Understanding tributyltin biodegradation in muddy sediment for the adaptation of an anti-siltation technique with bioremediation potential

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Contents

List of tables

List of figures

Abstract

Sediment siltation, the accumulation of sediment in navigable ways in ports, harbours and channels is a topic of concern for the management of these areas of high economic importance. Dredging, the conventional method to tackle siltation, has long been criticised for its high cost and detrimental environmental effects. Active Nautical Depth (AND) is one of the techniques which can be used in muddy ports and harbours to reduce dredging need and mitigate the environmental impact and cost of sediment management. The application of this method results in aeration of sediment and the proliferation of aerobic microorganisms. Many aerobes are known to degrade harmful contaminants commonly found in these highly polluted environments and therefore the application of AND could have a dual advantage of resolving siltation and contamination. The aim of this thesis is to evaluate the potential of AND to be used as a bioremediation technique, using tributyltin (TBT) as a model contaminant. The factors influencing TBT biodegradation in sediment were investigated in microcosm experiments, designed under different environmental scenarios. The variables used in the microcosms were temperature, agitation, aeration, mud type and time.

To explore the microbial community involved in TBT biodegradation, the 16S rRNA genes of total bacteria in different microcosms were sequenced and community analyses were performed. Isolation of TBT-degrading and TBT-resistant bacteria was evaluated by comparing two methods of isolation and cultivation (standard plating and iChip). iChip greatly enhanced the success of cultivation of sediment bacteria. Several TBT-degrading and TBT-resistant isolates could be maintained in full laboratory conditions. Only *Pseudomonas* were able to use TBT as sole carbon source but members of *Oceanisphaera* were reported for the first time as TBT-resistant bacteria.

The microbial community analyses highlighted a dominance of sulphate reducers and sulphide oxidisers in the sampled sediment, which likely persisted after aeration, spiking of

TBT and incubation of the microcosms. This study also reports for the first time the biodegradation of TBT at 4°C. At this temperature, biodegradation led to the accumulation of dibutyltin (which is also toxic), but it was degraded at 15°C. TBT biodegradation appeared to be inhibited by carbon and nitrogen amendment. This study suggests an applicability of AND throughout the year in temperate climates, including late winter when water temperatures are low. This contrasts with other studies which reported TBT degradation at warmer temperatures of up to 25-28°C. The parameters controlling TBT degradation are complex and field trials would be necessary to confirm and optimize the application of AND to a specific location before it can be adopted in the maritime industry as a bioremediation tool.

Declaration

I declare that that no portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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1. Introduction

1.1. Background

Two major and fundamental problems in ports and harbours are sediment contamination and sediment siltation. Port siltation is the accumulation of sediment in the navigable ways, and without intervention, would lead to significant problems for the safe passage of marine traffic. It is estimated that thousands of million tons of sediment are dredged each year (Bianchini et al., 2019), dredging being the conventional method to manage siltation. In parallel, ports and harbours are known to be extremely contaminated environments, and marine sediment is considered one of the main sources of contamination for the aquatic food chain (Lehoux et al., 2020). These two separate but inter-related fields both require technological innovation in order to reduce the costs and environmental impact of the maritime industry. The focus of this thesis is on a new technique developed to replace or reduce dredging, and which could have a dual benefit of also contributing to sediment remediation.

This technique, called Active Nautical Depth, consists of mixing and aerating muddy sediment *in situ* to fluidise the mud and make it navigable (so increasing under-keel clearance and navigable depth)(Kirby et al., 2008). The aeration promotes the growth of aerobic micro-organisms which then produce large quantities of extracellular polymeric substance (EPS). EPS production is a key to the sustainability of AND as it delays the mud reconsolidation, and the sediment thus remains navigable for longer (Kirby, 2011). The aeration is also of interest because numerous contaminants present in sediment are known to be biodegraded under aerobic conditions. Therefore, it is logical to consider the dual purpose of using AND to manage siltation with the additional benefit of eliminating contaminants that are common in highly polluted port and harbour environments.

AND is implemented by SEMASO, a company offering sediment management solutions, and who are partly funding this study. As context, SEMASO are seeking to investigate the

potential of applying AND in Liverpool Docks and to establish the bioremediation potential of the method through further research.

Among the list of aerobically biodegraded sediment contaminants, tributyltin (TBT) was chosen as a model for this study. TBT is a very toxic biocide that was previously used in antifouling paints (Ayanda et al., 2012). It is very persistent in anoxic sediment and is considered a legacy contaminant and remains a contemporary problem despite having been subjected to a global ban in 2008 (Egardt et al., 2017).

1.2. Scope and objectives

The overall aim of this study is to investigate the potential of AND for the bioremediation of TBT.

The two main objectives are to:

1) Determine the abiotic factors influencing TBT degradation by the native microbial community of sediment from Liverpool Dock.
2) Establish the key microbial communities associated with TBT resistance and biodegradation.

To fulfil the first objective, microcosm experiments were designed under different physical and environmental conditions of temperature, agitation, aeration, in septic and aseptic conditions and with carbon and nitrogen supplementation.

For the second objective, several approaches were applied. First, isolation, cultivation and identification of TBT-resistant and TBT-degrading bacteria were approached by standard plating and through the application of an alternative technique of isolation called the iChip. The microbial community present within the sediment was assessed through sampling over time and at different time of the microcosms.

1.3. Organisation of the thesis

This thesis has seven chapters with chapters 4 to 7 detailing the results and discussion arising from the experimental work conducted to achieve the above objectives. A list of references is provided at the end of the thesis.

Chapter 2 provides a literature review of the three mains thematic of this study: (i) sediment management, (ii) port contamination and remediation and especially TBT bioremediation, and (iii) sediment microbial communities.

Chapter 3 details the materials and methods used for the experimental work. It details in particular the methodological development required for the accurate measurement of organotin.

Chapter 4 provides the results and discussion of the microcosm experiments conducted to better understand the factors influencing TBT biodegradation in sediment.

Chapter 5 discusses the results of the microbial community analyses after 16S rRNA gene next generation sequencing of different sediment samples derived from the microcosm experiments.

Chapter 6 is a manuscript that was submitted to a peer reviewed journal and describes the use of two methods of isolation (standard plating and iChip) to cultivate TBT-resistant and TBT-degrading bacteria.

Chapter 7 summarizes the findings and provides a general discussion and conclusion on the advanced knowledge of TBT biodegradation in sediment and the applicability of AND as a bioremediation method. It also provides perspectives for future work.

2. Literature Review

2.1. Sediment management

2.1.1. Dredging

Most ports and harbours in the world experience siltation problems that have hindered ship navigation since ancient times. From Ancient Egypt, workers used to manually drag mud until the method improved when the first dredging machine was developed in 1796 (Knight and Lacey, 1843). Dredging consists of the excavation of the sediment from the target site, followed by its transport and disposal in a designated area, normally offshore by licence. Both the excavation and the disposal are strictly regulated and subject to legislation aimed at minimising environmental impact, especially because of the potential presence of harmful chemical contaminants. In England, the Marine Management Organisation (MMO) is the licencing authority for dredge disposal sites and operate under OSPAR[1] commission's guidelines (OSPAR, 2004).

2.1.1.1. Environmental impact of dredging

The negative impacts of dredging comprise effects related to the excavation method itself (locally) and to the impact of contaminated sediment manipulation (more widely). These effects can be categorized into three types: physical, chemical, and biological impacts, and are discussed below in relation to the dredging of non-contaminated and contaminated sediment.

When dredging non-contaminated sediment, different problems can be encountered. First, an increase in turbidity takes place at the excavation site and at the disposal site in the sea, which can affect photosynthetic activity. This can result in the widespread loss of seagrass vegetation (Erftemeijer and Lewis, 2006), a reduction in the production of phytoplankton

[1] From the unification and extension in 1992 of the OSlo and PARis conventions which occurred respectively in 1972 and 1974

and can also affect fish or membrane-feeding organisms through the clogging of gills and membranes (Balchand and Rasheed, 2000). A lethal effect on corals caused by turbidity and sedimentation at the disposal site has also been shown (Erftemeijer et al., 2012).

During the excavation, an abundance of nutrients is released into the water column. This causes a strong perturbation to the ecosystem, which can have an impact on the macrobenthic fauna by causing the population of native organisms to decrease in number (Ponti et al., 2009). The habitat is also modified during the process, with a change of the seabed surface at the excavation site and a potential change in sediment properties at the disposal site due to the import of non-local material. These changes can affect the ability of the benthic fauna to recover after the dredging perturbation (Cooper et al., 2011).

The acoustic impact must be considered too, the noise produced by dredging can be as high as 170-190 dB re 1 μPa^2m^2 at 50 Hz (Todd et al., 2015). These levels are thought to be too low to provoke physical damage to animals but they can induce stress, which may hinder their reproduction, modify their foraging behaviour and could have other detrimental consequences on their survival, for example, through diseases induced by toxin production (Pirotta et al., 2013; Todd et al., 2015). The overall consequence of these phenomena is a decrease in benthic faunal diversity after dredging operations (Barrio Froján et al., 2011; Kenny and Rees, 1996). q

In addition, the removal of sediment from the coastal system has a strong impact on the surrounding physical environment, leading to long-term changes to the adjacent shoreline indirectly through modifications of wave patterns and directly via the filling of the excavation hole by sediment transported from the beach (Demir Hüseyin et al., 2004). A secondary impact of dredging is the emission of greenhouse gas that occurs mainly during the transportation phase but also during the excavation itself. It has been estimated that dredging activities could release between 6.5 and 11.7 kg CO_2 per ton of dredged sediment (Bianchini et al., 2019).

For the dredging of contaminated sediment, the negative effects increase significantly (Manap and Voulvoulis, 2015). The resuspension of sediment during the excavation can result in the release of contaminants around the excavation site (Munawar et al., 1989) and the excavation exposes a new layer of potentially highly contaminated sediment. Some of

these contaminants, such as heavy metals, may even become more toxic after resuspension through an oxidation process (Roberts, 2012). The biological impact of contaminant exposure comprises three types: the organisms living in the sediment (benthic fauna), pelagic organisms (fish and plankton) and consumers (fish, birds, mammals and even humans) (Bridges et al., 2010). Strong increases in the bioavailability (Eggleton and Thomas, 2004) and bioaccumulation of contaminants have been reported after dredging activities (Hedge et al., 2009; Martins et al., 2012; Winger et al., 2000), which leads to the spreading of these toxic compounds through the entire food chain.

2.1.1.2. Regulation

In recognition of the significant environmental impacts of dredging, a range of rules and regulations have been implemented at local, national, and international level with the aim to control and reduce the negative effects of this process. Firstly, restrictions have been put in place by the London Convention (IMO, 1972) that "*prohibits the dumping of certain hazardous materials in the sea and requires a prior special permit for the dumping of a number of other identified materials and a prior general permit for other wastes or matters*". Several international convention agreements have followed (Abriak et al., 2006) and consequently, laws and directives have been created across the world with obligatory procedures in place before dredging is authorised. These include evaluation of sediment contamination, framing of contaminated sediment disposal and remediation, justification of dredging methods used and requirements for the follow-up monitoring of the dredged site.

The EU directives do not address dredging directly, but some of them have an impact on dredging projects although international conventions and guidelines prevail on EU law, which limits especially their impact on marine dredging (Mink et al., 2006). Mainly, the EU's Waste Framework Directives deal with the management of dredged sediment while the Habitat and Birds Directives have indirect consequences on dredging projects which are located near protected sites, forcing higher monitoring requirements and increasing their cost (Mink et al., 2006).

For the management of dredged sediment specifically, several disposal or recycling options are given depending on the physicochemical condition of the sediment, especially its

contamination state. For uncontaminated sediment, a beneficial use is usually targeted. Possible disposal solutions include sea deposit, using the sediment to support sediment-based habitats, shorelines and infrastructures, for habitat restoration such as wetlands, coastal features, beaches or even engineering use for example as capping material (OSPAR Commission, 2014), capping being a remediation methods consisting of physically isolating contaminated sediment from the surrounding water by the addition of clean layers of geologic materials and/or synthetic liners.

For contaminated sediment however the re-use is strictly regulated, and conventional options can only be considered after a decontamination treatment if the sediment then meets the specific requirements. If sufficient remediation cannot be achieved, contaminated sediment can be disposed in a Contained Disposal Facility (CDF), a Contained Aquatic Disposal (CAD) or most often at a landfill site. Such a disposal is very expensive and usually constitutes the main part of a dredging project's budget (Palermo and Hays, 2014).

In parallel to the implementation of restrictive laws, effort has been made to develop tools and methods of management to match the new regulations (Cooper, 2013). Different organisms such as the Central European Association (CEDA) or the Permanent International Association of Navigation Congresses (PIANC) provide resources for the selection of dredged-sediment management solutions. For the North East Atlantic, "*Guidelines for the Management of Dredged Material at Sea*" are described by OSPAR, with the most updated version dated from 2014 (OSPAR Commission, 2014). For dredging projects in general a wide range of concepts and decision-making frameworks have been proposed (Bates et al., 2015; Manap and Voulvoulis, 2014; Palermo et al., 2008) in an attempt to limit and reduce the detrimental environmental consequences. The complex legislation and the negative public perception of dredging make managing the process a challenge (Cutroneo et al., 2014; Hamburger, 2002). Conflicts can appear between the different stakeholders and projects are consequently subjected to delays or cancellation.

A further significant issue with dredging is its high cost, comprising the cost for the operation and the cost for the disposal. The cost can vary depending on the technology and equipment used, as well as the volume of sediment targeted, the distance to the disposal site and the presence of contaminants. Moreover, since ports and harbours are adapting

to enable the entry of larger vessels, the need for dredging increases in consequence and so does the associated cost (Kirby, 2011; Manap and Voulvoulis, 2015).

2.1.1.3. Port contamination and sediment remediation

Port and harbour activities generate many types of pollution: sewage and wastewater, petroleum and its derivatives, greenhouse gas emission and release of compounds from antifouling paints. The multiple sources of contamination and the usual enclosed configuration of ports and harbours result in limited circulation leading to high levels of contaminant accumulation in sediments and subsequent negative impact to aquatic life due to their toxicity. The presence of contaminants usually damages the ecosystem locally by affecting the development, reproduction and survival of many indigenous species. There are countless examples of evidence for the toxicity of pollutants found in ports and harbours. Tributyltin (TBT) is well-known for its endocrine disruptive action, first discovered by the appearance of malformations leading to the decrease in oyster populations. This caused severe problems to the oyster production market of the Arcachon Bay in France in the 70's (Alzieu, 2000). Since then, knowledge of TBT's high toxicity has increased and it is usually stated to be the most toxic substance deliberately delivered into the aquatic environment (more details on its toxicity are given in Section 2.2.3). Heavy metals also exert their toxicity in various organisms, by damaging tissue and DNA leading to numerous problems like growth inhibition, deformities or reduced fertility (Sharifuzzaman et al., 2016). In addition to their local impact, several contaminants, like PAHs, heavy metals, and organotin compounds, are known to bioaccumulate, which means that they can be transported along the food chain, affecting a wide range of organisms and can ultimately potentially be toxic towards humans (de Carvalho Oliveira and Santelli, 2010; Nikolaou et al., 2009; Sharifuzzaman et al., 2016).

Some of this pollution causes reversible damage when the contaminants degrade rapidly after introduction into the environment. Such pollutants are defined as non-persistent, which is the case for fertilizers, domestic sewage, or non-persistent pesticides. On the contrary, other contaminants are called persistent, because the damage that they cause is either irreversible or persists over a very long period. The main contaminants persisting in sediment are organotin compounds (OTCs), heavy metals, polychlorinated biphenyl (PCB) and polycyclic aromatic hydrocarbons (PAHs).

The vast majority of the methods designed for the remediation of contaminated sediment involve its dredging and placement *ex-situ* followed by a designated treatment. Most of the available treatments are physical and chemical. Thermal treatment such as incineration, as an example of physical treatment, is often used because of its efficiency but it consumes a lot of energy and has a high cost (Du et al., 2014). A classic chemical treatment is chemical oxidation, which uses oxidants such as Fenton's reagent, potassium permanganate or hydrogen peroxide to break down contaminants. It has been suggested however that incomplete reactions or side reactions may occur during chemical treatments, leading to the release of other potentially toxic compounds (Ferrarese et al., 2008; Finnegan et al., 2018).

Efforts have been made to find more environmentally friendly and cost-effective ways for the remediation of dredged contaminated sediment and bioremediation is an encouraging process in this regard. Bioremediation consists of the degradation of a contaminant as a result of the activity of a living organism. It usually involves contaminant breakdown by microorganisms (biodegradation) or by plants (phytoremediation). Bioremediation has been applied successfully as an *ex-situ* treatment for contaminated sediment (Chikere et al., 2016; Novak and Trapp, 2005; Rocchetti et al., 2014; Wu et al., 2014). For example, the remediation of crude-oil contaminated sediment has been achieved by biodegradation in bioreactors under different conditions with a maximum removal rate of 97.2% for a bioreactor biostimulated with fertilizers (Chikere et al., 2016).

A study conducted field trials using *Typha angustifolia* colonized dredged sediment and evaluated the impact of aeration on the phytoremediation of heavy metals. The aerated sediment was found to produce more plant biomass and therefore gave the best results in terms of metal removal even if waterlogged sediment was promoting plant metal uptake (Wu et al., 2014). It should be noted here that for the methods using phytoextraction, where an uptake of the contaminant is expected by plants, a risk of transmission by herbivores along the food chain exists. Other phytoremediation methods rely on the positive influence of plants on the microbial degrading activities in the surrounding environment, without any uptake of the contaminant by the plants. Bioremediation represents a low-cost method with few environmental impacts. Used *ex-situ*, however, it is still associated with the negative effects of dredging described above (*e.g.* strong

environmental impact, complex legislation, high cost) and remains unsustainable as the sediment is removed from its initial location. Consequently, developing *in-situ* solutions that do not require dredging for the remediation of contaminated sediment are most desirable.

A couple of options have been proposed for *in-situ* bioremediation of contaminated sediment, the simplest one being natural attenuation, which consists of leaving the environment to decontaminate itself and only monitoring the progress of degradation (Lofrano et al., 2017). Natural attenuation is usually a slow process and can be applied for low-risk contaminants. Biostimulation and bioaugmentation have therefore been used to speed the process of natural attenuation (see Section 2.2.4). Other innovative techniques have been proposed, often hybrids between physical, chemical and biological treatment, including for example, reactive capping, reactive barriers, or bioelectrochemical removal (Lofrano et al., 2017; Majone et al., 2015).

Recently, a field trial reported the successful use of immobilized microbial activated beads for the *in-situ* remediation of river sediment aiming at reducing nitrogen and organic carbon pollution (Fu et al., 2018). This study, however, represents an exception. Commonly, reviews of *in-situ* bioremediation cite the lack of application of the proposed methods, which are rarely brought to field trials, with the techniques only showing potential promise (Lofrano et al., 2017; Majone et al., 2015). This lack of application can be explained by several factors. There is a lack of consensus for the use of *in situ* bioremediation, due to uncertainty about the effectiveness, control and possible secondary effects (Majone et al., 2015). A need for the development of biomolecular tools for site investigation has also been emphasised (Majone et al., 2015). More research is consequently needed to overcome these barriers.

2.1.2. Alternative methods

Considering the environmental impact, the high cost, the constraining legislation and the conflicts related to dredging, research has been done to find alternatives (Kirby, 2011). Most alternatives found could be defined as “anti-siltation methods”, as they are designed to prevent sediment from accumulating in the targeted area. The major advantage of this kind of method is that a big part of the issue disappears, since there is no need for disposal

and no need for a dredging licence, although all sediment management projects are subject to approval.

2.1.2.1. Overview of different alternatives to dredging

The 43rd PIANC working group reviewed the different methods used as an alternative to dredging for sediment management in ports and harbours (Kirby, 2011). They categorised the techniques into three groups: Keep Sediment Moving (KSM), Keep Sediment Out (KSO) and Keep Sediment Navigable (KSN), also grouped as "sand by-passing plants", "anti-sedimentation structures" and "remobilising sediment systems" in a more recent review (Bianchini et al., 2019). A wide range of techniques have been created to adapt to specific situations but can nevertheless serve as useful examples, however, some of them can be considered as generic and they could be applied to different harbour configurations.

Keeping sediment out usually involves the design of structures that will physically prevent siltation by altering the effect of waves, currents, and sand movement. These structures have been stated to be less efficient for cohesive clay (Bianchini et al., 2019). Anti-sedimentation structures have been well described and comprise, sand traps, seawalls, current deflection walls, or even pile groynes (Bianchini et al., 2019; Kirby, 2011). It should be noted that these structures can potentially have negative impacts on the surrounding environment if they are not designed carefully. Similar to dredging, their modification of wave patterns can impact the shoreline and have a negative impact on the wildlife (Bianchini et al., 2019).

The second group of techniques, KSM, regroups the two categories called "sand by-passing plants" and "remobilising sediment systems" by Bianchini *et al* (2019). Sand by-passing plants consist of constantly transferring the sediment out of the channels, therefore preventing siltation in contrast to dredging which happens after siltation has occurred (Bianchini et al., 2019; Kirby, 2011). The physical transfer of sediment is performed through different pumping systems, which are adapted to port configurations (Bianchini et al., 2019). In Leer for example, slopes were created in the docks, so that gravity naturally leads the sediment to flow in a collection sump were an underwater pump collects it and discharges it into the estuary (Kirby, 2013, 2011). Remobilising systems, however, involve the resuspension of the sediment in order to put it back into the current for its evacuation

from the blocked areas. The most well-known method is water injection dredging, which uses a water-jet towards the seabed to create a density current which picks-up the sediment and takes it to a lower point (Bianchini et al., 2019).

The last category described in Kirby's 2011 review2011), KSN, is similar to the remobilising systems but differs in the point that it does not aim at evacuating the sediment from the port or harbours, but instead counts on the fact that some types of sediment are navigable when brought into suspension as a low density fluid (Kirby et al., 2008; Welp and Tubman, 2017). Keep sediment navigable plays around the concept of nautical depth and mostly involves the promising method called Active Nautical Depth (AND). It is a method emerging from the concept of "Passive Nautical Depth", which is a new way to define the depth in ports and harbours, using density parameters. AND derives from this new concept by the fact that fluid mud is created *in situ* by mixing and aerating the mud at the bottom of the sea which makes it navigable and therefore increases the nautical depth and under-keel clearance for ships. AND is the focus of investigation in this thesis and is discussed in more detail in the section 2.1.2.2.

2.1.2.2. Focus on Passive and Active Nautical Depth: Principles

The application of the concept, Passive Nautical Depth, has been one of the first steps implemented by ports and harbours around to world to reduce dredging need. It consists of changing the criteria defining the nautical bottom. The nautical bottom is defined as the level at which the physical characteristics of the bottom can cause either damage or unacceptable effects on controllability and manoeuvrability by contact with a ship's keel (Kirby, 2011; McAnally W. H. et al., 2016). Before the application of this concept, the depth was measured with a fathometer, which records the time for a sound pulse to be reflected from the bottom and back to the device. Depending on the rheological parameters (*e.g.* density, viscosity, *etc*.) of the sea bottom (especially in muddy bays and estuaries), the fathometer generates ghost echoes that can either be associated with a solid bed or with fluid mud that would be navigable. None of the instruments used are able to differentiate ghost echoes from real solid bed (McAnally et al., 2007). By precaution, ghost echoes are always considered to be associated with solid bed, which leads to a potentially unnecessary dredging of the fluid mud, resulting in a waste of money and additional pollution that could be avoided.

During Passive Nautical Depth, the depth should be defined by the parameters that permit discrimination between solid bed and fluid mud. The density criterion is generally used but density alone is not sufficient. Other parameters, such as shear stress, should be considered to establish whether the mud is fluid enough to be navigable (Wurpts, 2005). These parameters, however, are not easy to record routinely and different particle size arrangements (which are locally variable) also influence density, shear strength and therefore navigability. As a consequence, for each port the density at which the sediment is in a fluid mud state has to be determined. In muddy ports with low sand content the most often used density threshold is 1,200 $kg.m^{-3}$ (Welp and Tubman, 2017). The concept of Passive Nautical Depth is now widely used in the world's ports and harbours and permit reduced dredging use (McAnally W. H. et al., 2016). Nevertheless, it does not deal with the issue of chemical contaminants.

By derivation of the concept of Passive Nautical Depth, a new method to manage sediment in muddy ports and harbours has been developed, called Active Nautical Depth (Kirby et al., 2008; McAnally W. H. et al., 2016). The principle (see Figure 2-1) is to manipulate the fluid mud cloud to perpetuate its navigability by mixing and aerating it. Aeration is a critical step that determines the sustainability of the method. Indeed, the new aerobic state of the mud promotes the growth of aerobic microorganisms that start producing large amounts of extracellular polymeric substances (EPS). EPS are compounds, mainly polysaccharides and proteins but also DNA, excreted by bacteria to form a gel-like matrix in which cells are aggregated and immobilized and which has a main role of protection but is also favourable to communication between cells or carbon storage for example (Costa et al., 2018; Wingender et al., 1999). The production of EPS allows the cells to grow in a community called biofilms, or flocs at smaller scale, as opposed to their free-floating life or planktonic form. After AND, without EPS production, the mud would rapidly go back to its initial non-navigable state but with EPS the particles are kept in suspension longer (Pang Qi Xiu et al., 2018) and the fluid remains navigable for weeks. The physical properties of EPS also permit the hulls of vessels to pass through with minimal friction, thus facilitating navigability through the fluid mud cloud (Kirby et al., 2008).

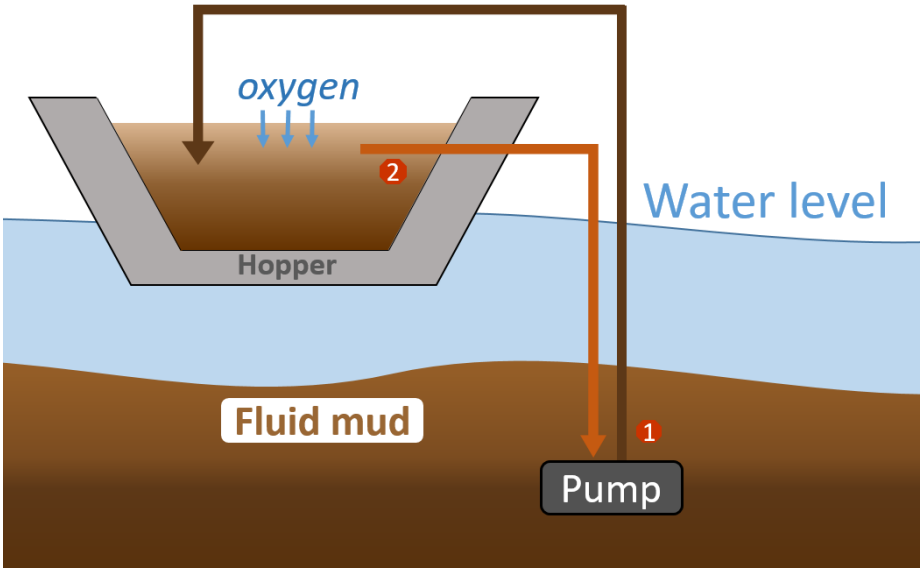


Figure 2-1: Active Nautical Depth principle (as applied in Emden).
Muddy sediment is pumped into a hopper dredger (1) where it is aerated before it is pumped back to the see bottom (2).

2.1.2.3. AND current application and worldwide applicability

Emden port (Ems estuary, Germany) was the first to experiment with AND in 2000. The method has been successfully applied and is well described in the literature (Kirby, 2011; McAnally W. H. et al., 2016; Wurpts, 2005). In this case, mixing is achieved by pumping the fluid mud with a low-power submerged dredge pump into a hopper dredger (see Figure 2-1). The pumping initially alters the physical conditions by breaking the inter-particle bonds and fluidizing the mud. This mud goes in the hopper and is exposed to the atmosphere, thus rapidly becoming aerobic and ready to be placed back to the sea-bottom. The fluid mud cloud remains in suspension for 3-4 months before the mixing episode has to be repeated (Kirby et al., 2008). In Emden's port configuration, the fluid mud cloud maintained by AND prevents exterior sediment from re-entering the basin, consequently reducing the need for dredging to zero where previously 4 million m^3 of sediment was dredged each year. Finally, as a result of the reduced need for maintenance dredging, the overall cost of sediment management decreased from €12.5 million per year to €4 million per year (Kirby, 2013).

Based on the successful results obtained following the implementation of AND in Emden port, an investigation of its potential to be up-scaled and used in other ports and harbours worldwide has been performed (Wurpts, 2005). There are some critical conditions necessary for AND to be successful and these include sediment particle size. A muddy

substrate with low sand content is required in the targeted area. According to Wurpts (Wurpts, 2005), AND should easily be applicable for a sand content of up to 10% with a particle size of between 60 and 200 μm. For sediment with a sand content exceeding 10%, however, the process can be refined. Indeed, the hopper dredger applied in Emden port has been designed in such a way that a sand extraction can be performed if required.

These application conditions are technically viable for many ports in the world with muddy sediment problems, such as Liverpool, Bristol, Leer, *etc*. (Wurpts, 2005) and feasibility studies could be performed to evaluate the possibility of applying AND as a sustainable method for sediment management (to replace or reduce dredging).

2.1.2.4. Potential for bioremediation

During AND, aerating the mud is a primary step to trigger the growth of microorganisms, which will produce the EPS necessary to keep the mud in a fluid state as long as possible but the proliferation of aerobic microorganisms may have other benefits. Indeed, numerous biochemical processes occur aerobically, notably the biodegradation of chemical contaminants (Brzeszcz and Kaszycki, 2018; Haritash and Kaushik, 2009; Levi et al., 2014). Consequently, as a beneficial side effect, aeration of the mud may favour bioremediation of sediment pollutants while reducing the production of other pollutants such as methane, ammonia, or hydrogen sulphide by anaerobic microorganisms.

Ports and harbours can be highly polluted environments and there is a need to find cheaper and more sustainable solutions for their remediation, rather than the deposition of dredged sediment in CDFs. Using AND for the bioremediation of contaminated sediment would be a good option since it would be applied *in situ* and therefore would not involve spreading of contamination or further pollution during transportation. Various studies have shown the aerobic biodegradation of contaminants commonly found in sediment. Several authors reported the aerobic biodegradation of contaminants such as pesticides (bentazone, dichlorprop, mecoprop, glyphosate), PAHs, alkanes, phthalate acid esters (PAEs), TNT, organotin compounds and nonylphenol in microcosm experiments involving sediment (Beolchini et al., 2014; Fahrenfeld et al., 2013; Levi et al., 2014, 2014; Li et al., 2015; Mulligan et al., 2001; Schurig et al., 2014; Wald et al., 2015; Wang et al., 2016; Z. Wang et al., 2015).

Other studies focussed on assessing the aerobic biodegradation of contaminants by specific microorganisms in pure culture which is also useful in a potential biostimulation approach (Cruz et al., 2007; Mulla et al., 2018; Y.-S. Wang et al., 2015). Even more interestingly, the beneficial effect of resuspension on the biodegradation of heavy metals and phenanthrene was reported (LeBlanc et al., 2006; Pourabadehei and Mulligan, 2016). All of these studies have clearly demonstrated the potential of microorganisms to degrade contaminants in conditions that can be obtained through AND (resuspension, aeration of sediment). Further research is necessary to evaluate specifically the potential applicability of AND for the remediation of contaminants found in ports and harbours. Ideally the aim would be to target a wide range of compounds to make AND a versatile method to manage and remediate sediment in multiple places around the world, but a first step in the investigation is to understand the factors contributing to degradation in single contaminant.

TBT, previously used in antifouling paints, is one of the major contaminants found in ports and harbours despite the global ban to which it was subjected after the discovery of its significant toxicity towards non-target organisms. As its biodegradation is stated to occur mainly through aerobic biodegradation, it could potentially be remediated through AND. Section 2 reviews TBT sources and distribution, the chemical properties and bioremediation potential.

2.2. Tributyltin (TBT)

2.2.1. Source and distribution

Organotin compounds were first used in the plastic industry in the 1940s. It was, however, after the discovery of their biocidal properties that their use was widened and TBT became the active component of antifouling paints on ships. Ship-fouling is the unwanted growth of various organisms on the hulls of ships, which hinders navigation and increases fuel costs (Champ, 2000). This led to its major application and to the ubiquitous presence of TBT in the marine environment. TBT can also be found in other biocides in the agriculture industry, in wood preservatives, in the textile industry or in the cooling systems of various industrial processes such as brewing (Cruz et al., 2015; Sousa et al., 2014).

Nevertheless, after the demonstration of its toxicity, TBT has progressively been subjected to various restrictions and finally to a global ban in 2008 (Sonak et al., 2009). It should be

noted that the U.S only ratified the Convention in August 2012. In May 2014, the signatories of the Convention represented 82% of the global shipping tonnage (Turner and Glegg, 2014).

Owing to its extensive use in antifouling paints, TBT can be widely found in marine water and sediment, particularly near ports and harbours. Despite the fact that a decrease in its concentration has been noted at several locations around the world after the global ban in 2008 (Arp et al., 2014; Kim et al., 2014; Langston et al., 2015), it is still a matter of concern in many others (Egardt et al., 2017; Erdelez et al., 2017; Lam et al., 2017). Concentrations as high as 2304 ng TBT/g sed dw were recorded in 2015 in Korea's coastal area (Lam et al., 2017) and 1942 ng Sn/g sed dw was measured in the port of Gdańsk in the Baltic Sea in 2018 (Filipkowska et al., 2018). This can be attributed to the very high persistence of TBT in anoxic sediment where it can remain for decades (in contrast to a couple months under aerobic conditions) and thus constitutes a stock that is released progressively over time (Antizar-Ladislao, 2008), especially during resuspension operations such as dredging and disposal (Langston et al., 2015).

Egardt *et al.* (2017) state also that high concentrations of TBT retrieved in recently deposited sediment suggests a continued use of the substance despite the global ban (Egardt et al., 2017). The same authors revealed that interviews of local boat owners in 2015 confirmed the illegal use of TBT-based paint. Evidence of this use has been shown elsewhere with reports of the sale of TBT-based antifouling paints by a U.S. company to Caribbean and South America as recently as 2014 (Turner and Glegg, 2014). As another example, the U.S. Department of Justice published a report in November 2018 stating that three men who had previously been charged for the manufacturing and selling of TBT based paint pleaded guilty (US Department of Justice, 2018). Therefore, TBT remains both a contemporary and legacy contamination problem.

2.2.2. Physicochemical properties

TBT is a xenobiotic compound from the organotin family of compounds, composed of three Butyl groups covalently linked to a central tin atom to which a 4th group is attached (Figure 2-1), which is most often an anion (Cl^-, F^-, HO^-). It can also form dimers such as bis (n-tributyltin) oxide (TBTO), which is the main compound used in TBT-based antifouling paints. In the aquatic environment, TBT is found as a mixture of these different compounds mostly bound to suspended material, dissolved organic matter and sediment particles. TBT is also subject to hydrolysis in water. Its speciation is mostly driven by pH and it is therefore found in cationic form TBT^+ in an acidic environment whereas it is neutral (TBTOH) at pH>7 (Fang et al., 2017).

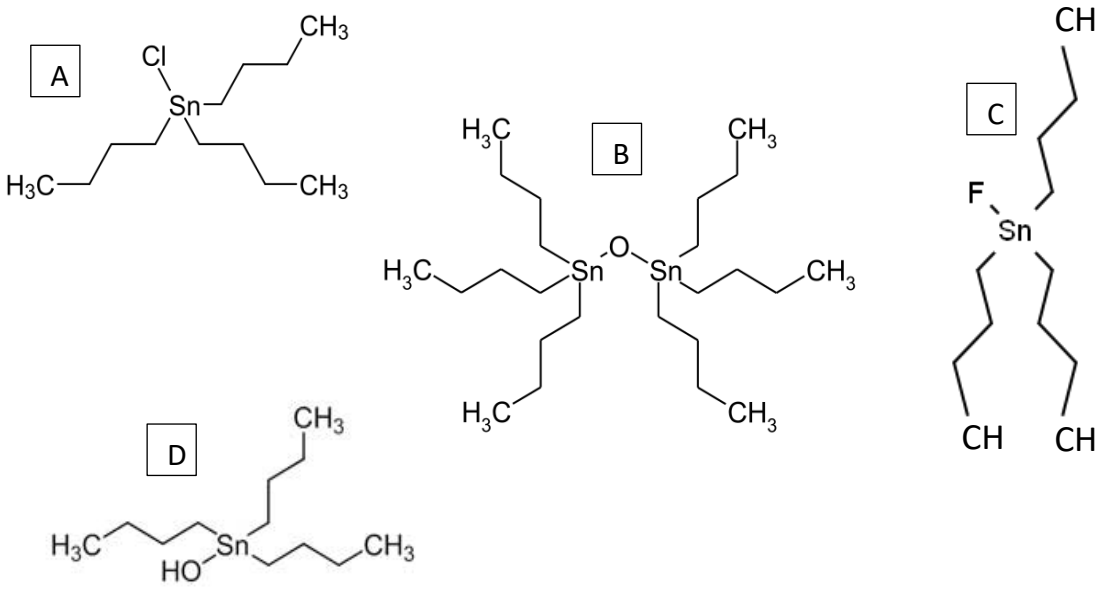


Figure 2-2 Structures of TBTCl (A), TBT2O (B), TBTF (C), and TBTOH (D).

TBTCl, the most used of the different TBT compounds in bioremediation studies, is poorly soluble in water. Its solubility ranges from 1 to 50 $mg.L^{-1}$ in seawater and 5 to 17 $mg.L^{-1}$ in distilled water depending on pH, salinity and ionic strength (Fang et al., 2017; Sunday et al., 2012). This explains its tendency to bind to suspended material and to the fine fraction of sediment corresponding to particles of size less than 62.5 µm (Langston and Pope, 1995). As with every organotin compound it is hydrophobic and its octanol/water partition coefficient (K_{ow}), which represents a measure of the tendency of a compound to move from an aqueous phase to lipids and is therefore an indicator of the bioaccumulative potential

of a compound, has been found to decrease with salinity and increase with pH (Cruz et al., 2015a; Fang et al., 2017).

TBT is categorized as a persistent chemical with a half-life ranging from 3 months in the water column to decades in anoxic sediment (Langston et al., 2015) and from 1 day to 4.4 year in soils depending on their many different characteristics such as pH, texture, organic matter content, aerobic or anaerobic condition and temperature (Cruz et al., 2015). A study conducted on a Canadian fjord with a low seawater temperature, a low exchange rate of deep waters and anoxic sediment estimated that the half-life of TBT was as high as 87 ± 17 years (Viglino et al., 2004). The high persistence of TBT makes it a threat over time. The stocks deposited in sediment are susceptible to biological or human induced resuspension events and act as a secondary source (Jokšas et al., 2019).

2.2.3. Toxicity

TBT is now considered as one of the most toxic compounds in aquatic environments. Its toxicity toward non-target organisms had been discovered in the 1980's after the observation of the stunting of the growth of oysters in oyster farms on the French Atlantic coast (Alzieu, 2000) and is now well documented. After being introduced into the environment, TBT enters the food chain and undergoes biomagnification, its toxicity accordingly affects a wide range of organisms, from bacteria to mammals, including humans. It is toxic at concentrations as low as 1 $ng.L^{-1}$ for the most sensitive organisms, which are molluscs and fishes (Lagadic et al., 2017), and 1 $\mu g.L^{-1}$ for bigger organisms (Hoch, 2001). TBT acts as an endocrine disruptor with its most notorious effect being the development of imposex, the masculinization of female organisms, in more than 200 of gastropod species (Shi et al., 2005). Imposex caused by TBT is so important that it is recommended to use it as a biomarker of TBT pollution (Sousa et al., 2014). More widely, TBT interacts with different receptors involved in various developmental, reproductive and metabolic pathways, thus affecting the survival of many marine species (Beaumont and Budd, 1984; Hagger et al., 2005; Santos-Silva et al., 2018).

The degradation intermediates of TBT are dibutyltin (DBT) and monobutyltin (MBT) (Figure 2-3) and have been subjected to less studies, but they are generally regarded as less toxic than the trisubstituted form. DBT is nevertheless known for its immunotoxic effect (Frouin

et al., 2008; Pagliarani et al., 2013). Most studies, however, show a significantly lower or no toxicity for MBT (Ferreira et al., 2013; Stasinakis et al., 2001; Yan et al., 2018). Upon complete degradation, TBT leads to inorganic tin which is stable and not known to possess any toxic properties.

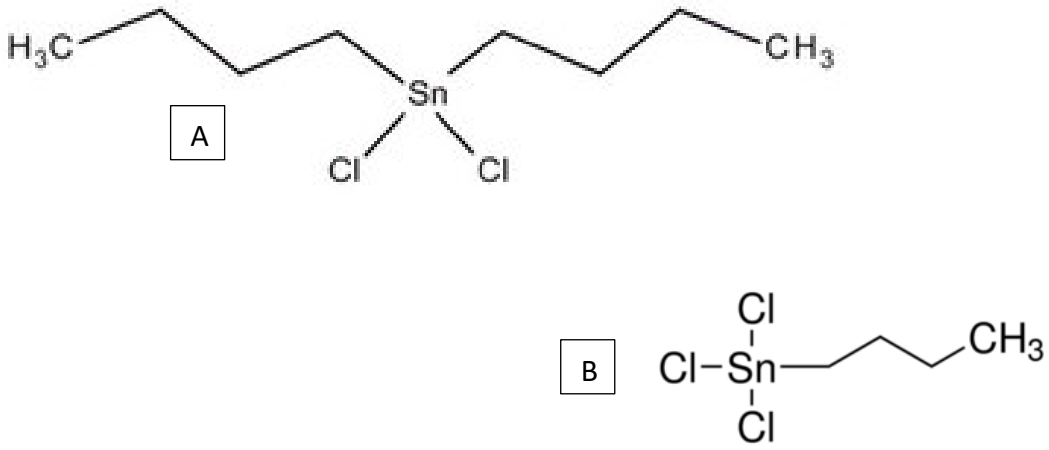


Figure 2-3: Structure of DBTCl (A) and MBTCl (B)

The toxicity of TBT towards microorganisms has been less studied but it was found that it has a negative effect on bacterial cell growth and metabolism through various mechanisms interfering with respiration, solute transport, biosynthesis of macromolecules and transhydrogenase reactions (Cruz et al., 2015). Since microorganisms are well known to rapidly adapt to their environment, it must be expected that bacteria have developed different mechanisms to tackle TBT's toxicity.

Some research has been undertaken to understand these resistance mechanisms. Some studies were able to identify genes that were overexpressed in the presence of TBT and found genes associated with enzymatic activity, transport and binding in one study (Cruz et al., 2012) and ribosomal protein ribosome modulation factor, cold-shock protein or even elongation factor Tu in another study (Fukushima et al., 2009). Elsewhere, efflux pumps were specifically identified in some bacteria of the *Pseudomonadaceae* and *Aeromonadaceae* families, which enable them to release TBT from the cells (Cruz et al., 2013; Hernould et al., 2008; Jude et al., 2004). The last mechanism that was highlighted for TBT resistance is its biodegradation as well as bacterial ability to use it as their sole carbon

source (D. Khanolkar et al., 2015; D. S. Khanolkar et al., 2015; Roy et al., 2004), which is of great interest for the bioremediation of TBT-contaminated material (see Section 2.2.4.2). The current knowledge about TBT biodegradation mechanism is reviewed in Section 2.2.4.1

A lot of studies were published during the last decade to progress the understanding of the toxicity mechanism(s) of butyltin compounds (especially TBT). These studies revealed a wide diversity of toxicity patterns, which vary greatly between species but a lot of questions are still unanswered, and it remains a highly relevant topic of research (Pagliarani et al., 2013). In particular, the TBT toxicity pathway is still unclear for various organisms and especially for microorganisms.

2.2.4. Remediation

Considering tributyltin's high toxicity and its ubiquitous distribution in the aquatic environment, even after the global ban, the remediation of TBT-contaminated sites remains necessary. Efforts have been made to better understand the mechanism of TBT degradation in order to develop appropriate remediation solutions.

2.2.4.1. Breakdown mechanism

The removal of tributyltin from the environment mainly occurs through sequential debutylation from TBT to inorganic tin, as follows with X being an anion (Cl^-, HO^-, F^-):

$(C_4H_9)_3$-Sn-X => $(C_4H_9)_3$-Sn-X_2 => $(C_4H_9)_3$-Sn-X_3 => Sn-X_4

For this reaction, the Sn-C bonds can be cleaved chemically or biologically. Chemical cleavage is enhanced by radiation (light, UV, etc.) and it is thought to be the major process for the degradation of OTCs in surface water. But the low transmission of UV in the ocean makes photolysis inefficient in deeper water or sediment, where biological degradation becomes prevalent.

The biodegradation of OCs is performed by microorganisms such as bacteria, fungi and algae. This degradation has been shown to be strongly dependent on oxygen (Dowson et al., 1996; Landmeyer et al., 2004; Sakultantimetha et al., 2010) although some studies described the occurrence of anaerobic degradation supported by nitrate reducing conditions (Yonezawa et al., 1994) and one study reported a faster degradation of TBT under anaerobic conditions (Maguire, 1987). Some specific conditions and bacterial

communities may favour an efficient anaerobic degradation of organotin compounds (Yonezawa et al., 1994) but the widely recorded strong persistence of TBT in anaerobic sediment around the world, the prevalence of studies showing a faster degradation in presence of oxygen (Barug, 1981; Landmeyer et al., 2004) and the fact that the vast majority of TBT-degrading microorganisms isolated so far are aerobic (Cruz et al., 2015; Finnegan et al., 2018) lend support for the conclusion that TBT degradation is most efficient under aerobic conditions.

The mechanism behind TBT biodegradation has not been entirely characterised, although it is widely accepted as being enzyme mediated. Some studies tried to provide more insights regarding the molecular factors surrounding organotin biodegradation. The successful use of enzyme extracts from a TBT-degrading bacterial isolate to degrade TBT has been described (Deviany et al., 2018), while another study emphasized the role of cytochrome P-450 for TBT degradation in the fungus, *Cunninghamella elegans*, implicating the role of cytochrome P-450 dependent enzymes (Bernat and Długoński, 2002). In parallel, a study showed a role of siderophores, which are iron-chelating compounds excreted by bacteria, in the Sn-C bond cleavage of triphenyltin and suggested that other siderophores rather than enzymes could be involved in TBT degradation (Inoue et al., 2003).

2.2.4.2. Remediation methods

The remediation of TBT from sediment, like most contaminants, can be achieved through physical chemical or biological treatment. A wide range of methods have been reviewed and compared (Du et al., 2014; Finnegan et al., 2018).

Essentially, thermal treatment is usually described as the most effective method for the remediation of highly contaminated material with more than 99% of TBT removal using a range of temperatures between 450 and 1000°C (Beuselinck and Valle, 2008; Song et al., 2005), but it is also considered to be very expensive. To mitigate this cost, a derivative of thermal treatment under high pressure has been proposed, enabling the use of lower temperatures and thus reducing energy consumption, and a pilot scale study has achieved >99.9% of removal (Mostofizadeh, 2001). Another study, however, which tested the efficiency of chemical, thermochemical and thermal treatment at bench scale, indicated that the cost of thermal treatment could be similar to traditional landfill disposal. It could

therefore be worth considering thermal treatment as a remediation solution for certain situations that would be identified on a case by case basis during cost-benefit analyses of the projects (Fergusson, 2014).

Chemical or electrochemical oxidation has also been shown to be efficient. The best result was obtained during a pilot scale study using 15% potassium permanganate, resulting in a 90% removal of TBT (Beuselinck and Valle, 2008). Chemical treatment is less expensive than thermal treatment but the use of chemicals could lead to unpredicted side reactions with other components of the sediment, which has not been assessed (Finnegan et al., 2018a).

It should be noted that during these physicochemical treatments, sediment is treated as waste, its physicochemical properties are altered, and the ecosystem is, therefore, potentially seriously damaged. The third option for the remediation of sediment, bioremediation, however, aims at using the biological activity of the sediment ecosystem to degrade contaminants. It can be further subdivided between phytoremediation, which uses plants and biodegradation, which uses microorganisms.

Several studies investigated the phytoremediation of TBT contaminated sediment and proposed a range of plant species that are efficient for TBT remediation (Carvalho et al., 2010; Lespes et al., 2009; Novak and Trapp, 2005; Qucani et al., 2004). Phytoremediation can be achieved through the uptake of TBT by plants or by the promotion of its biodegradation in the soil surrounding the plant as the roots create a favourable environment for the biodegradation activity of microorganisms or by a combination of both. The uptake of TBT has been shown in lettuce and willow, for example, where TBT is immobilized in the plant tissues (Lespes et al., 2009; Qucani et al., 2004). Another field-study, however, investigated the potential remediation of TBT contaminated sludge by plants in an agricultural setting and demonstrated a positive effect of several plant species on TBT degradation without uptake by the plants (Novak and Trapp, 2005). Finally, the ability of salt marsh plants to promote TBT degradation was evaluated *ex-situ* at both field laboratory scales, resulting again in a positive influence of the plants on TBT remediation (Carvalho et al., 2010).

Biodegradation, during which microorganisms are used for the remediation of contaminants, includes natural attenuation, biostimulation, bioaugmentation, or a

combination of biostimulation and bioaugmentation. Natural attenuation simply consists of letting the native microbial community degrade the targeted contaminants over time. Natural attenuation is usually efficient for moderate to low contamination. It is usually quite slow but has the advantage of being very cost effective and beneficial for the environment.

To boost natural attenuation, biostimulation or bioaugmentation can be used. Biostimulation involves the stimulation of the native degrading community by supplying more favourable conditions for the growth and activity of the microorganisms. This can be achieved, for example, by the addition of nutrients or oxygen. For bioaugmentation, microorganisms identified to be efficient at degrading a targeted contaminant are added to the native community. This implies that the added species successfully integrate with the natural community, by being able to survive in the habitat and compete effectively for nutrients with the indigenous population.

Bioremediation using microorganisms has been previously considered for the remediation of TBT contaminated sediment, as biodegradation is recognised to be the major pathway for the elimination of TBT from the aquatic environment. Natural attenuation of TBT was assessed in the field and in microcosms for two contaminated freshwater sediments characterised by a significant difference in organic matter content (Landmeyer et al., 2004). The microcosms showed a faster degradation in an organic rich sediment of a beaver pond compared to a sandy organic poor lake sediment. The same tendency was observed in the field but the overall degradation was approximately five times slower (Landmeyer et al., 2004). It was suggested that the controlled aerobic conditions in the laboratory were more favourable compared to the dynamic conditions of aerobic and anaerobic processes that occur in the field (Landmeyer et al., 2004).

Another study conducted as part of the TBT Clean Life Project, aiming at finding an integrated approach for the remediation of TBT contaminated material, and explored the natural attenuation of dredged sediment through lagooning (Pensaert et al., 2005). Lagooning is a remediation technique where excavated material is brought to a field and deposited as fluid mud to form a lagoon. The sediment is turned regularly for natural aeration and dewatering. It resulted in 68% of TBT degradation after 6 months, with a slowing down of the process after 3 months, which was explained by the drying out of the

sediment having a negative effect on microbial activity (Pensaert et al., 2005). The same study reported a slower degradation in the deeper layers of sediment from the unlagooned control (40% after 6 months) and no degradation at all in sediment stored in anoxic conditions, highlighting the importance of oxygen in the biodegradation of TBT.

The results of these field trials are encouraging and indicate that TBT bioremediation can be achieved in the field simply by the action of indigenous microbial degraders with the condition of a favourable oxygenation of the sediment. Nevertheless, natural attenuation, although environmentally friendly, is usually recognised as the slowest remediation strategy and a better understanding of the microorganisms and processes involved in natural attenuation can lead to improvement of the method by means of biostimulation or bioaugmentation. Consequently, many studies have focused on isolating TBT-degrading bacteria, fungi or even microalgae in order to characterise them and evaluate their efficiency to degrade TBT (Abubakar et al., 2015; Bernat and Długoński, 2002; Cruz et al., 2007; Hassan et al., 2018; Jeong et al., 2011; Kawai et al., 1998; D. S. Khanolkar et al., 2015a; Murthy et al., 2007a; Sakultantimetha et al., 2009; Sampath et al., 2012; Tsang et al., 1999).

All the TBT-degrading bacteria isolated in studies so far are members of the *Gammaproteobacteria*, with many representatives from the family Pseudomonadaceae. After isolation, some of these bacteria were used to perform microcosm studies using naturally contaminated or TBT-'spiked' sediment. A study examined the effect of biostimulation by the addition of inorganic nutrients and bioaugmentation by addition of a commercial mix of bacteria able to degrade xenobiotic compounds in slurry reactors containing TBT contaminated harbour sediment (Beolchini et al., 2014). They found that the combination of biostimulation and bioaugmentation was effective to boost the biodegradation of organotin compounds, with 50% of TBT removal after four weeks (Beolchini et al., 2014). The bioaugmentation alone did not show any significative degradation of TBT although a net increase in the concentration of biodegradation by-products occurred, the author did not find any explanation for this (Beolchini et al., 2014). Moreover, no results are shown for potential controls with no biostimulation or bioaugmentation at all, or with sterile sediment, the study therefore did not provide any insights regarding the ability of the indigenous community to degrade TBT.

Another study assessed the effect of bioaugmentation on estuarine sediment, using TBT-degrading bacteria isolated from the same sediment (Cruz et al., 2014). The bioaugmentation was proved to be efficient as a correlation between the amount of inoculum and TBT degradation was found. Interestingly, the authors also reported a higher degradation in autoclaved sediment, increasing as well with the inoculum of TBT-degrading bacteria, and suggested an effect of the autoclaving on TBT stability or a better ability of the inoculated strains to develop in the absence of the native community (Cruz et al., 2014).

TBT-spiked freshwater sediment was used elsewhere to evaluate the degradation ability of the indigenous microbial community. Microcosm experiments revealed 50% of TBT degradation in the non-autoclaved sample over a period of 150 days, whereas no change in TBT concentration occurred in the autoclaved sediment (Suehiro et al., 2006). Another study also used TBT-spiked freshwater sediment to conduct microcosm experiments over a period of 28 days. When the sediment was left undisturbed, a half-life of 578 days was calculated, which was reduced to 11 days when the sediment was aerated, and 9 days after addition of nutrients or TBT-degrading bacteria (Sakultantimetha et al., 2011). TBT half-lives reported by microcosm studies show highly variable results, with TBT half-lives ranging from a few days to several months, even under stimulated conditions.

Some consistent factors influencing TBT degradation can nevertheless be identified. Aeration undeniably enhances the biodegradation process, but the addition of nutrients or TBT-degrading microorganisms also seems to boost it. The variability of the results could be linked with the differences between microbial communities that are shaped by sediment physicochemical properties (see Section 3.2). More microcosm and field studies should be performed to better understand TBT biodegradation and assess the applicability of TBT bioremediation *in situ* for different locations with distinctive properties.

To conclude regarding the remediation of TBT-contaminated sediment, a wide range of methods can be chosen but it is important to note that most treatments are performed *ex situ* and therefore involve dredging of the material to remediate. Bioremediation overall represents a promising technique which, can potentially be performed *in situ*, reducing even more the potential environmental impact of the treatment by avoiding dredging and its environmental impacts (described in Section 1.1.1).

2.3. Sediment microbial communities

In the previous sections , the benefits of using *in situ* bioremediation as a method of remediation for contaminated sediment were discussed, compared to traditional physicochemical treatments or other bioremediation treatment performed *ex-situ,* as it is more environmentally friendly and cost effective (see Sections 1.1.3 and 2.4.2). Therefore, as *in situ* bioremediation mainly involves the stimulation of the degradation ability of the indigenous microbial community it is important to have an idea of the structure and distribution of sediment microbial communities, and the factors that affect them.

2.3.1. Overview of bacterial abundance, diversity and community structure in sediment

Sediment provides a habitat for a whole range of microscopic organisms from all three domains of life that are bacteria, archaea and eukaryotes. It is widely acknowledged that bacteria are the most active components of sediment microbial communities, outcompeting the other microorganisms due to their efficient and versatile metabolism (Nealson, 1997). Bacteria are also the most abundant group in this environment (Hong et al., 2019; Wei et al., 2016; Zhang et al., 2015), with the exception of some extreme environments where archaea, which were the first known extremophiles (Chaban et al., 2006), can sometimes outnumber bacteria (Korzhenkov et al., 2019). As a consequence, most studies assessing microbial communities in sediment have focused on bacteria and sometimes include archaea but often ignore eukaryotes. Because bacteria also constitute most of the microorganisms used for sediment bioremediation, this review concentrates on this group. Nevertheless, some recent studies advocate consideration of eukaryotes in sediment community analyses and suggest their inclusion in future studies (Ul-Hasan et al., 2019).

Globally, microorganisms play a critical role in nutrient cycling, and in the aquatic environment. Sediment is the last substrate environment where the matter that has not been degraded in the above water column can be transformed. This material, which is mostly organic matter resulting from the death of aquatic life or coming from anthropogenic sources is incorporated in the sediment at the critical site that is the sediment-water interface (Perliński et al., 2019). For most sediment and in the absence of

perturbations, oxygen is limited by its solubility in water and will be consumed by biochemical reactions involved in the degradation of deposited organic matter, which results in sediment becoming anoxic with depth (Nealson, 1997). Along this gradient, a stratified environment is created with layers where substrates are degraded by certain types of microorganisms, forming products for other groups of microorganism that will continue the degradation process (Nealson, 1997). These successive reactions are mostly observed for the mineralisation of organic matter in anoxic sediment because the anaerobic microorganisms are usually less versatile than aerobes and therefore depend on each other's activities (Laanbroek and Veldkamp, 1982).

The number of studies investigating the composition of sediment microbial communities has risen in recent years, especially linked to the increasing access to sequencing technologies. This information has not been reviewed recently for global sediment. One study synthesised the data produced so far for bacterial communities in seafloor sediment for deep-water and coastal water in 2011 (Zinger et al., 2011). This study revealed a fairly similar composition between deep seafloor and coastal sediment, with a global dominance of *Gammaproteobacteria*. Such microbes are a class holding a wide diversity of organisms with a range of different modes of metabolism, including bacteria capable of sulphur oxidation (Liu et al., 2015) and bacteria specialised in the degradation of high or low-molecular weight organic matter (Mahmoudi et al., 2015). The synthesis in 2011 also showed a dominance of *Deltaproteobacteria, Planctomycetes, Actinobacteria* and *Acidobacteria* in marine surface sediment. *Planctomycetes* and *Acidobacteria* were slightly more abundant in deep seafloor sediment whereas *Firmicutes* were more represented in coastal sediment and a terrestrial origin was suggested for this group as it is used as an indicator of human faecal contamination of water and is also common in soil (Zinger et al., 2011).

A study in 2000 reviewed the knowledge on "significant prokaryotes" from freshwater lake sediment, showing a dominance of *Betaproteobacteria, Gammaproteobacteria* and *Acidobacteria* (Spring et al., 2000). Those results, however, may not be representative as they were obtained by old methods, before the sequencing era, which have many biases mainly because they are restricted to the analysis of a part of the community whereas

sequencing now allows thousands to millions of sequences to be read, reducing considerably the chance to miss important organisms within the targeted community.

Recent studies assessing microbial diversity in freshwater lakes and reservoirs show a global dominance of *Proteobacteria* and especially *Gammaproteobacteria* (Bouzat et al., 2013; Huang et al., 2017; Kou et al., 2016; Li et al., 2019; Sorokin et al., 2014; Tamaki et al., 2005; Wobus et al., 2003; Zhang et al., 2019b). Chloroflexi (Bouzat et al., 2013; Huang et al., 2017; Kou et al., 2016; Tamaki et al., 2005; Wobus et al., 2003; Zhang et al., 2019b), *Acidobacteria* (Bouzat et al., 2013; Huang et al., 2017; Li et al., 2019; Tamaki et al., 2005; Wobus et al., 2003; Zhang et al., 2019b), *Bacteroidetes* (Bouzat et al., 2013; Huang et al., 2017; Kou et al., 2016; Li et al., 2016, 2019; Tamaki et al., 2005; Wobus et al., 2003), *Nitrospirae* (Bouzat et al., 2013; Huang et al., 2017; Kou et al., 2016; Li et al., 2019; Tamaki et al., 2005; Zhang et al., 2019b) and *Planctomycetes* (Li et al., 2016, 2019) are also often stated as the major groups of the sediments in these environments.

Estuaries are key interface ecosystems, where freshwater meets marine water, making them complex and dynamic environments to explore. Nevertheless, the studies clearly show, again, a predominance of *Proteobacteria*, mostly *Gammaproteobacteria* (Feng et al., 2009; Hong et al., 2019; Morris et al., 2019; Vidal-Durà et al., 2018; Zhang et al., 2014). *Bacteroidetes* is usually described as the second most abundant phylum(Feng et al., 2009; Morris et al., 2019; Zhang et al., 2014). *Acidobacteria* (Hong et al., 2019; Vidal-Durà et al., 2018; Zhang et al., 2014), *Firmicutes* (Feng et al., 2009; Hong et al., 2019; Morris et al., 2019; Vidal-Durà et al., 2018) and *Cyanobacteria* (Hong et al., 2019; Morris et al., 2019; Vidal-Durà et al., 2018; Zhang et al., 2014) are also often listed as the dominant groups of estuarine sediment.

The main bacterial groups constituting sediment communities described above have mostly been identified in surface sediment. As explained, sediment tends to have different characteristics with depth, becoming anoxic and holding different substrates. The subsurface sediment communities are therefore distinct from surface communities. Studies almost unanimously report an enrichment of *Chloroflexi* with depth (Brandt and House, 2016; Martino et al., 2019; Oni et al., 2015; Parkes et al., 2014; Schippers et al., 2012; Starnawski et al., 2017; Vidal-Durà et al., 2018). The other notable difference compared to surface sediment is the presence of *Atribacteria* (Martino et al., 2019; Oni et

al., 2015; Parkes et al., 2014; Petro et al., 2017; Schippers et al., 2012; Starnawski et al., 2017), which also increases in abundance with depth (Petro et al., 2019). *Atribacteria* is a recently proposed phylum, gathering JS1 and OP9 lineages, which remains mysterious especially due to the lack of pure culture studies but it is thought to play a key role in the carbon cycle, in particular in hydrocarbon-enriched environments (Liu et al., 2019). *Gammaproteobacteria* (Brandt and House, 2016; Petro et al., 2017; Vidal-Durà et al., 2018), *Actinobacteria* (Brandt and House, 2016; Martino et al., 2019), *Planctomycetes* (Brandt and House, 2016; Oni et al., 2015; Petro et al., 2017) and *Bacteroidetes* (Hong et al., 2019; Vidal-Durà et al., 2018) are also reported, but their abundance seems to decrease with depth (Hong et al., 2019).

Two other components that describe bacterial communities other than composition are diversity and abundance. The lack of reviews, the number of studies and the fact that they do not necessarily calculate the richness makes it hard to compare bacterial diversities in the major sediment ecosystems. The review by Zinger and co-workers in 2011 nevertheless revealed a higher diversity of the bacterial community in coastal sediment compared to deep-water sediment (Zinger et al., 2011), which probably reflects the influence of the terrestrial and freshwater environment but also a higher level of perturbation of the coastal environment due to anthropogenic activities.

A study recorded the bacterial diversity in freshwater, intertidal and marine sediments and observed a respective decrease in diversity but this could not be explained fully with the factors that were measured (Wang et al., 2012), although salinity is known to be negatively correlated with microbial diversity (see Section 3.2). It has been clearly shown, however, that bacterial diversity is higher in sediment than in water (Ul-Hasan et al., 2019; Zhang et al., 2019b; Zinger et al., 2011). This is explained by the nature of sediment, which provides a solid and stable habitat for bacteria to form biofilms. In addition, sediment minerals participate in bacterial redox reactions. It also holds a wide diversity of niches especially because of the stratification that occurs. For similar reasons, higher abundance of bacteria are reported in surface sediment compared to water in both marine and freshwater environments (Luo et al., 2019; Mahmoudi et al., 2015). In addition, a number of studies report a decrease of bacterial abundance with depth (Haglund et al., 2003; Hong et al., 2019; Pala et al., 2018; Petro et al., 2019; Starnawski et al., 2017). This can be mainly

attributed to the rarefication of nutrients, although active bacteria are still retrieved as deep as 2.5 km below the surface (Inagaki et al., 2015).

The general trends in terms of bacterial abundance, diversity and community composition of the main types of sediment ecosystems (subsurface sediment, surface sediment, deep marine and coastal sediment, freshwater sediment and estuarine sediment) have been described in this section. It is important, however, to remember that there is also high variation between locations within these categories. Indeed, numerous factors can potentially affect bacterial distribution over time and between sites such as the natural physico-chemical properties of the sediment, seasonal change, disturbance events or the presence of pollutants.

2.3.2. Factors shaping sediment microbial communities

As described in the previous section, many studies have assessed bacterial communities in different sediment environments, but even more research has focused on the abiotic factors shaping these communities. Sediment is a complex matrix and represents a wide diversity of environments and numerous drivers influence sediment bacterial diversity, abundance, and composition. This section will therefore present a broad picture of the impact of environmental parameters on bacterial communities in sediment.

Many studies have only recorded changes in the bacterial community composition. The influencing factors are sediment texture (Hamonts et al., 2014; Ibarra-Sánchez et al., 2020; Yao et al., 2019), pH (Chen et al., 2017; Li et al., 2019; Liu et al., 2015; Lu et al., 2019; Xiong et al., 2012; Yao et al., 2019), dissolved oxygen (DO) (Hong et al., 2019; Li et al., 2019; Sinkko et al., 2019), salinity (Hamonts et al., 2014; Klier et al., 2018; Pavloudi et al., 2016; Sorokin et al., 2014; Vidal-Durà et al., 2018; Yang et al., 2016), temperature (Acosta-González and Marqués, 2016; Hamonts et al., 2014; Pala et al., 2018; Zhang et al., 2014), nutrient level or type (Huang et al., 2017; Varliero et al., 2019; Zhang et al., 2014), trophic status (Villaescusa et al., 2010; Wan et al., 2017; Wobus et al., 2003), total phosphate (TP) (Li et al., 2019; Yao et al., 2019), total organic carbon (TOC) (Deviany et al., 2018; Hamonts et al., 2014; Klier et al., 2018; Oni et al., 2015; Powell et al., 2003; Rastelli et al., 2019; Zhang et al., 2019b), sediment organic matter (SOM) (Fagervold et al., 2014; Lu et al., 2019; Wan et al., 2017), total nitrogen (TN) (Dai et al., 2018; Huang et al., 2017; Ibarra-Sánchez et al.,

2020; Klier et al., 2018; Li et al., 2016; Lu et al., 2019; Pang Qi Xiu et al., 2018; Xiong et al., 2012; Yao et al., 2019; Zhang et al., 2019b) or more precisely ammonium (Chen et al., 2017; Vidal-Durà et al., 2018; Wan et al., 2017) and nitrate (Liu et al., 2015; Wan et al., 2017; Zinke et al., 2018), pollution (Acosta-González and Marqués, 2016; Catania et al., 2018; Chen et al., 2019; Hamonts et al., 2014; Powell et al., 2003), redox state (Sinkko et al., 2019) and water depth (Chen et al., 2019).

Some of the studies also assessed the influence of these factors on microbial diversity. While salinity (Ibarra-Sánchez et al., 2020; Sorokin et al., 2014; Vidal-Durà et al., 2018; Yang et al., 2016) and oil pollution (Acosta-González and Marqués, 2016; Catania et al., 2018) were negatively correlated to bacterial diversity, ammonium (Rastelli et al., 2019), nitrate (Liu et al., 2015; Zhang et al., 2014) and TOC (Chen et al., 2017; Rastelli et al., 2019) were found to increase this diversity. The trophic status (Wan et al., 2017; Wobus et al., 2003), pH (Chen et al., 2017; Liu et al., 2015; Xiong et al., 2012) and redox state (Wan et al., 2017; Wobus et al., 2003) also seem to have an effect on bacterial diversity in sediment.

The studies including an assessment of abundance are scarcer, but salinity (Perliński et al., 2019) and water depth (Zhang et al., 2014) were found to decrease bacterial abundance in sediment while TN (Zhang et al., 2014), SOM (Kou et al., 2016), TOC (Schippers et al., 2012; Zhang et al., 2014), trophic status (Villaescusa et al., 2010) and temperature (Pala et al., 2018) resulted in an increase in abundance.

The studies, however, rarely propose explanations of how these factors influence microbial communities, highlighting the complexity of the task, but some insights can emerge from our knowledge of microbial physiology. Most of these factors are directly or indirectly linked to each other. Many of them are related to energy sources and therefore shape the communities by selecting taxa whose growth depends on their uptake. Increased carbon source concentrations support overall growth within the community, hence the positive effects on bacterial abundance (Kou et al., 2016; Schippers et al., 2012; Villaescusa et al., 2010; Zhang et al., 2014). Similarly, nutrient availability limits bacterial diversity: increased ammonium, nitrate or organic matter concentrations can mitigate interspecies competition or hold various nutrient types, which would explain the positive effects on diversity that were reported (Chen et al., 2017; Liu et al., 2015; Rastelli et al., 2019; Zhang et al., 2014).

Oxygen also influences bacterial distribution and diversity. Strict anaerobes, for example, are inhibited in the presence of oxygen and strict aerobes in the absence of it, whereas facultative anaerobes may be represented in a wider range of dissolved oxygen concentrations or without oxygen at all. The deleterious influence of salinity on microbial community diversity and abundance is due to osmotic stress, which decreases cell-specific activity and growth efficiency (Perliński et al., 2019). Sediment pH would represent an integrated value of the sediment condition, being linked to several parameters such as salinity, mineral nutrient solubility and availability, or organic carbon chemical composition but it could also directly constrain bacterial physiology (Liu et al., 2015; Yao et al., 2019).

Temperature is well known to influence bacterial growth; physiological reactions being slowed down at low temperature. The impact of sediment grain size distribution is more difficult to understand. Sandy sediment for example holds lower organic content and the larger grain size leads to less surface area for bacterial attachment but it also favours nutrient circulation in pore water (Wang et al., 2013). The influence of pollution, the introduction of harmful materials into the environment, also depends on various factors, such as the level and the type of pollution. Strong pollution (defined by a high concentration of pollutant, which causes strong perturbations to the ecosystems) is reported to have a negative influence on bacterial diversity due to the toxicity towards most of the bacterial community, and only the resistant strains should survive but depending on their resistance mechanism they can help other taxa to survive thanks to their degradation abilities. One study reported, however, that medium levels of pollution increased bacterial diversity (Xiong et al., 2012). The authors explained this by the intermediate disturbance hypothesis, which states that intermediate disturbance leads to higher levels of diversity (Wilson, 1990). Additionally, hydrocarbon pollution of surface sediment in the Adriatic sea resulting in higher diversity of bacteria was explained by the subsequent availability of a higher diversity of carbon sources over a long period of time (Korlević et al., 2015).

Some studies observed seasonal changes within sediment microbial communities, which can be explained by the factors affected by seasonality. A lower temperature in winter, for example, can be responsible for lower bacterial abundance (Pala et al., 2018; Zhang et al., 2019a) whereas the increased growth of phytoplankton in summer leads to higher amounts

of organic matter in sediment and increased bacterial abundance (Perliński et al., 2019; Zhang et al., 2019a).

This section focussed on reviewing the abiotic factors shaping bacterial communities in sediment but it is important to note that biotic factors such as viruses and microbial interactions, which include competition, mutualism and commensalism, also play a role (Fagervold et al., 2014; Rastelli et al., 2019).

2.3.3. Isolation of bacteria from the environment

2.3.3.1. Relevance and limit

The advance in laboratory techniques, especially in the domain of molecular biology have been critical in boosting our understanding of microbial communities on Earth. Sequencing technologies allow us to obtain a complete description of microbial community composition in an ecosystem. Using 'omic' methods like metatranscriptomic or metabolomic, some inference on activities and functions of these microorganisms within the community can be made. Isolation and cultivation, however, remain useful as pure cultures are needed to obtain the genomic sequence of previously unknown microorganisms, and they allow us to easily characterise their metabolism. Cultivation is also an important way of finding and potentially producing new molecules of interest and the isolation of previously uncultured microorganisms is still actively used for biotechnological purposes (Dionisi et al., 2012). In particular, for bioremediation , widening our knowledge of the microbial processes involved in the degradation of pollutants is critical, and the isolation and successful cultivation of unknown bacteria is the starting point to achieve this objective (Bodor et al., 2020).

The challenge, however, when attempting to culture microorganisms is to find the appropriate conditions for their growth in the laboratory and, despite decades of experience in microbial cultivation, we are still unable to grow the vast majority of microorganisms (Rappé and Giovannoni, 2003). Estimates vary widely, mostly depending on the environmental origin of the sample to cultivate but cultivation efficiencies as low as 0.0007% (efficiency based on the comparison between colonies obtained on agar plates and the total number of bacteria in the sample, obtained by microscopy) have been found for desert samples (Hahn et al., 2019). Effort has therefore been made to improve our

current cultivation techniques as well as to find new ways to isolate bacteria from the environment, some of these methods are detailed in the next section (I.3.3.2).

2.3.3.2. Isolation and cultivation techniques

2.3.3.2.1. Standard isolation and improvement possibilities

The process of bacterial isolation consists of separating a bacterium from the rest of the microbial community and is usually coupled to the process of cultivation which consists of being able to maintain a pure culture of this bacterium in laboratory conditions. The most commonly used method for the isolation of bacteria involves plating dilutions of an environmental sample on Petri dishes containing a suitable medium and transferring a single colony, within which all the bacteria derive from the same cell, on another plate (Hahn et al., 2019). It clearly appears that conventional growth media may not contain the whole diversity of nutrients that most bacteria need, hence the fact that only a small percentage of them can actually be cultured using this technique, although other factors play a role (see section I.3.3.2.4).

Media composition can nevertheless be adapted depending on targeted organisms, for example the concentration of salt can be increased when attempting to isolate bacteria from seawater or particular sources of carbon or energy can be added if the aim is to isolate bacteria with specific physiology. Such media are described as “selective” and can be coupled with particular incubation conditions. As mentioned previously, many examples have observed the inability of certain bacteria to grow in the absence of others, emphasising the need for the production of a key substrate or growth factor by this ecological partner (Hahn et al., 2019).

One way to tackle this issue without carrying out fastidious experiments to identify the required compound is to use “environmental extract”, usually soil extract, which is a filter-sterilised solution produced from the washing of soil by different liquids such as distilled water or methanol, as a supplement for growth media (Nguyen et al., 2018). Designing a medium suitable for the cultivation of uncultured bacteria, however, remains a laborious task (Stewart, 2012).

Other approaches can be adopted to increase the chance of successful isolation, such as the addition of an enrichment step in liquid nutrient medium before plating. The

enrichment medium can be adapted to select for a group of interest within the microbial community. This raises the abundance of a targeted organism to increase the chance that it is present in the volume of liquid that will be plated and in turn, increasing the chance of obtaining a colony from it. Besides these considerations, it was acknowledged that the concentrations used for the nutrients in most media were significantly higher than environmental concentrations and were therefore selective for fast growing microorganisms as well as inhibiting "oligotrophic bacteria" (Watve et al., 2000). Low nutrient media have therefore been used for the isolation of previously uncultured bacteria (Kurm et al., 2019; Pulschen et al., 2017). A combination of low nutrient media with long incubation times and the extinction dilution method (Connon and Giovannoni, 2002; Rappé et al., 2002; Song et al., 2009). The extinction-dilution method consists of diluting an environmental extract until only a few bacterial cells remain in suspension, enabling pre-isolation of the cells before plating.

Extended incubation time has been a key parameter for the isolation of many novel microorganisms in the past (Davis et al., 2005). Moreover, some studies have observed the formation of microcolonies in agar media , that do not grow larger, and concluded that some microorganisms may never be able to form the conventional colonies expected during isolation processes (Ferrari et al., 2005). The micromanipulation of these small colonies has resulted in the successful isolation and cultivation of previously uncultured bacteria (Ferrari and Gillings, 2009). The agar used in conventional media has been found to slow and sometimes inhibit the growth of some bacteria (Tamaki et al., 2009), at least partially due to the production of hydrogen peroxide during the autoclaving of phosphate (which is extremely common in microbial media) with agar (Tanaka et al., 2014). The two components can consequently be autoclaved separately or gellan gum, for example, can be used as an alternative solidifying agent (Das et al., 2016; Pulschen et al., 2017).

The improvement of standard techniques of cultivation has been a success to isolate some previously uncultured microorganisms and to reduce the repeated isolation of the same species. The majority of microorganisms, however, still remain uncultured, with numerous phyla containing no cultivated representatives. Research has consequently been focused on developing more innovative methods for the isolation and cultivation of unknown species.

2.3.3.2.2. Innovative techniques

As it proves challenging to try to mimic environmental conditions in the laboratory and to provide a suitable medium for uncultivated bacteria to grow, a solution has been to return the potential isolates in their natural environments. The idea being to separate them from the *in-situ* microbial population by the means of semi-permeable membranes whose pores are too small for microorganisms to move from one side to another but permit the flux of smaller molecules that could be essential for the targeted microorganism's growth. The first models derived from this concept were called diffusion chambers (Kaeberlein et al., 2002).

For the first diffusion chambers, more precisely, a polycarbonate membrane with 0.03 µm pores was attached to the bottom of a stainless-steel ring (Figure 2-4A), which was then filled with agar supplemented with an appropriate dilution of the targeted environmental sample (Figure 2-4B) and sealed with a second membrane (Figure 2-4C) on the top (Kaeberlein et al., 2002). The chamber could then be placed in the desired environment (Figure 2-4D). Diffusion chambers, for practical reasons, are usually incubated in "simulated environments", which can be aquaria, buckets of soil or sediment or even living organisms such as marine sponges (Bollmann et al., 2010, 2007; Jung et al., 2016; Kaeberlein et al., 2002; Remenár et al., 2015; Steinert et al., 2014). After a certain incubation time, the growth of colonies can be observed on the agar. This first round of growth in *in-situ* conditions constitutes an adaptation phase for microorganisms, which may then allow the organisms to grow under laboratory conditions after sub-culturing on normal agar plates . Alternatively, they can be sub-cultured in a new diffusion chamber for more phases of adaptation (Bollmann et al., 2007).

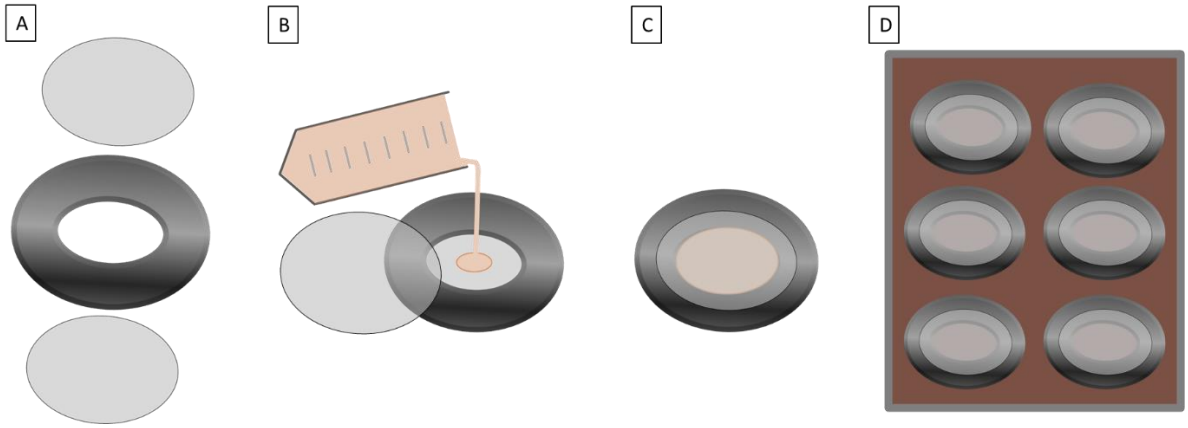


Figure 2-4: Diffusion chamber first prototype (Kaeberlein et al., 2002). *The diffusion chamber is composed of two polycarbonate membranes with 0.03µm diameter pores and a stainless-steel ring (A). After one of the membranes is attached to the bottom, the ring is filled with agar supplemented with the appropriate dilution of an environmental sample (B). The second membrane is then attached to the top (C) and the assemblage can be incubated in the source environment, for example sediment (D).*

Diffusion chambers inspired the design of several other devices, with the aim of improving them, or making them more specific to the isolation of atypical microorganisms. Simply by using a bottom membrane with bigger pore size of 0.2 µm, for example, the diffusion chamber can be turned into a trap that can be used for the isolation of filamentous Actinobacteria (Gavrish et al., 2008). In this case, uninoculated agar is placed in the trap before sealing, which is then placed on the soil where filamentous Actinobacteria can colonise it by passing through the bottom membrane.

Another study reported the isolation of 35 previously uncultured soil bacteria by designing a diffusion bioreactor, which uses a high volume of liquid medium inoculated with soil extract, imprisoned in an inner chamber pierced with several pores that are sealed by a semi-porous membrane and placed in a bigger outer chamber filled with soil (Chaudhary et al., 2019). In this study the authors used a low-nutrient medium to facilitate the isolation of slow-growing bacteria, which explains the necessity of using a high volume of medium to compensate for the consumption of nutrients during a long incubation time (Chaudhary et al., 2019).

These models of diffusion chambers or traps remain laborious to use by their design and size. Devices have therefore been created using the same concept but enabling a high throughput isolation of microorganisms. The first device developed was called iChip and was composed of an assemblage of three flat plates pierced by 384 mini holes. A central

plate is immersed in warm agar medium and inoculated with an environmental sample dilution (Figure 2-5A). Membranes are assembled on each side and the device is sealed with the two other plates screwed on each side (Figure 2-5B). Each of the 384 holes then act as diffusion chambers into which an isolated cell is expected to live and divide to form a mini colony (Berdy et al., 2017; Nichols et al., 2010).

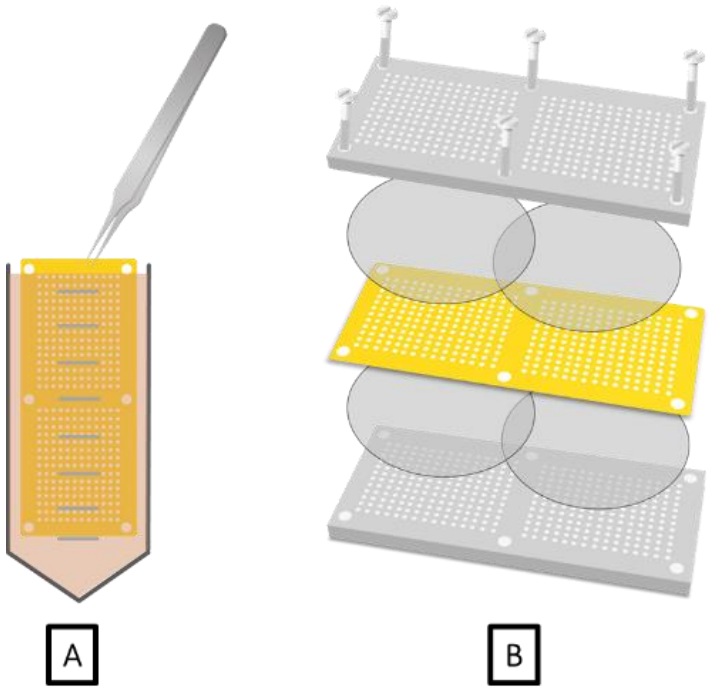


Figure 2-5: iChip assemblage. *A central plate is immersed in warm agar medium supplemented with the appropriate dilution of an environmental sample (A). Two polycarbonate membranes with pores of 0.03µm diameter are then added to each side of the central plates and the device is sealed with two external plates screwed on each side (B).*

As for the diffusion chamber, several rounds of adaptation may be needed to get a sustainable growth of previously uncultured bacteria on synthetic media after isolation using an iChip (Lewis et al., 2010). Other devices have been built on the same principle but with slightly different designs. A study developed a device with 96 through holes for the isolation of fungi, referred to as FIND (Fungal one-step IsolatioN Device (Libor et al., 2019)). Combining the principles of the iChip and the trap described above, another study reports the use of a minitrap for the isolation of human oral cavity bacteria (Sizova et al., 2012). These high throughput methods were successfully used for the isolation of a number of novel microbial strains from soil (Libor et al., 2019; Nichols et al., 2010), sediment (Libor et al., 2019), seawater (Libor et al., 2019; Nichols et al., 2010), or even human oral cavity

(Sizova et al., 2012) and notably enabled the discovery of a new antibiotic (Ling et al., 2015; Piddock, 2015).

All of the methods described above use polycarbonate membranes to separate the potential isolates from the environment. Some techniques have been developed using other porous materials and different designs. One study used the encapsulation of bacteria in polysulfone membranes coated agar spheres incubated in soil, wastewater or seawater. It was suggested that the small size of the spheres can be advantageous for the comparison of the growth of isolates in favourable microniches from their native environment (Ben-Dov et al., 2009).

Small spheres also present the advantage of facilitating the exchange of growth factors, which is one of the stated benefits of another device that was called the hollow-fibre membrane chamber (HFMC), which uses different pieces of hollow-fibre membranes as chambers into which inoculated liquid medium is poured before sealing and immersion in a liquid environment (Aoi et al., 2009). Fibres present high surface areas and, similar to the bioreactor presented above, the use of liquid medium means that the isolation does not rely on colony formation (Aoi et al., 2009), and may be able to lead to the cultivation of bacteria which do not have the capacity to form colonies on solid media.

2.3.3.3. Remaining challenges for the isolation and cultivation of uncultivated bacteria

The improvement of the standard culturing techniques as well as the design of non-standard methods for the isolation and adaptation of bacteria to growth under laboratory conditions was proved efficient to increase success in culturing previously unknown microorganisms from the environment. It has, moreover, highlighted that non-standard cultivation techniques, including the use of low nutrient media or innovative methods such as diffusion chambers, were more suitable for the cultivation of new genera and could recover a greater diversity of microorganisms whereas standard techniques usually lead to the re-isolation of the same strains (Hahn et al., 2019; Stewart, 2012).

We remain, however, still far from accessing the entire diversity of environmental microbial communities and some challenges persist. One of the greatest challenges, briefly mentioned previously, is the dependency of many strains on the production of molecules

by other members of the microbial community. This has been highlighted multiple times by the phenomenon of co-culture, when it was noted that some bacteria would be able to grow in laboratory conditions in the presence of a helper strain but not in pure culture (Garcia et al., 2014; Jezbera et al., 2009; Kaeberlein et al., 2002; Morris et al., 2008). While attempting to find the mechanisms of dependency, it has also been shown that some of the helper strains, instead of providing a growth factor or substrate, may modify the environment to make it suitable for the growth of the dependent strain. As an example, one study has demonstrated the protective role of helper strains against oxidative stress in a Cyanobacterium and were able to improve its culturability in pure culture by adding catalase to the growth medium (Morris et al., 2011, 2008). Independently of the co-culture phenomenon, a study used metatranscriptomics to find the appropriate conditions for the growth of a targeted bacterium, thanks to the RNA-transcript data they could identify its use of mucin as a carbon source and managed to isolate and grow the bacterium on mucin supplemented medium (Bomar et al., 2011).

Although impressive protocols for the fruitful identification of conditions and key factors needed for the isolation and cultivation of novel microorganisms have been used (Stewart, 2012), it cannot be ignored that they are laborious and time-consuming and must be adapted on a case by case basis. Overall, finding the appropriate conditions of pH, temperature, oxygen levels, nutrient types and concentration required for the growth of uncultured microorganisms in pure culture requires laborious exploration of unlimited combinations. Efforts to develop novel culturing techniques and innovative protocols for the identification of microbial growth requirements must therefore continue if we are to progress a better understanding of the microbial world.

2.4. Conclusion

This review described the challenges in the field of sediment management for ports and harbours, highlighting the issues linked with the most commonly used method that is dredging, especially in terms of environmental impacts. It therefore reviewed some alternative methods that have been developed recently and shed light on AND, a promising method that could combine sediment management with bioremediation. Among the numerous contaminants present in ports and harbours, the focus was on TBT, which is

often stated as the most toxic contaminant in the aquatic environment. It was banned in 2008 but is still recorded at high concentrations in many ports and harbours around the world. Some experiments aimed at the assessment of the factors influencing TBT biodegradation were described. Considering the potential ubiquity of TBT-microbial degraders and their ability to degrade TBT under aerobic condition, AND is proposed as a remediation method for TBT contaminated sediment. A more complete understanding of TBT biodegradation, however, is still needed in order to optimize the application of AND. This involves the isolation of different TBT-degraders, which has hitherto only been achieved using standard plating methods. The use of nonstandard isolation and culturing techniques could help to discover degraders among the uncultured bacterial phyla.

3. Experimental procedures and methodology development

3.1. Field Sampling

Several sampling campaigns were carried out throughout the project and were performed in different parts of the Liverpool Dock system with a double objective. First, to obtain muddy sediment to perform microcosm experiments using a material that would have the appropriate texture to perform AND. Second, to assess the contaminated status of different locations within Liverpool Docks, which explains why different docks were sampled each time. In addition, sampling TBT-contaminated mud would have been useful to assess TBT degradation in conditions that are the closest to the environmental ones. The sampling and storage of sediment as well as sieving can induce changes in biological and physicochemical characteristics, but the process of spiking a contaminant introduces further changes that can impact the outcome of biodegradation experiments (Northcott and Jones, 2000). In fact, several factors can differ between an *in-situ* contamination and a contamination created in laboratory by adding TBT to the mud and are detailed in section 3.2.

All samples were taken with a Van Veen grab sampler which collects the first 20 centimetres of the sediment layer (Figure 3-1).

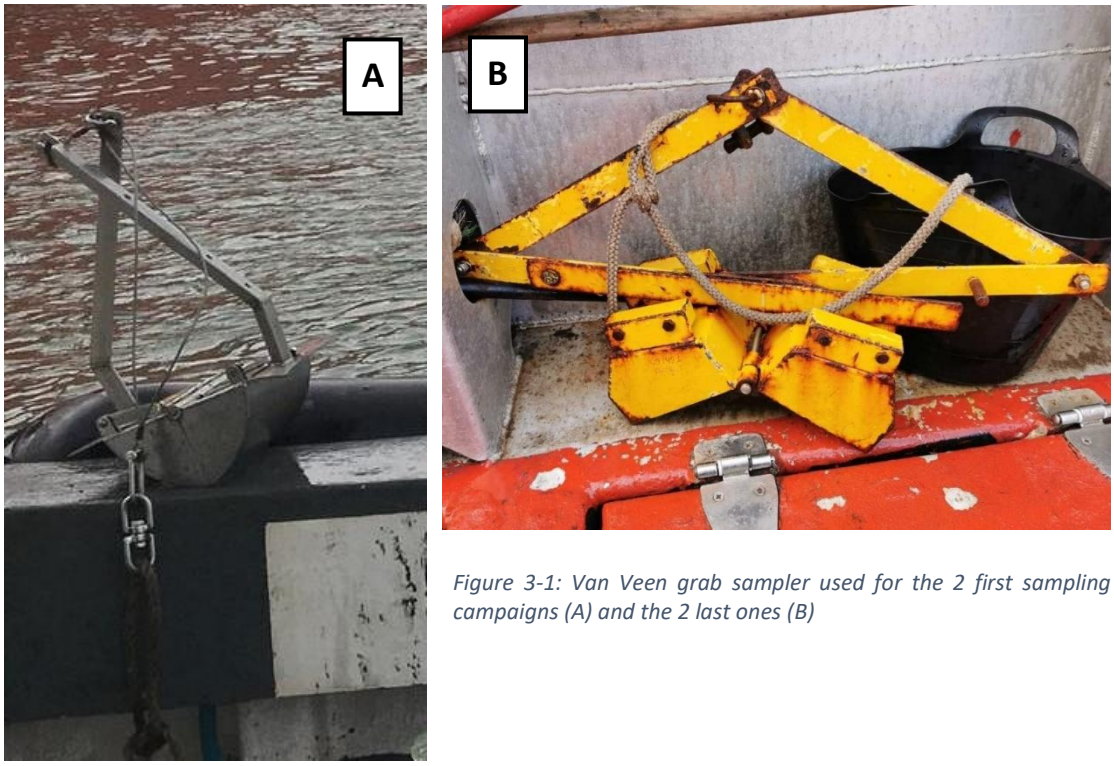


Figure 3-1: Van Veen grab sampler used for the 2 first sampling campaigns (A) and the 2 last ones (B)

A first sampling was performed in October 2018. Nine grab samples of approximately five kilos were taken in Gladstone Dock. A second sampling was performed in October 2019. Five grab samples of approximately five kilos were taken in Sandon and Bramley Moore Docks (Figure 3-2, Figure 3-3, Table 3-1).

It was initially planned to perform an assessment of the mud before and after some AND field trials in Liverpool Docks. Unfortunately, the field trials were delayed. As an alternative, it was decided to sample some sediment before and after dredging operations. Two supplementary samplings therefore occurred in February 2020 before and after water injection dredging and each time 10 grab samples of approximately 10 kilos were taken in Brocklebank Dock (Figure 3-2,Table 3-1).

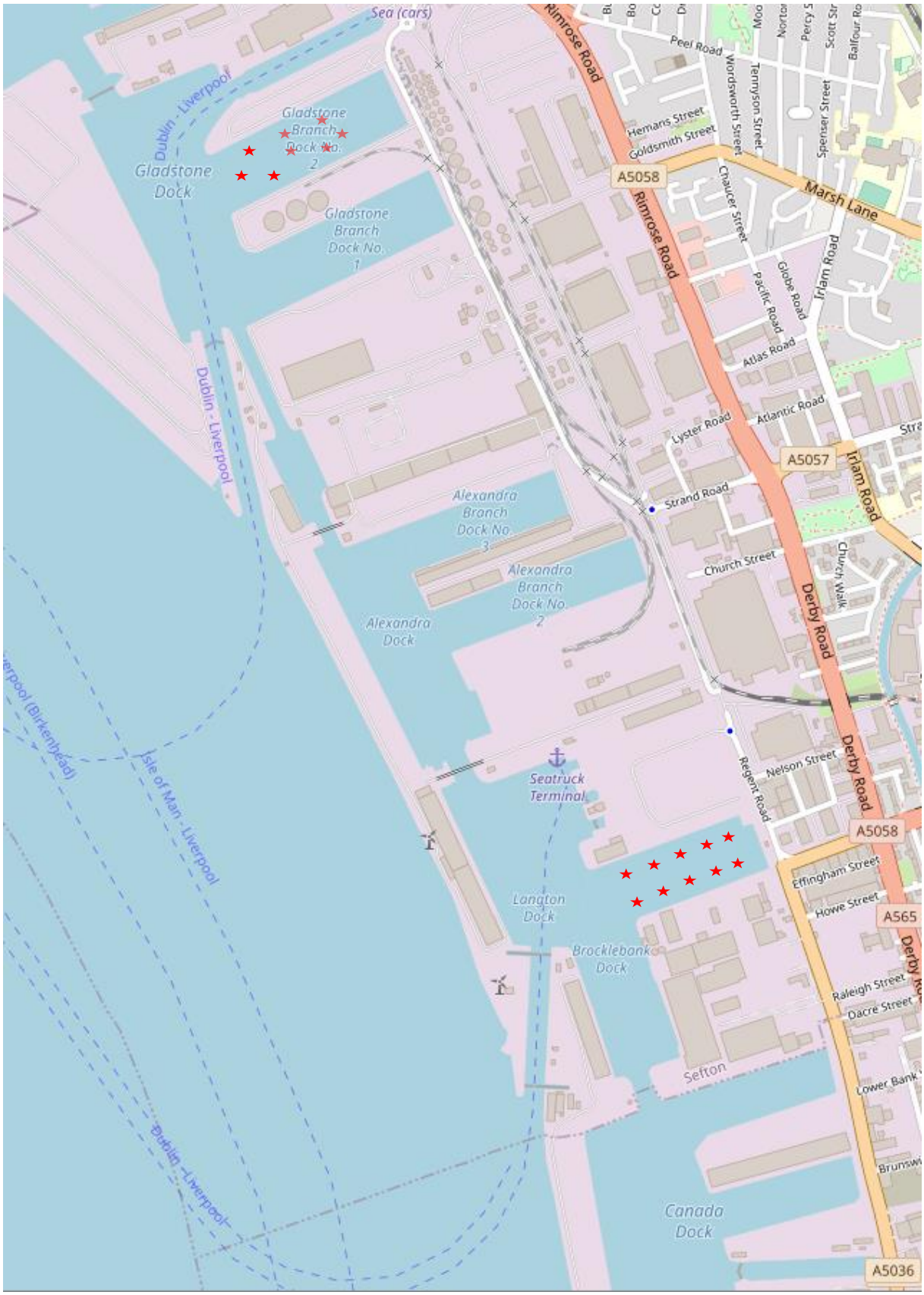


Figure 3-2: Liverpool Docks map representing Canada Docks and Brocklebank Dock.
Sample approximate locations are indicated by crosses. The exact coordinates are presented in table 1.

Figure 3-3: Liverpool Docks map representing Sandon Half Tide Dock and Bramley Moores Dock.
Sample approximate locations are indicated by red stars. The exact coordinates are presented in table 1.

Table 3-1: Sample list with their coordinates

Sample name	Sampling Date	Northing	Easting
1A	17/10/2018	395945.17	332514.54
2A	17/10/2018	395901.37	332513.19
3A (= sediment A)	17/10/2018	395895.06	332576.93
4A(= sediment A)	17/10/2018	395945.49	332596.66
5A	17/10/2018	395978.41	332584.1
6A	17/10/2018	395997.05	332652.81
7A	17/10/2018	395966.84	332700.73
8A	17/10/2018	395933.43	332653.27
9A	17/10/2018	395999.7	332760.19
A2	10/2019	392914.90	333295.14
B2	10/2019	392822.03	333255.97
E2 (= sediment B)	10/2019	392714.76	333364.33
H2	10/2019	392465.62	333332.31
K2 (= sediment C)	10/2019	392492.68	333552.24
B201	11/02/2020	394486,478	333230,627
B202	11/02/2020	394501,297	333297,886
B203	11/02/2020	394525,088	333297,886
B204	11/02/2020	394543,395	333408,603
B205	11/02/2020	394561,133	333469,209
B206	11/02/2020	394434,312	333260,509
B207	11/02/2020	394461,084	333311,826
B208	11/02/2020	394478,196	333374,483
B209	11/02/2020	394497,629	333426,75
B210	11/02/2020	394527,272	333480,671
B301	18/02/2020	333229,8	394483,9
B302	18/02/2020	333290,4	394500,1
B303	18/02/2020	333344,5	394524,6
B304	18/02/2020	333401,3	394546,2
B305	18/02/2020	333461,9	394566,8
B306	18/02/2020	333250,2	394433,3
B307	18/02/2020	333304	394448,8
B308	18/02/2020	333364,3	394471,5
B309 (= sediment D)	18/02/2020	333430,4	394487,1
B310	18/02/2020	333479,9	394528

3.2. Chemical analyses of the sediment samples

The pH of a 1:2.5 ratio of sediment:deionised water in 15 mL centrifuge tubes was measured with a pH probe after shaking them for 5 min and letting them settle for 30 min.

Total carbon, inorganic carbon and total nitrogen were analysed using an elemental microanalyser (Skalar Elemental Microanalyser). For this, according to the laboratory procedure, 2.5 g of each sample was oven dried for 72 hours at 60°C before sieving at 0.3 mm. The samples were kept in a desiccator until the day of analysis. A known quantity of approximately 0.25 g of each sample was then put in the analyser along with the appropriate reference compound: 0.1 g of glycine for nitrogen measurement and 0.3 g

calcium carbonate for carbon measurement. The difference between the values of Total Carbon and Inorganic Carbon measured by the elemental microanalyser is used to calculate the amount of organic carbon.

The particle size distribution was measured using a Beckman Coulter laser granulometer and the analyses performed using Gradistatv8 (Blott and Pye, 2001).

3.3. Microcosm experiments

In order to comprehend the environmental parameters influencing TBT biodegradation, literature was reviewed on pure cultures or consortia of TBT degrading bacteria, microalgae, or fungi (Bernat et al., 2013; Hassan et al., 2018; Murthy et al., 2007b; Tsang et al., 1999). The study of bacteria in synthetic media is limited as the conditions are very different from the natural environment and the effects observed during such experiments are not likely to reflect what happens in nature. A further step to mimic more closely the natural environment at the laboratory scale is the microcosm approach (Pritchard and Bourquin, 1984). In a microcosm, bacteria are believed to evolve in a small replication of the environment (Pritchard and Bourquin, 1984). This method however also has limitations and only field trials can confirm the patterns observed.

Microcosm experiments to study TBT biodegradation in sediment have been performed previously (Cruz et al., 2014; Finnegan et al., 2018b; Lee et al., 2012; Sakultantimetha et al., 2011; Suehiro et al., 2006; Tessier et al., 2007; Yonezawa et al., 1994), but most focus on freshwater settings or have added a specific bacterial strain with the aim to assess the potential of bioaugmentation for the remediation of TBT contaminated sediment (Cruz et al., 2014; Finnegan et al., 2018b; Sakultantimetha et al., 2011; Suehiro et al., 2006). In this study, since the wider objective is to evaluate the possibility of implementing *in situ* bioremediation in ports and harbours, microcosms were set up in order to assess the potential of the native microbial community to biodegrade TBT in different environmental conditions without bioaugmentation.

3.3.1. Environmental factors tested:

Microcosms were set up in different conditions to assess the factors influencing TBT (bio)degradation in the environment. The water temperature in Liverpool Docks ranges

from approximately 6°C in winter to 17°C in summer (data provided by Peel Ports). Consequently, the shaking incubators available for these experiments were set to 8°C as the closest possible representation of winter temperature and 15°C as the closest possible representation of summer temperature in the laboratory. Higher temperatures of 20 and 25°C were also tested to detect a potential plateau in TBT degradation, which is normally expected to increase with temperature (Sakultantimetha et al., 2010). The standard density chosen for mud substrates within the microcosms was 1.18 kg/m^3, which is the density of equilibrium of fluid mud after sediment resuspension in water (empirically verified) and should therefore correspond to the density of the fluid mud obtained through AND. In order to determine whether density is a factor controlling TBT degradation, other densities of 1.12 kg/m^3 and 1.15 kg/m^3 were tested.

As the presence of oxygen is a known key factor for TBT biodegradation (Pensaert et al., 2005; Sakultantimetha et al., 2011), a trial to provide different aeration levels within the microcosms was established by pumping air into the flasks at low and high rate with a vacuum pump. This however led to a substantial evaporation in the flasks, requiring the samples to be supplemented with sea water collected during the sampling campaigns (salinity of 27 PSU). Given the difficulty in obtaining a homogeneous aeration of the flasks or to maintain a consistent water content between the flasks, the system was later abandoned. Agitation was however used as another way to control the aeration status of the mud throughout these experiments. Therefore, some microcosms were incubated without agitation to determine if it slows the degradation of TBT.

Finally, a question remained about the possibility of providing supplementary sources of energy to the microorganisms in the sediment, in fact the literature reported contrasting effects of amendment on the biodegradation of various contaminants, sometimes enhancing it (Demirtepe and Imamoglu, 2019; Tang et al., 2019; Ye et al., 2013), other times hindering it (Z. Wang et al., 2015; Wong et al., 2002). As the first experiment did not show significant degradation levels, a carbon and nitrogen supplement was added to the samples to provide an energy source comparable with the natural environment.

3.3.2. Spiking procedure:

All aspects of soil and sediment organic compound spiking procedures have been discussed in detail by Northcott and Jones (2000). A compound usually has to be added to soil or sediment together with a solvent, which first introduces organic carbon to the matrix and then the solvents can be toxic by themselves or by modifying the solubility of other compounds (Northcott and Jones, 2000). These effects can therefore provoke changes in the microbial communities; however, they can be mitigated by solvent volatilization. It is therefore advised to consider this characteristic when choosing a solvent for spiking (Northcott and Jones, 2000). In the literature, ethanol was the most common solvent for microcosms of the microbial culture study of TBT degradation. This is despite the fact that ethanol is commonly used as an antiseptic, and is not always appropriate for the spiking of compounds in experiments studying microbial activities (Northcott and Jones, 2000). However, TBT being highly hydrophobic, there was a limited choice of solvents to be used for the purpose of these experiments. Ethanol solubility in water and volatility also makes it a good choice for the spiking of wet sediment, it was therefore selected for the current study.

Despite sampling different locations with the hope to obtain TBT contaminated mud to work with, the surface samples retrieved were not contaminated with organotin compounds. We therefore decided to carry on the experiment by adding TBT to the sediment. TBT was spiked at a concentration of 10 µg/ g dw sediment. This corresponds to a high level of contamination and was easy to detect. Previous tests problems were encountered with the development of an appropriate extraction and derivatization method to consistently measure organotin compounds at lower trace levels (section 3.4). A stock solution of tributyltin chloride was prepared at 1.2 g/L and added to the sediment at the chosen concentration. The mud was then mixed by hand for 10 minutes and left to equilibrate in the cold room at 4°C for 4 weeks.

3.3.3. Microcosms preparation:

For all the microcosm experiments, the mud was sieved at 2 mm. Approximately a month before the start of the experiments, the mud was resuspended with excess seawater. After 24 hours the excess water was removed. This process enabled the standardization of mud

density at 1.18 kg/cm^3. For the experiment involving different densities, three buckets of different density were prepared by mixing different volumes of mud and water, the densities were measured by weighing. The water content was then calculated in order to spike tributyltin chloride (Sigma-Aldrich) at 10 mg/g dw. The mud was thoroughly mixed by hand and allowed to equilibrate for at least 4 weeks at 4°C.

For the amended experiments, the mud was spiked with 5 g/kg dw of glucose (Sigma-Aldrich) and ammonium nitrate (Sigma-Aldrich) a week before the start of the experiments. On the first day of the experiments, 250 mL conical flasks were filled with 150 mL of mud. For the sterile microcosms, the flasks were filled up in advance and autoclaved for 2 times 40 min at 121°C, then kept in the cold room at 4°C until the start of the experiments.

3.3.3.1. Short-term microcosms without amendment:

Sterile and non-sterile flasks of mud from Gladstone Docks were incubated in triplicate at 8 and 25°C under agitation (Figure 3-4). Other triplicates of sterile and non-sterile flasks of mud were incubated at room temperature under different aeration patterns: closed flasks, flasks in which air was pumped through a vacuum pump at high rate, and flasks in which air was pumped with a vacuum pump at low rate (Figure 3-4). The flasks were incubated for 2 weeks with a sampling every day for the first week, and then once a week. The flasks incubated under high-rate air pumping dried out after the first weekend and were therefore not processed any further. The closed flasks however were incubated for 4 weeks in total.

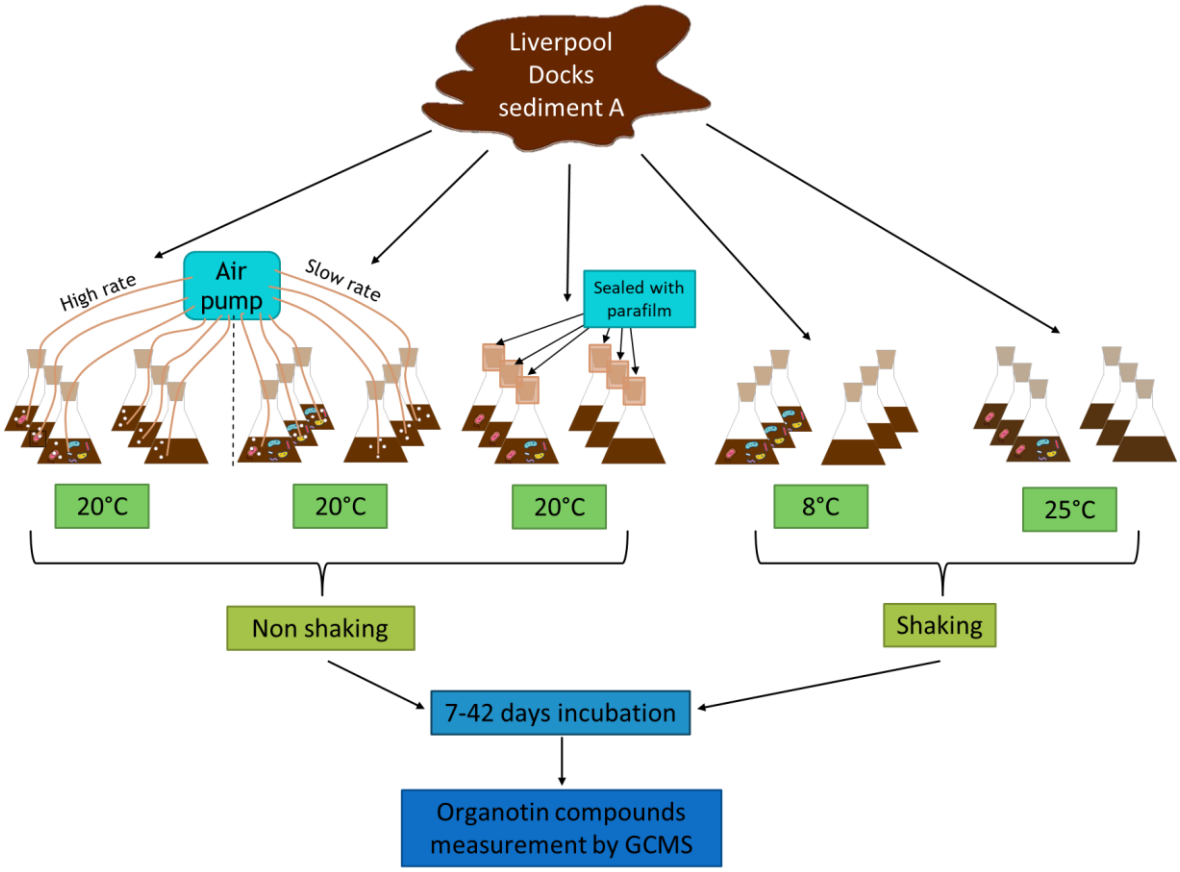


Figure 3-4: Short-term microcosm experiment set-up using TBTCl-spiked sediment. *For each condition of temperature, aeration and agitation, three replicates of non-sterile flasks (with colourful bacteria in the picture) and three replicates of sterile flasks were set-up.*

3.3.3.2. Short-term microcosms with amendment:

Sterile and non-sterile flasks of mud from Bramley Moore Dock were incubated in triplicates at 8, 15 and 20°C under agitation and at 20°C without agitation (Figure 3-5). Other triplicates of sterile and non-sterile flasks of mud from Sandon Docks were incubated at 20°C without agitation (Figure 3-5). The flasks were incubated for 35 to 42 days and sampled once a week.

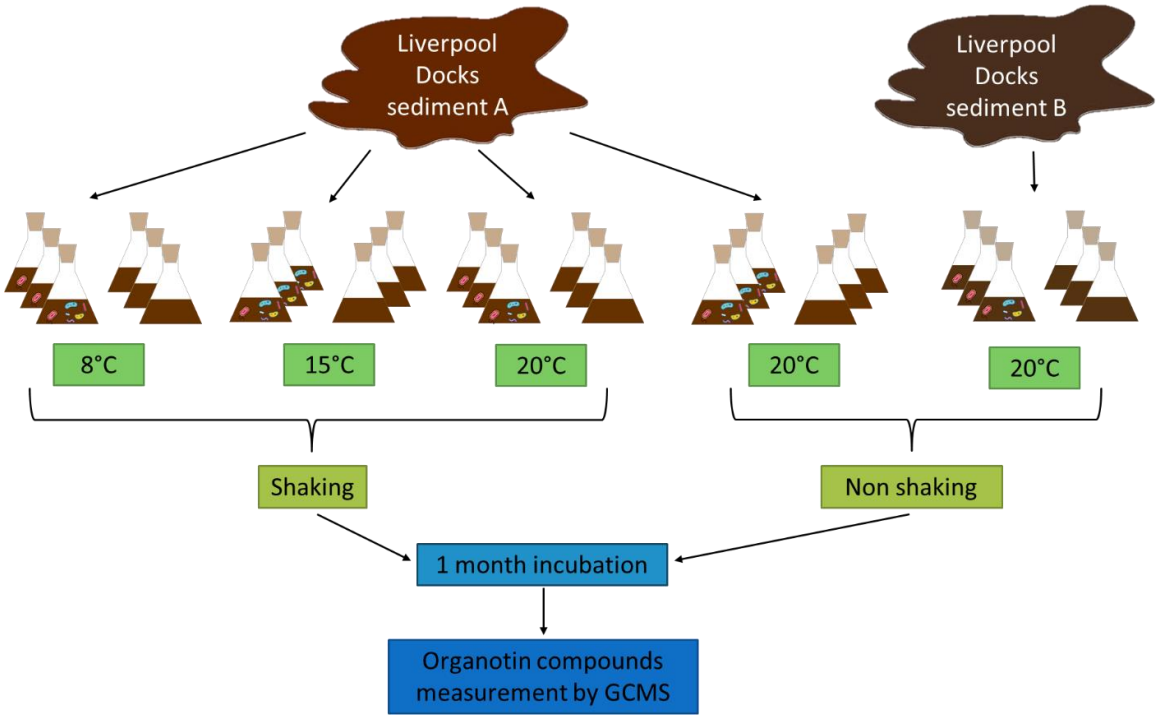


Figure 3-5: Short-term microcosm experiment set-up using TBTCl-spiked sediment supplemented in carbon and nitrogen

3.3.3.3. Mid-term microcosms at summer temperature:

Sterile and non-sterile flasks of mud from Bramley Moore Dock were incubated in triplicate at 15°C under agitation for 3 months (figure 3-6). Note that the flasks were removed after 1 day of incubation to be put in the cold store during 3 months during university closure due to the Covid-19 pandemic. They were subsequently sampled and placed again in the incubator after the reopening of the labs. The flasks were then sampled at the end of the experiment, 3 months after the second incubation.

3.3.3.4. Mid-term microcosms at different densities:

Sterile and non-sterile flasks of mud from Brocklebank Dock at the different densities of 1.15, 1.18 and 1.20 were incubated in triplicates at 20°C under agitation for 3 months (Figure 3-6). The flasks were sampled at the start and at the end of the experiment.

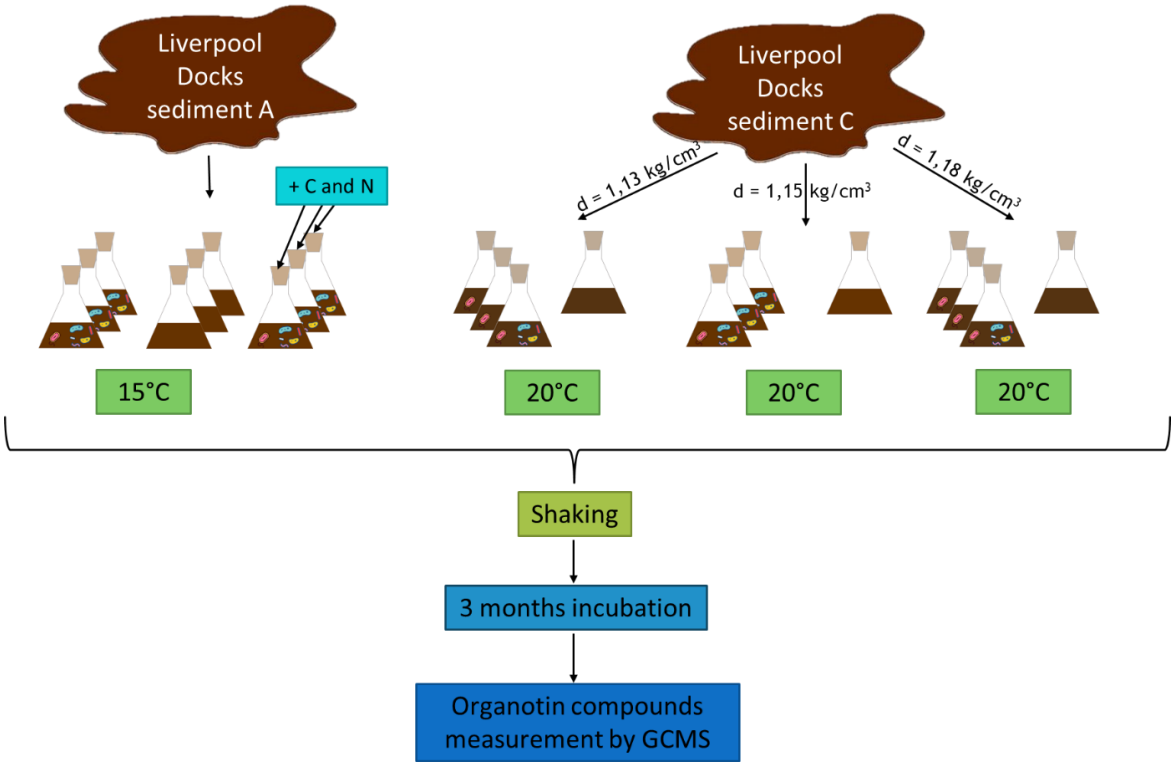


Figure 3-6: Mid-term microcosm experiment set-up using TBTCl-spiked sediment

3.3.4. Flask sampling and storage of the samples

Microcosm flasks were regularly sampled: for the short-term microcosms 12 – 16 g of mud was sampled whereas for the mid-term microcosms 20 - 30 g of mud was sampled into a weighing boat and put in a freezer at -20°C. During sampling, the dissolved oxygen of the flasks was measured with an ethanol washed dissolved oxygen probe. In addition, a subsample of 0.5 g of mud was transferred in 15 mL centrifuge tubes for bacterial enumeration. The obtained samples were then freeze-dried for 60 hours main drying and 12 hours final drying using a Lyocube freeze dryer (Christ Alpha 1-4 LSCplus). The freeze-dried samples were reduced into a fine powder using a mortar and a pestle and stored in plastic bags in a freezer at -20°C until further analyses.

3.3.5. Bacterial enumeration:

A viable count was performed at the different steps of the microcosms to check a potential change in the abundance of bacteria, and contamination of the controls. For this purpose, trypticase soy agar (TSA) medium was used as a standard rich growth medium that have

been used in previous studies of bacteria involved in TBT biodegradation (Beolchini et al., 2014; D. Khanolkar et al., 2015).

The 0.5 g of subsamples taken out during each subsampling of the microcosms was resuspended in 4.5 mL of sterile PBS then further diluted in ten-fold dilution series four times. A volume of 200 µL of the three last dilutions was spread on Petri dishes filled with TSA, which were consequently incubated at 37°C for 24 hours.

3.4. Organotin measurement method development

Many different analytical methods have been proposed over years for the detection and measurement of organotin compounds in environmental samples (de Carvalho Oliveira and Santelli, 2010; Dietz et al., 2007; Finnegan et al., 2018a), the most widely used being gas chromatography (GC) coupled with different detectors (Finnegan et al., 2018a): mass spectrometry (MS), flame photometry (FPD), inductively coupled plasma mass spectrometry (ICP-MS) and atomic emission spectrometry (AES). GC analyses of organotin compounds always require a derivatization step, which converts them to more volatile and thermostable compounds.

Several derivatizing reagents can be used: sodium borohydride ($NaBH_4$), tetraethylborate ($NaBet_4$) or Grignard reagent. The Grignard reagent was one of the first to be utilised but it is very time-consuming and requires special care and dry conditions to avoid reactions with water, alcohols or acid-ketones (Morabito et al., 2000) so it is now mostly replaced by $NaBet_4$. $NaBet_4$ is efficient and shows lower detection limits (Liscio et al., 2009), but presents the disadvantage of catching fire spontaneously in contact with air and therefore still involves complex handling and disposal procedures. Hydride generation using $NaBH_4$ can alternatively be used, being low-cost and straight forward (Yáñez et al., 2016). Therefore, an attempt to adapt organotin analysis by GCMS using $NaBH_4$ as a derivatizing reagent was firstly made and is described hereafter together with the final development of the analysis using $NaBet_4$ after obtaining poor results with the hydride generation method.

3.4.1. Material and methods:

3.4.1.1. Reagents:

Tropolone, trimethylpentane, triphenyltin chloride, tributyltin chloride, dibutyltin chloride, monobutyltin chloride were obtained from Sigma-Aldrich. The stock solutions of organotin compounds at 1000 µg/mL each were prepared monthly, and all the working solutions were made up fresh on the day of the experiments.

3.4.1.2. Initial extraction protocol:

1 g of freeze-dried sediment was placed in fresh 50 mL centrifuge tubes. Then 5 mL of 0.25% tropolone in diethyl ether is added followed by 5 g of anhydrous sodium sulphate and 200 µL of 10 µg/L internal standard (triphenyltin chloride). The tubes were shaken vigorously for 40 minutes on mechanical shaker after which the tubes were centrifuged down for 5 minutes at 3000 g and the ether layer was recovered into 50 mL Duran bottles. This procedure was repeated once without the addition of anhydrous sodium sulphate. The combined ether layers were then dried under nitrogen and recovered in 50 mL deionized water.

3.4.1.3. Alternative protocols for extraction:

Protocol 2:

1 g of freeze-dried sediment was placed in a fresh 50 mL centrifuge tube. Then 30 mL of 0.2% w/v NaCl at pH 1.7 was added together with 200 µL of 10 µg/L internal standard (triphenyltin chloride). The mixture was sonicated for 15 min and 5 mL of tropolone 0.5 % in diethylether was added. The tubes were then shaken vigorously on mechanical shaker after which the ether layers were recovered in 50 mL Duran bottles. The procedure was repeated from the addition of tropolone. Then the combined ether layers were dried under nitrogen and resuspended in 50 mL deionized water.

Protocol 3:

1 g of freeze-dried sediment was placed in a fresh 50 mL Duran bottles and 30 mL of 0.2%w/v NaCl at pH 1.7 was added together with 200 µL of 10 µg/L internal standard (triphenyltin chloride). The tubes were sonicated for 15 min and 30 mL of the supernatant

were recovered in 50 mL Duran bottles. For this protocol, the derivatization has been conducted with and without 0.5% tropolone in trimethylpentane.

Protocol 4:

1 g of freeze-dried sediment was placed in a fresh 50 mL Duran bottles and 20 mL of acetic acid in methanol (3:1) was added together with 200 µL of 10 µg/L internal standard (triphenyltin chloride, TPhT). The mixture was sonicated for 15 min, centrifuged down and 1 or 5 mL of supernatant was transferred to clean 50 mL Duran bottles.

Protocol 5:

1 g of freeze-dried sediment was placed in a fresh 50 mL Duran bottles and 30 mL of methanolic acetic acid (3%v/v acetic acid in methanol) was added together with 200 µL of 10 µg/L internal standard (triphenyltin chloride). The tubes were sonicated for 15 min, centrifuged down and the supernatant was transferred to clean 50 mL Duran bottles.

Protocol 6:

1 g of freeze-dried sediment was placed in a fresh 50 mL Duran bottles and 10 mL of 0.5 M tartaric acid in 20% methanol was added together with 200 µL of 10 µg/L internal standard (triphenyltin chloride). The tubes were sonicated for 15 min, centrifuged down and 5 mL of supernatant was transferred to clean 50 mL Duran bottles.

3.4.1.4. Derivatization

Using sodium borohydride:

The following solutions were added to the extracts: 6.25 mL sodium tetraethylborate (4% w/v in 0.1% NaOH), 4 mL of acetate buffer 2M (pH of the solution should be above 7 at this step) and 1.5 mL of hexane. The mixture was shaken for 30 min on mechanical shaker and the hexane layer was recovered in vials for further analyses by GCMS.

Using sodium tetraethylborate:

The following solutions were added to the extracts: 100 or 200 µL of 2%w/v $NaBet_4$, 1.5 to 20 mL of acetate buffer 1 M pH 5.4, and 1.5 mL of trimethylpentane. The mixture was shaken for 15 or 30 min on mechanical shaker and the trimethylpentane layer was recovered in vials for further analyses by GCMS.

3.4.1.5. *Final protocol for the extraction and derivatization of sediment samples:*

Extraction:

1 g of freeze-dried sediment was placed in a fresh 50 mL centrifuge tube and 20 mL of acetic acid in methanol (3:1) was added. The mixture was sonicated for 15 min, then left 5 minutes to settle down and 1 mL of supernatant was transferred to clean 50 mL Duran bottles.

Derivatization:

To 1 mL of sediment extract or TBT standard in acetic acid:methanol (3:1), 20 mL of 1 M acetate buffer pH 5.4, 200 µL of 10 µg/L internal standard (triphenyltin chloride), 200 µL of 0.2% $NaBet_4$ and 1.5 mL of trimethylpentane were added. The bottles were shaken for 30 minutes on a mechanical shaker. The trimethylpentane layer was then recovered in vials for further analyses by GCMS.

3.4.1.6. *GCMS parameters:*

Vials were analysed through GCMS using the parameters detailed in tables 3-2 and 3-3.

Table 3-2: GCMS parameters

Gas chromatograph	**Mass spectrometer**
Splitless	Mass filter: single quadrupole
Injected volume: 1 µl	Ion source: EI, 70 eV
Solvent delay: 4 mins	Quadrupole temperature: 150 °C
Initial oven temperature: 50 °C (held for 2 min)	Source temperature: 230 °C
Temperature ramp: 30 °C/min	Scan range (*m/z*): 41 – 500
Final oven temperature: 280 °C (held for 5 min)	

Table 3-3: Single Ion Monitoring (SIM) details

Ethylated organotin compound	Parent organotin compounds	SIM group start time (min)	Quantification ion (m/z)	Confirmation ion (m/z)
Butyltriethyl-tin	MBT	4	235	179
Dibutyldiethyl-tin	DBT	6.9	263	179
Tributylethyl-tin	TBT	7.5	291	177
Triphenylethyl-tin	TPhT	10.6	351	197

3.4.2. Results and discussion

Organotin compound derivatization using $NaBH_4$ has been proposed as an alternative to the use of more hazardous reagents like Grignard reagent and $Nabet_4$. A method developed for the derivatization and GCMS analysis of organotin compounds in water samples (Yáñez et al., 2016) was therefore used and modified to be applied to TBT-spiked sediment samples. Figure 3-7 represents one of the tests involving the extraction, derivatization, and analysis of TBT-spiked freeze-dried sediment replicates. It shows a very high variability in the detected TBT concentrations, with no detection at all for one of the replicates. This high variability is likely due to matrix interferences and has been previously described with hydride generation (Martin et al., 1994).

Although this method was shown to be suitable for the analysis of organotin compounds in water with a low detection threshold, consistent results could never be obtained from its application to sediment samples in the present study. As a consequence, further method development was conducted using sodium tetraethylborate. Higher peak values were obtained for the compounds of interest using this new derivatizing agent, but the inconsistency of the results persisted (Figure 3-8), together with high background noise, a signal interfering with the TBT peak as well as strong sulphur interferences (Figure 3-9). The extraction protocol was therefore questioned, and a range of tests performed to determine a better protocol.

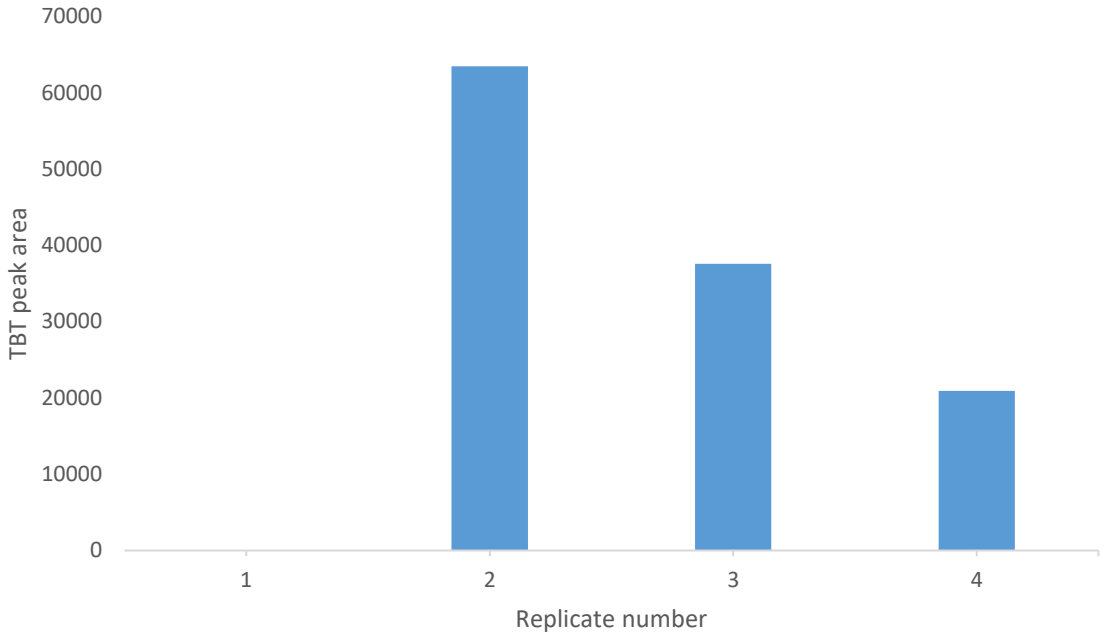


Figure 3-7: Peak Areas obtained after GCMS analyses of four replicates of one gram of sediment spiked with 25µg of TBT, using the initial procedure for extraction and $NaBH_4$ as derivatizing agent.

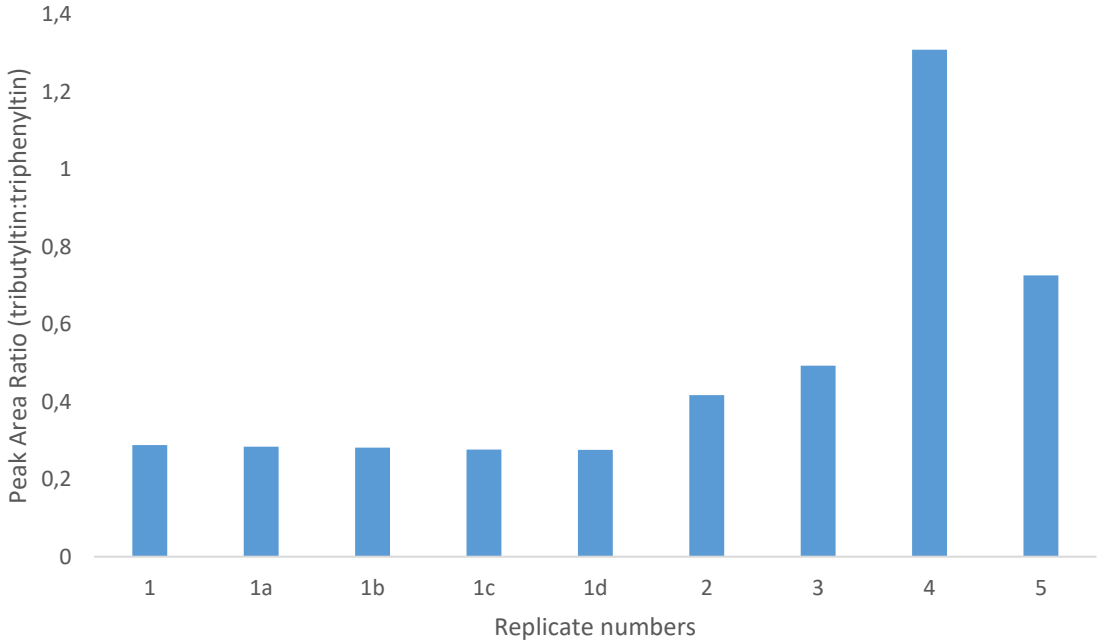


Figure 3-8: Histogram showing the peak area ratio between tributyltin and triphenyltin (internal standard) derivatized using NaBet4.

The bars 1, 1a, 1b, 1c, 1d correspond to the same standard analysed multiple times on the GCMS and account for the instrumental repeatability. Replicates 1, 2, 3, 4 and 5 were derivatized separately and account for experimental repeatability.

The tropolone used in the initial extraction protocol has a role of complexing agent in order to increase the recovery of compounds with shorter and fewer alkyl chains (in our case MBT for example). However, it leads to higher amounts of co-extracted compounds and may be responsible for the background interferences observed previously, so it was decided to test some protocols without tropolone. A study assessing the best extraction protocol for the analysis of organotin compounds by GCMS in sediment samples with high sulphur content suggested the use of tartaric acid (Flores et al., 2011), it was consequently tested to get rid of the sulphur interferences obtained previously.

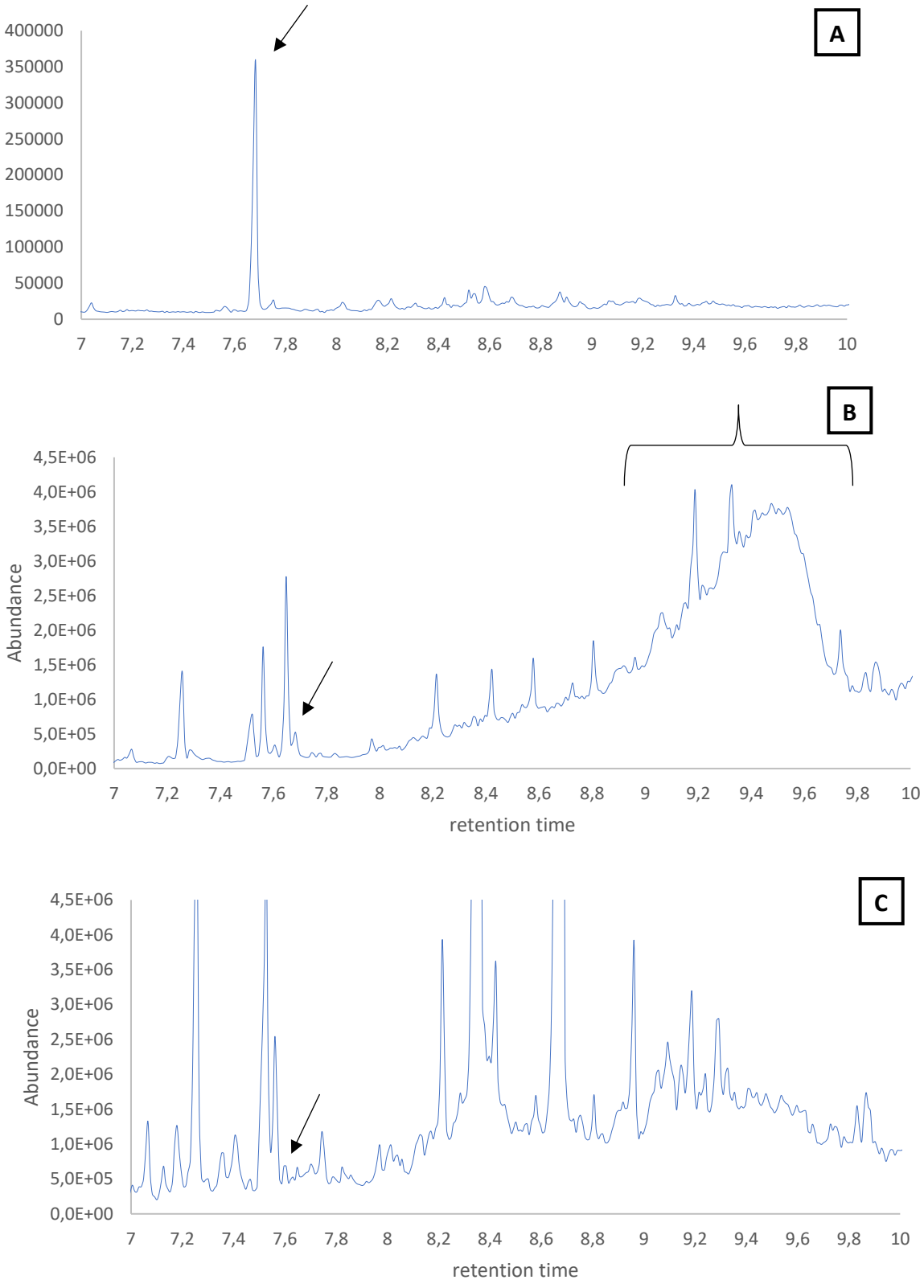
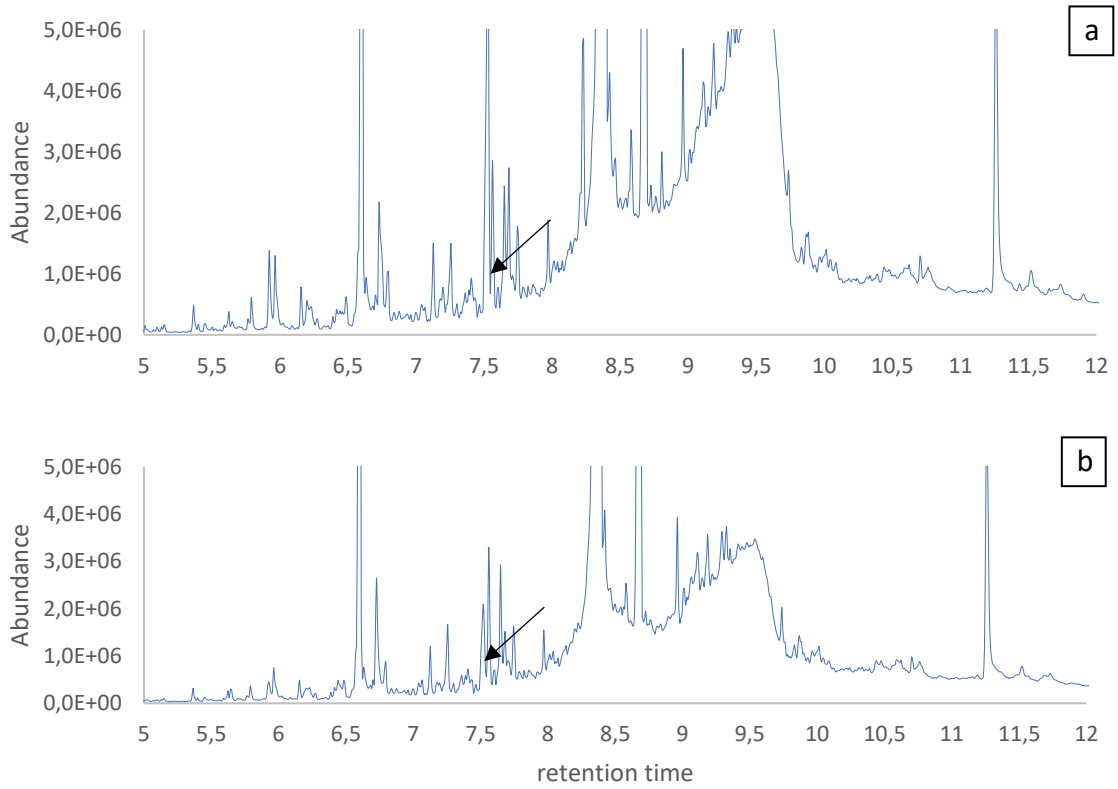


Figure 3-9: Chromatograms obtained from the total ion current (TIC) analyses of TBT of two sediments spiked with 50 (B) and 25 (C) ppm of TBT, extracted with the initial protocol for extraction and derivatized using NaBet4.
Chromatogram A shows the TIC analysis of TBT standard in water. Arrows show the location of the expected TBT peak (at 7.62 min), which is slightly visible whereas interfering with a co-extract peak for the chromatogram B, but indistinguishable from background noise for chromatogram C. The accolade shows sulphur interferences.

The chromatograms showing the results of the extraction and derivatization of TBT-spiked sediment using different extraction protocols are visible in Figure 3-10 and 3-11 respectively for the total ion current (TIC) and the single ion monitoring (SIM). Three protocols were already excluded by chromatogram plots. Protocol 2 and 3, which did not show distinguishable TBT peaks and presented a high background noise (Figure 3-10a, b and c). Protocol 5 demonstrated an acceptable TIC analysis (Figure 3-10e) but did not show a satisfactory peak for the internal standard on the SIM analysis (Figure 3-11b). The results were encouraging however for protocol 4 and 6 (Figure 3-10d and f, Figure 3-11a and b) so they were selected for further development. Protocol 6, using tartaric acid, was eliminated later as no sulphur interferences were observed with any of the new extraction protocols.



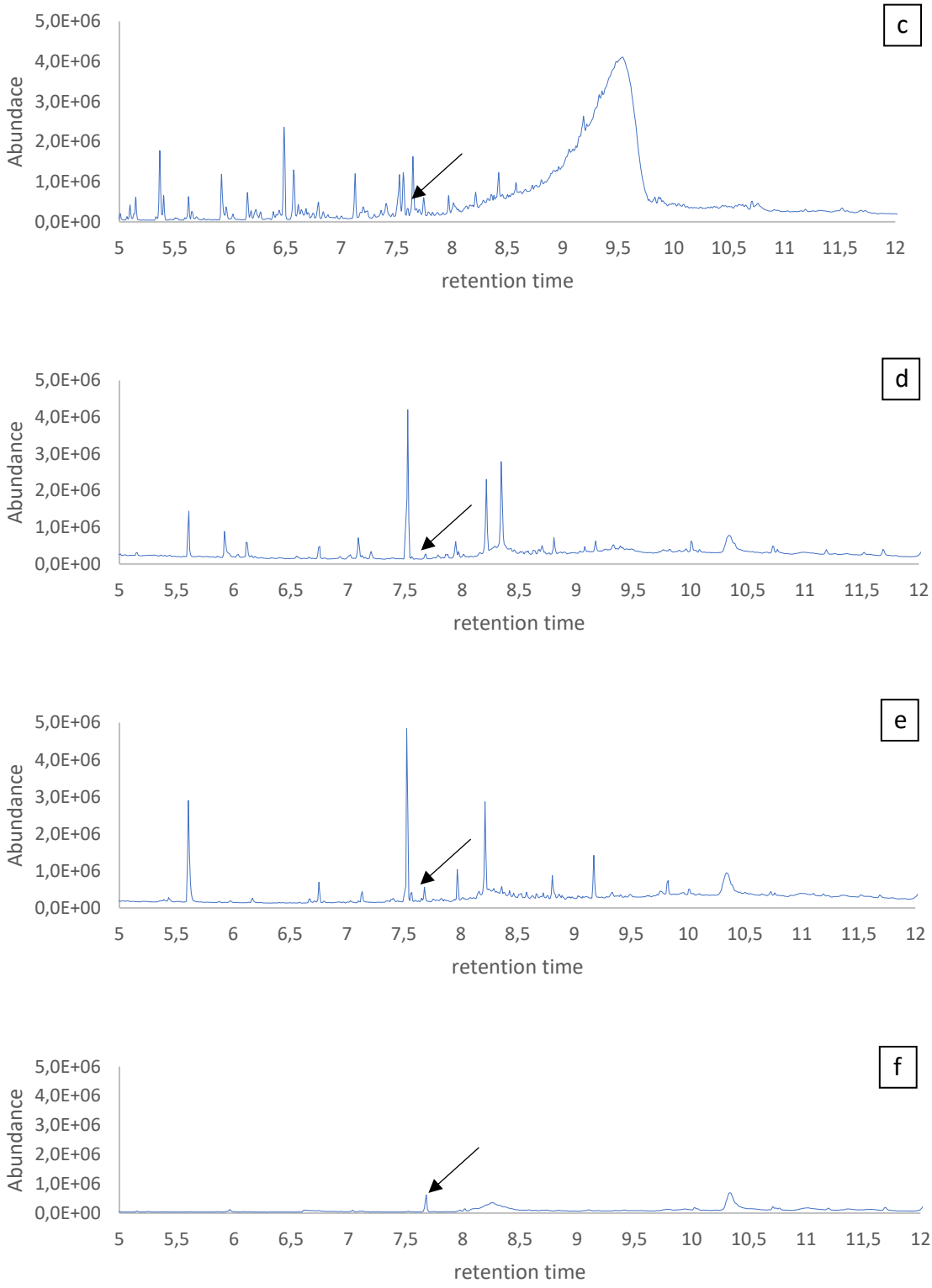


Figure 3-10: Chromatograms representing the TIC analyses of sediment spiked with 10ppm TBT and extracted following protocol 2, 3 (without tropolone), 3 (with tropolone), 4, 5 and 6 respectively for a, b, c, d, e and f.
The extracts were derivatized using $NaBet_4$. Arrows show the expected TBT peak location at 7.62 min. TPhT peaks, when present, are visible at 10.75 min and are designated by a star.

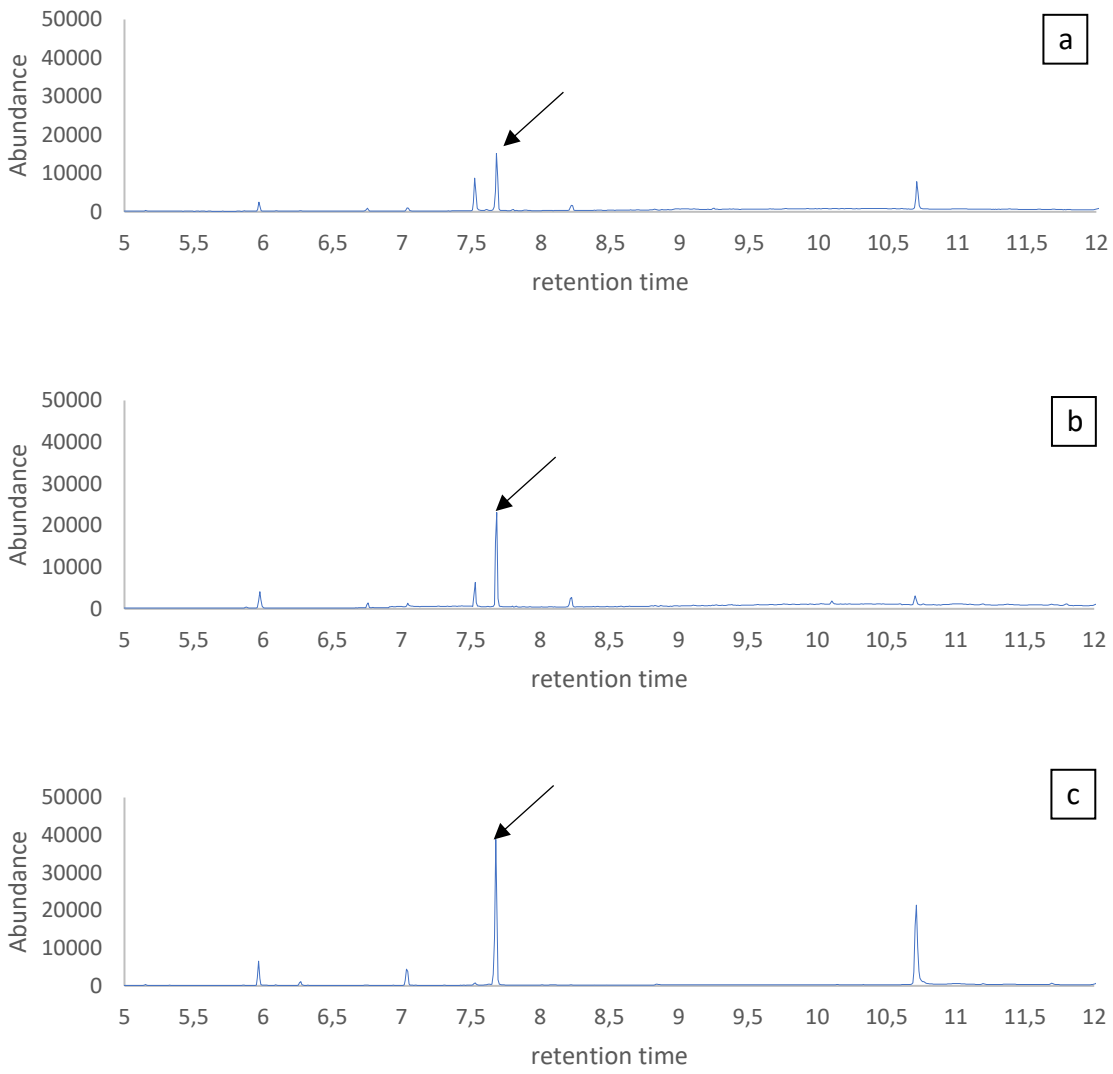


Figure 3-11: Chromatograms showing the single ion monitoring (SIM) analyses of sediment spiked with 10 ppm TBT and extracted using protocols 4, 5 and 6 respectively for a, b, and c.
The extracts were derivatized using $NaBet_4$. Arrows show TBT peaks (7.62 min). TPhT peaks can be seen at 10.75 min and are designated by a star.

For the next development steps, the volume of extract to be derivatized was decreased in order to be able to add a higher volume of acetate buffer, and thus obtain a better control of the pH of the reaction, which was adjusted to 5.4. A volume of 1 mL was selected as it led to the chromatogram of higher quality, with a very low background noise (Figure 3-12).

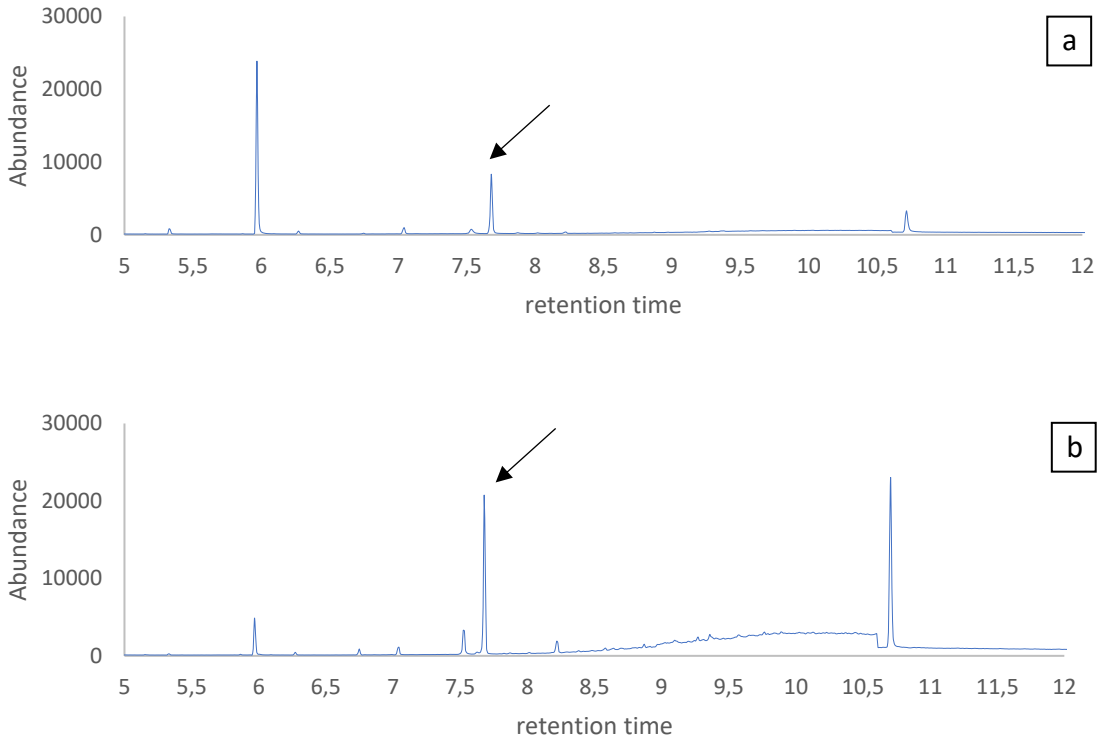


Figure 3-12: Chromatograms showing the SIM analyses of TBT spiked sediment extracted using protocol 5. *NaBet4 was used to derivatize 1 mL of extract (a) or 5 mL of extract (b). Arrows show the TBT peak. TPhT peaks can be seen at 10.75 min and are designated by a star.*

Some inconsistencies obtained with the internal standard value were fixed by adding a higher volume of NaBet4, as it was likely it was consumed by side reactions and therefore not in sufficient quantity to derivatize all our compounds of interest (TBT, DBT, MBT, TPhT).

As a result of numerous optimization steps, the procedure selected to carry on organotin analyses during this study was the extraction of organotin compounds with a mixture of acetic acid and methanol (3:1), and the derivatization of 1 mL of this extract using 200 µL of 2% $NaBet_4$ and 20 mL acetate buffer, with the addition of the internal standard during the derivatization step. For each run, a standard curve was made with the derivatization and analysis of organotin compounds standards dissolved in the extraction solvent and a home-made reference sediment, made by spiking sediment with a known amount of TBT, DBT and MBT, was used to calculate a recovery percentage which were used to correct the sample values.

Table 3-4 represents an example of the analysis of freeze-dried sediment samples taken from the microcosm experiments. The control sediment is a homemade reference sediment made by spiking TBT at 4 $\mu g.g^{-1}$ dw. On average, the recovery percentages

obtained for TBT, DBT and MBT were 97.1%, 98.6% and 84.5% respectively. The lower recovery percentage for MBT is likely due to a less efficient extraction from the sediment in absence of tropolone. The associated standard curves are shown in Figure 3-13.

Table 3-4: Peak Area ratios and calculated concentration of TBT, DBT and MBT obtained from the extraction, derivatization and GCMS analysis of sediment samples *coming from microcosm experiments and control samples made by spiking freeze-dried sediment by a mix of organotin at a final concentration of 4 µg.g^{-1} dw.*

Sample name	peak area TBT:TPhT	peak area DBT:TPhT	peak area MBT:TPhT	TBT (µg.g^{-1} dw)	DBT (µg.g^{-1} dw)	MBT (µg.g^{-1} dw)
Control sediment 1	0.03354	0.05305	0.08120	3.959	4.130	3.441
Control sediment 2	0.03226	0.04835	0.07836	3.805	3.756	3.319
Control sediment 3	0.03365	0.04882	0.07438	3.973	3.793	3.147
Sample 1.1	0.09859	0.02581	0.007338	12.16	1.993	0.2935
Sample 1.2	0.09516	0.02624	0.008609	11.73	2.027	0.3586
Sample 2.1	0.08544	0.02255	0.009878	10.53	1.730	0.4235
Sample 2.2	0.09162	0.02408	0.009770	11.29	1.853	0.4180

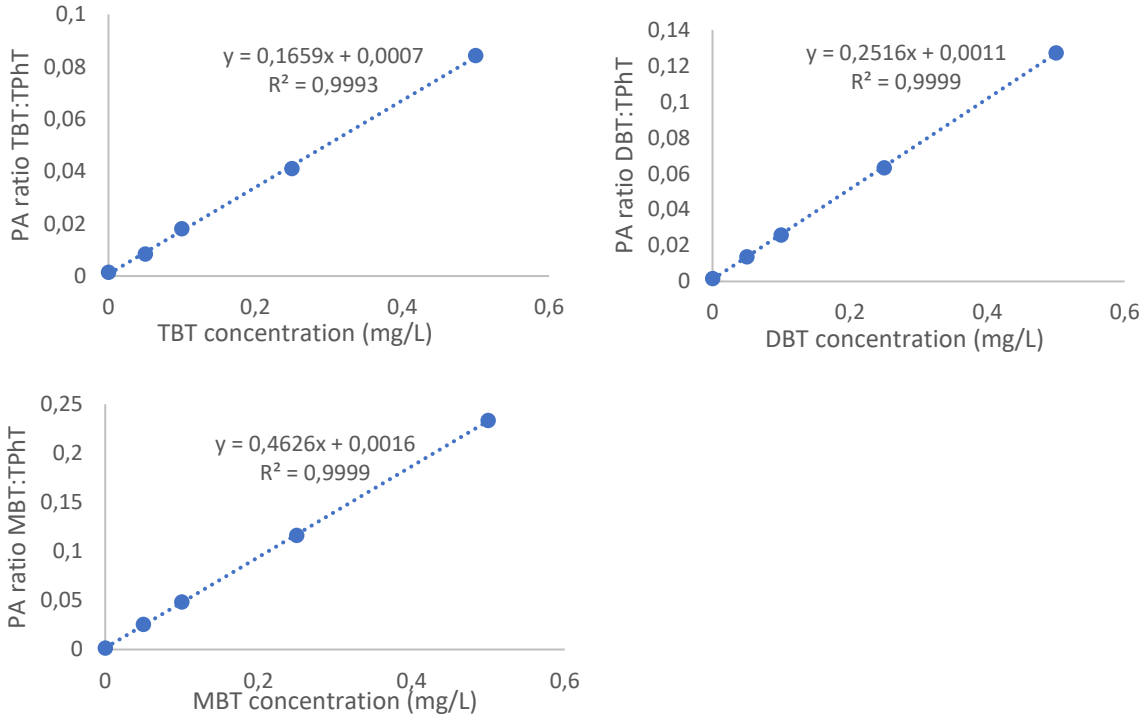


Figure 3-13: Standard curves obtained from the derivatization and GCMS analysis of TBT, DBT and MBT standards *during the run shown as an example in this section (Table 3-4).*

3.5. Statistical analyses for the organotin compounds' measurements

3.5.1. Issue with the arbitrary use of α = 0.05

Null hypothesis testing has now long been the dominant statistical approach in science. However, it is widely applied by convention without careful planification. In particular with regard to the significance level α which is usually arbitrarily set at 0.05. Yet, many studies have pointed out the inappropriate use of this significance level which can lead to wrong interpretations, sometimes at a high cost (Cascio and Zedeck, 1983; Field et al., 2004; Glaz and Yeater, 2020; Mudge et al., 2012; Orme and Tolman, 1986). In fact, depending on other parameters, especially sample size, the statistical power may be too low to efficiently detect any effect using alpha = 0.05, leading to a repeated acceptation of the null hypothesis. But in some cases, the cost of not detecting an effect that is present (type II error) can be very high whereas detecting an effect when there is none (type I error) would

not have much negative impact. This is for example the case of environmental management decisions, where a type II error can lead to substantial damages to the environment such as species extinction, water supplies pollution or strong decrease of fish stocks (Field et al., 2004).

To avoid this, it is advised to seek a maximised statistical power by first determining the minimum effect size that would be considered significant (Cascio and Zedeck, 1983). Then determine the appropriate sample size depending on the intended statistical power, effect size and alpha risk. However, studies are often constrained in term of sample size. In this case, to be able to achieve the intended statistical power, the solution is to reconsider the alpha level based on the importance of type I and type II error in the study (Cascio and Zedeck, 1983).

3.5.2. Choice of the alpha threshold:

In the present study, the sample size is constrained by the capacity of incubators and the microcosms were systematically run in triplicates. This is insufficient to ensure an acceptable statistical power and detect the potential medium or low effects. The risks associated with type I and type II errors were therefore considered.

In our case, we are measuring TBT degradation under several parameters that are relevant in the context of potentially applying AND as a method of bioremediation for TBT contaminated sediment. Therefore, the risk associated with a type I error would be to bring the tests to the next phase with more elaborated microcosms and/or field test to confirm the observed effects when actually these effects did not exist, implying the cost of these experimentations. Whereas the risk associated with the type II error would be to reject the possible application of AND as a method of bioremediation and keeping up with dredging

and the associated issues and cost, whereas AND would have potentially been effective. The consequence of type II error was therefore considered more important than type I error. We chose to increase the alpha level to 0.15 in order to offset the lack of power of the statistical analyses using the commonly used level of 0.05 which would have led to the incapacity of the tests to detect low or medium TBT biodegradation in the microcosms.

3.6. EPS measurement:

3.6.1. EPS extraction:

In order to quantify the production of EPS in some of the microcosms, extractions were performed on the subsamples of the short-term microcosms, using a protocol adapted from Underwood *et al*. (1995) and de Winder *et al.* (1999).

1 mL of NaCl 25% solution was added to 50 µg of freeze-dried sediment sample, mixed thoroughly, and incubated at room temperature for 1 hour. The mixture was centrifuged for 15 min at 14 000 rpm and the supernatant was taken (colloidal fraction). The pellet was then resuspended in 1 mL of EDTA 0.1 M and incubated at room temperature for 4 hours. The mixture was centrifuged again for 15 min at 14 000 rpm and the supernatant was taken out (capsular fraction).

3.6.2. Carbohydrate measurement:

Carbohydrates were measured using the phenol-sulphuric acid method adapted for microplate assays (Rasouli et al., 2014). A standard curve was made up ranging from 0 to 1.5 g/L of glucose. 40 µL of carbohydrate solution (test sample or standard) was added to a well of a 96-well plate, followed by 200 µL of concentrated sulphuric acid and finally 40 µL of phenol 6.5% and the microplate was shaken and left to incubate at room temperature in the dark for 2 hours. The absorbance was then measured at 495 nm.

Given the time required to perform these analyses, and the absence of observed variation, they were not performed on the other microcosm experiments.

3.7. Bacterial communities

A selection of subsamples from the microcosm experiments were chosen to perform a 16S rRNA gene sequencing in order to monitor temporal changes in bacterial communities during the experiment. The selected samples are displayed in Table 3-6, the microcosm samples were chosen due to the interesting TBT-degradation pattern.

Table 3-5: List of samples selected for next-generation sequencing

Name	Stage of storage/incubation at which the subsample was taken	replicate number	Carbon and nitrogen amendment	Source mud sample
E2	after sampling in the docks	0	no	E2
2102t0-4d	after spiking and 1 month of equilibration	2	no	E2
2102t0-15d	after 3 months in the cold store at 4 degrees (= 1st lockdown)	2	no	E2
2102t90-15d	after 3 months of incubation at 15 degrees	2	no	E2
2103t0-4d	after spiking and equilibration	3	no	E2
2103t0-15d	after 3 months in the cold store at 4 degrees (= 1st lockdown)	3	no	E2
2103t90-15d	after 3 months of incubation at 15 degrees	3	no	E2
3101t0-4d	after spiking and equilibration	1	yes	E2
3101t0-15d	after 3 months in the cold store at 4 degrees (= 1st lockdown)	1	yes	E2
30101t0-15d	after 3 months of incubation at 15 degrees	1	yes	E2
3102t0-4d	after spiking and equilibration	2	yes	E2
3102t0-15d	after 3 months in the cold store at 4 degrees (= 1st lockdown)	2	yes	E2
30102t0-15d	after 3 months of incubation at 15 degrees	2	yes	E2
3102t0-4d	after spiking and equilibration	3	yes	E2
3102t0-15d	after 3 months in the cold store at 4 degrees (= 1st lockdown)	3	yes	E2
30102t0-15d	after 3 months of incubation at 15 degrees	3	yes	E2

3.7.1. Library preparation:

The protocol advised by Illumina for the sequencing of 16S rDNA makes use of multiplexed primers that are constituted as followed:

- 515F fusion primer: 3'-P5 + i5 + NexTera consensus + Sequencing adaptor + Target region-5'

AATGATACGGCGACCACCGAGATCTACAC – **i5** – TCGTCGGCAGCGTC – AGATGTGTATAAGAGACAG - GTGCCAGCMGCCGCGGTAA

- 806R fusion primer: 3'-P7+ i7 + NexTera consensus + Sequencing adaptor + Target region-5'

CAAGCAGAAGACGGCATACGAGAT – **i7** – GTCTCGTGGGCTCGG – AGATGTGTATAAGAGACAG - GGACTACHVGGGTWTCTAAT

For the purpose of this study, the target region is the v4 region of the 16S ribosomal gene(Caporaso et al., 2011). The i5 and i7 are index sequences of 8 bp which can be associated to form a unique combination, a different combination is added to each sample and enables their separation during the bioinformatic analyses. These primers are very expensive and were not available in our laboratory. An alternative approach was therefore used, utilising multiplexed primers. These have a bridging tail already available in our laboratory with means of a supplementary PCR using simple primers that have the sequencing targeting the region of interest and the sequence attaching to the bridging tail of the multiplexed primers.

3.7.1.1. DNA extraction

The DNA was extracted using a DNeasy Power Soil Kit (Qiagen). The samples were extracted in two batches, for each batch an empty tube was used as a negative control. Separately again, *E.coli* DNA was extracted as a positive control along with another empty tube.

3.7.1.2. DNA amplification:

The first amplification was performed using the following primers:

515F_bg: ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT GTG CCA GCM GCC GCG GTA A

806R_bg: GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC TGG ACT ACH VGG GTW TCT AAT

The blue part anneals to the V4 region of 16S ribosomal gene while the green part of the primer is the sequencing primer that will be used to as a bridge and will be attaching to the primers of the 2nd PCR.

The reaction was prepared in a volume of 25 µL with 12.5 µL of Myfi mix, 1 µL of 10 pM of each primer and 2 µL of DNA. Reactions were then performed in a thermocycler with the following program: 95°C for 3 min of initial denaturation followed by 35 cycles at 95°C for 30 sec, 55°C for 30 sec and 72°C for 30sec, finishing with a final extension at 72°C for 5 min. The amplification of the samples was detected along with a DNA molecular weight standard (1 kb+, Invitrogen) by electrophoresis in a 2% agarose gel stained with SYBR Safe (Invitrogen) and visualized by transillumination by UV light.

The second amplification was performed using the same reagents and 8 cycles of the same program. The primers used this time are built with a sequence binding to the flow cell (orange), a barcode sequence (purple) that forms a combination differing for each sample, and the sequencing primer that was used as a bridge (green).

Example of reverse primer used in the 2nd PCR:

CAAGCAGAAGACGGCATACGAGATctggttGTGACTGGAGTTCAGACGTGT

3.7.1.3. Library Clean-up and Sequencing:

After checking the amplification again with an agarose gel electrophoresis, the samples were pooled together and cleaned using a magbind Kit (Omega) and a ratio beads:DNA of 1:1, they were then further purified by gel extraction (genJET, thermoscientific) and the quality was controlled in the TapeStation (High Sensitivity D1000 kit). Finally, to get the exact DNA concentration in the library, a qPCR was prepared (NEB Qant Kit for Illumina). The library was then diluted, pooled with Phix control (50% each) and loaded in an iSeq 100 reagent cartridge, which was then processed in the iSeq with a flow cell. The sequencing was done using the "generate FastQ" settings.

3.7.2. Sequence analyses:

The Illumina sequence reads were analysed using the obitools software (Boyer et al., 2016). FastQC was used to assess the quality of the reads, and obicut was used to trim reads with a minimum quality threshold of 28. Pair-end reads were aligned using illuminapairend, and

alignments with a quality score <40 were discarded. The aligned data set was demultiplexed using ngsfilter. The aligned reads were further filtered for length 280–300 bp (obigrep) and reads containing ambiguous bases 'N' were removed. The reads were then dereplicated using obiuniq, and a chimera removal step was performed using the uchime-denovo algorithm implemented in vsearch (Rognes et al., 2016). Molecular operational taxonomic unit (MOTU) clustering was carried out using an aggregation clustering algorithm implemented in swarm version 2.2.2 with default parameters (Mahé et al., 2014) with d value of 1. Taxonomic assignment for each MOTU was performed using the BLCA algorithm against a reference 16S database retrieved from the NCBI release. This algorithm uses a phylogenetic approach to assign MOTUs to the most conservative lowest common ancestor or monophyletic group.

3.8. Bacterial Isolation

A comparison of the cultivability of sediment bacterial community on TSA using the standard plating method and the iChip was carried out (Figure 3-14). Two types of sediment were used with both methods, TBT-spiked sediment and non-spiked sediment.

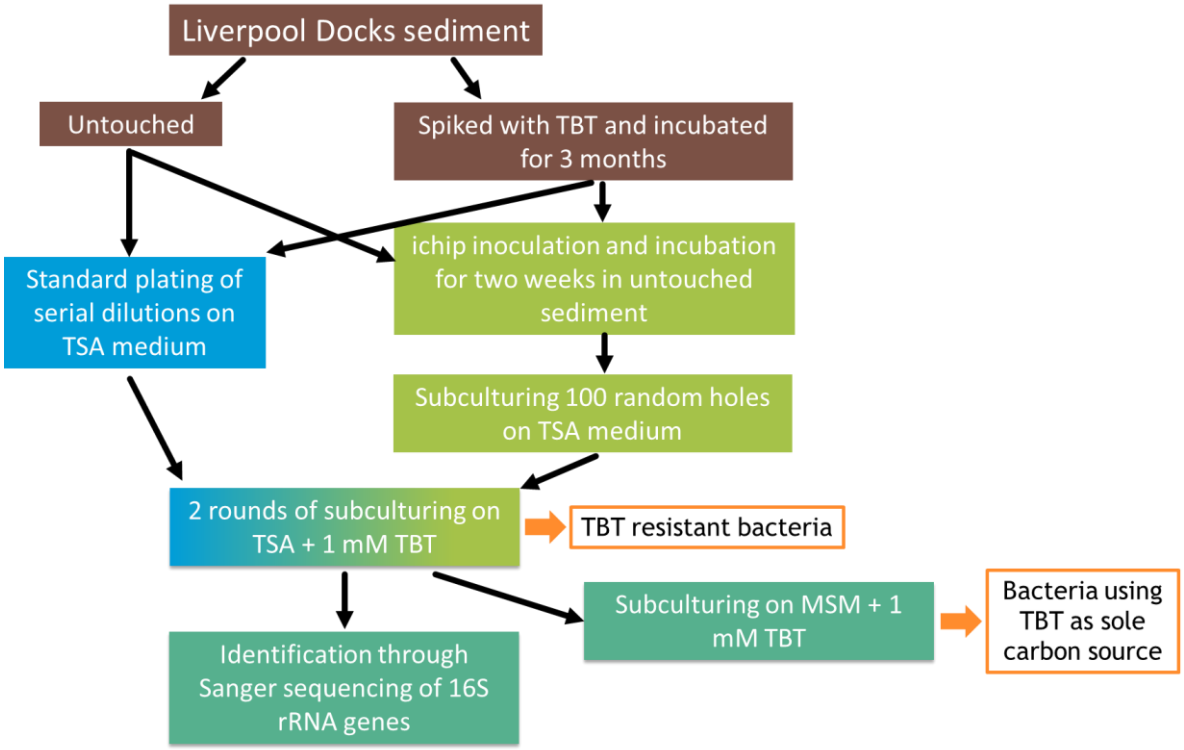


Figure 3-14: Diagram representing the experiments of cultivation and isolation of TBT resistant/degrading bacteria using the iChip and standard plating methods

3.8.1. Media:

TSA was used as a standard medium for bacterial growth. When looking at TBT-resistance phenotype, it was supplemented with TBTCl. For this purpose, a working solution of TBTCl in ethanol was prepared as a 1/10 dilution of the 97% TBTCl stock from Sigma and it was sterilized by filtration. Then the desired volume of TBTCl working solution was added to the medium after autoclaving and cooling to about 50°C. Typically, for TSA + 1 mM TBT, 940µL of TBTCl solution was added to 350 mL of fusion TSA medium and the bottle was agitated for homogenisation before pouring into Petri dishes. Note that TBT precipitates in the agar medium, leaving it cloudy.

For the screening of TBT use as sole carbon source phenotype, a minimum salt medium (MSM) was prepared with the following content per litre: 0.06 g ferrous sulphate; 12.6 g dipotassium hydrogen orthophosphate; 3.64 g potassium dihydrogen orthophosphate; 2 g ammonium nitrate; 0.2 g magnesium sulphate; 0.0012 g sodium molybdate; 0.0012 g manganese sulphate; 0.15 g calcium chloride; 15 g agar. After a couple of unsuccessful attempts, 1 L of medium containing only agar and the phosphate buffer was autoclaved, all the other elements were prepared in solution separately, filter sterilized and added to the fusion medium after autoclaving and before pouring into Petri Dishes.

3.8.2. Plating method:

Standard isolations of bacteria were performed using the plating method. Serial dilutions of sediment suspension with Phosphate Buffer Saline (PBS) were made and poured into Petri dishes containing TSA medium with or without 1 mM TBTCl.

3.8.3. iChip method:

3.8.3.1. Assemblage and incubation:

The iChips were manufactured at the engineering department of LJMU using the instructions provided by Nichols et al. (2010). Figure 3-15 displays pictures of an iChip used for these experiments dissembled (A) and assembled (B, C).

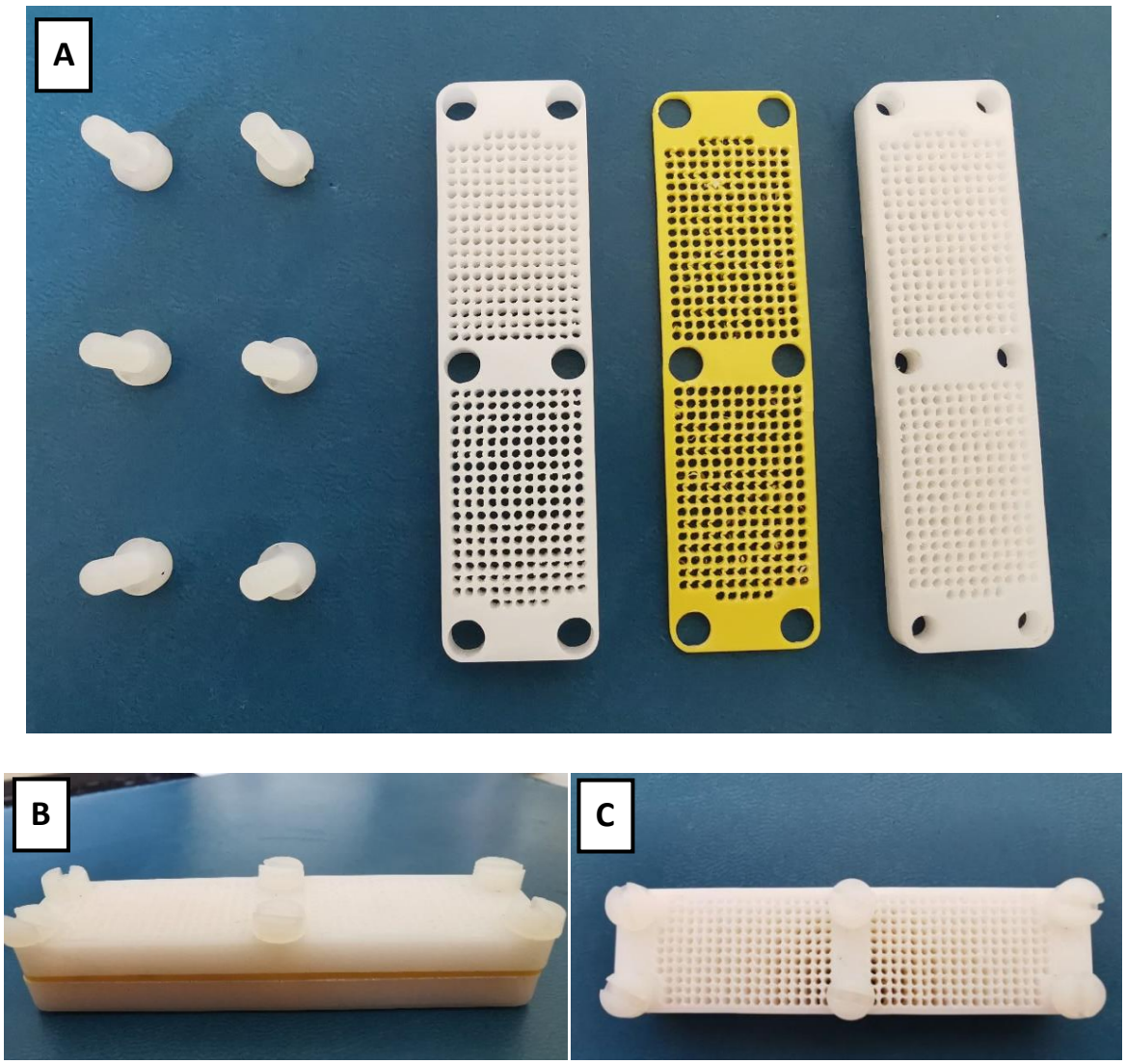


Figure 3-15: Example of an iChip that was used for the cultivation and isolation experiments

The iChip parts were sterilized in 70% ethanol for 15 min prior to the assembly. The central plate was immersed in a tube containing TSA at about 50°C, inoculated with sediment dilution. The iChip was then assembled with sterilized 0.03-µm-pore-size polycarbonate membranes and the outer plates, screwed tight. IChips assembled this way showed contamination during the seal check test, the assemblage was therefore improved by the addition of petroleum jelly and parafilm around the edges. The iChips were then immersed in a bucket of sediment kept at room temperature coming from Bramley Moore Dock for 2 weeks.

3.8.3.2. Control of the sealing:

Control ichips were assembled as above (first without petroleum jelly and parafilm, then with these) but using non-inoculated TSA to check the sealing. Once the sub-culturing of the agar plugs coming from the control ichips on TSA was not showing any growth, the assemblage was validated for the rest of the experiment.

3.8.3.3. Isolate recovery and sub-culturing:

After incubation, the iChips were taken out of the bucket, rinsed in sterile water and disassembled. The agar plugs were recovered using sterile unbent paper clips in wells from 24-well plates previously filled up with 1mL of TSA. The plates were incubated at room temperature in the dark for up to six weeks.

Colonies obtained from the subculturing of agar plugs from the ichips inoculated with sediment dilutions were subcultured in TSA medium with 1 mM TBT to check for the resistance phenotype.

3.8.4. Degradation phenotype screening:

The isolates that could grow on TSA + 1 mM TBT were further subcultured on MSM containing 1 mM TBT as sole carbon source. This method was used as a quick way to check for the ability to degrade TBTCl, but it does screen for the entire degrading community as TBTCl could also be degraded as a co-metabolism rather than for carbon source.

3.8.5. Identification through 16S rRNA gene Sanger sequencing:

3.8.5.1. DNA extraction

24 colonies growing on TSA + 1 mM TBT were selected to be further identified by 16S rRNA gene sequencing. Freshly grown colonies were resuspended in 30 µL of sterile water and heated at 95°C for 10 minutes to extract their DNA. The suspensions were then spun for 2 minutes in a benchtop centrifuge at maximum speed and the supernatant was used as template DNA for the PCRs.

3.8.5.2. DNA amplification

The amplification was performed using the following universal primers:

27F: AGA GTT TGA TCA TGG CTC A

1492R: TAC GGT TAC CTT GTT ACG ACT T

The reaction was prepared in a volume of 50 µL with 25 µL of ReadyMix™ (Sigma), 1 µL of 10 pM of each primer and 2µL of DNA. Reactions were then performed in a thermocycler with the following program: 94°C for 2 min of initial denaturation followed by 35 cycles at 94°C for 1 min, 58°C for 30 sec and 72°C for 1 min, finishing with a final extension at 72°C for 10 min. The amplification of the samples was detected along with a DNA molecular weight standard (1 kb+, Invitrogen) by electrophoresis in a 2% agarose gel stained with SYBR Safe (Invitrogen) and visualized by transillumination by UV light.

The DNA concentration was then measured using a Nanodrop. As all the concentrations were too low, the samples were evaporated and resuspended in the appropriate volume to obtain 25 ng/µL.

5 µL of each sample was then added to 5µL of primer, 24 tubes were prepared with the forward primer 27F and 24 others with the reverse primer 1492R at 5pmol/µL. The 48 tubes were barcoded using the LightRun barcodes from eurofins genomics and sent to the company for Sanger sequencing.

3.8.6. Sequence analyses

When the data were received, the ab1 files were checked for quality and the sequences appropriately corrected. The forward and reverse sequences of the same isolate were aligned and reassembled using BioEdit and the resulting FASTA sequences were analysed by BLAST using the total database, excluding uncultured/environmental sample sequences.

4. Environmental parameters influencing tributyltin biodegradation by natural microbial communities

4.1. Introduction

As an important process occurring during the application of AND for anti-siltation is the growth of aerobic organisms, it was hypothesised that it could be used for the bioremediation of a wide range of contaminants (Kirby, 2013). The biodegradation of sediment contaminants by aerobic microorganisms has been widely reported in the literature (Beolchini et al., 2014; Fahrenfeld et al., 2013; Levi et al., 2014; Li et al., 2015; Mulligan et al., 2001; Schurig et al., 2014; Wald et al., 2015; Wang et al., 2016; Z. Wang et al., 2015). However, much more research is needed to optimise and validate the application of AND for the bioremediation of port contaminants. The first step is to determine the degradation capacity of natural microbial communities in target ports. Being recognised as one of the most toxic compounds ever released in the aquatic environment, and still highly contaminating many ports and harbours around the world, TBT was selected as a model contaminant for this study, and Liverpool docks as the target area.

Therefore, the aim of this chapter is to assess the TBT degrading capacity of native microbial communities of muddy sediment sampled from Liverpool Docks under different environmental conditions. To achieve this objective, microcosms were set-up in different simulated conditions and TBT was measured over time. The variables considered were temperature, agitation, oxygenation and sediment density.

4.2. Results and discussion

4.2.1. Physical and chemical parameters of the sediments used:

The results of the physicochemical parameters of the different sediment used in the microcosms are presented in Table 4-1. All the sediments were slightly alkaline with a pH ranging from 7.7 to 8.3. As expected, the particle size analyses revealed fine-grained sediment. Sediment B and D were classed as mud while sediment C was classed as sandy

mud, it was therefore used as a "different type" of sediment in the short-term amended microcosm experiments. Missing textural and chemical data for sediment A and D are due to instrument disruptions and lack of time to redo the analyses after fixing.

Table 4-1: Sediment physicochemical parameters.

Sediment	pH	% Clay	% Silt	% Sand	Textural Group	Total Organic Carbon (TOC)	Total nitrogen (TN)	Total Carbon (TC)
A	7.7	-	-	-	-	-	-	-
B	8.3	14.3	79.5	6.2	Mud	3.12	0.26	3.92
C	8	8.9	72.6	18.5	Sandy Mud	2.87	0.30	4.08
D	7.8	13.1	81.0	5.9	Mud	-	-	-

4.2.2. Preliminary experiment: short-term microcosms without amendment

The degradation of TBT in sediment has been assessed in several studies and a wide range of half-lives found, ranging from 9 days to several months (Sakultantimetha et al., 2011). Consequently, a first batch of microcosms were set up as a preliminary experiment to assess TBT degradation potential and estimate its kinetics. Only TBT and carbohydrates were partially measured for the star t and the end of the experiment, although subsamples had been taken regularly. In this preliminary experiment, air was pumped into the mud at two different rates. In theory, this was an attempt to simulate the process of aeration in AND and to maintain sediment homogeneity. In practice, however, this had to be stopped after only a couple of days because it induced high evaporation and rapid drying of the mud. It was also clear by the colour of the mud that the system resulted in a patchwork of aerobic and anaerobic mud, which, in addition to the evaporation issues, make the results non-interpretable and the system was abandoned in further experiments.

For the microcosms involving sediment shaking at 8 and 25°C, TBT was measured at the start and end of the experiment (12th day) for only one or two biological replicates. No degradation was observed after 12 days of incubation, except for the non-sterile flasks

incubated at 8°C but only one replicate could be measured, so the statistical significance cannot be evaluated (Figure 4-1). The incubation in minimised aeration conditions, however, resulted in a reduced concentration of 57% of TBT in the flasks after 28 days (Wilcoxon test, p.value = 0.125, Figure 4-1). A degradation of 36.3% was also observed in the control involving autoclaved sediment (Wilcoxon test, p.value = 0.125, Figure 4-1). Bacterial contamination, however, was observed after 3 weeks of incubation in these control flasks. It is therefore not possible to attribute the degradation of TBT to a chemical or a biological process in these conditions.

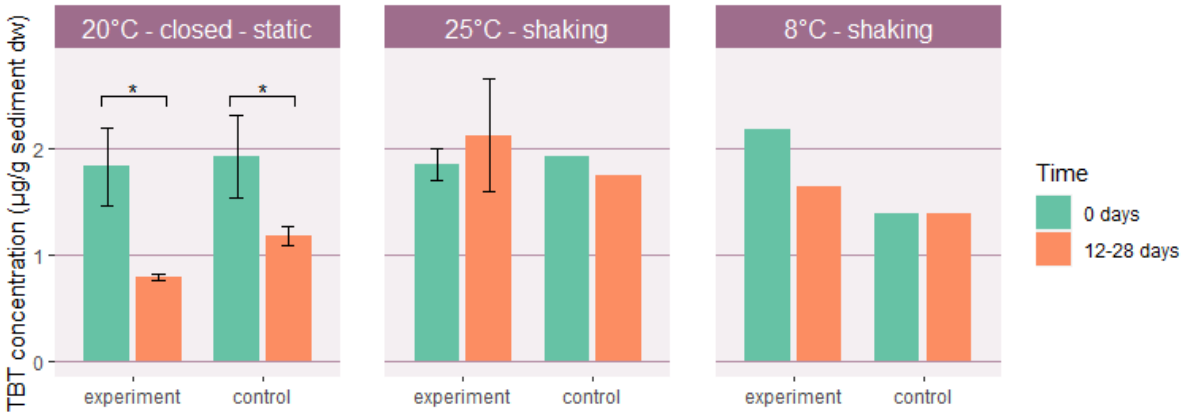


Figure 4-1: TBT concentration during the short-term microcosm experiment (preliminary)
The bar plots are the result of 3 biological replicates for the experiment involving closed flasks incubated at 20°C for 28 days without agitation, 1 replicate for the experiment involving sediment shaking for 12 days at 8°C and sterile sediment shaking for 12 days at 25°C and 2 replicates for the experiment involving nonsterile sediment shaking for 12 days at 25 °C. Error bars represent the standard deviation of the replicates. * represent the statistical significance calculated with a Wilcoxon test with a risk alpha = 0.15

The microcosm conditions chosen for these experiments aimed at mimicking processes occurring during AND (*i.e.* aeration and agitation), and one expected consequence of these processes when AND is applied in the field, is a major production of EPS (Kirby, 2011). This phenomenon is important for the sustainability of AND, as it acts to keep the created fluid mud in suspension for longer. The characteristic viscous aspect of fluid mud when EPS production occurs was not observed during this experiment, and the measurement of carbohydrates (which constitute most part of EPS) confirmed that no substantial production of EPS occurred (Figure 4-2, 3, 4). EPS production can be influenced by pH, temperature, dissolved oxygen and also carbon and nitrogen sources and ratios (Nouha et al., 2018). Even when EPS are produced, other factors influence their flocculation such as hydrodynamics, temperature or pH (Lai et al., 2018). The optimisation of EPS production in the microcosms fell beyond the scope of the project and was not pursued further but is an area of potential future research.

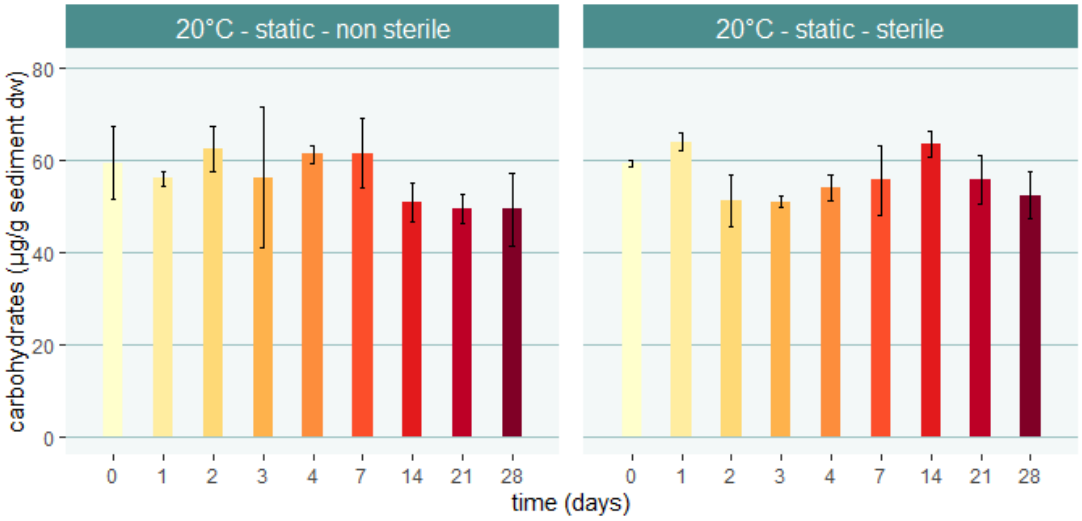


Figure 4-2: Carbohydrates measurements in subsamples of the sediment incubated at room temperature during 28 days without agitation. Error bars represent the standard deviation of 3 replicates.

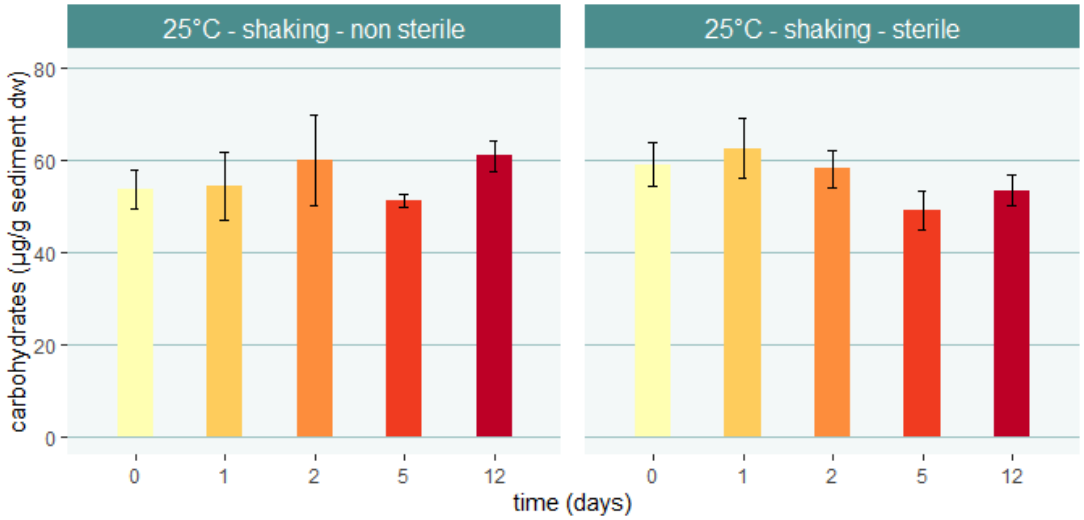


Figure 4-3: Carbohydrates measurements in subsamples of the sediment incubated at 25°C for 12 days with agitation. Error bars represent the standard deviation of 3 replicates.

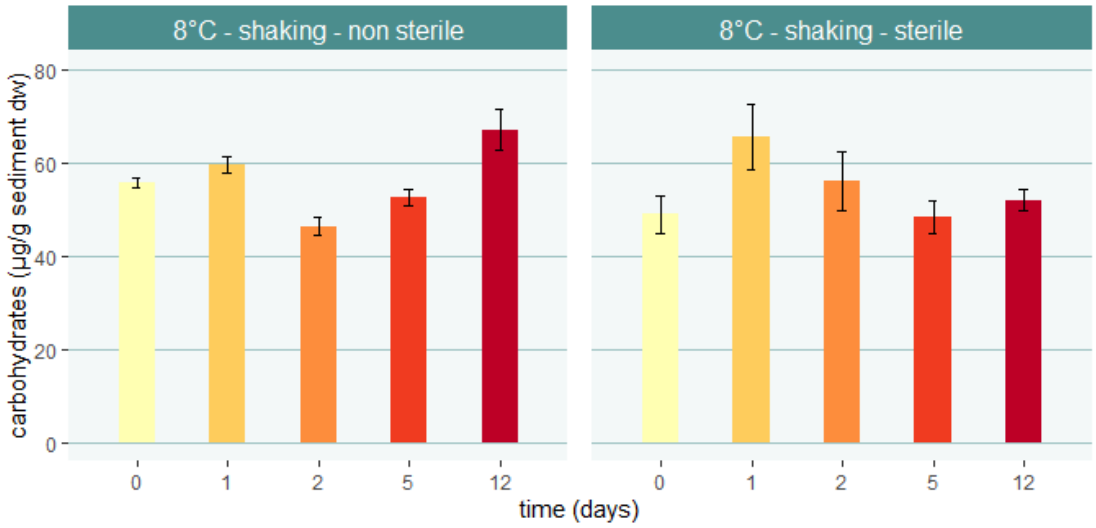


Figure 4-4: Carbohydrates measurements in subsamples of the sediment incubated at 8°C for 12 days with agitation. Error bars represent the standard deviation of 3 replicates.

4.2.3. Short-term microcosms with amendment

As at this time microcosms could not be incubated for more than a month, another series of microcosms were set up using sediment amended with glucose and ammonium nitrate, the hypothesis being that this amendment would boost microbial growth and increase TBT degradation. Biodegradation enhanced by nutrient addition has been shown for numerous sediment contaminants, including PAHs, TNT, glyphosate and triphenyltin (Dell'Anno et al., 2009; Demirtepe and Imamoglu, 2019; Fahrenfeld et al., 2013; Schiffmacher et al., 2016; Tang et al., 2019; Ye et al., 2013). For TBT especially, nutrient addition was reported to reduce TBT half-lives in microcosm experiments involving fresh-water sediment (Sakultantimetha et al., 2011). In these microcosm experiments, however, and despite longer incubation times, none of the conditions showed a significant degradation of TBT (Wilcoxon tests, p.values > 0.15, Figure 4-5).

Figure 4-5: TBT concentration at the start and the end of the short-term microcosm experiment using sediment supplemented in C and N. Each bar plots represents the mean of 3 biological replicates.

The products of degradation of TBT: DBT and MBT were also measured for this set of microcosms, and some more observations could be made (Figure 4-6). The presence of small amounts of DBT before microcosms incubation indicates that some degradation of TBT happens during the equilibration time (when sediment is left for a month in the cold store after the addition of TBT but before the actual incubation), this degradation was not quantified as no initial sample were taken before the equilibration. DBT degradation of 31% was measured for the sediment shaking at 20°C (Wilcoxon test, p-value = 0.125), which was not observed in the sterile control. This DBT degradation can be matched by an increase of MBT in the same flasks (Figure 4-7). A significant increase was also measured in the control, but it was significantly higher for non-sterile sediment (Wilcoxon test, p-value = 0.05). Again, contamination of the control flasks was observed in the 3rd week of incubation, therefore it is impossible to know if some DBT degradation occurred chemically or not, but microbial degradation did actually take place.

For the sediment shaking at 8°C, a significant DBT degradation of 36.2% and 51.5% was observed respectively for the control (in which no contamination was observed throughout the experiment) and the non-sterile flasks (Wilcoxon test, p-value= 0.125). Interestingly, in the sterile flasks, MBT degradation was observed (Wilcoxon test, p-value=0.125). This could mean that MBT is chemically degraded under these conditions, and a potentially higher degradation of DBT (no statistical significance) in the non-sterile flasks produced more

MBT, which masks the observation of its degradation in these flasks. No statistically significant DBT degradation was detected for sediment shaking at 15°C but this result is probably due to the high variability in the measurements.

DBT degradation was significantly higher at 8°C compared to 20°C (Wilcoxon test, p-value = 0.05). For sediment incubated without shaking, a slight DBT degradation was detected for one type of sediment and none for the other. The two sediment were sampled at different locations in the docks and had slightly different textures, the difference in degradation pattern could come from spatial variation in the sediment communities or be due to the nature of the sediment. No significant difference was detected between the DBT degradation rates for the experiment involving sediment under agitation and static sediment despite a clear difference in the oxygenation level (Figure 4-6).

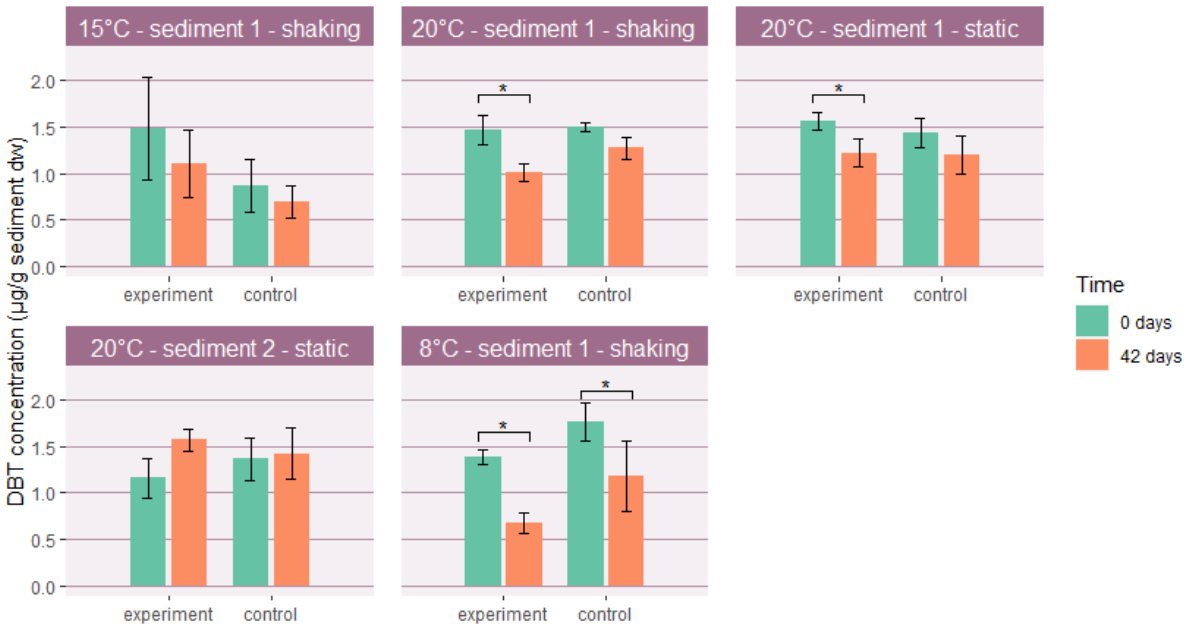


Figure 4-6: DBT concentration at the start and the end of the short-term microcosm experiment using sediment supplemented in C and N. Each bar plots represents the mean of 3 biological replicates. Statistical significance under the test of Wilcoxon is indicated by stars. * for a p-value ≤ 0.15; ** for a p-value ≤ 0.1 *** for a p-value ≤ 0.05.

Figure 4-7: MBT concentration at the start and the end of the short-term microcosm experiment using sediment supplemented in C and N. Each bar plots represents the mean of 3 biological replicates. Statistical significance under the test of Wilcoxon is indicated by stars. * for a p-value ≤ 0.15; ** for a p-value ≤ 0.1 *** for a p-value ≤ 0.05.

4.2.4. Mid-term microcosms:

Since very little statistically significant degradation could be obtained during a month of incubation, another series of microcosms were prepared with an objective of a 3-months incubation period. With the opportunity to use a refrigerating incubator for such a long period, some microcosms were incubated at 15°C (representing summer water temperature) with and without amendment. Another set was placed at room temperature (20°C) under agitation, using different sediment densities.

4.2.4.1. Density experiment:

During AND, sediment is fluidised by low pressure water injection. This effectively mixes the bottom layer substrate with oxygenated water, whereupon it is pumped back to the sea bottom at the equilibrium density of navigable fluid mud: 1.18 kg/m3. Therefore, microcosms were set-up at different densities in order to determine whether it has any influence on TBT degradation. Different TBT amounts were spiked in the sediment of different densities (from 3 to 10 µg/g sed dw), the figures are therefore representing TBT, DBT and MBT amounts as a percent of initial value, in order to facilitate the visual comparison. Note nevertheless that the difference in TBT amounts could constitute a bias

in the interpretation, as it could have an influence on microbial community degradation capacity through the potential difference in toxicity.

TBT degradation of 25.5%, 8.3% and 11.7% respectively at the densities of 1.12, 1.15 and 1.18 kg/m^3 (Figure 4-8 - Wilcoxon test, p-value = 0.125) was detected. The decrease in TBT was significantly higher for the lowest density (Wilcoxon test, p-value = 0.05), with degradation about 2.5 times higher at 1.12 kg/m3 compared to 1.15 kg/m3 and 1.18 kg/m3.

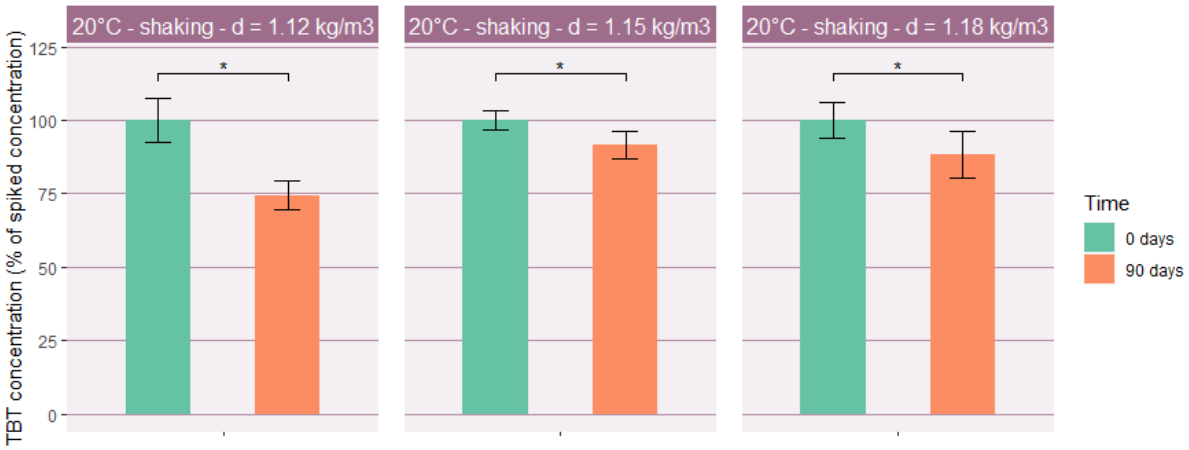


Figure 4-8: TBT amounts in sediment microcosms at the start and the end of 3 months of incubation at 20°C under agitation. Statistical significance under the test of Wilcoxon is indicated by stars. * for a p-value ≤ 0.15; ** for a p-value ≤ 0.1 *** for a p-value ≤ 0.05.

Degradation of DBT of 23.4%, 47.6% and 56.1% could also be observed respectively at the densities of 1.12, 1.15 and 1.18 kg/m^3 (Figure 4-9 - Wilcoxon test, p-value = 0.125). This time it was significantly higher by a factor of approximately 2.5 for the two highest densities compared to the lowest one (Wilcoxon test, p-value = 0.05).

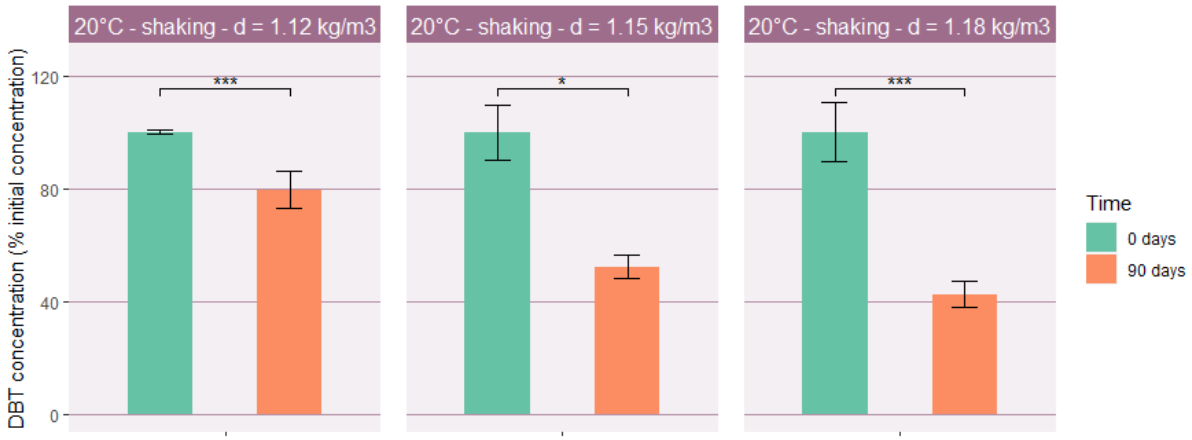


Figure 4-9: DBT amounts in sediment microcosms at the start and the end of 3 months of incubation at 20°C under agitation. Statistical significance under the test of Wilcoxon is indicated by stars. * for a p-value ≤ 0.15; ** for a p-value ≤ 0.1 *** for a p-value ≤ 0.05.

Finally, MBT increased in the three conditions with the highest increase in the lowest density (Figure 4-10 - Wilcoxon test, p-value = 0.05). Density seems to play a role in the degradation of TBT, with the lowest density resulting in more TBT degradation, which accumulates in the form of MBT at 20°C under agitation.

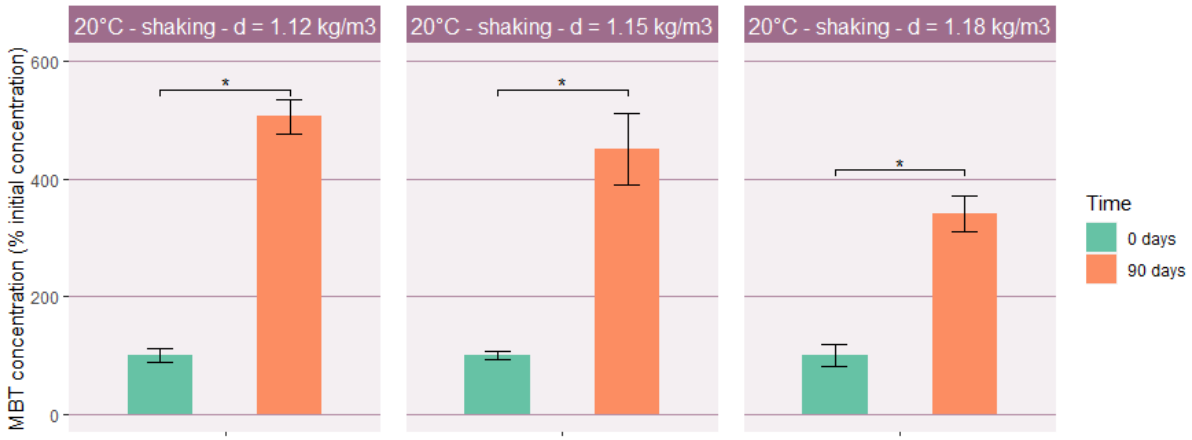


Figure 4-10: MBT amounts in sediment microcosms at the start and the end of 3 months of incubation at 20°C under agitation. Statistical significance under the test of Wilcoxon is indicated by stars. * for a p-value ≤ 0.15; ** for a p-value ≤ 0.1; *** for a p-value ≤ 0.05.

4.2.4.2. Influence of amendment

Since less TBT degradation than expected was observed during the experiment involving sediment supplemented with carbon and nitrogen. Another set of microcosms, therefore, was set up for a longer time of three months at 15°C (summer water temperature) with

and without supplementation to check the impact of the latter. The sediment was prepared as for the previous experiments but this time the flasks were put in the cold store after the first night of incubation, for a duration of three months, due to the closure of the labs during the Covid-19 pandemic. At the reopening of the labs, the flasks were put in the shaking incubator at 15°C for three months as initially planned. TBT, DBT and MBT concentrations were measured at the start, after the three months in the cold store and after the three months of incubation at 15°C.

In the non-sterile flasks, most TBT degradation happened during the incubation in the cold store (Figure 4-11). 27.6% and 36.8% of degradation were observed respectively for the non-amended and amended flasks. In the sterile flasks, TBT degradation was inhibited at 4°C but occurred during the incubation at 15°C (20.1%). This indicates that the degradation observed at 4°C in the other conditions was performed by the microbial community. Surprisingly, TBT amounts seemed to increase by 9.8% in the amended flasks during the period of incubation at 15°C. As TBT cannot be synthesised naturally, another phenomenon must explain this observation. One hypothesis is that some TBT was in a strongly bound form that could not be extracted during TBT measurement and was released in an extractable form during the incubation at 15°C. This means that part of the degradation observed at 4°C could be due to this phenomenon.

The significant increase in DBT (Wilcoxon test, p-value = 0.125, Figure 4-12) during this phase, however, supports the fact that TBT degradation did happen at 4°C. The difference in pattern of TBT amounts at the start and end of the period of incubation at 15°C in the non-sterile experiments suggests that the flasks without carbon and nitrogen supplementation are more prone to TBT biodegradation at this temperature, which would be in accordance with the previous results obtained for the amended short-term microcosms.

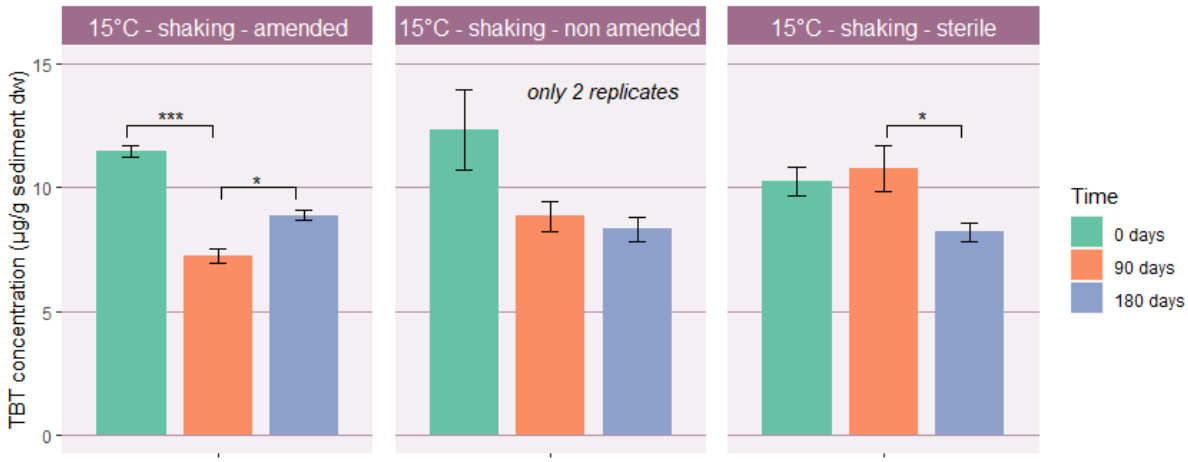


Figure 4-11: TBT amounts measured at different times of the mid-term microcosm experiments assessing the effect of amendment. Statistical significance under the test of Wilcoxon is indicated by stars. * for a p-value ≤ 0.15; ** for a p-value ≤ 0.1; *** for a p-value ≤ 0.05.

During the period of incubation at 4°C, DBT accumulated (by 46.5% and 38.9% respectively for the non-amended and amended conditions) in the non-sterile microcosms (Figure 4-12 - Wilcoxon test, p-value = 0.125) but it decreased during the incubation at 15°C (by 33.9% and 40.8% respectively for the non-amended and amended conditions). These findings suggest that biodegradation of DBT is inhibited at 4°C.

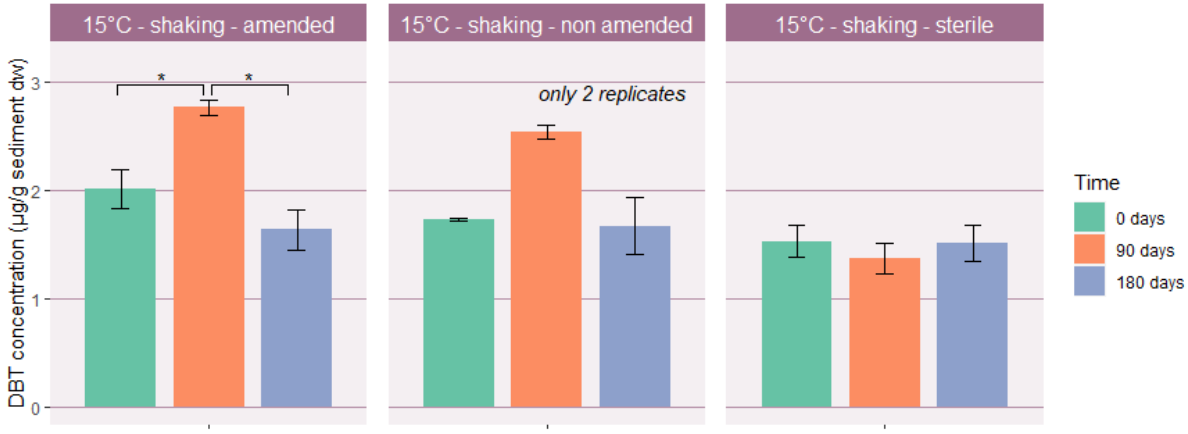


Figure 4-12: DBT amounts measured at different times of the mid-term microcosm experiments assessing the effect of amendment. Statistical significance under the test of Wilcoxon is indicated by stars. * for a p-value ≤ 0.15; ** for a p-value ≤ 0.1; *** for a p-value ≤ 0.05.

MBT amounts only increased significantly during the period of incubation at 15°C in the non-sterile flasks (Figure 4-13 - Wilcoxon test, p-value = 0.125) and during the whole experiment for the sterile flasks. MBT therefore does not seem to be degraded at all in

these conditions, but its accumulation does confirm the degradation of the other products, DBT and TBT.

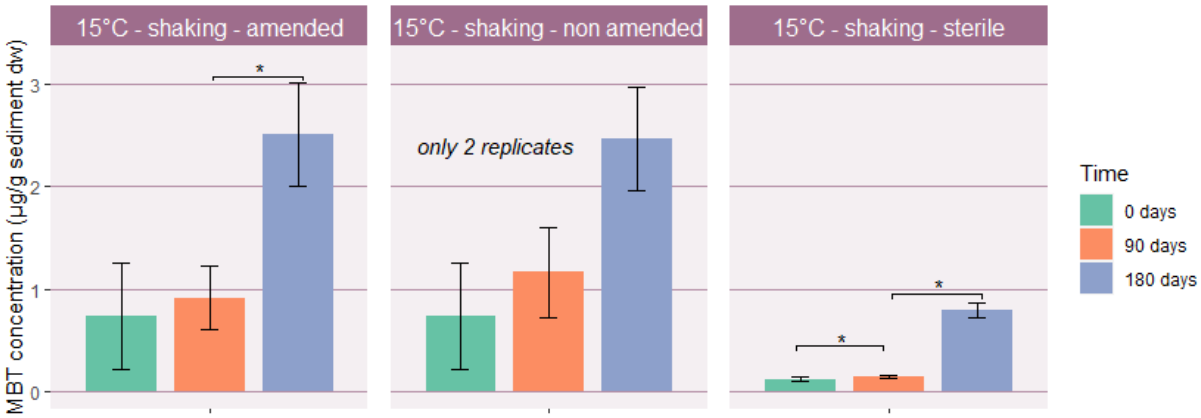


Figure 4-13: MBT amounts measured at different times of the mid-term microcosm experiments assessing the effect of amendment. Statistical significance under the test of Wilcoxon is indicated by stars. * for a p-value ≤ 0.15; ** for a p-value ≤ 0.1; *** for a p-value ≤ 0.05.

4.2.5. degradation :

4.2.5.1. Significance of the interpretations

The first aspect to consider is that the high variability in the measurement of OTCs and the low number of replicates prevents the use of powerful statistical tests and are obstacles to robustly detect changes in the concentrations. For this reason, the statistical risk in this study has been reconsidered (with low significance accepted at a risk of 15%, see section 3.5). The findings presented here represent a preliminary assessment of the environmental factors that play a role in the biodegradation of high concentrations of TBT by the native community of estuarine sediment and any firm conclusions require further replication and field trials. Therefore, the risk of getting false positives was re-evaluated and the threshold increased. Nevertheless, by measuring the degradation products of TBT as well, several indicators of potential degradation are suggested and reinforce the interpretations.

Furthermore, the amount of TBT used in this study corresponds to high TBT contamination that is extremely toxic and would deteriorate the native microbial community. Therefore, the patterns of degradation observed under the environmental conditions tested in the present microcosm experiments could differ for lower contamination levels.

4.2.5.2. Effect of temperature

In the present study, the effect of temperature on TBT degradation contrasted with the expectations. Although chemical degradation seemed to be positively influenced by higher temperatures as anticipated (Figure 4-8), biodegradation was lower for higher temperatures (Figure 4-1, 8). Microbial activities are reportedly higher for higher temperatures and generally considered to be inhibited at 4°C (Zaidi and Imam, 2008). Most previous microcosm studies focusing on TBT degradation did not assess the effect of temperature, only one study using water microcosms reported an increase of biodegradation with temperature, until it reached a plateau at 28°C (Sakultantimetha et al., 2011).

In the sediment and conditions used in this study, TBT biodegradation appears to be supported by psychrophilic microorganisms (Figure 4-14). Studies on TBT adsorption to sediment suggest that it increases for marine and estuarine sediment with increasing temperature in the range of 20°C to 40°C (Bangkedphol et al., 2009; Ma et al., 2000), meaning that its bioavailability would decrease with increased temperature. This phenomenon could explain the observations in the present study but cannot explain the contrast with other studies.

None of the microcosms studies performed previously assessed the effect of temperature on TBT biodegradation by the native community of sediment. The majority of the studies used room temperature or above (25-28°C) as a standard for their microcosm experiments. For some of them, this temperature is relevant as they used sediment from warm locations where the sea bottom temperature spans around 20-35°C all year round (Suehiro et al., 2006; Yonezawa et al., 1994). But for others, the sediment originated from Scotland, Portugal or Italy, where sea bottom can lower below 10 degrees in the winter (Beolchini et al., 2014; Cruz et al., 2014; Sakultantimetha et al., 2011). Contrary to the present study, they could measure TBT degradation at warmer temperature, but it would have been interesting to evaluate the degrading capacity of native communities at low temperature as well in these sediments, as it is closer to natural conditions. These results however demonstrate that other factors than temperature have a significant influence on TBT biodegradation rates.

Regarding the recorded increase in TBT in the amended flasks during the incubation at 15°C, which, again, cannot come from the synthesis of TBT, the hypothesis would be that

TBT was put in a less extractable form, or more strongly bound to the sediment during the incubation at 4°C, preventing its measurement through extraction, derivatisation and analysis by GCMS. The shaking and incubation at 15°C would then reverse the phenomenon.

No known process supports this hypothesis especially since at a pH of 7.8, TBT is mostly present in neutral form and attaches to sediment through hydrophobic bonds that are less robust than the links that TBT+ forms with sediment at a low pH. Studies showing an increase of TBT adsorption with temperature are also in contradiction with these observations. The factors influencing TBT adsorption to sediment, however, are multiple and complex and the conditions could not be controlled in real time during the incubation at 4°C.

Several parameters could have affected the behaviour of TBT. For example, it is unknown if the pH remained stable and above 6 and, if the pH decreased significantly, the speciation of TBT would change and the monocharged species would be the predominant species that forms stronger bonds with sediment. Yet, pH alone should not have an influence on the extractability of TBT as it uses a buffer that stabilises the pH and showed ranges of TBT recoveries from spiked sediment approximating 100% during the optimisation.

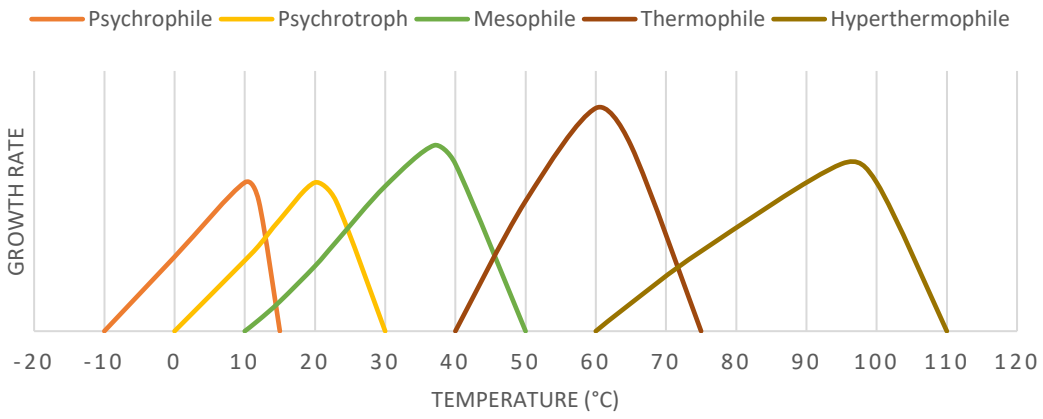


Figure 4-14: Growth curve of the different groups of microorganisms based on their temperature tolerance. Temperature limits are approximative.

Temperature had a slightly different impact on DBT degradation. It was inhibited at 4°C but occurred at 8°C, 15°C and 20°C. However, in sediment supplemented with carbon and

nitrogen it was higher at 8°C than 20°C. Like TBT, DBT biodegradation seems to be supported by microorganisms that are active at low temperatures but this time not as low as 4°C. As for TBT, the decrease in bioavailability due to a stronger adsorption of DBT to sediment with increasing temperature could also play a role in this pattern of degradation.

4.2.5.3. Effect of nutrient addition:

The fact that TBT degradation could not be observed at all in any of the conditions tested in the mid-term experiment using sediment supplemented with carbon and nitrogen, compared to the preliminary experiment, could have multiple causes. First, it is important to recall that the sediment used for this experiment was sampled at a different time and location within Liverpool Docks than the sediment used in the preliminary experiment. Although the physical parameters of the different sediment samples are similar, a potential seasonal or geographical effect cannot be excluded that could influence the microbial community composition and their capacity to degrade TBT. Given the ubiquity of known TBT degraders, however, such as the different members of *Pseudomonas* sp., it is reasonable to accept that such effects might not be the principal reasons for the absence of TBT degradation. A more likely explanation would be an inhibitory effect of the amendment, which has also been reported in the literature for TBT (Shizhong et al., 1989). It is known that, in some cases, providing readily available nutrient sources encourages microorganisms to use these as a priority and ignore more complex substrates (Z. Wang et al., 2015; Wong et al., 2002). This would also imply that the TBT-resistance mechanisms used by the bacteria growing in the flasks do not involve its degradation and probably involve efflux pumps instead.

For the long-term experiment, however, TBT degradation was not hindered at 4°C in the amended flasks but seemed to be inhibited again when the flasks were placed at 15°C. This could mean that a low temperature for the degrading community present in the flasks has a negative effect on the metabolism of glucose and not TBT, forcing the microorganisms to utilise the latter (Nedwell, 1999). However, when the temperature is increased, glucose becomes the preferred substrate. Another possibility is that the microorganisms able to degrade TBT at 4°C become unable to compete with other microorganisms that grow faster at 15°C. The subject of competition could be another parameter of growth (other than carbon source), like space, nitrogen source, oxygen or iron (Hibbing et al., 2010). The

microbial species for which the growth would be enhanced by the elevated temperature could rapidly lead to the depletion of key growth factors needed by the psychrophiles responsible for TBT degradation at 4°C, inhibiting the latter. Such a phenomenon could also explain the slowing down of TBT degradation in the non-amended flasks of the same experiment.

4.2.5.4. Effect of agitation:

The effect of shaking on TBT and DBT degradation is unclear. In the amended experiment, no difference could be detected between shaking and non-shaking flasks of the same sediment, and no degradation was observed for TBT. DBT degradation levels were only low, but it is possible for a difference to appear after a longer time of incubation. Reasonable levels of TBT degradation occurred in static conditions, however, during the preliminary experiment and during the 3 months of incubation at 4°C. Constant agitation, therefore, does not seem to be crucial for TBT degradation and could even hinder it, which could be attributed to the anaerobic or subaerobic TBT degradation in our experiment.

4.2.5.5. Effect of density:

The present study demonstrated a better TBT degradation at lower density, which is not surprising as a higher concentration of TBT dissolved in water can be expected in such conditions, increasing the fraction of TBT that is bioavailable for biodegradation. TBT has always been reported to degrade more rapidly in the water body than in sediment (Alzieu, 2000; Harino et al., 1997).

DBT degradation displayed the opposite pattern, which could be due to the higher transformation of TBT to DBT at lower density which interferes with the interpretation of DBT overall degradation. It must be noted that different TBT amounts were spiked for the different densities, therefore the conclusions are indicative.

4.2.6. Consequences for the use of AND as a bioremediation technique:

4.2.6.1. Favourable conditions for the bioremediation of TBT

If the patterns observed in the present study are confirmed (by further replicates and field trials), the influence of the parameters tested could have different implications for the potential use of AND for the bioremediation of TBT.

The apparent inhibition of TBT biodegradation by carbon and nitrogen supplementation indicates that AND could be applicable without further biostimulation by nutrients. It also means that the bioremediation efficiency could be altered in nutrient rich areas.

One of the most interesting observations in this study is the effect of temperature. An enhancement of TBT biodegradation by cold temperature was noticed. This temperature, however, did not trigger DBT biodegradation. Although less studied and usually assumed low, DBT toxicity seems of high concern too (Ferreira et al., 2013; St-Jean et al., 2002; Zhang et al., 2018), and only MBT consistently shows a clear reduced toxicity when studied (Dooley and Kenis, 1987; Ferreira et al., 2013; Marin et al., 2000). Thus, for an effective bioremediation of TBT, its degradation to MBT and inorganic tin must be targeted.

A compromise may be proposed to determine the best period of year to apply AND, if applied in Liverpool or areas of the same temperature. AND could be used when the water is warming up at the end of the winter, therefore TBT would be degraded at first, followed by DBT. The present results suggest that AND would not be efficient for the bioremediation of TBT in warm latitudes in sediment displaying similar properties as Liverpool Docks sediment.

Constant shaking does not seem to improve TBT or DBT degradation. When using AND, mud is only fluidised and oxygenated at the start and then repumped onto the sea bottom where it can remain with minimal disturbance. In fact, these processes were mimicked during the experiments where the most TBT degradation could be obtained: the mud was fluidised and oxygenised during TBT spiking, then left still in the flasks (for the preliminary experiment, or during the incubation at 4°C in the mid-term microcosms). This could mean that AND would not need to be applied frequently, a single passage should be sufficient to trigger the degradation of organotin compounds if the conditions are favourable.

These results constitute a good preliminary assessment of the favourable conditions for AND use as a bioremediation technique. The observed effects are only accountable for high TBT contamination in marine environments. More moderate levels of contamination could not be investigated due to detection limits, but the patterns of biodegradation could be different. In particular, the effect of temperature may be reconsidered and AND might be applicable at warmer locations with different microbial communities. Field trials are

necessary to obtain a better insight of the efficiency of AND use as an *in-situ* bioremediation technique.

The present study shows that TBT degradation can be obtained in conditions close to the field conditions in a reasonable time scale. A degradation of up to 4.2 µg TBT/g sed dw was observed over three months of incubation at 4°C after a period of resuspension and aeration. Most of the recent TBT contamination records span around 10^2 ng TBT/g sed dw, which is an order of magnitude less than the amount of TBT degraded in our experiments (Concha-Graña et al., 2021; Kuprijanov et al., 2021). Therefore, if the same rates were obtained in the field, many contaminated locations could be completely cleaned from TBT in the same amount of time or less.

4.3. Conclusion

In the present study the impact of different factors on TBT chemical degradation and biodegradation by the native microbial community of fine sediment sampled in Liverpool Docks was assessed in the conditions of a high contamination of 10 000 ng TBT/g sed dw.

Interestingly, temperature showed an inhibitory effect on TBT biodegradation, with higher degradation rates obtained at 4°C. Agitation did not seem to improve TBT or DBT degradation, but its inhibitory effect could not be assessed. Density plays a role on the degradation of TBT and DBT, with lower densities being favourable for TBT degradation but unfavourable for DBT degradation. Finally, carbon and nitrogen supplementation inhibited TBT biodegradation at temperatures above 8°C but had no effect at 4°C.

All of these are good indicators that AND can be efficiently used for TBT bioremediation in marine environment in the UK or other locations with similar temperate climates. More research is needed to confirm its applicability at a full scale, and to warmer locations in case of low to moderate TBT contamination. Of course, the biodegradation of many other toxic compounds should be investigated as the final aim would be to use AND to bioremediate port sediment from a wide range of contaminants commonly found in these highly polluted environments.

5. Influence of TBT spiking and sediment manipulation on sediment microbial communities

5.1. Introduction

Microbial communities in sediment may have the ability to degrade a wide range of contaminants introduced in the environment. As discussed in chapter II, the structure and activity of these communities depends on numerous factors such as the presence of the specific contaminants themselves, level of organic matter, temperature, pH or dissolved oxygen for example.

In order to investigate microbial community changes across the microcosm experiments, samples from different times of these experiments were selected for 16S rRNA genes next-generation sequencing and community analyses. Unfortunately, the quality of the reads was not sufficient to enable a demultiplexing of the samples. The only dataset available therefore represents all the sequences present in the different samples. This still enables a meaningful discussion about the biodegradability potential of the community as well as the potential influence of TBT spiking to be achieved.

5.2. Results and discussion

Because of a technical issue leading to poor quality of index reads after the iSeq sequencing, the demultiplexing of samples turned out to be impossible to achieve. This means that the workable dataset comprises the sequences of all the samples together. Due to this issue, the classic analyses could not be performed but the community structure as well as the presence of some taxa of interest can be discussed.

The set of samples comprised unprocessed sediment without treatment, sediment after spiking of TBT and amendment of carbon and nitrogen, and sediment after 3 to 6 months of incubation at 4°C then 15°C (Table 3-5). The TBT-spiking step kills a part of the community, but the subsequent carbon and nitrogen supplementation, aeration and incubation should trigger the growth of microorganisms. This theory is confirmed by the

enumeration performed for the isolation experiments where the number of cultivable bacteria obtained by standard plating was about 700 times higher for the sediment issued from the microcosms than the sediment that remained untouched after sampling (Figure X). By taking this into account, it can be assumed with caution that the community revealed in the dataset described in this chapter is more representative of the microbial community structure in the microcosms than the natural microbial community. Note however that all the microorganisms present here are also represented in the natural community as there was no input of microorganisms along the experiments.

5.2.1. Taxonomic composition of microbial communities

From the total sequences, 515260 were assigned to 25 Phyla while 169209 were unassigned (24.7%). *Proteobacteria* accounted for 89.8% of the classified sequences, followed by *Deinococcus-Thermus*, *Firmicutes*, *Bacteroidetes* and *Actinobacteria* which respectively represented 3.0%, 2.5%, 1.8%, 1.7% of the sequences (Figure 5-1). The rest of the Phyla represented each less than 1% of the sequences. Within *Proteobacteria*, *Gammaproteobacteria* (34.1%), *Deltaproteobacteria* (30.0%) and *Epsilonproteobacteria* (19.7%) were the most dominant groups (Figure 5-2). A predominance of *Proteobacteria* is not surprising as it was widely reported for estuarine sediment (Vidal-Durà et al., 2018; Zhang et al., 2014). However, in natural sediment their relative abundance usually spans from 40 to 70% (Vidal-Durà et al., 2018; Wei et al., 2016; Zhang et al., 2014) and it is rarely as high as observed presently, which is likely the consequence of mud manipulation (*i.e.*: TBT-spiking, aeration, supplementation in carbon and nitrogen). The effect of TBT spiking on microbial community was never investigated, but other studies have observed changes in microbial communities following the introduction of aerobically degraded contaminants such as PAHs, nonylphenol and bisphenol A as well as along biodegradation experiments (Mahjoubi et al., 2021; Wang et al., 2014; Yang et al., 2014). These investigations all observe a decrease in the bacterial diversity in parallel to an enrichment of *Gammaproteobacteria* with a relative abundance reaching more than 90% after addition of the toxic compound.

In this study, more *Deltaproteobacteria* than *Gammaproteobacteria* are recorded. *Deltaproteobacteria* are known to mainly hold sulphate reducing bacteria (SRB) which can thrive in organic-rich environments, as they metabolise organic matter to produce their

energy using sulphate as an electron acceptor in anoxic conditions. A high abundance of SRBs in our samples was confirmed by the analyses at the family level, which show many SRBs within the most abundant families (Desulfobulbacae 22.7%, Desulfobacteraceae 7.6%, Desulfovibrionaceae 4.5%, Figure 5-3). Their presence in sediment is usual but as they are anaerobes, they were not expected to thrive in the microcosms set up of this study. However, such populations would have been present in the native sediment due to the anoxic conditions at the time of sampling, and they could have persisted along the course of experiments. In fact, the members of SRBs were shown to possess different defensive strategies to survive the oxidative stress caused by a temporary presence of oxygen that is likely to happen in their natural environment (Dolla et al., 2006). This was also confirmed by a study of the microbial community shifts in sediment over different levels of oxygenation, which showed a relatively stable population of SRBs across the different levels of dissolved oxygen (Mori et al., 2018). The only study which performed community analyses in microcosms assessing the TBT degradation potential of sediment native community compared with bioaugmented sediment also reported a similar repartition within the phylum *Proteobacteria* (Cruz et al., 2014). This study could observe an enrichment of *Deltaproteobacteria* along the microcosms and concluded that members of this class could be involved in TBT biodegradation.

Finally, the most abundant family of this study was *Campylobacteraceae* (27.2%) which this time, along with the Thiovulaceae (4.6%) mostly consist of microaerophilic sulphur oxidising bacteria (SOBs). This would be in accordance with previous studies which reported the proliferation of SOBs following oxygenation of anoxic sediment (Broman et al., 2017; Ihara et al., 2017). Such a proliferation is regarded as beneficial as SOBs are able to eliminate the toxic hydrogen sulphide (H_2S) (Broman et al., 2017). Oxygenation was also shown to avoid production of methane (CH_4), the production of CH_4 and H_2S in anoxic sediment is a well-known issue and oxygenation has been recognised as one of the solutions to this problem (Bonaglia et al., 2019; Broman et al., 2017).

5.2.2. Implication for TBT resistance or degradation

As a high concentration of TBT was also spiked in the sediment (10 µg/g sed dw), a selection of TBT-resistant bacteria was undertaken. So, in addition to being able to resist to the introduction of oxygen, members of the SRBs, as well as SOBs, seem capable of tolerating

high contaminations of TBT. The potential persistence of SRBs, as observed here, after resuspension and aeration of mud (during AND for example) may not be representative of what would happen in the natural environment, as the system wouldn't be closed as in the microcosms, and there would be an input of aerobic microorganisms from the surroundings, especially through the resuspension event involved in AND manipulation.

Considering the biodegradation potential, these analyses of the microbial community during experiments of aerobic biodegradation of TBT do not highlight any enrichment of known degraders, or even unknown aerobic degraders as the most abundant groups highlighted are anaerobic or microaerophilic. Among known TBT-degraders, only the family *Pseudomonadacaea*e was represented in the non-negligible taxa (1,1%) but does not seem specifically enriched. A similar phenomenon was observed for the studies investigating microbial community changes along nonylphenol and bisphenol A biodegradation experiments during which, despite the observation of a clear change in microbial community, no known degraders were reported (Wang et al., 2014; Yang et al., 2014). Nevertheless, in the present study as well as the cited ones, a biodegradation of the target compounds was observed. This either emphasizes our extensively incomplete understanding of the natural degrading communities of these compounds or demonstrates that the degrading organisms may display their biodegradation activity even if they are rare members of the community.

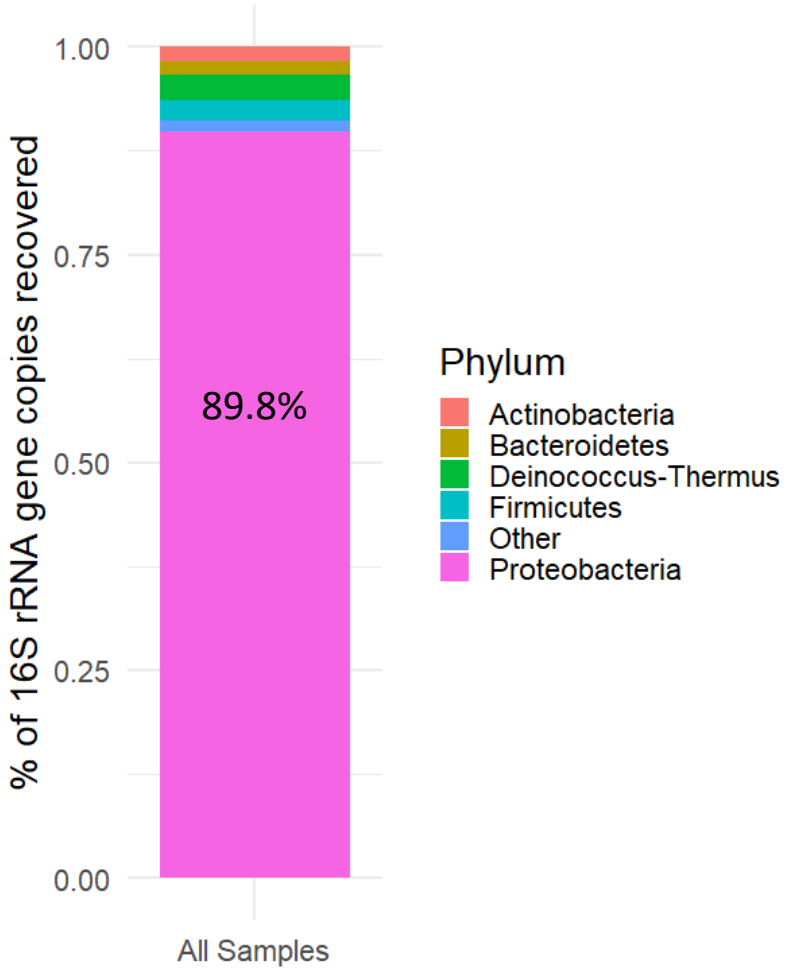


Figure 5-1: Relative abundance of sequences at the phylum level
Phyla with <1% abundance were summarized as "Other".

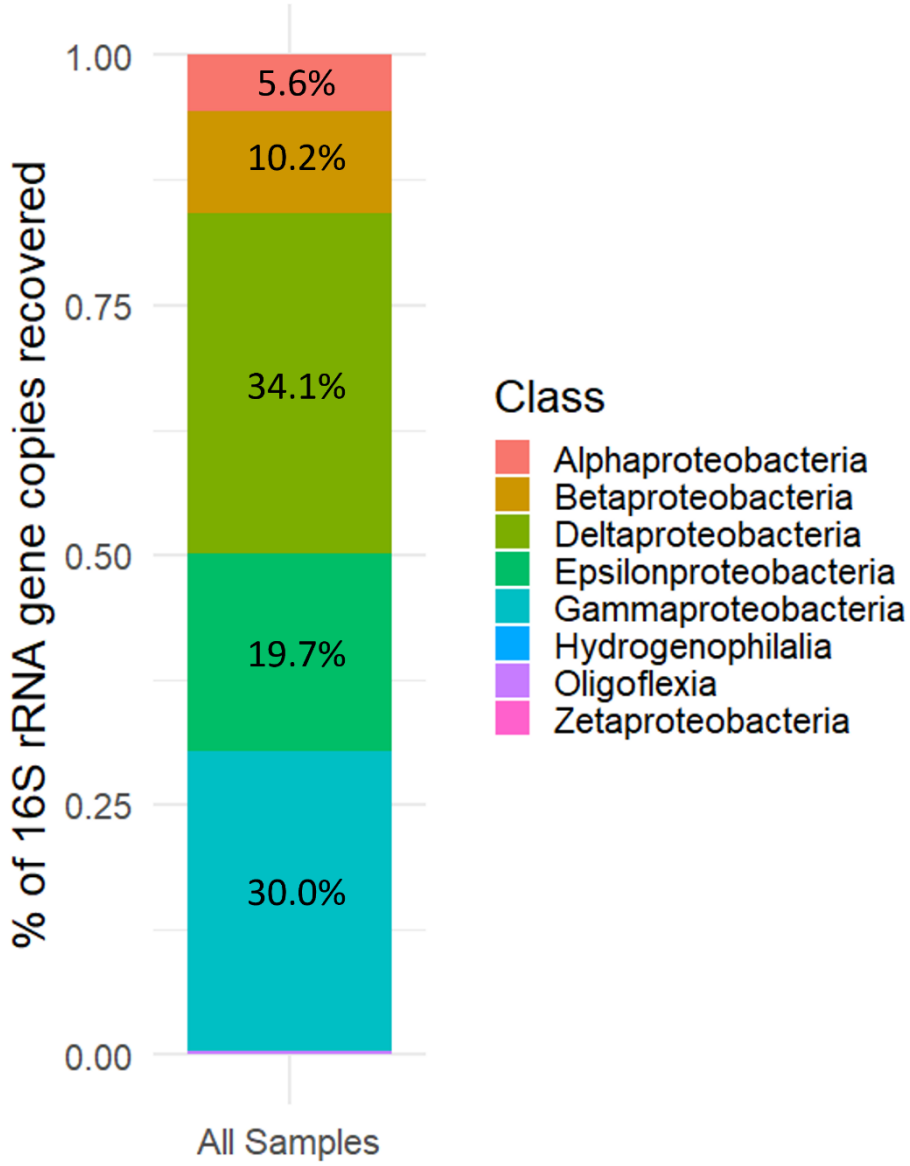


Figure 5-2: Relative abundance of Proteobacteria sequences at the class level

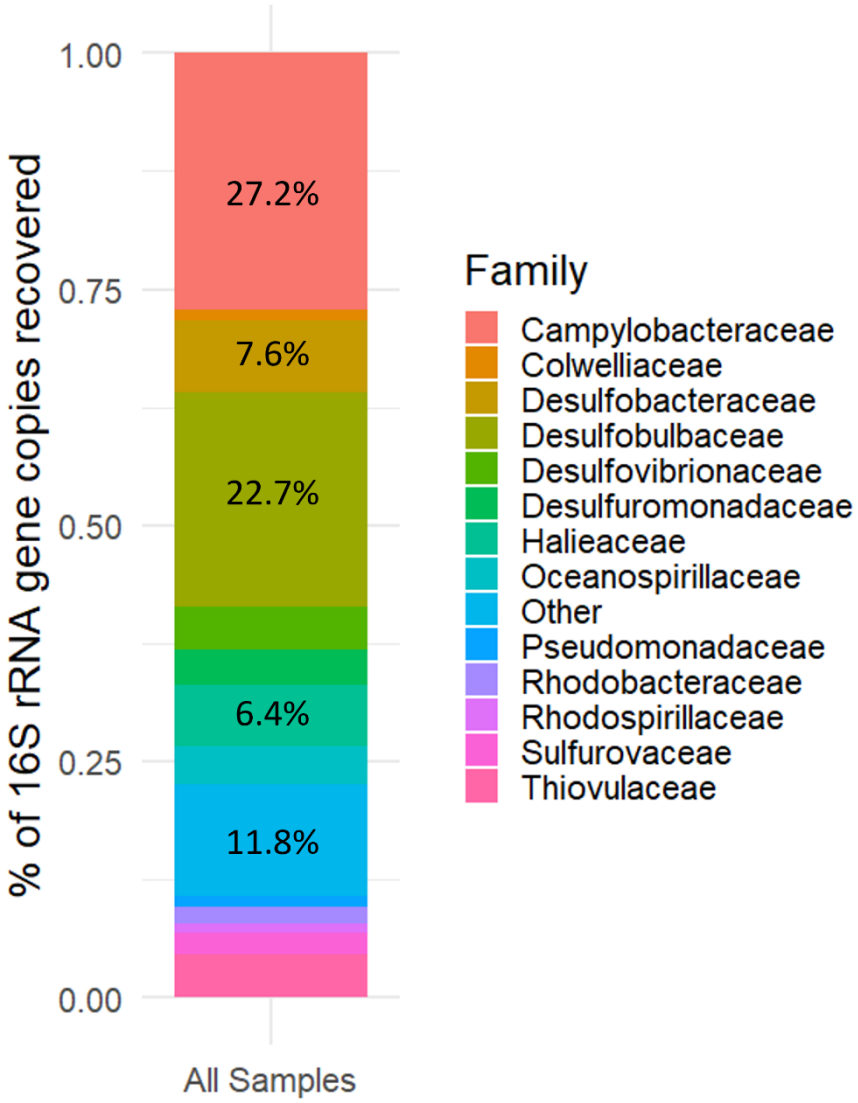


Figure 5-3: Relative abundance of sequences at the Family level
Phyla with <1% abundance were summarized as "Other". Only sequences classified as Proteobacteria and with >99% identity were analysed (50.0% of the sequences that were classified at the phylum level).

5.3. Conclusion:

In conclusion, despite the challenge imposed by the technical failure to demultiplex the samples, some elements of the microbial community of the total samples have been identified. Because a greatly higher abundance of total bacteria was reported in the microcosms compared to native sediment, the community was considered representative of the microbial community composition in the microcosms. While the sediment was aerated, a high proportion of the taxa are anaerobes. The proliferation of microaerophilic

SOBs after sediment oxygenation is consistent with previous studies, but no enrichment of aerobes was observed. This result is surprising as a shift in microbial communities towards aerobic microbial communities was expected after resuspension and aeration of the mud, but it can be explained by the anaerobic origin of the sediment used and the closed set-up of the microcosms. It would however reveal a high capability of the SRBs to resist long periods of oxygenation.

6. iChip increases the success of cultivation of TBT-resistant and TBT-degrading bacteria from estuarine sediment.

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Abstract:

Standard methods of microbial cultivation only enable the isolation of a fraction of the environmental bacteria. Numerous techniques have been developed to increase the success of isolation and cultivation in the laboratory, some of which derive from diffusion chambers. In a diffusion chamber, environmental bacteria in agar medium are put back in the environment to grow as close to their natural conditions as possible, only separated from the environment by semi-permeable membranes. In this study, the iChip, a device that possesses hundreds of mini diffusion chambers, was used to isolate tributyltin (TBT) resistant and degrading bacteria. IChip was shown to be efficient at increasing the number of cultivable bacteria compared to standard methods. TBT-resistant strains belonging to *Oceanisphaera sp*., *Pseudomonas sp.*, *Bacillus sp*. and *Shewanella sp*. were identified from Liverpool Docks sediment. Among the isolates in the present study, only members of *Pseudomonas sp*. were able to use TBT as a sole carbon source. It is the first time that members of the genus *Oceanisphaera* have been shown to be TBT-resistant. Although iChip

has been used in the search for molecules of biomedical interest here we demonstrate its promising application in bioremediation.

Keywords:

Bacterial isolation; isolation chip, high throughput isolation, bioremediation, tributyltin

6.1. Introduction:

Tributyltin is an organotin compound that has been used widely as a biocide in antifouling paints. It is therefore highly toxic and has been shown to be a major threat to aquatic ecosystems. Due to its toxicity, it was subjected to a global ban in 2008 (Sonak et al., 2009b). However, TBT is still a major concern in many locations around the world (Filipkowska and Kowalewska, 2019). In fact, it is still authorised in a small number of countries (Turner and Glegg, 2014), and it is suspected to be used illegally in many others because of its high efficiency (Egardt et al., 2017). The main concern is its high persistence in anoxic sediments and as such, is a pernicious legacy contaminant. Indeed, TBT is hydrophobic and strongly binds to organic matter and sediment where it can remain for decades (Langston et al., 2015). Sediment therefore acts as a secondary source of contamination during resuspension events, causing more disturbance to aquatic ecosystems. There is therefore a need to remediate sediment contaminated with TBT.

Traditional remediation techniques such as incineration (Song et al., 2005) or electrochemical oxidation (Beuselinck and Valle, 2008) are usually regarded as efficient but costly. In addition, they can cause environmental issues as they involve the excavation of sediment, which causes problems of contaminant spreading and further pollution due to carbon emissions during transportation (Manap and Voulvoulis, 2015b). The more environmentally friendly approach is bioremediation by the use of biological organisms to remediate a material. In particular, *in situ* bioremediation removes the need for excavation plus the associated cost and environmental issues linked to it. Bioremediation can be further subdivided into phytoremediation, when using plants, or biodegradation, when using microorganisms. The latter is especially interesting for *in situ* bioremediation of port sediment. Biodegradation includes natural attenuation, biostimulation and bioaugmentation. Natural attenuation consists of using the native microbial community to naturally degrade harmful contaminants (Lofrano et al., 2017). Biostimulation aims at

boosting the degrading activity of the microbial community by providing more favourable conditions, for example by the addition of nutrients, or through oxygenation. Finally, bioaugmentation consists of adding specific microorganisms to decontaminate the material. The added microorganisms are selected for their exceptional abilities to efficiently degrade the contaminants of interest.

The use of bioremediation requires a comprehensive understanding of the degradation pathways and kinetics, the microbial communities involved in the degradation as well as the most favourable conditions for the growth and degrading activity of the microorganisms involved. A first step towards this objective is to proceed with the isolation and cultivation of the microbial degraders. Thus, research has been carried out to isolate and characterise TBT-resistant and degrading microorganisms (Cruz et al., 2015b). The identified microbes include *Chlorella* species (Jin et al., 2011; Tsang et al., 1999) and fungi such as *Cunninghamella elegans* or *Cochliobolus lunatus* (Bernat et al., 2013; Bernat and Długoński, 2002). In addition, many bacteria have been studied for their TBT degradation ability, such as *Aeromonas molluscorum*, *Enterobacter cloacae* and numerous species of *Pseudomonas* (Finnegan et al., 2018a).

Despite this, it is well-known that only a small proportion of microbes have been discovered so far. Indeed, it is estimated that more than 99% of bacteria remain unknown (Locey and Lennon, 2016). The main reason for this is our inability to cultivate them in the laboratory. Classic methods of isolation and cultivation, that were used for the isolation of TBT-degrading bacteria so far, failed to provide the appropriate conditions for the growth of the majority of the environmental bacteria and are biased towards the same species. Nevertheless, some techniques have been developed to improve the success of cultivation of novel species, usually by mimicking as accurately as possible the natural environment (Bodor et al., 2020; Hahn et al., 2019). Among these, the diffusion chamber concept was of special interest. In diffusion chambers, microorganisms are trapped in agar while in contact with their natural environment with semipermeable membranes. The membranes ensure that cells cannot move in or out of the diffusion chamber but small molecules that may be necessary for microbial growth can enter the chamber (Kaeberlein et al., 2002). On the basis of this concept, iChip was created, acting like hundreds of mini diffusion chambers and therefore allowing the high-throughput isolation of bacteria (Nichols et al., 2010). IChip

allowed the cultivation of species of bacteria different to those isolated with standard plating methods (Nichols et al., 2010).

The present study used iChip for the isolation and cultivation of TBT-resistant and TBT-degrading bacteria in order to develop a better understanding of TBT degrading communities. A comparison of the standard plating and iChip techniques was performed to isolate microbes of interest in the field of bioremediation. This was done by measuring the difference in culturability of sediment bacteria using the standard method of plating compared to iChip and screening of TBT-resistant/degrading bacteria among the obtained isolates. Using iChips enabled the isolation of a higher number of bacteria but the method is not sufficient to successfully maintain these in laboratory conditions. A selection of isolates of interest were subsequently identified by 16S rRNA gene Sanger sequencing. For the first time, members of the genus *Oceanisphaera* were found associated with TBT resistance.

6.2. Results:

6.2.1.1. Abundance of cultivable bacteria on TSA medium:

The abundance of cultivable bacteria increased significantly (prepared sediment: p-value = 0.003, native sediment: p-value = 0.007) when using one round of culturing in iChip compared to standard plating on TSA plates (Figure 6-1). The number of CFU increased by a factor of 5.5 and 9.5 for the experiment involving untouched sediment and prepared sediment respectively (Figure 6-1). A higher abundance of cultivable bacteria was also observed for method when using prepared sediment compared to untouched sediment.

6.2.2. Proportion of TBT-resistant bacteria cultivated using iChip compared to standard plating

From untouched sediment, no TBT resistant bacteria could be grown using the standard method of plating sediment dilutions on TSA in petri dishes. However, the proportion of TBT-resistant bacteria among the isolates firstly grown on TSA without TBT was not checked. Note as well that no isolates could be obtained when preparing an iChip using TSA containing 1 mM TBT.

Nevertheless, TBT-resistant proportions among iChip isolates on TSA using the two different types of sediment can be compared. A higher proportion of TBT-resistant bacteria was found for prepared sediment (p-value= 0.038), with 38.2% of TBT-resistant isolates obtained from prepared sediment compared to 16.3% for untouched sediment (Figure 6-1).

6.2.3. Proportion of bacteria using TBT as sole carbon source

Although the mean number of bacteria capable of using TBT as the sole carbon source appeared higher for prepared sediment compared with untouched sediment (9.3% and 2.0% of the isolates respectively), no statistical difference could be detected due to the high variability within the triplicates (Figure 6-1, p-value = 0.36).

6.2.4. Identification of the isolates through 16S rRNA genes Sanger sequencing

After several rounds of cultivation, some isolates could not be recovered. The remaining isolates growing on TSA after four rounds of cultivation were therefore further identified. 18 of them came from iChip experiment using prepared sediment, two came from iChip experiment using untouched sediment and four came from standard plating isolation (Table 6-1).

As shown in Table 6-1, after the Sanger sequencing of 16S rRNA genes, four distinctive genera were identified by BLAST analyses: *Pseudomonas sp*.; *Shewanella sp*.; *Bacillus sp*. and *Oceanisphaera sp*. All of them were able to grow on TSA +1mM TBT for at least 4 subculturing attempts. However, some of them stopped growing after this 4th step, but they could still grow on TSA without TBT.

The four isolates coming from standard plating were identified as *Pseudomonas sp.*.. In the names of the isolates, the first letter represents the label of an iChip (α, β, γ, Y). A correlation seems to be observed between the isolate's genera and iChip experiment.

6.3. Discussion:

6.3.1. IChip increases the abundance of culturable bacteria:

The period of culturing in iChip constitutes a good adaptation step prior to growth of bacteria on synthetic media. While a bacterium is trapped in TSA in an iChip buried in

sediment, molecules that may be necessary for their growth can diffuse across the polycarbonate membranes and into the medium. As the growing conditions are closer to those of the natural environment, it is not surprising greater cultivation success is achieved. The real benefit of using the technique is the fact that, after sub-culturing iChip agar plugs on TSA in full laboratory conditions, a much greater variety of bacteria are able to grow, compared to the attempts at isolation without using the intermediate step in iChip.

The mechanisms behind this adaptation are unclear. It is also important to note that among the initial isolates, which could grow after the direct subculturing from iChip, a number of others failed to grow after a couple of subculturing attempts. As our interest was focused on TBT resistant bacteria, only these were subcultured. Failure to maintain bacterial isolates after subculturing is often described but there are a lack of explanations for this issue (Hahn et al., 2019; Overmann et al., 2017). As the subculturing was performed on TSA + 1 mM TBTCl, some hypotheses can be proposed to explain this lack of growth, in addition to an unknown cause. First, the subculturing may have been delayed, and the bacteria could not be recovered after being kept in the fridge for a few weeks. Second, during the subculturing, a very small quantity of key molecules necessary for the growth of some isolates may have been utilised during the initial subculturing stages but eventually became depleted. Finally, given the selectivity of the medium used, the bacteria could simply have lost their ability to grow in the presence of TBT. This explanation was confirmed for some of the isolates, which after the fourth subculturing stage could be grown on TSA but not on TSA + 1 mM TBTCl. This loss of resistance is most likely to occur through the loss of a plasmid, therefore suggesting that the resistance genes are located on a plasmid for at least some of these strains. Plasmid loss is a well-studied phenomenon due to the wide use of plasmids in research but our understanding remains incomplete (Carroll and Wong, 2018). Plasmids are usually well maintained in the presence of a selective pressure, here TBT, but if the isolation plates are kept long enough for TBT degradation to occur, the selective pressure could be reduced around the isolates, which would increase the chance of plasmid loss (Hanak and Cranenburgh, 2001).

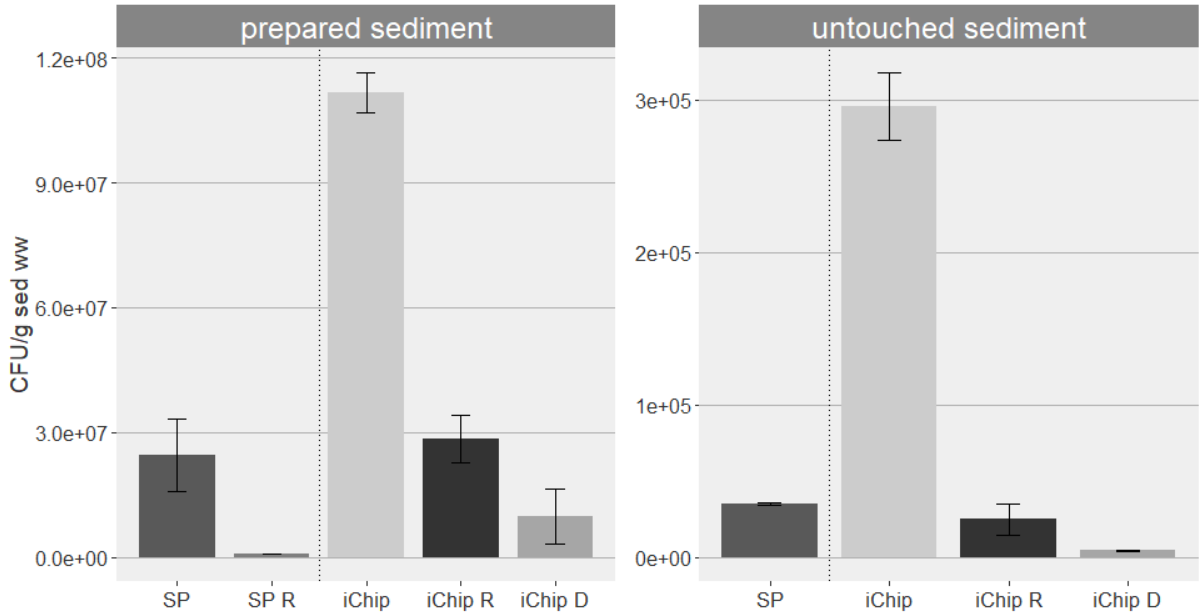

Figure 6-1: Difference in cultivability between standard plating and iChip method using prepared or untouched sediment

SP: CFU numbers obtained by Standard Plating; SP R: TBT-resistant CFU numbers obtained by Standard Plating on TSA + 1 mM TBT; iChip: CFU obtained after one round of iChip and subculturing on TSA; iChip R: TBT-resistant CFU numbers from the subculturing of isolates coming from iChip; iChip D: CFU numbers for cells able to use TBT as sole carbon source from the subculturing of isolates coming from iChip. Results shown represent the mean of triplicates and the error bars are the standard deviations.

6.3.2. A higher proportion of TBT-resistant bacteria are found among isolates obtained from prepared sediment:

In the literature, bacteria are usually called resistant when growing on a medium containing a biocide concentration that kills 90% of the population (Cruz et al., 2015b). For the purpose of this study, however, TBT-resistant bacteria are those bacteria that grow on a medium containing 1 mM TBTCl. Observing a higher proportion of TBT-resistant bacteria among the isolates obtained from prepared sediment compared to the ones obtained from untouched sediment is to be expected.

Different mechanisms can lead to bacterial resistance to toxic compounds. There are four main categories: 1) efflux of the compound; 2) uptake limitation; 3) modification of the compound's target and 4) compound inactivation. Determining the resistance mechanism used by the bacteria isolated in this study would require further testing. Previous studies of TBT-resistant bacteria have been able to identify some genes and molecules involved in the resistance mechanisms. Transcriptomic studies have looked at the difference in gene expression in the presence of TBTCl. (Bernat et al., 2014) reported a clear change in

membrane phospholipid composition as well as production of peroxidase. The peroxidase could have a protective role against the generation of reactive oxygen species that have been reported to play a critical role in TBTCl toxicity. Efflux pumps have been identified as a basis of the resistance in two bacterial species, coded by the operon *tbtABM* in some *Pseudomonas stuzeri* strains (Jude et al., 2004) and coded by the gene *SugE* in *Aeromonas molluscorum* (Cruz et al., 2013).

For a bioremediation purpose, the mechanism of most interest is the degradation of the compound. A quick way of checking for degradation ability is to provide TBT as sole carbon source in the growth medium. Therefore, further tests were carried out to identify this type of TBT-degrader among the isolated strains.

6.3.3. Some of the isolates are able to use TBT as the sole carbon source

As a straightforward way of screening TBT-degrading bacteria, the TBT-resistant isolates were subcultured on a medium containing TBT as the sole carbon source. Growth on this medium demonstrates the ability of the bacteria to use TBT as a sole carbon source.

The high variability of the results prevented the detection of a statistical difference between the proportion of isolates able to use TBT as sole carbon source in prepared sediment and untouched sediment. A higher number of bacteria using TBT as the sole carbon source in the prepared sediment would be an expected result as the presence of TBT will have favoured a population of bacteria that was adapted to the presence of such a biocide. TBT degradation and its use as a carbon source is thought to happen through sequential debutylation but the enzymes responsible for this degradation have never been clearly identified (Cruz et al., 2015b). In parallel, siderophores produced by *Pseudomonas chlororaphis* have been shown to be responsible for Tin-C cleavage using triphenyltin (TPT), diphenyltin (DPT) and dibutyltin (DBT) as the substrates and may have the same effect on TBT (Inoue et al., 2003). For siderophores, as well as enzymatic degradation, however, TBT may not be the intended target and its degradation could result from co-metabolism. It is important to emphasise that bacteria, which are not able to use TBT as the sole carbon source could still have the ability to degrade it. Further tests would be necessary to resolve this.

6.3.4. iChip reveals members of Oceanisphaera, Bacillus, Shewanella and Pseudomonas as TBT-resistant bacteria, and members of Pseudomonas as TBT-degrading bacteria

The loss of the resistance ability for some of the isolates after a couple rounds of subculturing on TSA + 1mM TBT would suggest a plasmidic location of the resistance genes. These include the only *Bacillus* sp. isolate, the two *Shewanella* sp. isolates, some of the *Pseudomonas* sp. and *Oceanisphaera* sp. isolates.

The remaining *Oceanisphaera* sp. isolates were still maintained on TSA + 1mM TBT but could not grow on MSM + 1mM TBT, which means that they were not capable of using TBT as the sole carbon source. . At this stage it cannot be determined if they are still capable of TBT degradation by another mechanism. TBT could be degraded by an adverse reaction of enzymes secreted by the bacteria without utilisation of the degradation product. Nevertheless, this is the first time that members of the genus *Oceanisphaera* have been shown to be capable of TBT resistance. *Oceanisphaera* members have been repeatedly isolated from coastal and marine sediment (Cho and Lee, 2016; Romanenko et al., 2003; Shin et al., 2012; Zhou et al., 2015), the present study therefore shows their presence in estuarine sediment too.

Finally, many of the isolates belonging to *Pseudomonas* sp. were able to use TBT as the sole carbon source. This result is not surprising as *Pseudomonas* members have often been reported as TBT-resistant and as TBT-degraders (Ebah et al., 2016; D. S. Khanolkar et al., 2015b; Roy et al., 2004; Yáñez et al., 2015). In addition they are also known to degrade a wide range of other sediment contaminants (Wasi et al., 2013).

It is interesting to note that all of the isolates coming from the same iChip experiments belong to the same genera, although the small numbers of representatives for some of the iChips prevents any statistically significant conclusions to be made.

Table 6-1*: Details of the isolates identified by Sanger sequencing of the 16S rRNA genes* This table describes the different isolates and the techniques used for their obtention as well as their growth capacities when the identification was performed and the result of the identification. All of these isolates could grow on TSA + 1 mM at the 1st subculturing.

		Growth on the following medium after 4th subculturing			
Isolate	Isolation technique used	TSA	TSA + 1 mM TBT	MSM + 1 mM TBT	Identification
β2A3	iChip - prepared sediment	+	+	+	Pseudomonas sp.
β2B2	iChip - prepared sediment	+	+	+	Pseudomonas sp.
β5A5	iChip - prepared sediment	+	+	+	Pseudomonas sp.
β5A6	iChip - prepared sediment	+	+	+	Pseudomonas sp.
β5C4	iChip - prepared sediment	+	+	+	Pseudomonas sp.
β5C5	iChip - prepared sediment	+	+	+	Pseudomonas sp.
β5C3	iChip - prepared sediment	+	+	+	Pseudomonas sp.
3A1	standard plating	+	+	+	Pseudomonas sp.
3A2	standard plating	+	+	+	Pseudomonas sp.
I13b	standard plating	+	+	+	Pseudomonas sp.
α4D6	iChip - prepared sediment	+	+	-	Oceanisphaera sp.
α4A2	iChip - prepared sediment	+	+	-	Oceanisphaera sp.
α3D4	iChip - prepared sediment	+	+	-	Oceanisphaera sp.
α1C3	iChip - prepared sediment	+	+	-	Oceanisphaera sp.
7A	standard plating	+	+	-	Pseudomonas sp.
α1B6	iChip - prepared sediment	+	-	-	Oceanisphaera sp.
α1D5	iChip - prepared sediment	+	-	-	Oceanisphaera sp.
β2C5	iChip - prepared sediment	+	-	-	Pseudomonas sp.
β2B6	iChip - prepared sediment	+	-	-	Pseudomonas sp.
β2D5	iChip - prepared sediment	+	-	-	Pseudomonas sp.
β5B5	iChip - prepared sediment	+	-	-	Pseudomonas sp.
γ1D4	iChip - prepared sediment	+	-	-	Bacillus sp.
Z3D5b	iChip - untouched sediment	+	-	-	Shewanella sp.
Z3D5a	iChip - untouched sediment	+	-	-	Shewanella sp.

6.3.5. Discussion on the use of iChip for the isolation of uncultured bacteria:

Owing to its design, iChips are useful tools for the high throughput isolation of bacteria from a wide range of environments. In iChip, bacterial cells can easily be isolated from one another, and their growth is facilitated by the close proximity to the environment. One of the issues stated for the cultivation of unknown bacteria is that the fast-growing species outcompete the slow growing or rare species on the culture plates but in iChips each bacterial cell occupies one of the many through holes, giving more chance for these species to successfully develop. IChip, however, will not solve every issue. For example, the subculturing is later done in full laboratory conditions, and as this paper shows, not all the bacteria that have been able to grow in iChip are adapted for further growth on synthetic medium. Ideally, a coupling of iChip and the use of alternative media and growth conditions could lead to the best results. The need for key growth factors that are normally not present in the classic incubation media may persist after subculturing out of the iChip, and media supplemented with different types of molecules would still be useful. On the contrary, the nutrient-rich media classically used have sometimes been pointed out as inhibitory to some types of bacteria referred as 'oligophilic-' which would only develop on nutrient-poor media (Watve et al., 2000). Lowering the temperature of incubation is also usually suggested and this was done in the present study where all the incubation steps were performed at 20°C.

6.4. Experimental Procedure:

6.4.1. Sediment sampling and preparation

Sediment samples were taken from Liverpool Brocklebank Docks. One sample remained untouched in a cold room, stored in the dark at a temperature of 4°C, while another sample was sieved at 2 mm and spiked with 10 µg TBTCl / g dw sediment and thoroughly mixed by hand before being put back in the cold store for 4 weeks as an equilibration step. After that equilibration step, the mud was incubated at 20°C for 3 months. At the end of this incubation period, the sample was used for the present study and will be referred as "prepared sediment" for the rest of this paper. When using sediment stored directly after sampling and not processed further, the term "untouched sediment" will be used.

6.4.2. Sediment dilution and standard plating

Serial dilutions of the two types of sediment were plated on Tryptic Soy Agar (TSA) and TSA + 1mM TBT in order to calculate the abundance of bacteria capable of growth in standard laboratory conditions. After inoculation of different sediment dilutions in triplicates, the agar plates were incubated at room temperature for 3 to 5 days before the enumeration of colonies was performed.

The result of this enumeration was used to calculate the appropriate dilution for the inoculation of one "cultivable" bacterial cell in 10% of the iChip through-holes (10^2 bacteria per mL).

6.4.3. iChip assembly and incubation:

IChips were manufactured in the general engineering workshops of Liverpool John Moores University using the instructions provided by Nichols et al. (2010). Figure 6-2B indicates all of the components of an iChip, the central plate and the two external ones, which are pierced with a multitude of through-holes arranged in two arrays, in this case two arrays of 192 through-holes. Before assemblage, all the components were sterilized by immersion in 70% ethanol for 15 minutes. They were then allowed to dry under a sterile hood after which the central plate was immersed in molten agar (Figure 6-2A) containing the appropriate sediment dilution as a means to load one cultivable bacterial cell in 10% of the through-holes (10^2 cultivable cells/mL). Once the agar solidified on the central plate, the excess was removed using a sterile microscope slide and 8 sterile polycarbonate membranes disks of 27cm diameter with 0.03µm diameter pores were placed on each side. The external plates were finally mounted at the bottom and top of the central plates and the whole assemblage was screwed together (Figure 6-2B). To avoid any leaking from the sides, petroleum jelly was applied to seal the edges of the iChip, which was then protected with a fine band of parafilm. After assemblage, the iChips were immersed in a bucket of sediment and stored at 20°C for a week (Figure 6-2C).

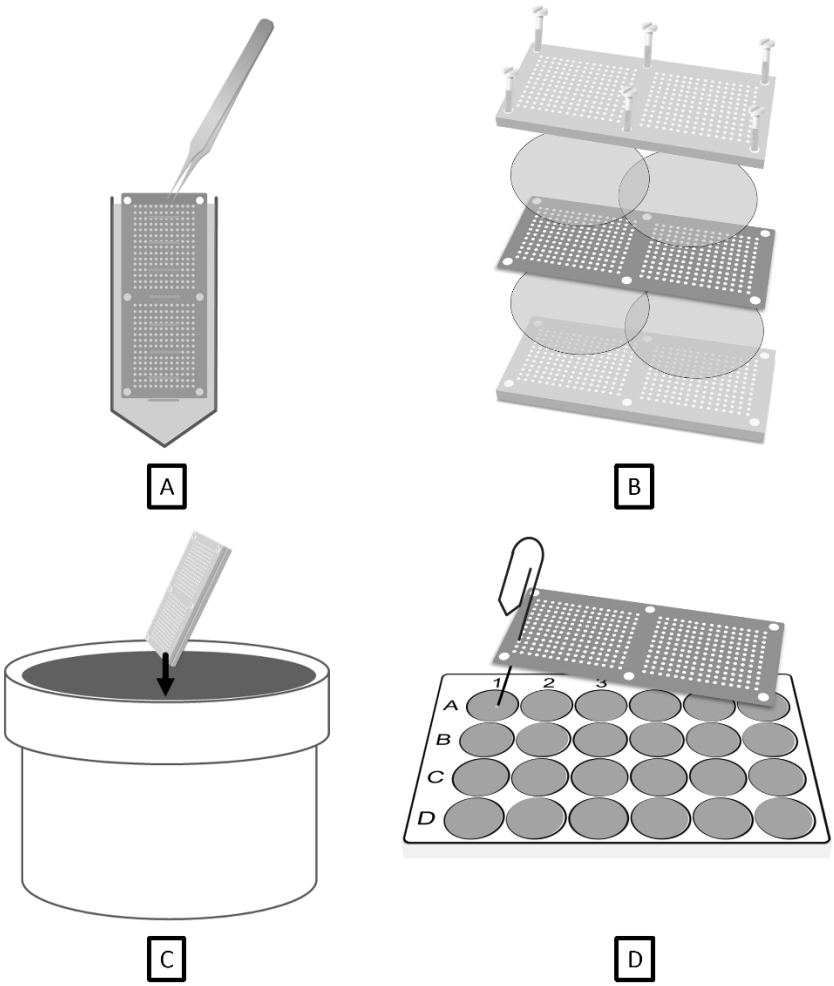

Figure 6-2: Steps to sediment bacteria isolation and cultivation using an iChip
The central plate is loaded with fusion agarose medium inoculated with sediment bacterial dilution (A). The iChip is then assembled with 0.03 µm polycarbonate membranes and the external plate, screwed together (B), and immersed in a bucket of muddy sediment for 2 weeks (C). After incubation, the iChip is thoroughly rinsed with sterile water, disassembled and sterile gauge clips are used to deposit each agar plug in a well of a 24-well plate filled up with TSA.

6.4.4. Isolate recovery:

After the incubation period, the iChips were thoroughly rinsed in sterile distilled water and disassembled. About one hundred random cores were retrieved from each iChip using a sterile and unbound gauge paper clip and gently crushed on the surface of TSA medium in 24-well plates (Figure 6-2D). The 24-well plates were incubated for several weeks at room temperature in the dark. The percentage of positive wells at this step was used to calculate the difference in cultivability between iChip and standard plating.

6.4.5. Screening for TBT resistance and use as sole carbon source:

Each isolate that could be grown on the 24 well plates containing TSA from the iChip cores were subcultured on TSA + 1 mM TBT to screen for the resistance phenotype.

The isolates that could grow on TSA + 1 mM TBT were further subcultured on Minimal Salt Medium (MSM) containing 1 mM TBT as the sole carbon source. MSM was prepared with the following compounds per litre of distilled water: 0.06g ferrous sulphate; 12.6g dipotassium hydrogen orthophosphate; 3.64g potassium dihydrogen orthophosphate; 2g ammonium nitrate; 0.2g magnesium sulphate; 0.0012g sodium molybdate; 0.0012g manganese sulphate; 0.15g calcium chloride; 15g agar. 1L of medium containing only agar and the phosphate buffer was autoclaved, all the other elements were prepared in solution separately, filter sterilized and added to the fusion medium after autoclaving and before pouring into petri dishes.

6.4.6. Identification of the isolates:

6.4.6.1. DNA extraction

24 colonies growing on TSA + 1 mM TBT were selected to be further identified by 16S rRNA gene sequencing. 20 isolates coming from the isolation through iChip, and 4 isolates obtained using the classic method of isolation. Freshly grown colonies were resuspended in 30 µL of sterile water and heated at 95°C for 10 minutes to extract their DNA. The suspensions were then spun down for 2 minutes in a benchtop centrifuge at maximum speed and the supernatant was used as template DNA for the PCRs.

6.4.6.2. DNA amplification

The amplification was performed using the following universal primers: 27F (AGAGTTTGATCATGGCTCA) and 1492R (TACGGTTACCTTGTTACGACTT). The reaction was prepared in a volume of 50 µL in total, with 25 µL of ReadyMix™ (Sigma), 1 µL of 10 pM of each primer and 2µL of DNA. Reactions were then performed in a thermocycler with the following program: 94°C for 2 min of initial denaturation followed by 35 cycles at 94°C for 1 min, 58°C for 30 sec and 72°C for 1 min, finishing with a final extension at 72°C for 10 min. The amplification of the samples was detected along with a DNA molecular weight standard (1 kb+, Invitrogen) by electrophoresis in a 2% agarose gel stained with SYBR Safe (Invitrogen) and visualized by transillumination by UV light.

The DNA concentration was then measured using a Nanodrop. As all the concentrations were too low, the samples were evaporated and resuspended in the appropriate volume to obtain 25 ng/µL. 5 µL of each sample were then added to 5 µL of primer at 5pmol/µL, 24

tubes were prepared with the forward primer 27F and 24 others with the reverse primer 1492R. The 48 tubes were barcoded using the LightRun barcodes from eurofins genomics and sent to the company for Sanger sequencing.

6.4.6.3. Sequence analyses

The ab1 files received from Sanger sequencing were checked for quality and the sequences appropriately corrected. The forward and reverse sequences of the same isolates were aligned and reassembled using BioEdit and the resulting FASTA sequences were analysed by BLAST using the total database, excluding uncultured/environmental sample sequences.

6.4.7. Statistical analyses:

All statistical analyses were performed using R Studio. Significant differences in the cultivability of bacteria using the two methods were calculated with a Student's t-test. Statistical significance was assumed when the p-value was below or equal to 0.05.

6.5. Conclusion:

This study confirms the potential of iChip to improve environmental bacteria cultivability success. It was effective to improve the cultivability of sediment bacteria from both native sediment and sediment after it was spiked with TBT and incubated for 3 months. We suggest that adaptations should be made regarding the conditions of cultivation and medium composition after the subculturing from iChip, in order to obtain a higher chance of maintaining the isolates in laboratory conditions. Such a tool has a strong potential in the cultivation of unknown organisms, and therefore has implications in many fields. It was previously shown fruitful for the discovery of new antibiotics and this study now demonstrated an extension of the applicability of iChip with its beneficial use in the field of bioremediation. Using iChip, TBT- resistant and TBT-degrading bacteria could be successfully isolated from sediment sampled from Liverpool Docks. For the first time, TBT-resistant bacteria were highlighted among the genus *Oceanisphaera sp*. These strains were not capable of using TBT as the sole carbon source, but more research is required to determine if they have the ability to degrade TBT as a co-metabolism.

7. General discussion and conclusion

7.1. Summary of the key findings

The objective of this study was to better understand the physical and environmental factors and the microbial actors controlling TBT-(bio)degradation in muddy sediment. This investigation was conceived in order to gain insights into the potential application of AND as a bioremediation method for the maritime industry.

The series of microcosm experiments in a scenario of high TBT contamination (Chapter III) revealed the following key points:

- TBT-biodegradation was inhibited by carbon and nitrogen supplementation
- TBT degradation was higher at 4°C without sediment agitation
- DBT accumulated at 4°C (as a by-product of TBT degradation) but was subject to biodegradation at 15°C with sediment agitation
- Over a 3 months period (the longest duration of experimental observations within the study), the rate of TBT biodegradation was relatively slow, with a maximum total of 40.8% recorded. But as the contamination level used was very high, the amount of TBT eliminated (4.2 µg/g sed dw) is superior to many contamination levels currently recorded around the world.

The microbial community analysis (Chapter 5) emphasised a likely reduction of diversity following TBT-spiking and aeration, without specific enrichment of aerobes or known TBT-degraders, but a likely proliferation of SOBs. The vast majority of the bacterial community was composed of bacteria involved in the sulphur cycle.

In the last result chapter (Chapter 6), bacterial isolation using two methods is described. These isolations were performed to reveal new members of the TBT-degrading or TBT-resistant community. All the isolates capable of degrading TBT as the sole carbon source were members of *Pseudomonas*. Among TBT-resistant isolates, members of *Oceanisphaera* were retrieved, showing a TBT-tolerance for the first time in bacteria belonging to this genus.

7.2. Implication for the bioremediation of TBT contaminated sediment

7.2.1. TBT-degrading bacteria cultivation

One interesting finding from the microbial community analysis was that the main taxa known to degrade TBT (mainly *Enterobacteriaceae*, in particular from the genus *Pseudomonas*) were not particularly enriched during the microcosm experiments. Adding to this, the isolation experiments did reveal a repeated isolation of *Pseudomonas* species from the same sediment. This demonstrates once again the bias of laboratory cultivation towards certain bacteria that are adapted to laboratory conditions despite not being dominant in the studied environment. And this still applied while a different method of isolation, the iChip, was attempted in order to retrieve a different diversity of bacteria.

In this study, the main taxa observed in the microbial community analyses were either sulphate reducers or sulphide oxidisers and would have required specific growth conditions to be isolated and maintained in the laboratory. Sulphate reducers for example only grow in the lab under strict anaerobic conditions with specific nutrients. It is therefore not surprising that these abundant taxa were not represented in the isolates recovered from the microcosms. However, as the microcosms were mainly maintained under aerobic conditions, aerobes were expected to be dominant in the samples at the time when the isolation experiments were performed. Hence why the isolations were performed under aerobic conditions.

Methodological approaches to cultivation remain suboptimal for a full characterisation and understanding of the degrading microbial community of a sample. This can impact indirectly on bioremediation studies when assessing the degrading capacity of the natural community of a sample by looking for previously isolated degraders, whereas impactful degraders could be microorganisms that are presently 'uncultivable'.

7.2.2. *In situ* bioremediation

The first step towards the development of a bioremediation solution is to establish the parameters influencing the biodegradation rate, in a quantitative way, in order to optimize the application of the method. Progressing the understanding of these parameters can

provide both spatial and temporal insights regarding the applicability (i.e. where, under what conditions, and when might biodegradation be optimal). Such knowledge would also contribute to potential biostimulation approaches. Although preliminary, the results of the microcosms enable an evaluation of the potential application of AND to the bioremediation of TBT (chapter III). The results of the carbon and nitrogen supplementation, and different shaking condition suggest that the oxygen provided during mud resuspension would be sufficient and that no nutrient biostimulation is required to ensure TBT biodegradation. This study is the first to report non-negligible biodegradation of TBT at low temperature in microcosms (4°C), which suggests an application of AND in late winter might be successful.

In contrast, other studies have reported the degradation of TBT at warmer temperatures of 25-28°C (Beolchini et al., 2014; Cruz et al., 2014; Sakultantimetha et al., 2011; Suehiro et al., 2006; Yonezawa et al., 1994). The factors causing this contrast is unclear, although it could be explained by the physical properties of the sediment or by the microbial community composition for example. The only study to assess the microbial community associated with TBT degradation in microcosm experiments reported a rather similar microbial composition to this investigation, with the exception of a higher abundance of *Bacteroidetes* (Cruz et al., 2014). No studies have been able to isolate members of this genus as TBT-degrading bacteria, but the possibility that this class could be a TBT-degrader cannot be eluded given the barriers to bacterial isolation described in this study. It is therefore uncertain whether the higher abundance of *Bacteroidetes* in the microcosms performed by Cruz et al. (2014) is the key to TBT degradation at warmer temperature, or if other factors such as the physicochemical parameters of the associated sediments are the determinants of this process.

Numerous studies have achieved the successful isolation of TBT-degrading bacteria claiming that, in addition to the acquisition of fundamental knowledge, the isolates can be used in the second approach of bioremediation using bacteria, which is bioaugmentation (Cruz et al., 2007; Ebah et al., 2016; Finnegan et al., 2017; Hassan et al., 2018; D. S. Khanolkar et al., 2015a; Roy et al., 2004; Sampath et al., 2012). In this study, the different isolates selected based on their TBT resistance and degradation capabilities were identified through Sanger sequencing of their 16S rRNA genes and classified at the genus lever but no further characterization and tests were done yet. Many studies have evaluated the

degradation capacity of their isolates and attempted to identify the most efficient strains (Finnegan et al., 2017; Kawai et al., 1998; Sakultantimetha et al., 2009; Sampath et al., 2012). Some have tested their potential use in bioaugmentation through microcosm experiments (Beolchini et al., 2014; Cruz et al., 2014; Sakultantimetha et al., 2011). One of these investigations did not show any improvement in TBT degradation rate after bioaugmentation alone but a beneficial effect of the combination of bioaugmentation and biostimulation (Beolchini et al., 2014) while the two others reported a reduced half-life of TBT after bioaugmentation (Cruz et al., 2014; Sakultantimetha et al., 2011). Cruz et al. (2014) also observed that the microbial community structure remained unchanged after the addition of the degrading strain. All of these studies display encouraging results for TBT biodegradation in sediment or by bacteria isolated from sediment in laboratory conditions but are not sufficient to confirm the application of such bioremediation solutions into industrial practice without field trials (7.1.2).

7.3. Research perspective

7.3.1. Further steps towards the application of AND for bioremediation

While not studied for the present project, as beyond the scope of investigation, there remain a number of issues and topics that may be considered for further investigation before validating the use of AND for bioremediation.

An important aspect is the successful degradation of the other contaminants that are likely to be present in the sediment within ports and harbours. TBT was chosen as a model for this study as a persistent and common legacy contaminant relating to shipping, but in practice, the aim would always be to degrade a wide range of contaminants present in ports. Evidence shows that a lot of these contaminants are aerobically degraded, like TBT, and are therefore good candidates to also be removed using AND. However, it would require significant investment to fully investigate the behaviours of other pollutants to establish whether they similar degradation patterns.

Furthermore, the use of AND for the bioremediation of strongly contaminated locations would need to be approached with caution as this could lead to the release of toxic compounds into the surrounding waters for a certain period of time before contaminant biodegradation. Indeed, it could be argued that causing a strong perturbation of the

ecosystem in order to sustainably clean an area might be an acceptable compromise compared to leaving these highly contaminated locations as they are but facing regular resuspensions and perturbations caused by ship traffic or natural events.

Some remediation methods such as capping, a method consisting in isolating sediment from the surrounding aquatic environment using clean layers of geologic materials and/or synthetic liners, are especially designed to tackle the passive resuspension of substrate mud by ship traffic, but they are only suitable for contaminants that are degraded anaerobically, and therefore cannot be applied to a wide range of contaminants. The fact that some contaminants are specifically degraded in different conditions of oxygenation also complicates the development of bioremediation solutions as they consequently must be adapted to the local 'cocktail of contaminants'. Similarly, substrate composition is also important in determining physico-chemical conditions, with muds much more likely to be anaerobic (and more suited to AND) than sandy sediments. If AND were to be applied to a site where aerobically degraded contaminants are present alongside anaerobically degraded contaminants in high quantity this would lead to the resuspension of the latter without any hope of future degradation, which would represent a bigger threat to the ecosystem and make the remediation effort counterproductive.

It is important to consider the impact of oxygenation. Oxygenation of anoxic sediment is usually considered beneficial due to the reduction of methane and H2S production, especially in zones where sulphate reduction is active (Broman et al., 2017), which could be the case in Liverpool Docks given the high prevalence of SRBs in the sediment. But the proliferation of aerobes also results in higher respiration rates. The oxygenation of anoxic areas with high organic carbon loads could therefore result in major release of carbon dioxide (CO_2) (Tong et al., 2016). It is necessary to take into account this potential production of $C0_2$ by respiration following AND and compare it to the reduction in CO_2 emissions if dredging need is reduced.

In addition, resuspending sediment certainly constitutes a perturbation of the port ecosystem, mostly because of the turbidity caused by the fluid mud cloud. Note that during AND, this turbidity is more localised than the one observed during dredging excavations, as the fluid mud is pumped back to the sea bottom where it forms a layer of navigable mud without mixing with the above water (Figure 7-1). In addition, it is important to remember

that ports are by their very nature, highly perturbated, anthropogenic environments, with ship traffic, maintenance work and contamination, causing frequent disturbance to the ecosystem (Darbra et al., 2005). Such a method being used as a replacement for dredging could still mitigate the disturbance as all the issues linked with transportation and disposal (e.g. cost, carbon emissions etc.) are eliminated.

AND could find its best value when actually used routinely, predominantly as a sediment management method, whilst at the same time eliminating moderate levels of contamination as they are introduced in ports and harbours through the inherent activities. It would therefore avoid their accumulation to toxic levels, while preventing sediment accumulation in the navigable waterways.

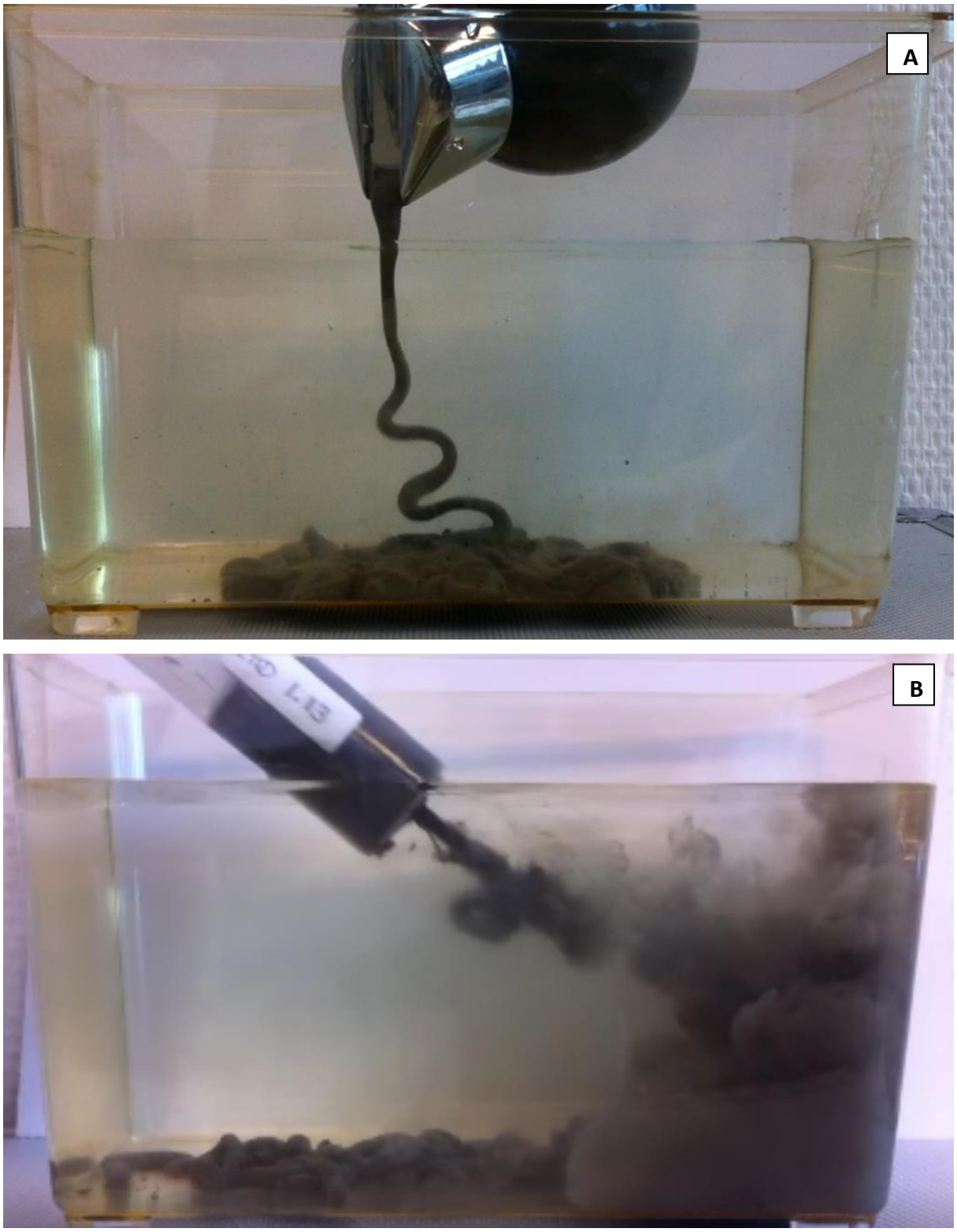

Figure 7-1: Fluid mud release tests depending on mud density. When the fluid mud has a density of 1.18, density obtained in AND, it gently sinks at the bottom of the water without generating much turbidity (A). Whereas a lower density of 1.13 which is closer to the density of sediment involved in dredging, generates a lot of turbidity (B).

7.3.2. The importance of field tests to bring in-situ bioremediation to application

The contrasting results among studies assessing the factors influencing TBT biodegradation in sediment, as well as the high variability in the observed TBT degradation rates demonstrate, as is usual for contaminant biodegradation studies, that the biogeochemical processes involved are driven by a complex sum of factors that are difficult to disentangle and understand. Perhaps, while waiting for laboratory studies to unravel all the mechanisms involved in TBT biodegradation, the most efficient way to determine the bioremediation potential of a certain microbial community is to run field tests *in situ*. In microcosms, many factors differ from the real conditions in the environment and can bias the study. For example, the impact of sample collection, transport and storage, suppression of natural nutrient fluxes, controlled temperature, etc., each result in compromise, and the efficacy of results arguably delays the development of practical bioremediation solutions.

For the remediation of TBT-contaminated sediment, studies have been restricted to the laboratory scale and have never been scaled-up despite the encouraging results. Although one could conclude that this illustrates that the application of *in situ* biostimulation and bioaugmentation in the field is unrealistic, successful field trials for the bioremediation of other sediment contaminants proves otherwise.

For bioaugmentation, one interesting study reported the slow but encouraging biodegradation of PCBs following a bioaugmentation experiment with active charcoal loaded with both aerobic and anaerobic degraders (Sowers et al., 2018). Bioamended charcoal was dispersed over the water of contaminated freshwater ponds and PCB concentrations were monitored for 409 days. This study showed no impact of the bioaugmentation on the native microbial community structure and reported over 50% of total PCB degradation over the period. Their cost assessment also revealed that this technique was cheaper than the other bioremediation techniques used for PCB contaminated sediment remediation, including *in sit*u methods, to the exception of natural attenuation.

For biostimulation, there are two types of approaches depending on the oxygen status of the target sediment. Studies have first focused on anaerobic biodegradation as sediment is usually considered anoxic. In this case, biostimulation consisted of providing energy

sources suitable to anaerobic metabolisms, such as acetate (Perelo, 2010). But with more recent investigations on resuspension and the aerobic degradation of various sediment contaminants (Levi et al., 2014; Li et al., 2015; Wald et al., 2015), different types of biostimulation have been investigated, from simple oxygenation to the addition of nutrients typical of aerobic metabolism. One study reported the success of sediment mixing and the continuous addition of a nutrient solution and hydrogen peroxide to groundwater in order to biostimulate the *in situ* aerobic biodegradation of phenol (da Silva et al., 2012).

Such studies demonstrate the realistic applicability of *in situ* bioaugmentation and biostimulation solutions if research puts the effort in up scaling their tests. But field-scale studies for the biodegradation of sediment contaminants are still rare and research should focus on these rather than multiplying microcosm experiments which are not exact representatives of the real scale solutions and are not sufficient to fully develop an applied bioremediation solution.

For the application of AND in bioremediation specifically, the same conclusion applies. Several microcosm studies have demonstrated an efficient TBT biodegradation in conditions close to the ones involved when applying AND. Further nutrient addition and bioaugmentation approaches could be envisaged if AND was to be used for the bioremediation of highly contaminated sites. But the present study, in addition to previous microcosm studies, suggests that the initial aeration step should be sufficient to trigger the biodegradation of small contaminations if AND is simply applied as an anti-siltation technique, and various contaminants could be degraded. This use would make AND a method of choice for port and harbours' sediment management but there remains significant inertia with a persistence of traditional methods and a lack of investment in innovation. This study has clearly identified a need for field tests to optimize the application of AND. Specific site conditions should be used to develop optimisation criteria as a means to prioritise target sites with maximum potential.

7.3.3. Progressing the understanding of the microbial community involved in TBT biodegradation

The ongoing limitations of the available bacterial isolation and cultivation methods discussed throughout this thesis show that there is still room for methodological innovation

in this field if we are able to cultivate and characterise all (or at least more) of the microbial diversity on Earth. A combination of different techniques such as the iChip with variable cultivation conditions (lower temperature, different media) could lead to more success in the discovery of new bacteria but is not likely alone to resolve the entire problem. If the need to isolate different bacteria persists, as is the case not only in bioremediation but also for example for the discovery of new metabolites that can be useful in medicine, then it will be necessary to completely revolutionise the cultivation techniques as a research priority.

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