAMAMOTO, Y. 2010. Assessment of antifouling biocides contaminations in Maizuru Bay, Japan. *Archives of Environmental Contamination and Toxicology,* 58, 684-693.

El FANTROUSSI, S. & AGATHOS, S. N. 2005. Is bioaugmentation a feasible strategy for pollutant removal and site remediation? *Current Opinion in Microbiology,* 8, 268-275.

ERRÉCALDE, O., ASTRUC, M., MAURY, G. & PINEL, R. 1995. Biotransformation of butyltin compounds using pure strains of microorganisms. *Applied Organometallic Chemistry,* 9, 23-28.

ESCHENBACH, A., HÖHNE, J., SÖDING, Z. & LUTHER, G. 2001. The steam stripping process: A remediation technique for TBT- and PAH-contaminated dredged sediments and soils. *In:* 2001 International Containment & Remediation Technology Conference and Exibition, Orlando, Florida.

EUROPEAN UNION 2008. Directive 2008/105/EC of the European Paliament and of the Council on environmental quality standards in the field of water policy, amending and subsequently repealing Council Directives 82/176/EEC, 83/513/EEC, 84/156/EEC, 84/491/EEC, 86/280/EEC and amending Directive 2000/60/EC of the European Paliament and of the Council. *Official Journal of the European Union.*

EWEIS, J.B. 1998. *Bioremediation principles*, Boston, McGraw-Hill.

FENT, K. 1991. Bioconcentration and elimination of tributyltin chloride by embryos and larvae of minnows *Phoxinus phoxinus*. *Aquatic Toxicology,* 20, 147-158.

FENT, K. & LOOSER, P. W. 1995. Bioaccumulation and bioavailability of tributyltin chloride - influence of ph and humic acids. *Water Research,* 29, 1631–1637.

FORSYTH, D. S., SUN, W. F. & DALGLISH, K. 1994. Survey of organotin compounds in blended wines. *Food Additives & Contaminants,* 11, 343-350.

FRENCH, C. E., NICKLIN, S. & BRUCE, N. C. 1998. Aerobic degradation of 2,4,6-trinitrotoluene by *Enterobacter cloacae* PB2 and by pentaerythritol tetranitrate reductase. *Applied Environmental Microbiology,* 64, 2864-2868.

FRTR 2008. *In situ biological treatment for soil, sediment, bedrock and sludge* [Online]. Federal Remediation Technologies Roundtable. Available: http://www.frtr.gov/matrix2/section3/3_1.html [Accessed 11 July 2008].

GADD, G. M. 2000. Microbial interactions with tributyltin compounds: Detoxification, accumulation, and environmental fate. *Science of the Total Environment* 258, 119-127.

GADD, G. M., GRAY, D. J. & NEWBY, P. J. 1990. Role of melanin in fungal biosorption of tributyltin chloride. *Applied Microbiology and Biotechnology,* 34, 116-121.

GIPPERTH, L. 2009. The legal design of the international and European Union ban on tributyltin antifouling paint: Direct and indirect effects. *Journal of Environmental Management,* 90, S86–S95.

GOH, C. L. 1985. Irritant dermatitis from tri-n-butyl tin oxide in paint. *Contact Dermatitis,* 12, 161-163.

GOMEZ-ARIZA, J. L., BELTRAN, R., MORALES, E., GIRALDEZ, I. & RUIZ-BENITEZ, M. 1995. Acid extraction treatment of sediment samples for organotin speciation, occurrence of butyltin and phenyltin compounds on the Cadiz coast, south-west Spain. *Applied Organometallic Chemistry,* 9, 51-64.

GUENGERICH, F. P. 1993. *Metabolic reactions: Types of reactions of cytochrome P450 enzymes,* London, Springer-Verlag.

GURUGE, K. S., TANABE, S., IWATA, H., TAKSUKAWA, R. & YAMAGISHI, S. 1996. Distribution, biomagnification, and elimination of butyltin compound residues in common cormorants (*Phalacrocorax carbo*) from Lake Biwa. *Japanese Archives of Environmental Contamination and Toxicology,* 31, 210-217.

HADIBARATA, T. & TACHIBANA, S. 2009. Enhanced chrysene biodegradation in presence of a synthetic surfactant. *In:* OBAYASHI, Y., ISOBE, T., SUBRAMANIAN, A., SUZUKI, S. & TANABE, S. (eds.) *Interdisciplinary studies on environmental chemistry - Environmental research in Asia.* TERRAPUB.

HALL, L. W., BUSHONG, S. J., HALL, W. S. & JOHNSON, W. E. 1988. Acute and chronic effects of tributyltin on a Chesapeake Bay copepod. *Environmental Toxicology & Chemistry* 7, 41-46.

HALL, L. W., SCOTT, M. C., KILLEN, W. D. & UNGER, M. A. 2000. A probabilistic ecological risk assessment of tributyltin in surface waters of the Chesapeake Bay watershed. *Human and Ecological Risk Assessment,* 6, 141-179.

HARINO, H., FUKUSHIMA, M., KUROKAWA, Y. & KAWAI, S. 1997a. Degradation of the tributyltin compounds by the microorganisms in water and sediment collected from the harbour area of Osaka City, Japan. *Environmental Polution,* 98, 163-167.

HARINO, H., FUKUSHIMA, M., KUROKAWA, Y. & KAWAI, S. 1997b. Susceptibility of bacterial populations to organotin compounds and microbial degradation of organotin compounds in environmental water. *Environmental Pollution,* 98, 157-162.

HARINO, H., OHJI, M., WATTAYAKORN, G., ARAI, T., RUNGSUPA, S. & MIYAZAKI, N. 2006. Occurrence of antifouling biocides in sediment and green mussels from Thailand. *Archives of Environmental Contamination and Toxicology,* 51, 400-407.

HARINO, H., YAMAMOTO, Y., EGUCHI, S., KAWAI, S., KUROKAWA, Y., ARAI, T., OHJI, M., OKAMURA, H. & MIYAZAKI, N. 2007. Concentrations of antifouling biocides in sediment and mussel samples collected from Otsuchi Bay, Japan. *Archives of Environmental Contamination and Toxicology,* 52, 179-188.

HARRIS, J. R. W. & CLEARY, J. J. 1987. Particle water partitioning and organotin dispersal in an estuary. *In:* Proceeding of the fourth international organotin symposium.
HEROULT, J., NIA, Y., DENAIX, L., BUENO, M. & LESPES, G. 2008. Kinetic degradation processes of butyl- and phenyltins in soils. *Chemosphere,* 72, 940-946.
HICKS, P. 1999. The use of oxygen release compound (ORC ®) for enhanced bioremediation. *In:* UZOCHUKWU, G. A. & REDDY, G. B. (eds.) *Proceedings of the 1998 National Conference on Environmental Remediation Science and Technology*. Columbus: Battelle Press.
HOCH, M. 2001. Organotin compounds in the environment - an overview. *Applied Geochemistry,* 16, 719-743.
HOCH, M., ALONSO-AZCARATE, J. & LISCHICK, M. 2003. Assessment of adsorption behaviour of dibutyltin (DBT) to clay-rich sediments in comparison to the highly toxic tributyltin (TBT). *Environmental Pollution,* 123, 217-227.
HONGXIA, L., GUOLAN, H. & SHUGUI, D. 1998. Toxicity and accumulation of tributyltin chloride on Tilapia. *Applied Organometallic Chemistry,* 12, 109-119.
HORIGUCHI, T., IMAI, T., CHO, H. S., SHIRAISHI, H., SHIBATA, Y., MORITA, M. & SHIMIZU, M. 1998. Acute toxicity of organotin compounds to the larvae of the rock shell, *Thais clavigera*, the disk abalone, *Haliotis discus discus* and the giant abalone, *Haliotis madaka*. *Marine Environmental Research,* 46, 469-473.
HOWARD, A. & GKENAKOU, E. 2008. Remediation of tributyltin-contaminated sediments and water. *5th International SedNet conference.* Oslo, Norway.
HUANG, J. H. & MATZNER, E. 2004. Degradation of organotin compounds in organic and mineral forest soils. *Journal of Plant Nutrition and Soil Science,* 167, 33-38.
HUANG, J. H., SCHWESIG, D. & MATZNER, E. 2004. Organotin compounds in precipitation, fog, and soils of a forested ecosystem in Germany. *Environmental Pollution,* 130, 177-186.
HUANG, Y., ZHU, L. Y., QIU, X. C. & ZHANG, T. W. 2010. Effect of bis(tributyltin) oxide on reproduction and population growth rate of calanoid copepod *Schmackeria poplesia*. *Chinese Journal of Oceanology and Limnology,* 28, 280-287.
HUNZIKER, R. W., ESCHER, B. I. & SCHWARZENBACH, R. P. 2001. pH dependence of the partitioning of triphenyltin and tributyltin between phosphatidylcholine liposomes and water. *Environmental Science & Technology* 35, 3899-3904.
IMO 2002. *International Convention on the Control of Harmful Anti-fouling Systems on Ships* [Online]. Available: http://www.imo.org/conventions/mainframe.asp?topic_id=529 [Accessed 08 July 2010].
INABA, K., SHIRAISHI, H. & SOMA, Y. 1995. Effects of salinity, pH and temperature on aqueous solubility of four organotin compounds. *Water Research,* 29, 1415-1417.

INOUE, H., TAKIMURA, O., FUSE, H., MURAKAMI, K., KAMIMURA, K. & YAMAOKA, Y. 2000. Degradation of triphenyltin by a fluorescent pseudomonad. *Applied and Environmental Microbiology*, 66, 3492-3498.
INOUE, S., OSHIMA, Y., NAGAI, K., YAMAMOTO, T., GO, J., KAI, N. & HONJO, T. 2004. Effect of maternal exposure to tributyltin on reproduction of the pearl oyster (*Pinctada fucata martensii*). *Environmental Toxicology and Chemistry,* 23, 1276-1281.
INOUE, S., OSHIMA, Y., USUKI, H., HAMAGUCHI, M., HANAMURA, Y., KAI, N., SHIMASAKI, Y. & HONJO, T. 2007. Effect of tributyltin on veliger larvae of the Manila clam, *Ruditapes philippinarum*. *Chemosphere,* 66, 1353-1357.
IPCS 1990. *Environmental health criteria 116: Tributyltin compounds* [Online]. Available: http://www.inchem.org/documents/cicads/cicads/cicad14.htm [Accessed 11 August 2010].
JARERAT, A., TOKIWA, Y. & TANAKA, H. 2004. Microbial poly(L-lactide)-degrading enzyme induced by amino acids, peptides, and poly(L-amino acids). *Journal of Polymers and the Environment,* 12, 139-146.
JAYASHREE, R. & VASUDEVAN, N. 2007. Effect of tween 80 added to the soil on the degradation of endosulfan by *Pseudomonas aeruginosa*. *International Journal of Environmental Science and Technology,* 4, 203-210.
JOYCE, E., PHULL, S. S., LORIMER, J. P. & MASON, T. J. 2003. The development and evaluation of ultrasound for the treatment of bacterial suspensions: A study of frequency, power and sonication time on cultured *Bacillus* species. *Ultrasonics Sonochemistry,* 10, 315-318.
KARAGUNDUZ, A., GEZER, A. & KARASULOGLU, G. 2007. Surfactant enhanced electrokinetic remediation of DDT from soils. *Science of the Total Environment,* 385, 1-11.
KARLSSON, J., BREITHOLTZ, M. & EKLUND, B. 2006. A practical ranking system to compare toxicity of anti-fouling paints. *Marine Pollution Bulletin,* 52, 1661-1667.
KAWAI, S., KUROKAWA, Y., HARINO, H. & FUKUSHIMA, M. 1998. Degradation of tributyltin by a bacterial strain isolated from polluted river water. *Environmental Pollution,* 102, 259-263.
KIM, S. K., KIM, J. H., HAN, J. H. & YOON, Y. D. 2008. Inhibitory effect of tributyltin on expression of steroidogenic enzymes in mouse testis. *International Journal of Toxicology,* 27, 175-182.
KING, R. B., LONG, G. M. & SHELDON, J. K. 1997. *Practical environmental bioremediation,* London, Lewis Publishers.
KLEIN, P. H. 1996. *Microbiology,* Iowa, Brown Publishers.
KONNO, N., TSUNODA, M. & SUGITA-KONISHI, Y. 2005. Effect of tributyltin compound on N-methyl-D-aspartate (NMDA) receptors in brain of preweanling mice. *Environmental Health and Preventive Medicine,* 10, 335-337.
KRIEG, N. R. & HOLT, J. G. 1984. *Bergey's Manual of Systemic Bacteriology,* Baltimore, The Williams & Wilkins Co.
KRZYWONOS, M., CIBIS, E., MISKIEWICZ, T. & KENT, C. A. 2008. Effect of temperature on the efficiency of the thermo- and mesophilic aerobic batch

biodegradation of high-strength distillery wastewater (potato stillage). *Bioresource Technology,* 99, 7816-7824.
KUBALLA, J., DESAI, M. V. M. & WILKEN, R. D. 2004. Influence of humic substances on sorption and methylation processes of inorganic- and organo-tin species. *Applied Organometallic Chemistry,* 9, 629-638.
KWOK, K. W. & LEUNG, K. M. 2005. Toxicity of antifouling biocides to the intertidal harpacticoid copepod *Tigriopus japonicus* (Crustacea, Copepoda): Effects of temperature and salinity. *Marine Pollution Bulletin,* 51, 830-837.
LANDMEYER, J. E., TANNER, T. L. & WATT, B. E. 2004. Biotransformation of tributyltin to tin in freshwater river-bed sediments contaminated by an organotin release. *Environmental Science & Technology,* 38, 4106-4112.
LANGSTON, W. J. & POPE, N. D. 1995. Determinants of TBT adsorption and desorption in estuarine sediments. *Marine Pollution Bulletin,* 31, 32-43.
LASCOURREGES, J. F., CAUMETTE, P. & DONARD, O. F. X. 2000. Toxicity of butyltin, phenyltin and inorganic tin compounds to sulfate-reducing bacteria isolated from anoxic marine sediments. *Applied Organometallic Chemistry,* 14, 98-107.
LAUGHLIN, R. B., FRENCH, W. & GUARD, H. E. 1986. Accumulation of bis(tributyltin) oxide by the marine mussel *Mytilus Edulis*. *Environmental Science & Technology,* 20, 884-890.
LEE, R. F., VALKIRS, A. O. & SELIGMAN, P. F. 1989. Importance of microalgae in the biodegradation of tributyltin in estuarine waters. *Environmental Science & Technology,* 23, 1515-1518.
LEI, L., KHODADOUST, A. P., SUIDAN, M. T. & TABAK, H. H. 2005. Biodegradation of sediment-bound PAHs in field-contaminated sediment. *Water Research,* 39, 349-361.
LENS, P., GROTENHUIS, T., MALINA, G. & TABAK, H. 2005. *Soil and sediment remediation,* London, IWA Publishing.
LEUNG, K. M., GRIST, E. P., MORLEY, N. J., MORRITT, D. & CRANE, M. 2007. Chronic toxicity of tributyltin to development and reproduction of the European freshwater snail *Lymnaea stagnalis* (L.). *Chemosphere,* 66, 1358-1366.
LI, J. L. & CHEN, B. H. 2009. Surfactant-mediated biodegradation of polycyclic aromatic hydrocarbons. *Materials,* 2, 76-94.
LI, S. X., WEI, D., MAK, N. K., CAI, Z., XU, X. R., LI, H. B. & JIANG, Y. 2009. Degradation of diphenylamine by persulfate: Performance optimization, kinetics and mechanism. *Journal of Hazardous Materials* 164, 26-31.
LIMA, D., VIANA, P., ANDRÉ, S., CHELINHO, S., COSTA, C., RIDEIRO, R. & SOUSA, J. P. 2009. Evaluating a bioremediation tool for atrazine contaminated soils in open soil microcosms: The effectiveness of bioaugmentation and biostimulation approaches. *Chemosphere,* 74, 187-192.
LIU, S. M., HUANG, H. T., HSIA, M. P. & CHANG, C. F. 2008. Tributyltin degradation in estuarine sediments and the isolation of tributyltin-resistant bacteria from these sediments. *Journal of the Fisheries Society of Taiwan,* 35, 315-327.
LOCH, J. P. G., GREVE, P. A. & VAN DER BERG, S. 1990. Accumulation and leaching of the fungicide fentin acetate and intermediates in sandy soils. *Water, Soil, and Air Pollution,* 53, 119-129.

LOOSER, P. W., BERTSCHI, S. & FENT, K. 1998. Bioconcentration and bioavailability of organotin compounds: Influence of pH and humic substances. *Applied Organometallic Chemistry,* 12, 601-611.

LOUPPIS, A. P., GEORGANTELIS, D., PALEOLOGOS, E. K. & KONTOMINAS, M. G. 2010. Determination of tributyltin through ultrasonic assisted micelle mediated extraction and GFAAS: Application to the monitoring of tributyltin levels in Greek marine species. *Food Chemistry,* 121, 907-911.

LU, J., JIN, Q., HE, Y. L. & WU, J. 2008. Enhanced anaerobic biodegradation of nonylphenol ethoxylates by introducing additional sulfate or nitrate as terminal electron acceptors. *International Biodeterioration & Biodegradation,* 62, 214-221.

LUAN, T. G., JIN, J., CHAN, S. M. N., WONG, Y. S. & TAM, N. F. Y. 2006. Biosorption and biodegradation of tributyltin (TBT) by alginate immobilized *Chlorella vulgaris* beads in several treatment cycles. *Process Biochemistry,* 41, 1560-1565.

MAIER, R. M., PEPPER, I. L. & GERBA, C. P. 2009. *Environmental Microbiology,* London, Academic Press.

MCKAY, D. 2001. *Multimedia environmental models: The fugacity approach,* London, Lewis Publishers.

MCKINSEY, P. C., BRIGMON, R. L., BERRY, C. J., ROSSABI, J., PHIFER, M., POPPY, T. E., MARTIN, D. B., HEITKAMP, M. A. & LOEFFLER, F. E. 2003. Bioremediation of trichloroethylene-contaminated sediments augmented with a *Dehalococcoides consortium*. *In:* MAGAR, V. S., KELLEY, M. E. (eds.) *The seventh international in situ and on-site bioremediation symposium*, Orlando, Florida.

MEADOR, J. P. & RICE, C. A. 2001. Impaired growth in the polychaete *Armandia brevis* exposed to tributyltin in sediment. *Marine Environmental Research,* 51, 113-129.

MILLER, B. & BOYLE, J. 2003. Assessment of TBT impacts and concentrations in Loch Ryan, Scotland. Glasgow: Tidal Waters, Scottish Environmental Protection Agency.

MINCHIN, D., FLOERL, O., SAVINI, D. & OCCHIPINTI-AMBROGI, A. 2006. *Small craft and the spread of exotic species,* Dordrecht, Springer.

MISUMI, I., YADA, T., LEONG, J. A. C. & SCHRECK, C. B. 2009. The effect of in vitro exposure to tributyltin on the immune competence of chinook salmon (*Oncorhynchus tshawytscha*) leukocytes. *Archives of Environmental Contamination and Toxicology,* 56, 229-237.

MOCHIDA, K., ITO, K., KONO, K., ONDUKA, T., KAKUNO, A. & FUJII, K. 2010. Effect of tributyltin oxide exposure on the F-0 and F-1 generations of a marine teleost, the mummichog *Fundulus heteroclitus*. *Fisheries Science,* 76, 333-341.

MOORMAN, T. B., COWAN, J. K., ARTHUR, E. L. & COATS, J. R. 2001. Organic amendments to enhance herbicide biodegradation in contaminated soils. *Biology and Fertility of Soils,* 33, 541-545.

MORA, S. J. D., STEWART, C. & PHILLIPS, D. 1995. Sources and rate of degradation of tri(n-butyl)tin in marine-sediments near Auckland, New-Zealand. *Marine Pollution Bulletin,* 30, 50-57.

MOSLEMY, P., NEUFELD, R. J., MILLETTE, D. & GUIOT, S. R. 2003. Transport of gellan gum microbeads through sand: An experimental evaluation for encapsulated cell bioaugmentation. *Journal of Environmental Management,* 69, 249-259.

MURPHY, T., MOLLER, A. & BROUWER, H. 1995. In situ treatment of Hamilton Harbour sediment. *Journal of Aquatic Ecosystem Health,* 4, 195-203.

MURTHY, R. K., CABRAL, L., VIDYA, R. & DUBEY, S. K. 2007. Isolation and biological characterization of a tributyltin chloride degrading marine bacterium, *Vibrio* sp. from Bombay High Oil Field, India. *Current Science,* 93, 1073-1074.

NHAN, D. D., LOAN, D. T., TOLOSA, I. & DE MORA, S. J. 2005. Occurrence of butyltin compounds in marine sediments and bivalves from three harbour areas (Saigon, Da Nang and Hai Phong) in Vietnam. *Applied Organometallic Chemistry,* 19, 811-818.

OBBARD, J. P., NG, K. L. & XU, R. 2004. Bioremediation of petroleum contaminated beach sediments: Use of crude palm oil and fatty acids to enhance indigenous biodegradation. *Water, Air and Soil Pollution,* 157, 149-161.

OHJI, M., ARAI, T., MIDORIKAWA, S., HARINO, H., MASUDA, R. & MIYAZAKI, N. 2007. Distribution and fate of organotin compounds in Japanese coastal waters. *Water, Air, and Soil Pollution,* 178, 255-265.

OHJI, M., TAKEUCHI, I., TAKAHASHI, S., TANABE, S. & MIYAZAKI, N. 2002. Differences in the acute toxicities of tributyltin between the *Caprellidea* and the *Gammaridea* (Crustacea: Amphipoda). *Marine Pollution Bulletin* 44, 16-24.

OLIVEIRA, R. C. & SANTELLI, R. E. 2010. Occurrence and chemical speciation analysis of organotin compounds in the environment: A review. *Talanta,* 82, 9-24.

OLSON, G. J. & BRINCKMAN, F. E. 1986. Biodegradation of tributyltin by Chesapeake Bay microorganisms. Gaithersburg: Surface Chemistry and Bioprocesses Group, National Bureau of Standards.

OMAE, I. 2003. Review: Organotin antifouling paints and their alternatives. *Applied Organometallic Chemistry,* 17, 81-105.

OSPAR COMMISSION 2006. Overview assessment: Implementation of PARCOM Recommendation 87/1 on the use of tributyl-tin compounds and PARCOM Recommendation 88/1 on measures to reduce organotin compounds reaching the aquatic environment through docking activities.

OSPAR COMMISSION 2008. Overview of contracting parties' national action levels for dredged material (2008 Update).

ÖSTBERG, T. L., JONSSON, A. P., BYLUND, D. & LUNDSTRÖM, U. S. 2007. The effects of carbon sources and micronutrients in fermented whey on the biodegradation of n-hexadecane in diesel fuel contaminated soil. *International Biodeterioration & Biodegradation,* 60, 334-341.

PANAGOULA, B., PANAYIOTA, M. & ILIOPOULOU-GEORGUDAKI, J. 2002. Acute toxicity of TBT and IRGAROL in *Artemia salina*. *International Journal of Toxicology,* 21, 231-233.

PIISPANEN, A. 2004. Vuosaari harbour project publication: Removal of tributyltin from the seabed in Vuosaari harbour. *In:* SUNIMENTO, M. & SUNIMENTO, T. O. (eds.). Helsinki.
PRESCOTT, L., HARLEY, J. & KLEIN, D. 2002. *Microbiology,* London, Mcgraw-Hill.
PUMURE, I., RENTON, J. J. & SMART, R. B. 2010. Ultrasonic extraction of arsenic and selenium from rocks associated with mountaintop removal/valley fills coal mining: Estimation of bioaccessible concentrations. *Chemosphere,* 78, 1295-1300.
QUEVAUVILLER, P., DONALD, O. F. X. & ETCHEBER, H. 1994. Butyltin distribution in a sediment core from Arcachon harbour (France). *Environmental Pollution,* 84, 89-92.
RADWAN, S. S. & AL-MUTEIRIE, A. S. 2001. Vitamin requirements of hydrocarbon-utilizing soil bacteria. *Microbiological Research,* 155, 301-307.
RADWAN, S. S., AL-MAILEM, D., EL-NEMR, I. & SALAMAH, S. 2000. Enhanced remediation of hydrocarbon contaminated desert soil fertilized with organic carbons. *International Biodeterioration & Biodegradation,* 46, 129-132.
RAJAN, S. S. 2003. *Microbial physiology,* New Dheli, Anmol Publications Pvt. Ltd.
REED, J., WALDOCK, M. J., JONES, B., BLAKE, S. J., ROBERTS, P., JONES, G., ELVERSON, C., HALL, S. & MURRAY, L. A. 2001. Remediation techniques applied to reduce paint-derived tributyltin (TBT) in dredged material. *In:* HINCHEE, R. E., PORTA, A. & PELLEI, M. (eds.) *The First International Conference on Remediation of Contaminated Sediments, Venice, Italy*. Battelle Press.
REIN, H. & JUNG, C. 1993. *Metabolic reactions: Mechanisms of substrate oxygenation,* London, Springer-Verlag.
REVATHI, P. & MUNUSWAMY, N. 2010. Effect of tributyltin on the early embryonic development in the freshwater prawn *Macrobrachium rosenbergii* (De Man). *Chemosphere,* 79, 922-927.
ROBERTS, D. P., MARTY, A. M., DERY, P. D., YUCEL, I. & HARTUNG, J. S. 1996. Amino acids as reduced carbon sources for *Enterobacter cloacae* during colonization of the spermospheres of crop plants. *Soil Biology & Biochemistry,* 28, 1015-1020.
RODRÍGUEZ, J. G., SOLAUN, O., LARRETA, J., BELZUNCE SEGARRA, M. J., FRANCO, J., IGNACIO GARCÍA ALONSO, J., SARIEGO, C., VALENCIA, V. & BORJA, A. 2010. Baseline of butyltin pollution in coastal sediments within the Basque Country (northern Spain), in 2007-2008. *Marine Pollution Bulletin,* 60, 139-151.
ROY, U. & CABRAL, L. J. 2002. Effect of succinate and glycerol on biodegradation of tributyltin chloride (TBTC). *In:* SETAC2002, Salt Lake City, Utah.
ROY, U., DUBEY, S. K. & BHOSLE, S. 2004. Tributyltin chloride-utilizing bacteria from marine ecosystem of west coast of India. *Current Science,* 86, 702-705.
RULKENS, W. 2005. Introduction to the treatment of polluted sediments. *Reviews in Environmental Science and Bio/Technology,* 4, 213-221.
SAKULTANTIMETHA, A., KEENAN, H. E., DYER, M., BEATTIE, T. K., BANGKEDPHOL, S. & SONGSASEN, A. 2009. Isolation of tributyltin-

degrading bacteria *Citrobacter braakii* and *Enterobacter cloacae* from butyltin-polluted sediment. *Journal of ASTM International,* 6, 369-377.
SALAZAR, M. H. 1986. Environmental significance and interpretation of organotin bioassays. *Oceans,* 18, 1240-1245.
SAMBROOK, J. & RUSSEL, D. W. 2001. *Molecular cloning: A laboratory manual,* New York, Cold spring habor laboratory press.
SARKAR, D., FERGUSON, M., DATTA, R. & BIRNBAUM, S. 2005. Bioremediation of petroleum hydrocarbons in contaminated soils: Comparison of biosolids addition, carbon supplementation, and monitored natural attenuation. *Environmental Pollution,* 136, 187-195.
SARTORIUS 2010. *VoluPAC™ reproducibly measures biomass of Saccharomyces cerevisiae cultures: Application notes* [Online]. Available: http://www.sartorius-stedim.com/Biotechnology/Lab_Technologies/Lab_Cell_Culture/Disposables/Application_Notes/Appl_VoluPac_SL-4053-e.pdf [Accessed 12 August 2010].
SCHNOOR, J. L. 1996. *Environmental modeling: Fate and transportation of pollutants in water, air and soil,* New York, John Wiley & Sons.
SCHUBERT, P., ROSENBERG, E. & GRASSERBAUER, M. 2000. Comparison of sodium tetraethylborate and sodium tetra(n-propyl)borate as derivatization reagent for the speciation of organotin and organolead compounds in water samples. *Fresenius' Journal of Analytical Chemistry,* 366, 356-360.
SCRAGG, A. 1999. *Environmental Biotechnology,* Essex, Longman.
SEGALL, M. 1997. *The P450 catalytic cycle* [Online]. Theory of Condensed Matter group, University of Cambridge. Available: http://www.tcm.phy.cam.ac.uk/~mds21/thesis/node49.html [Accessed 14 November 2008].
SEMPLE, K. T., MORRISS, W. J. & PATON, G. I. 2003. Bioavailability of hydrophobic organic contaminants in soils: Fundamental concepts and techniques for analysis. *European Journal of Soil Science*, 54, 809-818.
SEPA 2010. *Tributyltin compounds: Scottish pollution release inventory* [Online]. Available: http://apps.sepa.org.uk/spripa/Pages/SubstanceInformation.aspx?pid=167 [Accessed 09 July 2010].
SHIM, W. J., JEON, J. K., HONG, S. H., KIM, N. S., YIM, U. H., OH, J. R. & SHIN, Y. B. 2003. Accumulation of tributyltin in olive flounder, *Paralichthys olivaceus*: Its effect on hepatic cytochrome P450. *Archives of Environmental Contamination and Toxicology,* 44, 390-397.
SHIM, W. J., YIM, U. H., KIM, N. S., HONG, S. H., OH, J. R., JEON, J. K. & OKAMURA, H. 2005. Accumulation of butyl- and phenyltin compounds in starfish and bivalves from the coastal environment of Korea. *Environmental Pollution,* 133, 489-499.
SHIMASAKI, Y., OSHIMA, Y., INOUE, S., INOUE, Y., KANG, I. J., NAKAYAMA, K., IMOTO, H. & HONJO, T. 2006. Effect of tributyltin on reproduction in Japanese whiting, *Sillago japonica*. *Marine Environmental Research,* 62, S245-S248.
SHIMASAKI, Y., OSHIMA, Y., YOKOTA, Y., KITANO, T., NAKAO, M., KAWABATA, S., IMADA, N. & HONJO, T. 2002. Purification and

identification of a tributyltin-binding protein from serum of Japanese flounder, *Paralichthys olivaceus*. *Environmental Toxicology & Chemistry,* 21, 1229-1235.
SIGMA-ALDRICH 2007. *Tributyltin products* [Online]. Available: http://www.sigmaaldrich.com [Accessed 17 July 2010].
SINGH, A. & TURNER, A. 2009. Surfactant-induced mobilisation of trace metals from estuarine sediment: Implication for contaminant bioaccessibility and remediation. *Environmental Pollution,* 157, 646-653.
SINGH, K. & JOSHI, L. 1991. Analysis of organotin uptake in *Escherichia coli* K-12. *Ecotoxicology and Environmental Safety,* 21, 235-239.
SINGLETON, D. R., RICHARDSON, S. D. & AITKEN, M. D. 2008. Effects of enrichment with phthalate on polycylic aromatic hydrocarbon biodegradation in contaminated soil. *Biodegradation,* 19, 577-587.
SMITH, B. S. 1981. Male characteristics on female mud snails *Nassarius obsoletus* caused by antifouling bottom paints. *Journal of Applied Toxicology*, 1, 22-25.
SONAK, S., PANGAM, P., GIRIYAN, A. & HAWALDAR, K. 2009. Implications of the ban on organotins for protection of global coastal and marine ecology. *Journal of Environmental Management,* 90, S96-S108.
SOUSA, A., GÉNIO, L., MENDO, S. & BARROSOI, C. 2005. Comparison of the acute toxicity of tributyltin and copper to veliger larvae of *Nassarius reticulatus* (L.). *Applied Organometallic Chemistry*, 19**,** 324-328.
STÄB, J. A., TRAAS, T. P., STROOMBERG, G., VAN KESTEREN, J., LEONARDS, P., VAN HATTUM, B., BRINKMAN, U. A. T. & COFINO, W. P. 1996. Determination of organotin compounds in the food web of a shallow freshwater lake in the Netherlands. *Archives of Environmental Contamination and Toxicology,* 31, 319-328.
STANIER, R. Y., DOUDOROFF, M. & ADELBERG, E. A. 1970. *The microbial world,* NJ, Prentice-Hall.
STASINAKIS, A. S. & THOMAIDIS, N. S. 2010. Fate and biotransformation of metal and metalloid species in biological wastewater treatment processes. *Critical Reviews in Environmental Science and Technology,* 40, 307-364.
STASINAKIS, A. S., THOMAIDIS, N. S., NIKOLAOU, A. & KANTIFES, A. 2005. Aerobic biodegradation of organotin compounds in activated sludge batch reactors. *Environmental Pollution,* 134, 431-438.
SUDARYANTO, A., TAKAHASHI, S., IWATA, H., TANABE, S. & ISMAIL, A. 2004. Contamination of butyltin compounds in Malaysian marine environments. *Environmental Pollution,* 130, 347-358.
SUEHIRO, F., KOBAYASHI, T., NONAKA, L., TUYEN, B. C. & SUZUKI, S. 2006. Degradation of tributyltin in microcosm using Mekong River sediment. *Microbial Ecology,* 52, 19-25.
SUTHERSAN, S. S. 1999. *Remediation engineering : Design concepts,* Boca Raton, CRC Press LLC.
SUZUKI, S. & FUKAGAWA, T. 1995. Tributyltin-resistant marine bacteria: a summary of recent work. *Journal of Industrial Microbiology*, 14, 154-158.
SUZUKI, S., FUKAGAWA, T. & TAKAMA, K. 1992. Occurrence of tributyltin-tolerant bacteria in tributyltin-containing or cadmium-containing seawater. *Applied and Environmental Microbiology,* 58, 3410-3412.

SWACKHAMER, D. L. & SKOGLUND, R. S. 1993. Bioaccumulation of PCBs by algae - kinetics versus equilibrium. *Environmental Toxicology and Chemistry,* 12, 831-838.
SWINDOLL, C. M., AELION, C. M. & PFAENDER, F. K. 1988. Influence of inorganic and organic nutrients on aerobic biodegradation and on the adaptation response of subsurface microbial communities. *Applied and Environmental Microbiology,* 54, 212-217.
TAHA, H. M., SAID, H. A., ABBAS, N. H. & KHALEAFA, A. F. M. 2009. Biosorption and biodegradation of the antifouling compound tributyltin (TBT) by microalgae. *American-Eurasian Journal of Scientific Research,* 4, 1-6.
TAKAHASHI, S., MUKAI, H., TANABE, S., SAKAYAMA, K., MIYAZAKI, T. & MASUNO, H. 1999. Butyltin residues in livers of humans and wild terrestrial mammals and in plastic products. *Environmental Pollution,* 106, 213-218.
TAM, N. F. Y., CHONG, A. M. Y. & WONG, Y. S. 2002. Removal of tributyltin (TBT) by live and dead microalgal cells. *Marine Pollution Bulletin,* 45, 362-371.
TAM, N. F. Y., WONG, Y. S. & CHONG, A. M. Y. 2001. Toxic effect of tributyltin (TBT) on different green microalgal species. *In:* CHEN, F. & JIANG, Y. (eds.) *Algae and Their Biotechnological Potential.* Dordrecht, Springer.
TAN, K. H. 1998. *Principles of soil chemistry,* New York, Marcel Dekker, Inc.
TANG, C. H., HSU, C. H. & WANG, W. H. 2010. Butyltin accumulation in marine bivalves under field conditions in Taiwan. *Marine Environmental Research,* 70, 125-132.
TARIGHIAN, A., HILL, G. & LIN, Y. H. 2001. Lag phase model for transient growth of *Pseudomonas putida* on phenol. *The Canadian Journal of Chemical Engineering,* 79, 732-736.
THAKUR, I. S. 2006. *Environmental biotechnology: Basic concepts and applications,* New Dheli, I.K. International Pvt. Ltd.
THE NATIONAL ARCHIVES 1998. *The Surface Waters (Dangerous Substances) (Classification) (Scotland) Regulations 1998* [Online]. Available: http://www.legislation.gov.uk/uksi/1998/250/made [Accessed 08 October 2010].
THE SCOTTISH GOVERNMENT 2008. *ANNEX 9: Surface water quality - Environmental Quality Standards for priority substances and other dangerous substances* [Online]. Available: http://www.scotland.gov.uk/Publications/2008/06/26111138/16 [Accessed 25 August 2010].
TIANO, L., FEDELI, D., SANTONI, G., DAVIES, I. & FALCIONI, G. 2003. Effect of tributyltin on trout blood cells: Changes in mitochondrial morphology and functionality. *Biochimica Et Biophysica Acta-Molecular Cell Research,* 1640, 105-112.
TODAR, K. 2008. *The Growth of Bacterial Populations* [Online]. Available: http://www.textbookofbacteriology.net/growth_3.html [Accessed 9 August 2010].
TOKIWA, Y. & CALABIA, B. P. 2006. Biodegradability and biodegradation of poly(lactide). *Applied Microbiology and Biotechnology,* 72, 244-251.

TSANG, C. K., LAU, P. S., TAM, N. F. Y. & WONG, Y. S. 1999. Biodegradation capacity of tributyltin by two *Chlorella* species. *Environmental Pollution,* 105, 289-297.
UNEP 2000. Bibliography for a selected number of persistent toxic substances. *GEF: Regionally based assessment of persistent toxic substances.* United Nations Environment Programme.
UNGER, M. A. 1988. Sorption behaviour of tributyltin on estuarine and freshwater sediments. *Environmental Toxicology and Chemistry,* 7, 907-915.
UNITED NATIONS 2005. Inclusion of chemicals in Annex III of the Rotterdam Convention: Review of notifications of final regulatory actions to ban or severely restrict a chemical: Tributyl tin compounds. Geneva: Rotterdam Convention on the Prior Informed Consent Procedure for Certain Hazardous Chemicals and Pesticides in International Trade Chemical Review Committee.
USEPA 1997. Toxicological review: Tributyltin oxide. *Integrated Risk Information System.* Washington D.C.: U.S. Environmental Protection Agency.
USEPA 2005. Contaminated sediment remediation guidance for hazardous waste sites. U.S. Environmental Protection Agency.
USEPA 2008a. *In situ groundwater bioremediation* [Online]. U.S. Environmental Protection Agency. Available: http://www.epa.gov/swerust1/cat/INSITBIO.HTM [Accessed 24 April 2008].
USEPA 2008b. Revised production chemistry chapter for the tributyltin (DC20460). Washington, DC, USA: U.S. Environmental Protection Agency.
USEPA 2009. National Recommended Water Quality Criteria. Office of Water & Office of Science and Technology, U.S. Environmental Protection Agency.
USGS 2008. *Electron acceptor* [Online]. U.S. Geological Survey. Available: http://toxics.usgs.gov/definitions/electron_acceptor.html [Accessed 05 November 2008].
USGS 2009. *Impact of organic contaminants on the evolution of aquifer geochemistry* [Online]. Geological Survey. Available: http://water.usgs.gov/ogw/pubs/ofr0289/imc_organic.htm [Accessed 05 December 2010].
ÜVEGES, M., RODRÍGUEZ-GONZÁLEZ, P., GARCÍA ALONSO, J. I., SANZ-MEDEL, A. & FODOR, P. 2007. Isotope dilution analysis mass spectrometry for the routine measurement of butyltin compounds in marine environmental and biological samples. *Microchemical Journal,* 85, 115-121.
VAN DER KERK, G. J. M. 1975. Present status of the use of organotin compounds. *Chemiker-Zeitung*, 99, 26-32.
VELLA, A. J. & ADAMI, J. P. T. 2001. Preliminary evidence for in vitro methylation of tributyltin in a marine sediment. *Applied Organometallic Chemistry,* 15, 901-906.
VISOOTTIVISETH, P., KRUAWAN, K., BHUMIRATANA, A. & WILAIRAT, P. 1995. Isolation of bacterial culture capable of degrading triphenyltin pesticides. *Applied Organometallic Chemistry,* 9, 1-9.
VOGT, C., NOWAK, C., DIOGO, J. B., OETKEN, M., SCHWENK, K. & OEHLMANN, J. 2007. Multi-generation studies with *Chironomus riparius* -

effects of low tributyltin concentrations on life history parameters and genetic diversity. *Chemosphere,* 67, 2192-2200.
VOULVOULIS, N. & LESTER, J. N. 2006. Fate of organotins in sewage sludge during anaerobic digestion. *Science of the Total Environment,* 371, 373–382.
WILLIAMS, D. E., LECH, J. J. & BUHLER, D. R. 1998. Xenobiotics and xenoestrogens in fish: Modulation of cytochrome P450 and carcinogenesis. *Mutation Research,* 399, 179-192.
WORLD HEALTH ORGANIZATION 1990. Tributyltin compounds: Environmental health criteria 116. Geneva, Switzerland: United Nations Environment Programme - World Health Organization.
WORSZTYNOWICZ, A., KUCHARSKI, R., SAS-NOWOSIELSKA, A. & RZYCHOŃ, D. 2008. Fate and transport of chemical contaminants in soil: A general introduction. *In:* BARNES, I. & KHARYTONOV, M. M. (eds.) *Simulation and assessment of chemical processes in a multiphase environment*. Dordrecht, Springer.
WUERTZ, S., MILLER, C. E., PFISTER, R. M. & COONEY, J. J. 1991. Tributyltin-resistant bacteria from estuarine and freshwater sediments. *Applied and Environmental Microbiology,* 57, 2783-2789.
XIA, W. X., LI, J. C., ZHENG, X. L., BI, X. J. & SHAO, J. L. 2006. Enhanced bioremediation of diesel oil in seawater supplemented with nutrients. *Engineering in Life Sciences,* 6, 80-85.
YEBRA, D. M., KIIL, S. & DAM-JOHANSEN, K. 2004. Antifouling technology - past, present and future steps towards efficient and environmentally friendly antifouling coatings. *Progress in Organic Coatings,* 50, 75-104.
ZHANG, J. L., LI, R. X. & WANG, C. G. 2006. Effect of tributyltin exposure on growth and activities of ATPase in juvenile *Tilapia nilotica*. *Xiamen Daxue Xuebao (Ziran Kexue Ban),* 45, 692-695.
ZHANG, J. L., ZUO, Z. H., HE, C. Y., CAI, J. L., WANG, Y. Q., CHEN, Y. X. & WANG, C. G. 2009. Effect of tributyltin on testicular development in *Sebastiscus marmoratus* and the mechanism involved. *Environmental Toxicology and Chemistry,* 28, 1528-1535.

Appendices

Appendix A: Media for isolation of TBT-degrading microorganisms

1. Minimal medium (g/L):

Na_2HPO_4…… 15.0

KH_2PO_4……....3.0

NaCl…….0.5

NH_4Cl……...1.0

$MgCl_2$……..0.19

$CaCl_2$……...0.01

Trace elements solution……..1 ml

Agar powder (if required)…...15.0

Final pH 7.4 with TBTCl at 8 mg/L

Trace elements solution (g/100 ml):

H_3BO_3……11.4

$ZnSO_4.6H_2O$……....2.2

$MnCl_2.4H_2O$…….0.5

$FeSO_4.7H_2O$…….0.5

$CoCl_2.6H_2O$……0.16

$CuSO_4.5H_2O$……...0.16

$Na_2MoO_4.2H_2O$……..0.11

EDTA……...5.0

2. Glycerol medium (g/L):

K_2HPO_4…….1.0

KH_2PO4……1.0

$(NH_4)_2SO4$……....1.0

$MgCl_2$……0.4

Yeast extract……..0.5

Glycerol……...1 ml

Agar powder (if required)…...15.0

Final pH 6.8 with TBTCl at 55 mg/L (130µM)

Appendix B: API 20E Reading Table

Appendix Table B1 API 20E Reading Table.

Tests	Active ingredients	Reactions/Enzymes	Results	
			Negative	Positive
ONPG	2-nitrophenyl-βD-galactopyranoside	β-galactosidase (Ortho NitroPhenyl-βD-Galactopyranoside)	colourless	yellow (1)
ADH	L-arginine	Arginine dihydrolase	yellow	red/orange (2)
LDC	L-lysine	Lysine decarboxylase	yellow	red/orange (2)
ODC	L-ornithine	Ornithine decarboxylase	yellow	red/orange (2)
CIT	trisodium citrate	CITrate utilisation	pale green/ yellow	blue-green/ blue (3)
H2S	sodium thiosulphate	H_2S production	colourless/ greyish	black deposit/ thin line
URE	urea	UREase	yellow	red/orange (2)
TDA	L-tryptophane	Tryptophane DeAminase	TDA / immediate	
			yellow	reddish
IND	L-tryptophane	INDole production	JAMES / immediate	
			colourless	
VP	sodium pyruvate	acetoin production	pale green/ yellow	pink
		(Voges Proskauer)	VP1 + VP2 / 10 min	
			colourless	pink/red
GEL	gelatin (bovine origin)	GELatinase	no diffusion	diffusion of black pigment
GLU	D-glucose	fermentation / oxidation (GLUcose) (4)	blue/blue-green	yellow/greyish yellow
MAN	D-mannitol	fermentation / oxidation (MANitol) (4)	blue/blue-green	yellow
INO	inositol	fermentation / oxidation (INOsitol) (4)	blue/blue-green	yellow
SOR	D-sorbitol	fermentation / oxidation (Sorbitol) (4)	blue/blue-green	yellow
RHA	L-rhamnose	fermentation / oxidation (RHAmnose) (4)	blue/blue-green	yellow
SAC	D-sucrose	fermentation / oxidation (SACcharose) (4)	blue/blue-green	yellow
MEL	D-melibiose	fermentation / oxidation (MELibiose) (4)	blue/blue-green	yellow
AMY	amygdalin	fermentation / oxidation (AMYgdalin) (4)	blue/blue-green	yellow
ARA	L-arabinose	fermentation / oxidation (ARAbinose) (4)	blue/blue-green	yellow

(1) A very pale yellow should also be considered positive.
(2) An orange colour after 36-48 hours incubation must be considered negative.
(3) Reading made in the cupule (aerobic).
(4) Fermentation begins in the lower portion, oxidation begins in the cupule.
(5) A slightly pink colour after 10 minutes should be considered negative.

Appendix C: 16S gene sequencing of the isolated TBT-degraders with 27F primer

B1:CGGGGGATAACTACGCGAAAGCGTAGCTAATACCGCATACGCCCTACGGGGGAAAGCAGGGGATCGCAAG
ACCTTGCACTATTGGAGCGGCCGATATCGGATTAGCTAGTTGGTGGGGTAACGGCTCACCAAGGCGACGATCCGT
AGCTGGTTTGAGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGG
GAATTTTGGACAATGGGGGAAACCCTGATCCAGCCATCCCGCGTGTGCGATGAAGGCCTTCGGGTTGTAAAGCA
CTTTTGGCAGGAAAGAAACGTCATGGGCTAATACCCCGTGAAACTGACGGTACCTGCAGAATAAGCACCGGCTA
ACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCA
GGCGGTTCGGAAAGAAAGATGTGAAATCCCAGGGCTTAACCTTGGAACTGCATTTTTAACTACCGGGCTAGAGT
GTGTCAGAGGGAGGTGGAATTCCGCGTGTAGCAGTGAAATGCGTAGATATGCGGAGGAACACCGATGGCGAAG
GCAGCCTCCTGGGATAACACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGT
CCACGCCCTAAACGATGTCAACTAGCTGTTGGGGCCTTCGGGCCTTGGTAGCGCAGCTAACGCGTGAAGTTGACC
GCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGG
ATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGTCTGGAATGCCGAAAAGATTTGGCAGTGCTC
CCAAGAAAACTGGAACACAGGGG

B2:NNNGGTAGCTACCATGCAGTCGAACGGTAGCACAGAGGAGCTTGCTCCTTGGGTGACGAGTGGCGGACGG
GTGAGTAATGTCTGGGAAACTGCCCGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTC
GCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGG
TAACGGCTCACCTAGGCGACGATCCCTAGCAGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTC
CAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTA
TGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGAGGAGGAAGGTGTTGTGGTTAATAACCGCAGCAATTGA
CGTTACTCGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAAT
CGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGA
ACTGCATCCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAG
AGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGG
AGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTG
GCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACG
GGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCA
GAGAACTTAGCAGAGATGCTTTGGTGCCTTCGGGAACTCTGAAACA

B3-1:GAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGAAGGGGGATAACTACTGGAAACGGTA
GCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGG
GATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACA
CTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGA
TGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGTTGTGGTT
AATAACCGCAGCAAGTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGG
AGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCC
CCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGT
AGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCA
GGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGG
TTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAA
ACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAAAACCTT

ACCTACTCTTGACATCCAGAGAACTTAGCAGAGATGCTTTGGTGCCTTCGGGAACTTTGAAACAGGTGCTGCATG
GCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCC

B3-2:AGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACG
GCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGAC
TCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAG
AAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGTTGTGGTTAATAACCGCAGCAATTGACGTTAC
CCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATT
ACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCAT
TCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTG
GAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAAC
AGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCG
GAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCC
GCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACT
TAGCAGAGATGCTTTGGGGCCTTCGGGAACTCTGAAACAGGtGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAA
TGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGAACTCAAAGGAAA
CTGCCAGTGATAAACTGGGAGG

B4:GTAAAGCACTTAAAGTTGGGAGGAAGGGCAGTTACCTAATACGTGATTGTTTTGACGTTACCGACAGAATA
AGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTA
AAGCGCGCGTAGGTGGTTAGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGA
CTGACTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACC
AGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGAT
ACCCTGGTAGTCCACGCCGTAAACAATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTAGTGGCGCAGCTAACGC
ATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGG
TGGAGCATGTGGTTTAATTCGAAGCAACGCGAAAAACCTTACCAGGCCTTGACATCCAATGAACTTTCTAGAGAT
AGATTGGTGCCTTCGGGAACATTGAAACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTA
AGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCCCGTAATGGGGGGCACTCTAAGGAAACTGCCGGTG
ACAAACCGGAAGAAGGGGGGGATGAAA

B5:AGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGG
GCCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCT
AGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGG
GAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTA
CTTTCAGCGGGGAGGAAGGTGTTGTGGTTAATAACCGCAGCAATTGACGTTACCCGCAGAAGAAGCACCGGCTA
ACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCA
GGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGT
CTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGG
CGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC
CACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACC
GCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGG
TTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTAGCAGAAATGCTTTGGTGCCT
TCGGGAACTCTGAAACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAA

Appendix D: Calculation of limit of detection (LOD)

Appendix Table D1 Calculation data of LOD for TBT determined by GC-MS.

Concentration (mg/L)	Peak area of TBT over peak area of internal standard, y_i	Signal from regression line, [$\hat{y}$] $\hat{y}$ = 0.0131X + 0.0176 R^2 = 0.9987	y-residuals $[y_i - \hat{y}]^2$
10	0.1544	0.1486	3.35×10^{-5}
20	0.2701	0.2796	9.10×10^{-5}
30	0.4156	0.4106	2.50×10^{-5}
40	0.5338	0.5416	6.12×10^{-5}
50	0.6767	0.6726	1.67×10^{-5}

Calculation

To obtain the calculation, substitute a and $S_{y/x}$ in LOD signal = a + $3S_{y/x}$

When: a is intercept of regression line

$S_{y/x}$ is $\sqrt{\frac{\Sigma[y_i - \hat{y}]^2}{n-2}}$, n = 5

Therefore: LOD signal = (0.0176) + 3(0.008709334)

= 0.043728001

From the formula of regression line is Y = 0.0131X + 0.0176

When: Y = LOD signal, and X = LOD

Therefore: LOD = 1.99 mg/L

Appendix Table D2 Calculation data of LOD for DBT determined by GC-MS.

Concentration (mg/L)	Peak area of DBT over peak area of internal standard, y_i	Signal from regression line, [$\hat{y}$] $\hat{y} = 0.01X + 0.0044$ $R^2 = 0.9975$	y-residuals $[y_i - \hat{y}]^2$
2	0.0242	0.02440	5.54×10^{-8}
4	0.0443	0.04440	4.00×10^{-9}
6	0.0664	0.06440	4.03×10^{-6}
8	0.0822	0.08440	4.78×10^{-6}
10	0.1055	0.10440	1.24×10^{-6}

Calculation

To obtain the calculation, substitute a and $S_{y/x}$ in LOD signal = $a + 3S_{y/x}$

When: a is intercept of regression line

$S_{y/x}$ is $\sqrt{\frac{\sum[y_i - \hat{y}]^2}{n-2}}$, n = 5

Therefore: LOD signal = (0.0044) + 3(0.001835483)

= 0.009906449

From the formula of regression line is Y = 0.01X + 0.0044

When: Y = LOD signal, and X = LOD

Therefore: LOD = 0.55 mg/L

Appendix Table D3 Calculation data of LOD for MBT determined by GC-MS.

Concentration (mg/L)	Peak area of MBT over peak area of internal standard, y_i	Signal from regression line, [$\hat{y}$] $\hat{y}$ = 0.0143X + 0.0038 R^2 = 0.9934	y-residuals $[y_i - \hat{y}]^2$
2	0.0324	0.0325	1.06×10^{-8}
4	0.0571	0.0611	1.62×10^{-5}
6	0.0955	0.0897	3.32×10^{-5}
8	0.1183	0.1183	4.00×10^{-8}
10	0.1447	0.1469	5.00×10^{-6}

Calculation

To obtain the calculation, substitute a and $S_{y/x}$ in LOD signal = a + $3S_{y/x}$

When: a is intercept of regression line

$S_{y/x}$ is $\sqrt{\frac{\sum[y_i - \hat{y}]^2}{n-2}}$, n = 5

Therefore: LOD signal = (0.0038) + 3(0.004261801)

= 0.016685403

From the formula of regression line is Y = 0.0143X + 0.0038

When: Y = LOD signal, and X = LOD

Therefore: LOD = 0.89 mg/L

Appendix E: Calculation of weight of carbon sources for biomass determination

Glycerol medium containing 1 ml of glycerol/L (d 1.26 g/ml) gives 1.26 g of glycerol/L.

Molecular weight of glycerol ($C_3H_5(OH)_3$) is 92.09 g/mol, and of carbon is 12.011 g.

Therefore; $$\%C = \frac{12.011 \times 3}{92.09} \times 100$$

$$= 36.03\ \%$$

Then 1.26 g contains; $$C = \frac{1.26}{100} \times 36.03$$

$$= 0.49\ g$$

To obtain 0.49 g of carbon; $$X = \frac{0.49}{\%C} \times 100$$

Where %C is %carbon, and X is the weight of the carbon sources required.

Appendix Table E1 Data of the carbon sources for biomass determination.

Carbon supplement	Molecular weight (g/mol)	Carbon weight (g/mol)	%C	Weight/volume per litre
Tween 80 (d 1.06 g/ml)	1310	768.70	58.68	0.783 ml
Lauryl sulphate (SLS)	288.38	144.13	49.98	0.980 g
Salicylate	160.11	84.08	52.51	0.933 g
Acetate	82.03	24.02	29.28	1.673 g
Citrate	294.10	72.07	24.50	2.000 g
Succinate	270.00	48.04	17.79	2.754 g
Tartrate	230.08	48.04	20.88	2.347 g
Glucose	198.17	72.07	36.37	1.347 g
Sucrose	342.3	144.13	42.11	1.164 g
Xylose	150.13	60.06	40.00	1.225 g

Appendix F: Bacterial growths of SED and B3 for screening of pH and temperature for the study of their effect to degradation

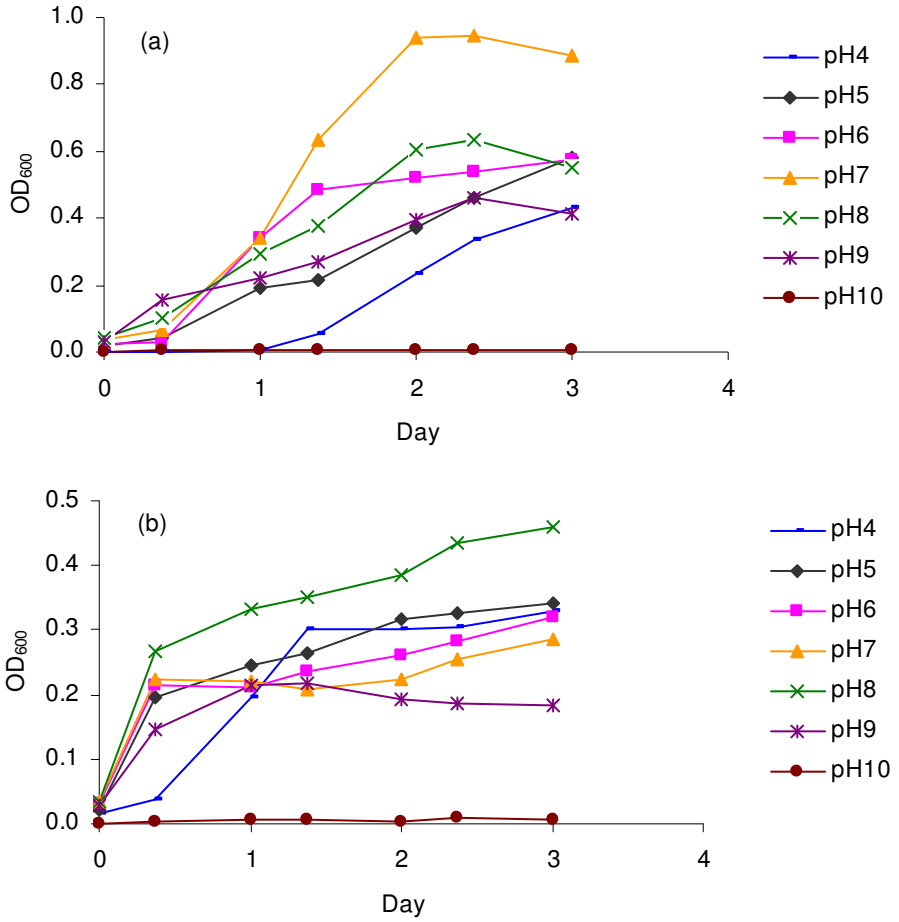


Figure F1 Growth curves showing OD_{600} of (a) SED; and (b) B3 in glycerol medium at different pH during 3 days of incubation.

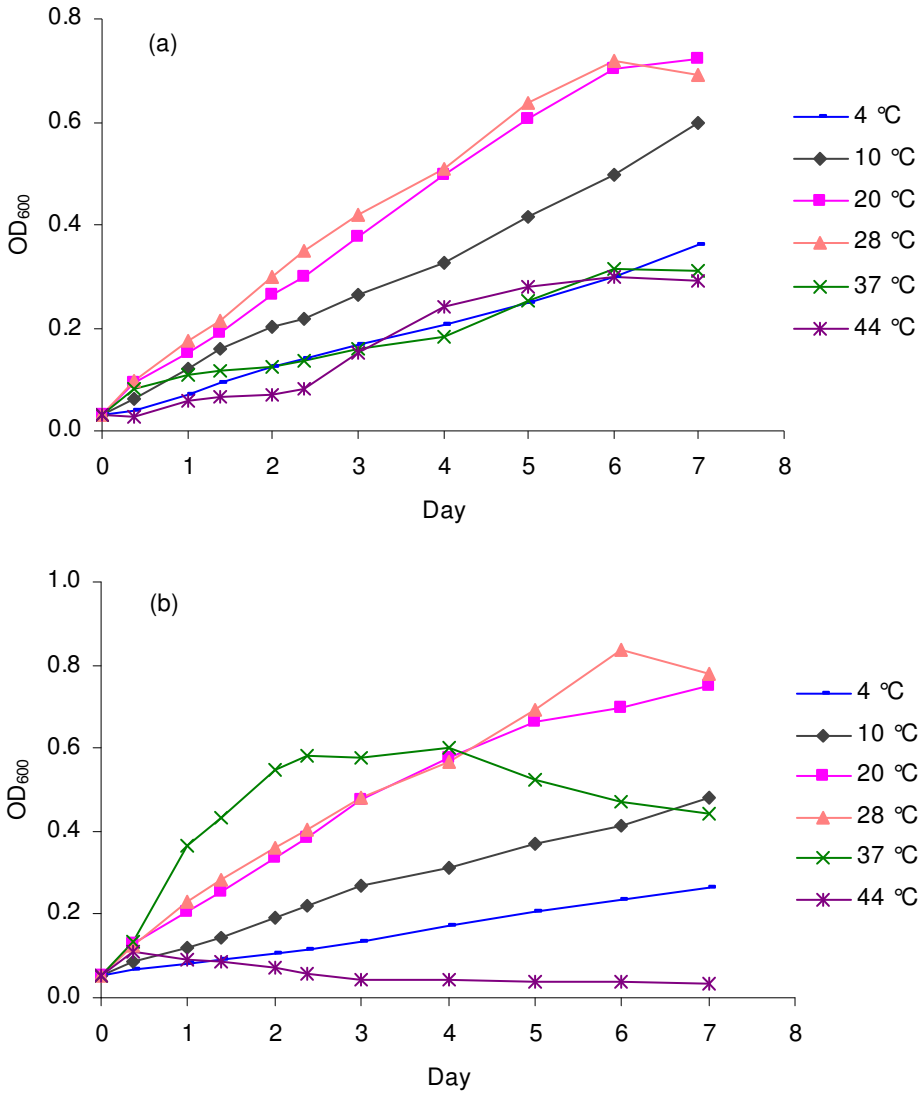


Figure F2 Growth curves showing OD_{600} of (a) SED; and (b) B3 in glycerol medium at varying temperatures during 7 days of incubation.