Biotechnological potential of impacted scenarios for the restoration of TBT contaminated environments

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Resumo

O Tributil-estanho (TBT) é um composto organoestânico bastante utilizado na formulação de tintas de barcos como anti-incrustante. Devido à sua ampla aplicação industrial e consequente descarga para o ambiente, este composto tem sido reconhecido como um problema ambiental de escala global devido à sua toxicidade em organismos marinhos. Tendo sido por esta razão considerado como a substância mais tóxica alguma vez introduzida no ambiente marinho.

Os microrganismos presentes em locais historicamente contaminados possuem a capacidade de tolerar contaminantes e eventualmente de os degradar, o que pode constituir um fator chave no restabelecimento de ambientes contaminados. No entanto, os subprodutos resultantes da degradação de um poluente poderão ser tão ou mais tóxicos que o composto original. A deteção de um composto através de procedimentos químicos analíticos, embora importante, não fornece dados sobre a sua relevância ecológica, visto que estes poderão não estar relacionados com a sua ecotoxicidade.

Neste estudo, bactérias resistentes ao TBT foram recolhidas em 7 portos de pesca Portugueses (Póvoa de Varzim, Leixões, Aveiro, Figueira da Foz, Peniche, Setúbal e Sines) e posteriormente isoladas na presença de concentrações crescentes de TBT (0.1, 1, e 3mM). As bactérias tolerantes à concentração mais elevada foram caracterizadas por genotipagem (REP-PCR) e o seu potencial de bioremediação foi avaliado em águas contaminadas em laboratório.

A percentagem de microrganismos resistentes variou entre 0.08% (Setúbal) e 7.67% (Peniche). A análise de REP-PCR revelou um total de 111 perfis genéticos distintos, sendo que Peniche foi o local com menos variabilidade, enquanto Figueira da Foz foi o local com maior variabilidade. Os isolados selecionados foram usados em ensaios de bioremediação e o seu potencial de bioremediação foi avaliado através de ensaios ecotoxicológicos com o gastrópode *Gibbula umbilicalis*. Os ensaios ecotoxicológicos realizados sugerem que algumas bactérias marinhas são capazes de reduzir a toxicidade em águas contaminadas com TBT.

Este estudo contribuiu com novos dados sobre a resistência ao TBT, no entanto, estudos mais aprofundados na área da bioremediação do TBT mediada por bactérias marinhas são ainda necessários, nomeadamente na compreensão dos mecanismos associados à resistência e na identificação de vias e genes responsáveis pela sua degradação.

Palavras-chave: Tributil-estanho, resistência ao TBT, bactérias marinhas, ecotoxicologia, genotipagem

Abstract

Tributyltin (TBT) is an organotin compound commonly used as an antifouling agent in marine paint formulations. Due to its wide industrial application and its consequent discharge into the environment, TBT pollution is recognized as major environmental problem at a global scale, being recently considered to be the most toxic substance ever introduced into the marine environment.

Microorganisms from historically contaminated sites are able to tolerate pollutants and even degrade them, which may be a key factor in the restoration of contaminated environments. Nevertheless, byproducts resulting from the degradation process might be more or less toxic than the parent compound to ecological relevant species. The determination of the substance presence by analytical chemistry, although essential, may not present ecological relevance, as it might not be related to its ecotoxicity.

In this study, TBT-resistant bacteria collected from 7 Portuguese ports (Póvoa de Varzim, Leixões, Aveiro, Figueira da Foz, Peniche, Setúbal and Sines) were isolated in increasing concentrations of the toxicant (0.1, 1, and 3mM of TBT) and those growing at the highest concentration were characterized by genomic fingerprinting (REP-PCR) and tested as potential bioremediation tool in laboratory contaminated media.

The percentage of TBT-resistant isolates varied between 0.08% (Setúbal harbor) and 7.67% (Peniche). REP-PCR analysis revealed a total 111 distinct genetic profiles, being Peniche the location with lower variability while Figueira da Foz had the highest variability. Selected isolates were used to bioremediate waters contaminated waters, and their potential as bioremediation tools was assessed through ecotoxicological testing with the gastropod *Gibbula umbilicalis*. Ecotoxicological testing suggested that some TBT-resistant bacteria are able to reduce the toxicity of TBT contaminated waters.

This study contributed to the understanding of TBT resistance, however more intensive and focused research in the area of TBT bioremediation mediated by marine bacteria is still needed, particularly on the mechanisms behind TBT resistance and on the identification of pathways and genes responsible for TBT degradation.

Keywords: Tributyltin, TBT resistance, marine bacteria, ecotoxicology, genomic fingerprinting

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1. Background

1.1 Pollution of the marine environment

The history of marine contamination goes as back as to the geologic beginning of the planet. Nevertheless, population growth and increasing human activities in coastal regions have generated a greater contaminant input threatening significantly and dangerously global biodiversity and the productivity of marine ecosystems (Jenssen, 2003).

The ocean covers more than 70% of the Earth's surface and therefore is a large source of biological diversity (Targett et al., 2002). However marine pollution did not receive much attention until adverse consequences on organisms and ecosystems were evident (Islam and Tanaka, 2004). Since then, marine pollution has become a global concern but even so, most developed nations are still producing substantial pollution loads and the trends are expected to increase (Islam and Tanaka, 2004). Annually, an estimated 300 Mt of chemicals find their way into receiving watercourses mainly through industrial effluents (Schwarzenbach et al., 2006).

In this scenario, mitigating the risks that pollutants pose to the environment and human health is a major global concern and one of the greatest challenges for the 21st century. In recent years many organizations made several efforts to help raise awareness to take steps to halt biodiversity loss (e.g. the EU biodiversity strategy to 2020 and WWF).

In general, abnormalities suspected to be caused by exposure to toxic environmental contaminants have been largely found and reported in marine life (Tanabe, 2002). For example, as reported by Tanabe (2002), since 1960s sterility, abortion, stillbirths, diseases and strandings are increasing in marine mammals like pinnipeds and cetaceans all over the world, with most of these abnormalities taken place during the 1980s and the 1990s due to the incidence of organochlorines and other toxic contaminats. Other undesirable consequences of pollution include the loss of nesting sites of turtles (Kasparek et al., 2001), mortality, malformation and abnormal chromosome division of fish embryos (Longwell et al., 1992) and the decline in populations of seabirds (Yorio et al., 2010).

Many pollutants accumulate in marine organisms, and later enter the food chain, and therefore humans are often exposed to persistent organic pollutants (POPs) such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and some heavy metals (Jenssen, 2003). Therefore today's concern is focused not only towards the impacts on wildlife and the environment but also towards human health.

1.2 Degradation processes of pollutants

As stated above, many compounds discharged into the aquatic environment are toxic and persistent, not being easily removed during wastewater treatments (Peñuela and Barceló, 1998). Once in the environment, most pollutants may undergo volatilization, photolysis, chemical oxidation, adsorption or biodegradation (Haritash and Kaushik, 2009). Most of modern society's environmental pollutants like organic and many inorganic chemicals are subject to biodegradation, *i.e.*, an enzymatic degradation through the activities of living organisms (Crawford and Crawford, 2005).

Biodegradation is an important but poorly understood process that is crucial to all mitigation strategies. Biodegradation may be applied to remove or detoxify pollutants that threaten public health and to restore the environment, in a process termed bioremediation (Crawford and Crawford, 2005). Usually, this process is carried out by microorganisms such as bacteria, yeasts and microalgae or by plants and seaweeds (Bonaventura and Johnson, 1997).

This approach, which is potentially more cost-effective than traditional techniques such as incineration of soils, requires an understanding of how organisms transform chemicals (and knowing the toxicity of the degradation products), how they survive in polluted environments and how they should be employed in the environment (Crawford and Crawford, 2005).

Among the current wide-ranging research in bioremediation, some studies are focusing on identifying organisms that possess the ability to degrade specific pollutants (Fingerman and Nagabhushanam, 2005).

Recent advances in molecular biology techniques have provided sensitive, rapid and quantitative analytical tools for identifying and characterize microorganisms at species

and subspecies levels (Adiguzel et al., 2009; Girones et al., 2010). Genomic fingerprinting of microorganisms i.e., the capability to differentiate individuals of the same phylotype, have significantly evolved in the last years, by a series of methods that take avail of the variations found in their DNA. Among these methods, repetitive sequence based polymerase chain reaction (rep-PCR) is based on the usage of PCR primers complementary to interspersed repetitive sequences (Adiguzel et al., 2009). This technique enables the amplification of differently sized DNA fragments lying between these elements, producing clearly resolvable bands by agarose gel electrophoresis (Versalovic et al., 1991). BOX, ERIC (enterobacterial repetitive intergenic consensus) and REP (Repetitive Extragenic Palindromic) elements are examples of evolutionarily conserved repetitive sequences (Adiguzel et al., 2009). Rep-PCR fingerprinting is a well-established technique for the differentiation of species genetically close to each other (Adiguzel et al., 2009; Xie et al., 2008). Compared with other genomic fingerprinting techniques, such as RFLP (restriction fragment length polymorphism) and RAPD (random amplified polymorphic DNA), the reliability, reproducibility, low cost, high resolution and discrimination makes rep-PCR advantageous especially when the targeting closely related strains (Rademaker and de Bruijn, 1997;Trindade et al., 2003; Xie et al., 2008). However, although this technique has already been applied extensively in taxonomy studies, relatively little work has been done to determine the efficacy of using rep-PCR to characterize the diversity of environmental samples (Xie et al., 2008).

The correct identification and classification of microorganisms is of extreme practical importance for environmental biotechnology studies. With several detoxificating microorganisms already identified, studies are being conducted to identify the mechanisms whereby some pollutants are being removed from the environment (Fingerman and Nagabhushanam, 2005). Several mechanisms have been proposed like immobilization, which refers to the removal of contaminants, typically metals, by means of adsorption or bioaccumulation by various microorganisms or plants (Evans and Furlong, 2010a). Another important mechanism of detoxification is the transformation of toxic species into inactive forms by reduction, methylation or precipitation (Cloirec and Andrès, 2005). Yet, another mechanism of degradation consists on using the pollutant as a nutrient source.

Plants are also capable to remove degrade or sequester hazardous contaminants from the environment, in a process known as phytoremediation (Glass, 2005). Although

some phytoremediation applications are believed to be carried out by rhizosphere bacteria, the focus of phytoremediation is to use plants for this purpose (Glass, 2005). For example, Radwan et al. (2000) reported that plants like the leguminous *Vicia Faba* can be used to remove hydrocarbon from contaminated soils. Plants are also naturally capable of accumulating large amounts of metals, pesticides, solvents and various organic chemicals, (Evans and Furlong, 2010b; Glass, 2005) making them a potential solution to restore contaminated environments.

1.3 Endocrine Disruptor Compounds and Organotins

In recent years there has been increasing concern about the possible consequences of environmental exposure to a group of chemicals that are suspected to disrupt the normal endocrine functioning of wildlife populations, causing adverse health effects in an intact organism, its offspring, or (sub) population, the Endocrine Disruptor Compounds (EDCs) (Hagger et al., 2006; Lemos et al., 2010a, 2011a). Some known EDCs, like chlordane compounds (CHLs) and polychlorinated biphenyls (PCBs) have already attracted great attention due to their severe toxicity, high accumulation potential in the body and persistence in the environment, posing a great ecological risk (Tanabe, 2002). Also, in 2000 the European Commission (Groshart and Okkerman, 2000) published a list of potential EDCs based on their persistence, evidences of endocrines disruption and wildlife and human exposure. This list includes industrial chemicals such as plasticizers (e.g. bisphenol A), flame retardants (e.g. PBBS) or biocides with antifouling properties (organotin compounds).

Organotins are the most toxic pollutants for aquatic life known so far (Fent, 2006). The discovery of the first organotin compound, di-ethyltin diiodide, was made by Edward Frankland in 1849 and, until 1940s, the importance of these compounds was merely scientific. Since then, many reports were published mentioning several practical applications of organotins, such as PVC stabilizers (Thoonen et al., 2004) and as antifouling agents (Dubey and Roy, 2003). Those are still the major applications of organotins nowadays.

Of all know organotins, some of the most toxic are tributyltin (TBT) compounds like tributyltin oxide (TBTO) and tributyltin chloride (TBTCl) (Carfi' et al., 2008). TBT compounds are organic derivatives of tin (Sn^{4+}) characterized by the presence of covalent bonds between three carbon atoms and a tin atom (Antizar-Ladislao, 2008). While inorganic forms of tin are regarded as non-toxic, these lipid-soluble organotins can be highly toxic (Gadd, 2000).

Tributyltin has been used extensively since 1960s as a toxic chemical for various industrial purposes such as slime control in paper, as a wood preservative (Antizar-Ladislao, 2008) and as a polyvinyl chloride (PVC) stabilizer (Mimura, et al., 2008). In the 1970s, TBT paints replaced copper-based paints due to a superior performance in terms of efficacy and duration (Sonak, 2009). Since then, TBT has been used mostly as an antifouling agent in marine paint formulations to prevent the attachment of barnacles and slime on boat hulls and aquaculture nets (Kannan et al., 1998). In fact, TBT compounds are the main active ingredients in biocides used to control a broad spectrum of organisms (Fent, 2006), as it acts as a biocide for fungi, bacteria and insects (Mimura, et al., 2008).

Due to its widespread use as an antifouling agent in boat paints, TBT is a common contaminant of marine and freshwater ecosystems. Due to high boat traffic, ports and harbors waters and sediments are historically contaminated with TBT (Champ, 2003).

Tributyltin damaging consequences to marine ecosystems were recognized in early 1980s as the cause for the decline of oysters (Alzieu et al., 1982) Recently it has been considered by some authors to be the most toxic substance ever introduced into the marine environment (Antizar-Ladislao, 2008; Guo et al., 2010; (Sonak, 2009). This issue will be further discussed below.

1.4 Toxic effects of TBT

Since that TBT is used in a variety of industrial processes, its subsequent discharge into the environment, its fate, toxicity and human exposure are topics of current concern. It has been reported that TBT causes impairments in growth, development and reproduction, which ultimately may lead to the extinction of some populations (Guo et al., 2010).

Tributyltin from hulls and nets can be adsorbed onto suspended particles in the water (Gadd, 2000), sediment and biota. Subsequently it is readily incorporated into the tissues of filter-feeding zooplankton, invertebrates and eventually higher organisms such as fish and mammals where it accumulates (Antizar-Ladislao, 2008). Nonetheless, research undertaken since the early 1970s has shown that TBT is very toxic to a large number of aquatic organisms than those targeted (Antizar-Ladislao, 2008).

Tributyltin is known as an endocrine disruptor that promotes adverse effects in diverse organisms from snails to mammals (Guo et al., 2010). Two of the best-documented adverse impacts of TBT in non-target organisms are imposex and intersex in prosobranch snails (Matthiessen and Gibbs, 1998). Imposex occurs when male sex characteristics and organs, such as penis and vas deferens, are superimposed on normal female gastropods (Pavoni et al., 2007). The first clues linking TBT to imposex were reported in 1970 for *Nucella lapillus* in the UK (Antizar-Ladislao, 2008). Since then several studies have related TBT to the worldwide decline of marine molluscs is coastal areas due to imposex (Pavoni et al., 2007). Imposex has also been associated with reduced fecundity, sterility, population declines, and local extinctions of gastropod populations (Barroso et al., 2002; Tewari et al., 2002) Additionally, marine invertebrates are extremely sensitive to TBT, and imposex can be observed in gastropods at concentrations lower than 1 ng TBT L^{-1} (Gooding et al., 2003). Other major toxic effects of TBT include neurotoxicity and immunotoxicity (Asakawa et al., 2010).

These are perhaps the most complete examples of ED studies caused by TBT in wildlife populations. Although invertebrates dominate over 95% of the known animal species (McClellan-Green et al., 2007) other examples for ED in invertebrates are scarce and limited to laboratory studies.

At higher concentrations TBT may also be lethal to several marine and freshwater species. Short and Thrower (1987) reported that the 96 hour LC_{50} for juvenile Chinook salmon, (*Oncorhynchus tshawytscha*) is 1.5 μg L^{-1}. TBT also appears to have distinct effects on different life cycle stages. For example, the 96 hours LC_{50} for larval Pacific oysters, *Crassostrea gigas*, is 1.6 μg L^{-1}, whereas the value for adults of the same species is 282.2 μg L^{-1} (Thain, 1983).

In a study conducted by Guo et al. (2010) on Western clawed frog embryos, *Xenopus tropicalis* the results suggested that TBT might be the cause of several malformations. These include the loss of eye pigmentation, enlarged trunks and bent tails, in the presence of 50 ng L^{-1} of TBTCl after 24 hours of exposure. This is particularly relevant since the levels of TBT in open water, bays, estuaries, lakes and freshwater harbors are commonly higher than 50 ng L^{-1}. Highest TBT values can be found near marinas and seaports. Although TBT levels have been decreasing in the last decades, mainly due to restrictions in its use, it is still present at ng L^{-1} levels (Fent, 2006).

Concerning phytotoxicity, Turner et al. (2010) showed that concentrations of antifouling paint particles of few mg L^{-1} significantly reduced the photosynthetic response of the marine macroalgae, *Ulva lactuca*.

Furthermore, TBT has also been linked to malfunctions of mitochondria in some species (Antizar-Ladislao, 2008). Due to its persistence in sediments, gastropods that live on sediment may not recover for many years (Galante-Oliveira et al., 2010). Due with its lipophilicity, it tends to accumulate in oysters, mussels, crustaceans, molluscs, fish, and algae favouring the bioconcentration up the marine predators' food chain (Cruz et al., 2010; Santos et al., 2009).

1.5 Human exposure to TBT

To what concerns human health, two main routes of exposure are generally proposed: (1) direct ingestion of contaminated seafood and (2) indirect exposure from household items containing butyltin compounds (Azenha and Vasconcelos, 2002). Studies have shown that organotins leach from PVC and related materials, resulting in contamination of foodstuff and beverages like drinking water and wine (Antizar-Ladislao, 2008; Chien et al., 2002; Forsyth and Jay, 1997). Although in recent years there has been a significant increase of public concern on possible harmful effects of TBT on human health, there is still limited data available on organotin deposition in humans. Most information is estimated or extrapolated from rodent toxicity studies. Several experiments have suggested that potential adverse effects of organotins in humans includes cardiovascular, respiratory and reproductive deficiencies (Antizar-Ladislao, 2008). *In vitro* studies also suggest that organotins may compromise the immune response in humans by affecting survival,

proliferation and differentiation of lymphocytes B and reducing natural killer cells (NK cells) activity (Azenha and Vasconcelos, 2002).

Based on immunological studies, a Tolerable Daily Intake (TDI) value for TBT of 0.25 μg kg body weight^{-1} day^{-1} was established (Antizar-Ladislao, 2008). This TDI was based on rat immunotoxicity, and takes a safety factor of 100 to account for some uncertainties in human-rat toxicity extrapolation (Santos et al., 2009). It is now internationally accepted and it has been adopted by the World Health Organisation (WHO, 1999) and by European Food Safety Authority (EFSA, 2004).

In order to improve the understanding of the risk involved in the consumption of seafood, it has been suggested the calculation of the tolerable average residue levels (TARL), which is defined as the daily amount of TBT in seafood that is tolerable for the average consumer (Santos et al., 2009). Based on average seafood consumption, the TARL for Portuguese consumers is 93 ng g wet weight^{-1}. Santos et al. (2009) reported that four bivalve samples selected from Portuguese markets displayed butyltin levels above TARL, which indicates that bivalve consumers may be at risk, making TBT contamination more than "just" an environmental issue of concern.

However more research is still needed to elucidate which is the ideal method for estimating organotin destination and its consequences in humans, as well to uncover its mechanism of toxicity in humans.

1.6 TBT levels in water and regulation on its use

As a result of field evidences of negative ecological impact of organotins, in 1989 the European Union published a directive (89/677/CEE) banning TBT on ships smaller than 25 m (Santos et al., 2002). This legislation was adopted by Portugal a few years later in 1993 (D.L. 54/93) (Santos et al., 2009). On the assumption that TBT concentrations in the open sea were too low to cause effects there were not many restrictions on the use of organotins in larger ships. However, a similar impact in the open sea as in coastal areas has been shown for TBT with incidence of imposex being correlated with shipping density (Santos et al., 2002). The International Maritime Organization banned the application of TBT-based paints in 2003 and called for a global agreement for total prohibition of the

presence of organotins on ship hulls in 2008 (Antizar-Ladislao, 2008; Wang et al., 2010). Albeit these restrictions, TBT is still present in water and sediments (Fent, 2006).

Levels in harbor and port waters prior to restrictions on TBT use in antifouling paints have shown levels higher than 0.5 μg L^{-1} in North American and European marinas. For example, in 1986, TBT concentrations in Wroxham Broad (Norfolk, England) and at the nearby River Bure boatyard (Norfolk, England) were 0.9 μg L^{-1} and 1.5 μg L^{-1}, respectively (Waite et al., 1989). They were significantly higher than in open surface waters, bays and estuaries where commonly values of up to 50 ng L^{-1} were observed (Fent, 2006). Highest TBT levels are still found near harbors and ports due to painting operations. (Fent, 2006).

Albeit this regulation for the use of TBT, recent studies still indicate levels higher than those reported to cause effects at a global scale: for example, 13 ng L^{-1} in South Korea coastal waters (Sidharthan et al., 2002), an average of 6.8 ng L^{-1} in several Japanese coastal waters (Takeuchi et al., 2004), and up to 200 ng L^{-1}in Corsica, Western Mediterranean (Michel et al., 2001).

Present restrictions will not immediately remove TBT and its degradation products from the marine environment. TBT can be expected to remain in waters and sediments for long periods of time because of the low degradation rates in anoxic sediments and their widespread presence (Cruz et al., 2010; Santos et al., 2009).

1.7 Bioremediation of TBT

Once released from an antifouling coating, the fate of TBT will depend on physical and chemical reactions as well on biological activity it is subjected to (Gadd, 2000). Tributyltin is susceptible to degradation in water with half-lives ranging from few days to several months in water (Cooney, 1988). Reported degradation half-lives for TBT include 2 months in seawater (5.5 months in marine sediment), 4 to 19 days in estuarine waters and 6 days in freshwater (4 months in freshwater sediment) Adelman D et al., (1990).

Maguire et al. (1983) demonstrated that TBT dissolved in water does not volatizes after 2 months in the dark at 20ºC. Their results from experiments in water have also indicated that abiotic degradation of TBT is limited to photolysis in surface water (Maguire

et al., 1983, 1985).

Several microorganisms have been reported to present resistance to organotins, such as *Aeromonas molluscorum* Av27 and *Aeromonas molluscorum* G.N1.24, two bacteria isolated from an estuarine environment, in Ria de Aveiro (NW Portugal) (Cruz et al., 2007). These microorganisms may have an important role in organotins' environmental cycle and may be applied as TBT indicators in contaminated waters. Reactions known to be carried out by bacteria and algae include accumulation, release and degradation of TBT (Gadd, 2000; Luan et al., 2006).

Under favourable conditions TBT may be biodegraded through successive dealkylation to produce dibutyltin (DBT), monobutyltin (MBT), and ultimately inorganic tin, becoming progressively less toxic in the process (Table 1.1) (Dubey and Roy, 2003).

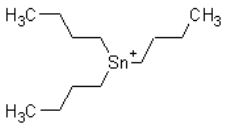
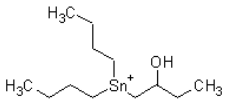
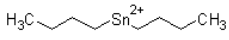
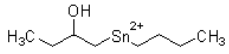
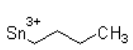
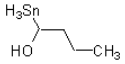
Dibutyltin is less toxic than TBT and its toxicity action is by blocking the absorption of oxygen in the mitochondria, while MBT has no reported toxic effect on mammals (Antizar-Ladislao, 2008). However, information on the mechanisms of TBT degradation mediated by microorganisms in marine environments is still very limited (Antizar-Ladislao, 2008). Some mechanisms have been proposed that could be involved in TBT degradation by bacteria, like metabolic consumption as a carbon source (Kawai et al., 1998) or bioaccumulation into the cell (Cruz et al., 2007). Because of its persistence and slow degradation it is valuable to use microorganisms for TBT degradation.

Given the function played by bacteria in biogeochemical cycles, the identification and characterization of TBT pollutant-degrading bacterial strains is crucial. Knowledge of their physiology and genetics is fundamental for their future application as natural decontamination agents. For example, several studies reported that most TBT-resistant bacteria possess plasmids, which might codify for TBT resistance. (Baya et al., 1986; Cruz et al., 2007; Wuertz et al., 1991). Moreover, TBT-resistance has also been associated to resistance to drugs, heavy metals and other contaminants (Suehiro et al., 2007; Baya et al., 1986; Suzuki et al., 1992; Wuertz et al., 1991).

1.8 Importance of ecotoxicological assays in Environmental Biotechnology

Bioremediation processes should be taken into account when removing pollutants from the marine environment, as it is emerging as an effective and economically viable alternative to traditional techniques. However, from biodegradation processes can result unexpected outcomes and consequences. For example, a xenobiotic pollutant might be mineralized, transformed to another compound that may be toxic and bound to natural materials in soils, sediments or waters (Crawford and Crawford, 2005).

Table 1.1
TBT Degradation Pathway through consecutive dealkylation by enzyme action

Compound	Formula	Chemical Structure	Enzyme
Tributyltin (TBT)	$C_{12}H_{27}Sn^{+}$		
		⇩	TBT dioxygenase
β-hydroxybutyl-dibutyltin	$C_{12}H_{27}OSn^{+}$		
		⇩	DBT dioxygenase
Dibutyltin (DBT)	$C_8H_{18}Sn^{2+}$		
		⇩	
β-hydroxybutyl-butyltin	$C_8H_{18}OSn^{2+}$		
		⇩	MBT dioxygenase
Monobutyltin (MBT)	$C_4H_9Sn^{3+}$		
		⇩	
β-hydroxybutyltin	$C_4H_{12}OSn^{3+}$		
		⇩	
	Sn^{4+}	Sn	

Adapted from "Biocatalysis/Biodegradation Database" University of Minnesota; http://umbbd.msi.umn.edu/ last updated 12 October 2011 and Antizar-Ladislao (2008).

After the treatment with bacteria, it is important to assess if the pollutant was degraded into a less toxic compound and/or less bioavailble. Analytical chemistry provides information on abundance or presence of a particular substance in a sample, although no information is provided on its toxicy. Hence it is also necessary to perform ecotoxicological assays. These assays are very useful to understand the effects of any chemical toxicants on ecologically relevant species (Lemos et al., 2010b). With these tests it is possible to observe the effect of a toxicant on ecologically relevant species, and therefore evaluate the pollutant and its degradation products toxicity. Gastropods and bivalves are among the most sensitive organisms to the toxic effects of organotins (Santos et al., 2002).

Gibbula sp. is a genus of sea snails, marine gastropods of the family Trochidae inhabiting the upper intertidal zone on rocky shores. Many of them are very tolerant to sewage discharges and heavy metals, living around the high tide mark with only brief periods of immersion (Ali and Bream, 2010). As prosobranch snails, *Gibbula sp.* can be used as biomarker of TBT environmental concentrations (Galante-Oliveira et al., 2010). These gastropods are known to be the animal group most sensitive to TBT, with several malformation symptoms being reported (Nehring, 2000). Also, this group of gastropods is largely affected by imposex and intersex, making them a focus group for TBT effects.

2. Objectives

The present study aimed to isolate TBT-resistant bacteria collected from Portuguese ports and evaluate their ability to bioremediate TBT into less toxic compounds through ecotoxicological assays.

The specific aims of this study were:

- Isolate bacteria highly resistant to TBT;
- Characterize TBT-resistant bacteria by REP-PCR and test them as potential bioremediation tool in laboratory contaminated waters through ecotoxicological assays;
- Optimize acute ecotoxicological assays for TBT using *Gibbula umbilicalis* as a model organism and calculate the median lethal concentration (LC_{50}).

3. Materials and Methods

3.1 Sampling locations and collection

The water samples were collected from 7 Portuguese ports (Figure 3.1):

Location 1 – Póvoa de Varzim (V; 41.376120,-8.766945);
Location 2 – Leixões (L; 41.195238,-8.684177);
Location 3 – Aveiro (A; 40.645899,-8.727098);
Location 4 – Figueira (F; 40.146848,-8.849176);
Location 5 – Peniche (P; 39.355422,-9.375479);
Location 6 – Setubal (St; 38.521228,-8.887277);
Location 7 – Sines (S; 37.950219,-8.864599).

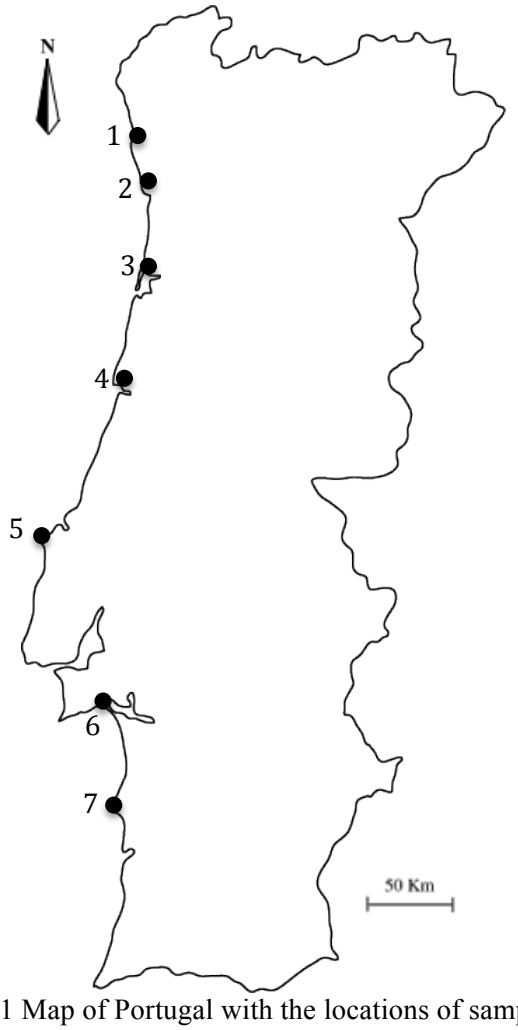


Figure 3.1 Map of Portugal with the locations of sampling sites.

These ports were selected because they are amongst the Portuguese ports with highest marine traffic. Also, because they are located in different latitudes, it is expected to have distinct physicochemical properties, and different bacterial communities. All samples were collected during low tide using a Van-Dorn water sampler 50 cm above the sediment level. Water parameters (pH, salinity, temperature, dissolved oxygen and depth) were measured with a Hanna HI 9828 (Hanna Instruments, Italy) multiparameter meter. A total of 2 L was collected at each sampling site, of which 1 L were used for chemical analysis (500 mL for heavy metals analysis and 500 mL for butyltins (TBT, DBT and MBT) analysis). Samples were transported to the laboratory in an icebox and processed within 8 h.

3.2 Chemical analysis of butyltins

Chemical analyses of butyltins were made at the Laboratório de Análises do Instituto Superior Técnico (Lisbon, Portugal). Samples were derivatised with tetraethylborate and analysed by GC-MS, using an RTX-5MS column (30mm x0.25 mm x 0.25 μm). Monobutyltin chloride, Dibutyltin chloride and tributyltin chloride were used as standards. Quantification was achieved by comparison with an internal standard.

3.3 Chemical preparation of TBT

TBT (tributyltin chloride, purity 96%; Sigma-Aldrich, Spain) stock solution was prepared in absolute ethanol and stored in the dark at room temperature.

3.4 Selective medium preparation

Microbial selective growth medium was prepared with Tryptic Soy Agar (TSA) medium (Sharlau, Spain) supplemented with 1.5% (w/v) NaCl (Sharlau, Spain). TSA was also supplemented with 0.1, 1 and 3 mM TBT by adding the proper volume of TBT stock solution and adjusting the total volume of ethanol in media to 1% (v/v). This procedure was performed under sterile conditions.

3.5 Microorganism isolation

Membrane filtration technique was used for the processing of all water samples. Briefly, water samples were filtered in vacuum through a membrane filter with pore size 0.45 μm and 47 mm in diameter (Sartorius Stedim Biotech, Germany), in which

microorganisms get concentrated on its surface. Different volumes were filtered of each sample, to ensure that for every concentration colony forming units (cfu) in the filter were between a countable number of 9 and 90, and only those included in this range were considered. These filters were then placed on the top of the medium in 50 mm diameter plates and incubated at 30 °C for 48 hours. Three replicates of each sample and concentration were made. Colony forming units were then counted and all 3mM TBT-resistant bacteria of 1 random plate per location were isolated and purified by streaking technique.

3.6 Extraction of genomic DNA

Bacterial stocks were maintained both in TSA containing 3mM of TBT at 4ºC and in glycerol (Sharlau, Spain) stocks at -80ºC Tryptic Soy Broth (TSB) (Sharlau, Spain) with 20% (v/v) of glycerol. Before the extraction process fresh cultures were prepared.

The extraction began with the inoculation of one colony in 500 µL of TSB, which was incubated overnight, at 30ºC and 200 rpm in an orbital shaker (heidolph titramax 1000). Subsequently, 250 µL of the culture were transferred into a sterile microtube. Cells were pelleted by centrifugation for 5 min at 15680 *g* and resuspended in 50 µL TE buffer (10 mMTris, 1 mM EDTA). Cell lysis was induced with 5 µl of Lysozyme (from hen egg white, 10 mg/mL; Sigma, Switzerland) followed by 1 hour incubation at 37ºC and then by 50 µL of Lysis Solution (Genomic DNA purification kit, Fermentas, Germany) followed by a 10 minute incubation at 65ºC. One hundred µL of chloroform (Fisher Scientific, United Kingdom) was added and thoroughly mixed with the lysate before spin 5 min at 15680 *g*. Aqueous phase was then transferred to another microtube, mixed with 100 µL of isopropanol (Panreac, Spain) and centrifuged at 15680 *g* during 10 min. Afterwards the supernatant was removed and the pellet washed with 100 µL of 70% (v/v) ethanol. After centrifugation (5 min, 15680 *g*) the supernatant was discarded and the pellet dried at room temperature. Lastly the pellet was resuspended in 50 µL of TE buffer and stored at -20ºC.

DNA quality and extraction efficiency were evaluated through electrophoresis in 0.8% (w/v) agarose gel at 80 V for 80 min in 1xTAE buffer (40 mM Tris, 20mM acetic acid, 1 mM EDTA) and stained in ethidium bromide solution (10 µg Etbr /L 1× TAE buffer).

3.7 Rep-PCR genomic fingerprinting

Rep-PCR was conducted to obtain the genomic fingerprinting of the isolated bacteria. This technique allowed to verify the diversity of the samples, but also enabled to select only the isolates with distinct genetic patterns for subsequent assays. The primers used for this reaction were: REP1R (5' IIIICGICGICATCIGGC 3') and (REP2I 5' ICGICTTATCIGGCCTAC 3') (Stab Vida, Portugal) (Adiguzel et al., 2009). Briefly, 24 µL of reaction cocktail was prepared as follows: 11.15 µL of ultrapure water, 5 µL of 5x buffer (Promega, USA), 3 µL of $MgCl_2$ (25mM) (Promega, USA), 1.5 µL of dNTP solution (2mM of each dNTP (Bioline, UK), 1.25 µL of DMSO (99.5%; Sigma, USA), 1 µL of each primer at 10mM and 0.1 µL of *taq* DNA Polymerase (Promega, USA). Lastly, 1 µL of extracted DNA was used as a template in a 25 µL reaction mixture. A negative control (no DNA) was included in each PCR assay. The whole process was conducted on ice and in sterile environment. PCR amplification reactions were performed with a thermocycler (MyCycler Thermal Cycler, BIO-RAD) using the following conditions: an initial denaturation at 95 °C for 5 min followed by 30 cycles consisting of denaturation at 94ºC for 1 min, annealing at 40ºC for 1 min and extension at 65ºC for 8 min. A final extension at 65ºC for 16 min was performed before cooling at 15ºC. The PCR products were then stored at -20ºC. Twenty microliters of the PCR products were examined by agarose gel (1.5% w/v) at 80v for 150 min and stained in ethidium bromide solution and analyzed by Dice/UPGMA cluster analysis, after image acquisition of a Gel-Doc (Bio-Rad).

3.8 Dice/UPGMA cluster analysis of REP-PCR genomic fingerprints

Banding patterns were analyzed with the software GelCompar II (version 3.0; Applied Maths, Kortrijk, Belgium). Relationships between the PCR-generated patterns were examined through cluster analysis using the unweighted pair-group method with average linkages (UPGMA) (Tacão et al., 2005). Similarity between fingerprints was calculated with the Dice coefficient. Patterns with more or equal to 94% similarity were regarded as the same - this similarity level was determined using control samples in all agarose gels.

3.9 Bacterial growth curve assessment

All selected isolates (different phylotypes) were transferred to 6 mM media. After this, isolates that grew in these conditions were then grown in triplicates in a 96 well plate with increasing concentrations of TBT (0, 1, 3 and 6 mM in TSB), and incubated at 160 rpm on a orbital incubator (Stuart Scientific SI50), at 30ºC. Isolates were previously grown until reaching an O.D. of 0.600 to standardize growth conditions. After this, same isolates were grown. Absorbance was read at 570nm in a Labsystems Multiskan EX well plate reader (Helsinki, Finland) at every 30 minutes until reaching the stationary phase. After this growth curves were plotted and bacterial generation time was calculated.

3.10 Acute test of tributyltin with *Gibbula umbilicalis*

The test organism used in this assays was the sea snail *Gibbula umbilicalis* (da Costa, 1778), collected in the coast of Peniche (Portugal; 39.368773, -9.378371) during low tide. The tested organisms were then acclimated in glass tanks with clean seawater (adjusted to 34 PSU with distilled water) at 20 ± 1 ºC for 15 days with a photoperiod of 16h:8h light:dark, and fed *ad libitum* with the macroalgae *Ulva lactuca* (Linnaeus, 1753) collected in the Mondego estuary during low tide (Portugal; 40.118491, -8.830175). Prior to conducting an ecotoxicological test, organisms with approximate shell length sizes were chosen (10 ± 2 mm) and kept for 24 hours without food. Organisms were individually exposed in 60 mL glass flasks filled to the top and covered with *tulle* netting to prevent their escape but assuring proper aeration and animal submersion and left in same conditions described above for cultures, but unfed. The sea snails were exposed to increasing dilutions of the media with clean seawater to obtain nominal concentrations of: 0.01; 0.05; 0.2; 0.5; 1; 2; 5; 10; 25; 50; and 100 µg L^{-1}. Eight replicates were used for each concentration and after 48, and 96 hours, mortality was checked and the LC_{50} determined.

3.11 Bioremediation assays

To select isolates for bioremediation assays, selected microorganisms were exposed to a high concentration of TBT (6 mM) in TSA medium for 48 hours. A total of 66 isolates grew in these conditions and their growth curve was measured until stationary phase. To determine the potential of the selected microorganisms to bioremediate TBT contaminated

media, glass bottles were filled with 250 mL of sterile seawater (34 PSU) contaminated with 500 μg L^{-1} of TBT. Selected bacteria were grown overnight in TSB and the inoculum was then individually added to these contaminated media - 0.1% TSB, O.D. = 0.600 at 570 nm. To one treatment 0.1% of sterile TSB was added. Simultaneously, glass bottles with 250 mL of sterile seawater and each bacterial inoculum (0.1% TSB, O.D. = 0.600 at 570 nm) were kept in the same conditions to assess possible toxic effects of each tested bacteria. Three replicates were made for each of the mentioned treatment. This apparatus was kept in the dark for 48 hours at 20 ± 1 ºC. After that period the bottle content was filtered (0.45 μm pore size, cellulose nitrate filter) transferred to dark glass bottles and kept at -20ºC until chemical analysis were made. For the ecotoxicological testing, replicates were pooled and stored in the cold until used (in less than 8 h).

3.12 Ecotoxicological testing of potentially bioremediated media

To assess different toxicity between potentially bioremediated and non-bioremediated treatments, an acute test with *Gibulla sp.* was performed (same condition as described in 3.10). The sea snails were exposed to increasing dilutions of the media with clean seawater to obtain dilutions of: 20; 12.2; 7.6; 4.6; 2.8; 1.8; 1; 0.7 and 0.4% of the initial media. These concentrations were chosen after prior range-finding tests (results not shown). Eight replicates were used for each concentration and after 48, 72, and 96 hours, mortality was checked and the LC_{50} determined.

3.13 Statistical analysis

One-way analysis of variance (ANOVA) was used to analyze data among treatments, followed by Dunnett test (when applicable) to discriminate significant differences between treatments and controls. When applicable, results are presented as mean ± SEM. These analyses were performed using GraphPad Prism 5 for Mac. Median lethal concentration was determined using Probit analysis (Finney, 1971) with StatPlus for Mac. The significance level was inferred at $P<0.05$ for all statistical tests.

4. Results

4.1 Physicochemical properties of port and harbor samples

The physicochemical properties (pH, salinity, temperature, conductivity, dissolved oxygen (DO) and collection depth of sampled waters are described in table 4.1.

Table 4.1
Physicochemical properties of near-sediment waters from sampling sites

Physicochemical properties	Póvoa de Varzim	Leixões	Aveiro	Figueira da Foz	Peniche	Setúbal	Sines
pH	8.7	8.9	8.1	8.3	7.8	8.8	9.0
Salinity (‰)	34.5	34.2	37.4	37.3	31.8	27.9	34.8
Temperature (ºC)	13.6	14.4	11.5	13.5	17.8	15.8	16.1
Conductivity ($WS.cm^{-1}$)	46.3	46.3	52.9	46.2	43.8	39	47.3
Dissolved Oxygen (%)	92.5	48.0	82.0	76.0	69.3	99.9	99.0
Depth (m)	3.0	8.0	7.5	5.0	6.5	3.5	4.0

4.2 Chemical analysis of butyltins of port and harbour samples

The concentration of butyltins (MBT, DTB and TBT) was below 50 ng L^{-1} on all near-sediment sampled waters.

4.3 Bacterial isolation and percentage of resistant bacteria

Bacterial growth was witnessed for all seawater samples at every TBT concentration tested and the results are represented in the form of colony forming units per milliliter (cfu mL^{-1}) (figure 4.1 – a to g).

It is possible to observe for all sampled locations that with increasing concentrations of TBT, the number of colony forming units per milliliter decreases and consequently the percentage of resistant bacteria compared to the control is also lower (values above bars on figure 4.1). At 0.1 mM, Póvoa de Varzim had the highest percentage of resistants' (45.46 ± 6.83%) while Setúbal had the lowest (2.56 ± 0.65%). Regarding

1 mM and 3mM concentrations, Peniche had the highest percentage rate (8.20 ± 0.53% and 7.67 ± 1.82% respectively) as well the highest number of colony forming units per milliliter values (410 ± 26 cfu mL^{-1} and 383 ± 91 cfu mL^{-1} respectively). On the other hand Aveiro (0.26 ± 0.09%) and Setúbal (0.08 ± 0.03%) had the lowest percentage of resistants' for 1 mM and 3 mM, respectively.

Aveiro also had the lowest cfu mL^{-1} in the control, while Peniche had the highest. Statistically significant differences were observed for all locations between selective media and control groups (ANOVA, Dunnett's test, $P < 0.05$).

A total of 157 isolates resistant to 3mM TBT were isolated from the 7 ports and used in subsequent assays.

4.4 Rep-PCR genomic fingerprinting

Due to high TBT pressure, it was expected that despite the high number of bacteria isolates there would be a low level of variability. Thus all isolates were subjected to rep-PCR genomic fingerprinting using primer sets corresponding to REP elements. Several bacteria exhibited similarities on their profiles. For this purpose rep profiles with more or equal to 94% similarity were regarded as the same (this similarity level was determined using control samples in all agarose gels) (Figure 4.2). The sample with the highest variability was Figueira da Foz (21 out of 23 different isolates) and the sample with the lowest was Peniche (5 out of 30 different isolates). Similarity among isolates ranged from 37.5% to 100%. A total of 111 isolates were acknowledged as different through this genomic fingerprinting technique.

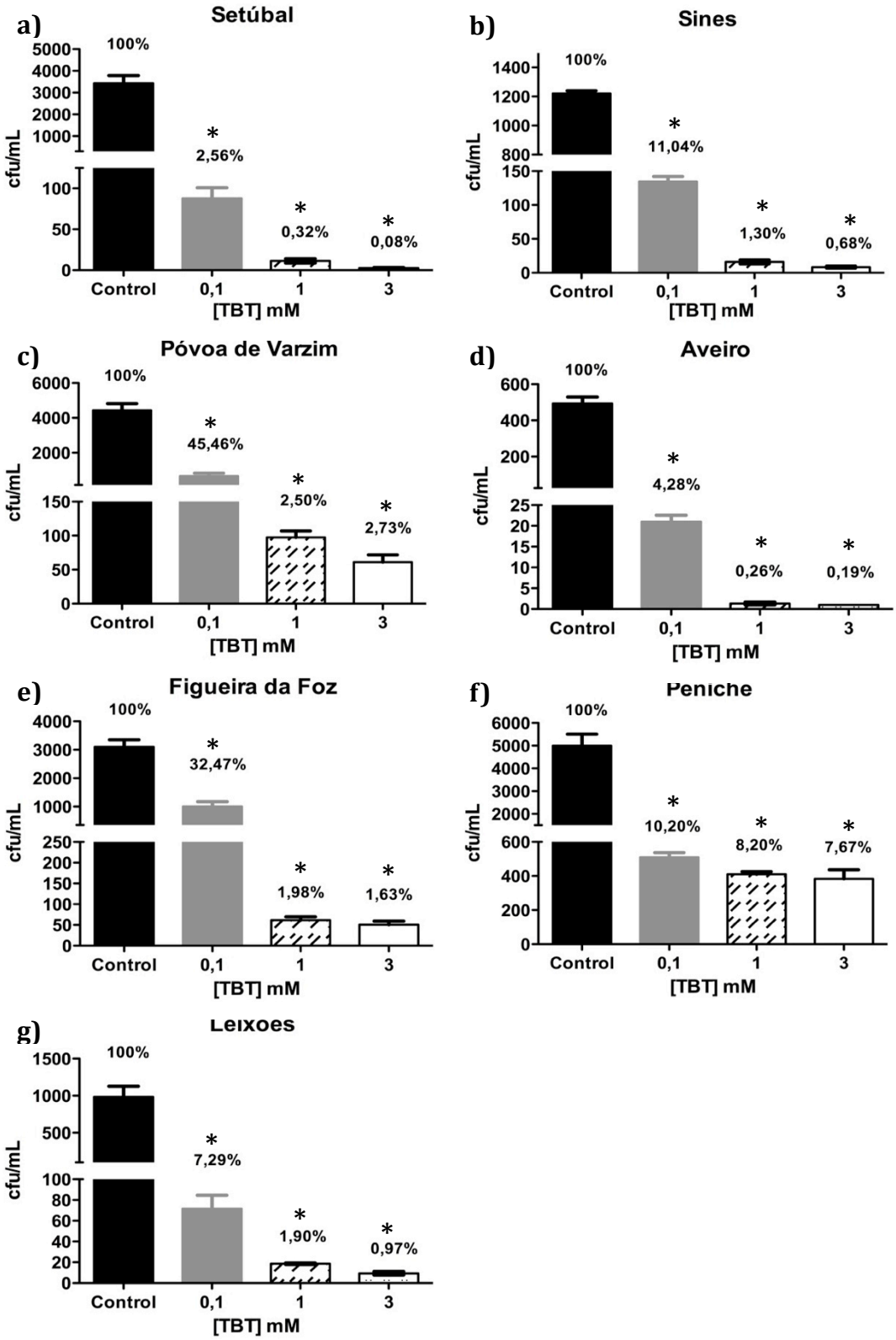
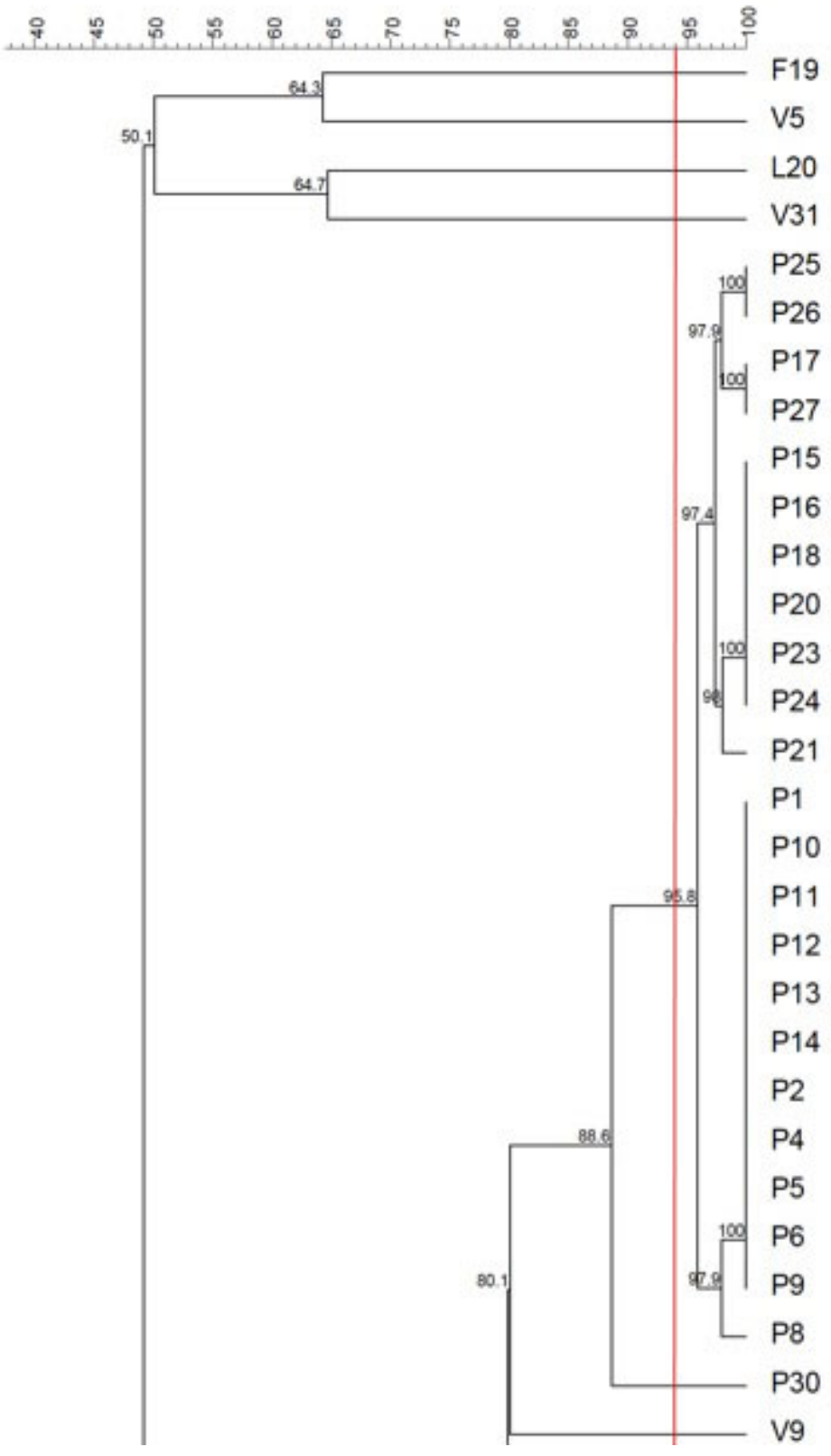
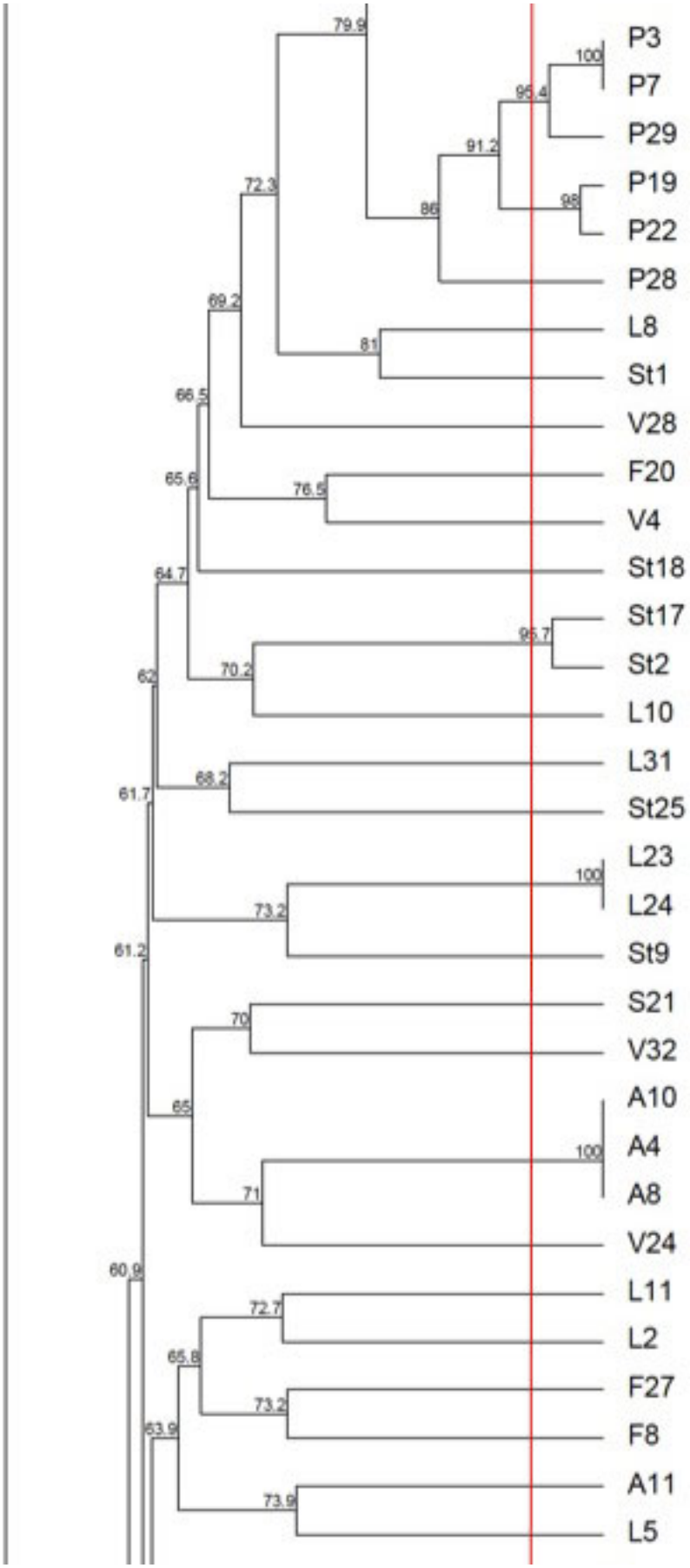


Figure 4.1 – Concentration of isolates (cfu mL-1) at increasing concentrations of tributyltin (0, 0.1, 1 and 3mM) for all sampling sites (a – Setúbal; b – Sines; c – Póvoa de Varzim; d – Aveiro; e Figueira da Foz; f – Peniche; g – Leixões). Asterisk (*) indicates signifcant diferences between treatments and control with $p \leq 0.05$.

Dice (Opt:0.73%) (Tol 1.0%-1.0%) (H>0.0% S>0.0%) [0.0%-100.0%]
rep

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P7
P29
P19
P22
P28
L8
St1
V28
F20
V4
St18
St17
St2
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St25
L23
L24
St9
S21
V32
A10
A4
A8
V24
L11
L2
F27
F8
A11
L5
79.9
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95.4
91.2
72.3
86
98
69.2
81
66.5
65.6
76.5
64.7
95.7
70.2
62
68.2
61.7
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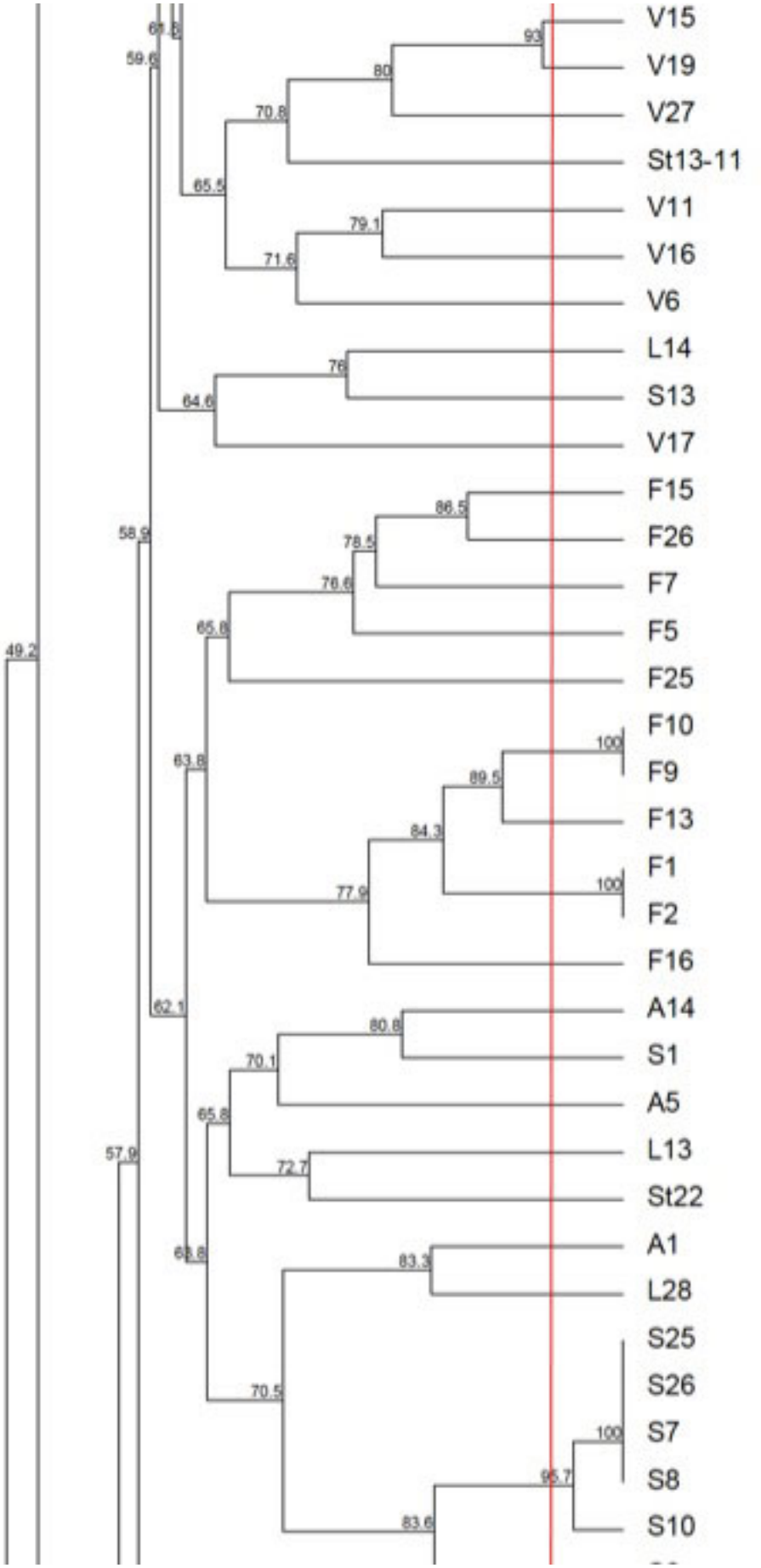
73.9


V15
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V11
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V17
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F7
F5
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F9
F13
F1
F2
F16
A14
S1
A5
L13
St22
A1
L28
S25
S26
S7
S8
S10
93
80
70.8
65.5
79.1
71.6
76
64.6
86.5
78.5
76.6
65.6
49.2
58.9
100
89.5
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83.3
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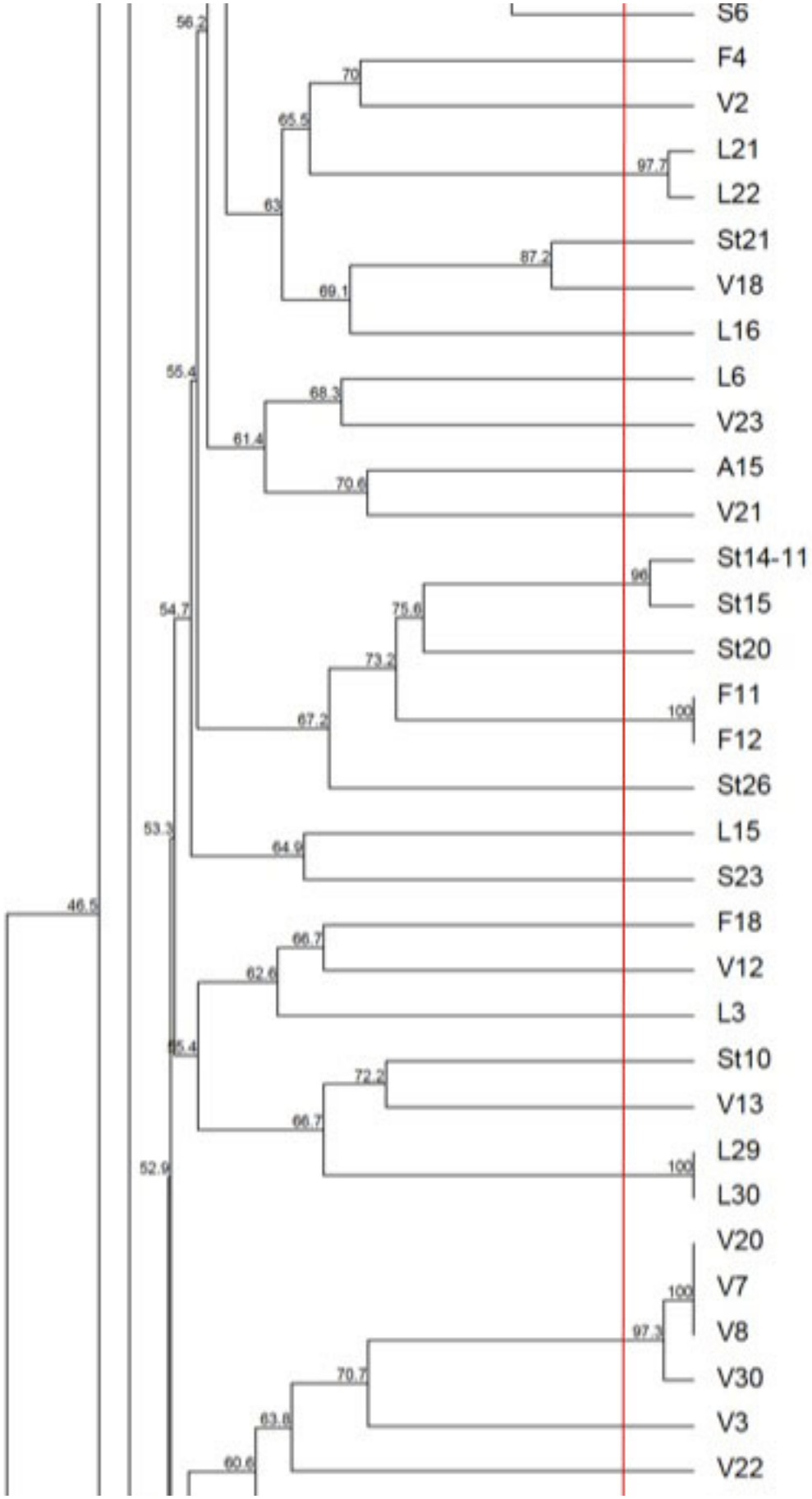
100
96.7
83.6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V23
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F12
St26
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L30
V20
V7
V8
V30
V3
V22

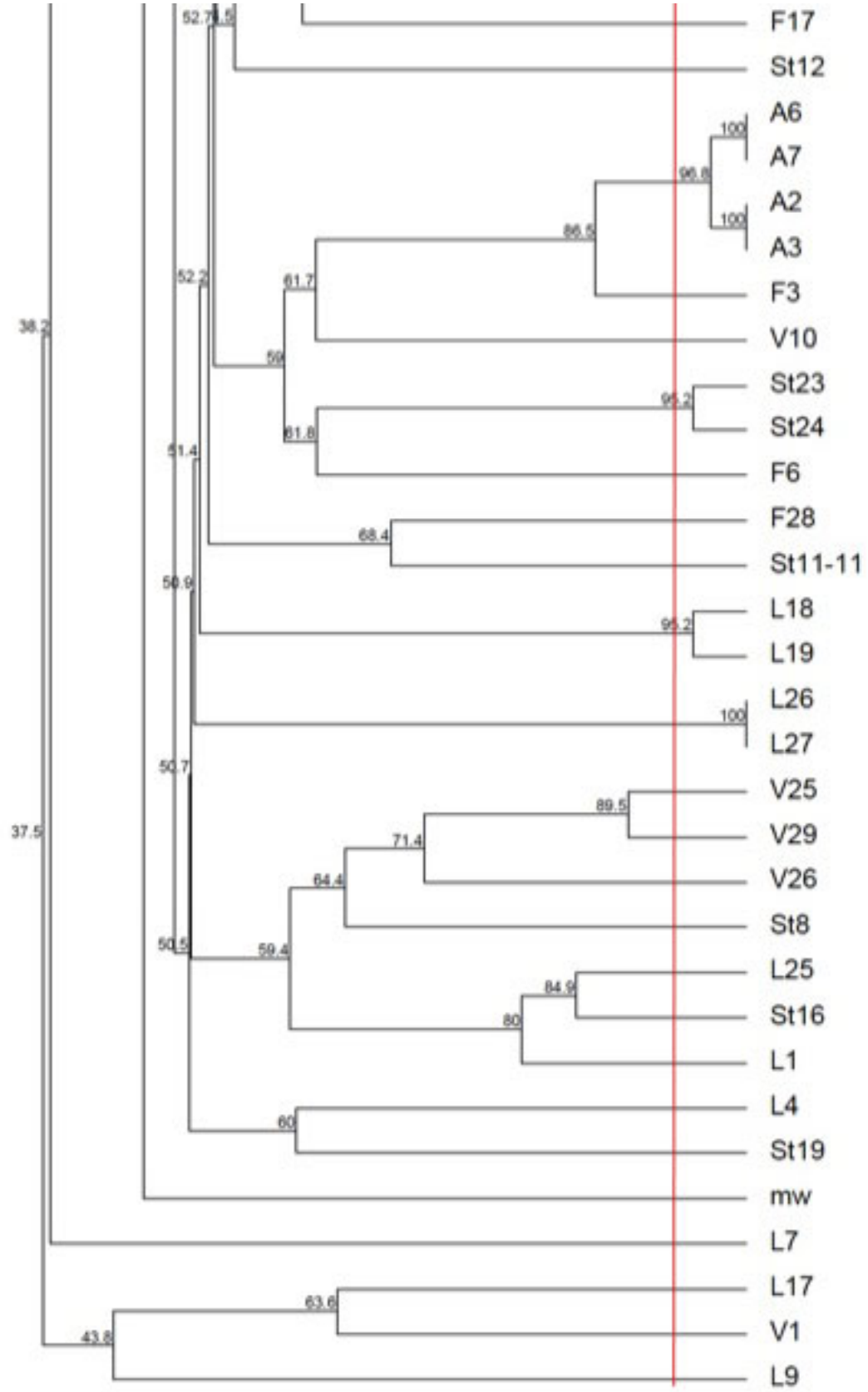

Figure 4.2 – Dendrogram showing genetic relatedness of isolated bacteria determined by analysis of REP-PCR fingerprint patterns using Dice similarity coefficient and UPGMA cluster methods. Based on control samples, isolates clustered together to the right of red line (94%) are regarded as being the same phylotype.

4.5 Bacterial growth quantification in increasing tributytin concentrations

As previously stated selected isolates were grown in 96 well plates in increasing concentrations of TBT (from 1mM to 6mM). A selection of 5 isolates for subsequent assays was made according to their sample location and higher growth in the presence of the highest concentrations of TBT. Bacteria selected for bioremediation assays were the ones previously labeled as S13, F3, L31, St11 and V11. The growth curves of these bacteria are represented in figure 4.3 and generation times are presented in table 4.2 (for the remaining growth curves please refer to the annex).

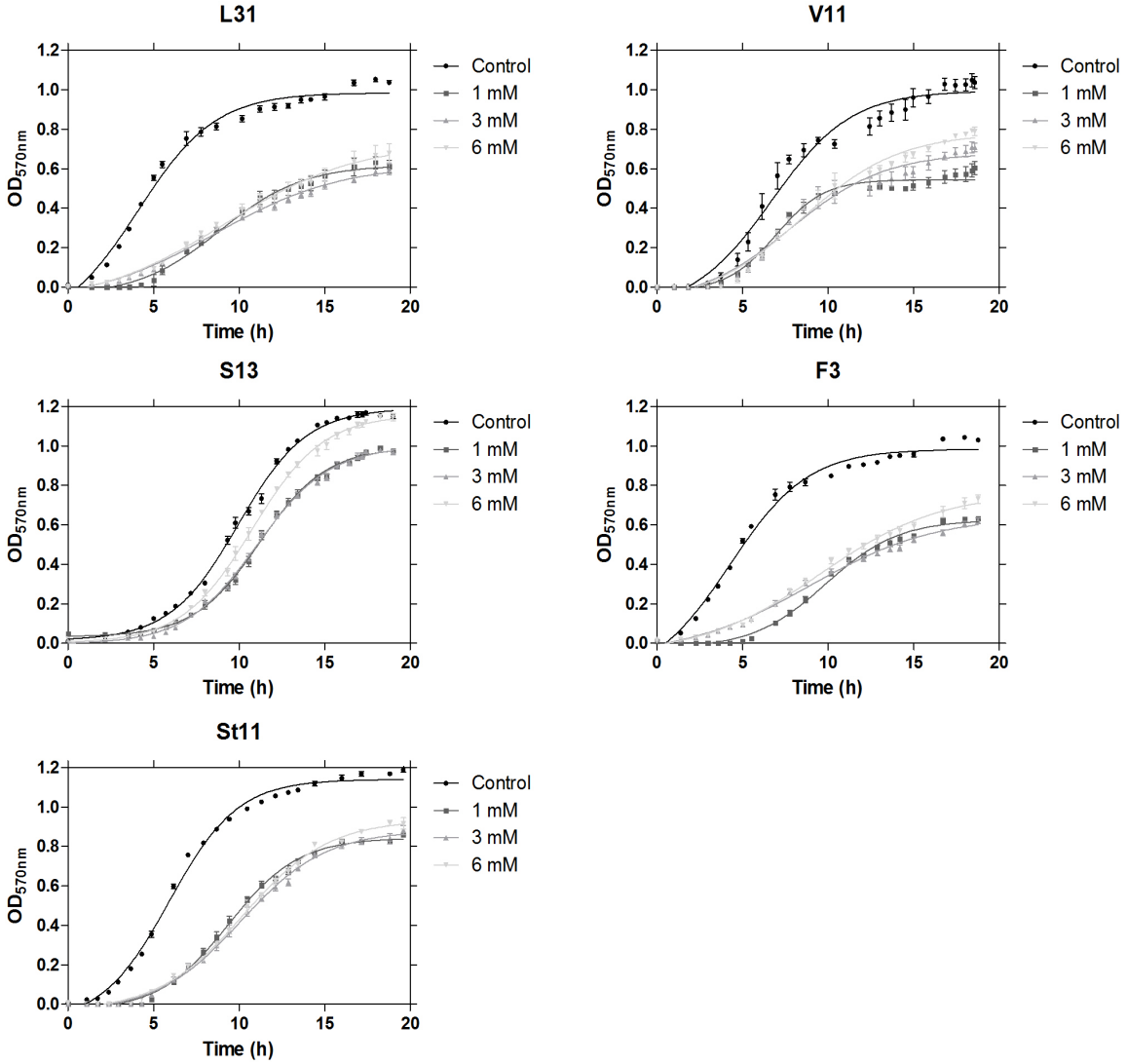

Figure 4.3 – Growth curves of bacteria selected for bioremediation assays (a- L31; b- S13; c – F3; d – St11; e- V11) in the presence of tributyltin. Graphics plotted as mean ± SEM.

Table 4.2
Generation time of selected isolates exposed to tributyltin and tributyltin-free media.

Isolate	Generation time (h)			
	Control	1 mM	3mM	6mM
L31	1.62	2.06	2.72	2.57
S13	3.13	3.15	2.81	3.10
F3	1.39	1.45	2.66	2.39
St11	2.40	2.15	2.68	3.57
V11	2.24	3.06	2.96	2.90

It is possible to observe that the growth of all isolates was affected by the presence of TBT, being S13 the less affected isolate. No specific pattern was detected between generation times of all isolates.

4.6 Tributyltin acute ecotoxicology assays

Median lethal concentration was calculated for *Gibbula umbilicalis* exposed to TBT contaminated contaminated seawater. The LC_{50} (confidence interval) values for 48 and 96h were 61.45 µg L^{-1} (39.85 µg L^{-1} - 124.92 µg L^{-1}) and 15.69 µg L^{-1} (9.59 µg L^{-1}- 25.56 µg L^{-1}), respectively.

4.7 Bioremediation and ecotoxicology assays

Median lethal concentration was calculated for *Gibbula umbilicalis* exposed to contaminated and potentially remediated water. The LC_{50} values as well the 95% confidence interval for each treatment are presented in table 4.3. The LC_{50} values are presented as a percentage of the initial contaminated solution (500 µg L^{-1}).

After 48 hours of exposure it was only possible to determine the LC_{50} for the control treatment (TBT aerated for 48 h) with a LC_{50} of 16.83% (12.26% - 40.07%). For all treatments with the bacteria the LC_{50} was above the highest concentration tested (20% of the initial solution). After 72 hours, the control treatment had the lowest LC50 value, 8.58% (6.70% – 10.88%), compared to all other treatments. For the treatment with S13 the LC_{50} was still above test concentrations. After 96 hours, once again the control treatment had the lowest LC_{50}, 5,07% (4.01% - 6.58%), while the treatments with L31 [11.19% (8.35% - 16.15%)], V11 [13.66% (9.80% - 25.02%)] and S13 [19.78% (15.13% - 145.97%)] had the highest values of LC_{50}.

Table 4.3
Median Lethal concentration values for all treatments after 48, 72 and 96 hours exposure in *Gibbula umbilicalis*

Hours after exposure	Treatment	LC50 (%)	95% Confidence Interval
48h	Control (TBT)	16.83	12.26 - 40.07
	S13 + TBT	>20	n.d.
	F3 + TBT	>20	n.d.
	L31 +TBT	>20	n.d.
	St11 + TBT	>20	n.d.
	V11 +TBT	>20	n.d.
72h	Control (TBT)	8.58	6.70 - 10.88
	S13 + TBT	>20	n.d.
	F3 + TBT	17.45	11.33 - 61.75
	L31 +TBT	12.84	9.49 - 20.32
	St11 + TBT	9.46	6.55 - 15.98
	V11 +TBT	17.38	12.14 - 50.57
96h	Control (TBT)	5.07	4.01 - 6.58
	S13 + TBT	19.78	15.13 - 145.97
	F3 + TBT	9.48	6.36 - 17.47
	L31 +TBT	11.19	8.35 - 16.15
	St11 + TBT	6.23	4.41 - 9.18
	V11 +TBT	13.66	9.80 - 25.02

As mentioned in section 3.10, glass bottles with seawater plus bacterial inoculum were kept in the same conditions as the other treatments. *Gibbula umbilicalis* individuals were exposed to increasing dilutions of these solutions. It was observed that these solutions at 100% concentration, for S13 and L31 inoculums, killed all tested individuals. However below 20% of the initial solution (corresponding to the highest dilution tested on bioremediation treatments) no mortalities were registered during the 96 hour period in any treatments (Table 4.4).

Table 4.4
Percentage of *Gibbula umbilicalis* mortality registered in treatments with bacterial inoculum and seawater after 48, 72 and 96 hours

Treatment	Concentration (%)	48h mortality (%)	72h mortality (%)	96h mortality (%)
S13 + Salt water	100	100	100	100
	20	0	0	0
	10	0	0	0
	5	0	0	0
L31 + Salt water	100	100	100	100
	20	0	0	0
	10	0	0	0
	5	0	0	0
F3 + Salt water	100	0	0	0
	20	0	0	0
	10	0	0	0
	5	0	0	0
St11 + Salt water	100	0	0	0
	20	0	0	0
	10	0	0	0
	5	0	0	0
V11 + Salt water	100	0	0	0
	20	0	0	0
	10	0	0	0
	5	0	0	0

5. Discussion and Conclusions

In order to isolate and evaluate TBT-resistant bacteria, Portuguese port and harbor water samples were exposed to high concentrations of TBT in TSA medium. Previous studies have reported these heavily impacted sites as sources of bacteria with tolerance and degrading capabilities (Cruz et al., 2007). In the same study for samples collected in the Ria de Aveiro (Portugal) 3 mM resistant bacteria were regarded as highly resistant. In this study we searched for 0.1; 1 and 3 mM resistant bacteria. According to the data shown in figure 4.1 it is possible to observe that the concentration of TBT in the medium has influence on bacteria growth. The number of cfu mL^{-1} in the media with and without the contaminant is significantly different, supporting that the media with TBT limit the organisms that are able to grow and are very selective when compared to the control. In all sampled locations, there was a decrease in the number of cfu mL^{-1} as the concentration increases, meaning also that the number of resistant bacteria is concentration dependent. For all sampled locations, at 0.1 mM the microorganisms' growth was already affected with a decline of at least 50% of cfu mL^{-1} when compared to the control, implying that this concentration is already considerably toxic (ANOVA, Dunnett's test, $P < 0.05$).

Seaport waters and sediments are due to be historically contaminated with several pollutants such as heavy metals, PCBs and PAHs (Rank, 2009; Lepland et al., 2010), making these environments more selective for bacteria since they have to develop mechanisms to adapt and be stable in such conditions (Ferrer et al., 2007). TBT resistance mechanisms are usually associated to the resistance of drugs, organic solvents and heavy metals (Suheiro et al., 2007), which might explain the presence of high numbers of TBT-resistants' in all locations and concentrations. In Peniche, it is possible to observe that the percentage of resistant bacteria did not vary much within TBT concentrations (0.1 – 3 mM). Peniche was the also the location where there was the highest percentage of resistant bacteria at the highest concentration tested. These results might be explained by the possibility of this location having the highest levels of contaminants and also because the initial pool of microorganisms was the highest from all samples tested (5000 ± 513 cfu mL^{-1}), increasing the chances to find more resistants'.

It may be expected that there is a connection between the occurrence of TBT-

resistant bacteria and the levels of TBT in water and sediment; however some studies demonstrated that this supposition is not correct. Suehiro et al. (2007) reported that the occurrence of TBT-resistant bacteria was not correlated with the occurrence of TBT in sediment from and near the Mekong River in Indochina. In the same line of work, Wuertz et al. (1991) reached a similar conclusion in estuarine samples from Boston Harbor, suggesting other factors and chemicals might determine whether populations become resistant to TBT. For example, Suzuki et al. (1992) demonstrated that there is a correlation between cadmium and TBT tolerance. Wuertz et al. (1991) observed that their TBT-resistant organisms were all resistant to copper and most of them were also resistant to other heavy metals like lead or zinc. Moreover, most TBT-resistant bacteria have multiple antibiotic resistances (Baya et al., 1986; Wuertz et al., 1991) Hence it is clear that resistance to metals and antibiotics are common among TBT-resistant organisms (Suehiro et al., 2007; Wuertz et al., 1991). However, further monitoring and experimental studies are required to elucidate the mechanism of TBT-resistant bacteria occurrence (Suehiro et al., 2007). In the present work the water samples were analyzed for their content in butyltins (TBT, DBT, and MBT) but quantifiable levels were not found (<50 ng L^{-1}). Despite this, we cannot exclude that although not quantifiable, due to this chemical extremely high toxicity, small concentrations might be available and thus conferring resistance to these microorganisms. Also, it cannot be excluded that these environments could have been previously contaminated with TBT and the natural occurring bacteria were able to restore these contaminated environments, and therefore justifying their presence in this areas and the non-quantifiable butyltins. Moreover, further work must be done in analytical chemistry to quantify other contaminants that, as stated above, might confer bacteria TBT tolerance (e.g., heavy metals).

In this study it was noticed that some of TBT-resistant isolates contained plasmids in high number (data not shown). Similar results were perceived by Baya et al. (1986) and by Wuertz et al. (1991), they found that many TBT-resistant isolates, besides having multiple antibiotic and heavy-metal resistances also contained plasmids. These plasmids might play an important role in TBT resistance and possibly in the transfer of TBT resistance between microorganisms (Cruz et al., 2007; Miller et al., 1995). However genetic studies on TBT-resistant and degrading bacterial strains are limited and the involvement of plasmids in resistance to organotins has not been properly addressed to

date. Nonetheless, Miller et al. (1995) reported that the plasmid pUM505, known to encode chromium resistance in *Pseudomonas aeruginosa*, also confers resistance to TBT. Understanding the involvement of plasmids in bacterial resistance to TBT will be important for further applications of TBT-degrading bacteria *in situ* and genetic engineering for *ex-situ* use.

Nevertheless, TBT resistance is not exclusively associated with plasmids, since not all TBT-resistant bacteria contain plasmids. Jude et al. (2004) described the mechanism of TBT resistance in *Pseudomonas stutzeri* 5MP1. This resistance is associated with the presence of an operon called tbtABM (Jude et al., 2004), which is linked to multidrug resistance such as the resistance-nodulation-cell division (RND) efflux pumps system (Suehiro et al., 2007). These pumps are the most important multidrug resistance efflux system in gram-negative bacteria and they export antibiotics, biocides, dyes, detergents, organic solvents and heavy metals (Suehiro et al., 2007). This mechanism might explain the resistance to TBT of several bacteria, especially gram-negative bacteria. However there is no evidence that this mechanism might be associated with TBT degradation or removal from surrounding environment. Fukagawa and Suzuki (1993) also characterized a chromosomal gene involved in TBT resistance in a strain of *Alteromonas sp*. that is devoid of any plasmids.

To characterize 3 mM resistant bacteria, rep-PCR method was used in this study for molecular typing of 157 isolates. This technique is based on the amplification of DNA fragments lying between interspersed repeated sequences and DNA primers corresponding to one of these sequences (REP elements) were used. Although this technique has been widely applied in diversity and taxonomic analysis of various bacteria species, relatively little work has been done to determine the efficacy of using rep-PCR to identify diversity of environmental isolates (Xie et al., 2008).

Relationships between the PCR-generated patterns were examined through cluster analysis (Figure 4.2). Among the 157 isolates examined, 111 different banding patterns were identified. Peniche port, despite having the highest rate of tolerants had the lowest diversity, with only 5 distinct banding patterns identified out of 30 isolates. Additionally, the similarity between these isolates was above 79%, suggesting that these isolates are genetically close to each other. This lower diversity might be explained with adverse

conditions resulting from contamination by marine traffic. It is known that in stressed environments, the diversity is usually lower due to selectivity of surrounding environment (Haller et al., 2011). However this is just a presumption since there is no available data of contamination in Peniche's port and the results of chemical analysis on heavy metals of collected samples are not yet available. Moreover, there are not isolates from different sampling sites clustering together. This suggests that all samples locations have distinct microbial communities. As mentioned before, Peniche's port had the highest percentage of resistant bacteria at higher concentrations of TBT, however the genomic fingerprinting analysis revealed that the diversity of this sample was indeed very low. These results suggest that the TBT-resistant bacteria are very common in this location, which might be associated with a previously contamination of TBT as usual nearby ports and harbors. However the initial diversity of the sample could also be low, which could explain the high percentage of resistants in this location. This analysis of diversity and abundance of microbial community on the sample is currently being evaluated by Denaturing Gradient Gel Electrophoresis (DGGE).

As mentioned above, this genomic fingerprining method is based on the use of primers corresponding to naturally occurring repetitive elements in bacteria. Besides REP, some examples of other repetitive sequences are BOX, ERIC and $(GTG)_5$ (Masco et al., 2003) The corresponding protocols are referred to as BOX-PCR, ERIC-PCR and $(GTG)_5$-PCR, respectively, and together with REP-PCR this protocols are collectively termed rep-PCR (Braem et al., 2011; de Bruijn et al., 1996. Because these sequences are located in distinct positions, these banding patterns generated form each protocol is different (de Bruijn et al., 1996). So the combined analysis of patterns generated by each protocol, increases discrimination between isolates (Vinuesa et al., 1998), meaning that it is possible that isolates that clustered together in this experiment might generate different patterns in other protocols and correspond to different bacteria. However, this technique allowed identifying at least 111 different profiles form a high selective medium, suggesting that there is a lot of diversity among TBT-resistant bacteria, increasing the possibility that some of these isolates are not yet described by the literature as TBT-resistants'. Nonetheless, these results support that REP-PCR is a powerful molecular technique for characterization of TBT-resistant bacterial strains. This work also demonstrates that rep-PCR technique might be applied to environmental isolates, as Adiguzel et al. (2009) and Xie et al. (2008)

have previously demonstrated.

Ecotoxicological testing proved to be a great tool to evaluate acute toxicity of TBT on test specie but also valuable to assess the effectiveness of the bioremediation assays. Acute ecotoxicological testing revealed that the LC_{50} (CI) values of TBT on *Gibbula umbilicalis* for 48 and 96 hours were 61.45 µg L^{-1} (39.85 µg L^{-1} - 124.92 µg L^{-1}) and 15.69 µg L^{-1} (9.59 µg L^{-1} - 25.56 µg L^{-1}), respectively. These results broaden the knowledge of the effects of TBT on gastropods, especially on prosobranch gastropods. These results suggest that *Gibbula umbilicalis*, however, is not as susceptible to TBT as most marine gastropods, or marine organisms in general. For example, acute toxicity tests of TBT exposure on *Thais clavigera*, *Haliotis discus discus* and *Haliotis madaka* indicated that the 48 hour LC_{50} values was of 5.6 µg L^{-1}, 5.4 µg L^{-1}, 3.9 µg L^{-1}, respectively (Horiguchi et al., 1998). Nehring, (2000) compiled information on acute toxicity and chronic effects of TBT in gastropods and compared those data to TBT levels found in the environment (Figure 5.1). It is clear that environment concentrations of TBT were high enough to cause adverse effects on some organisms.

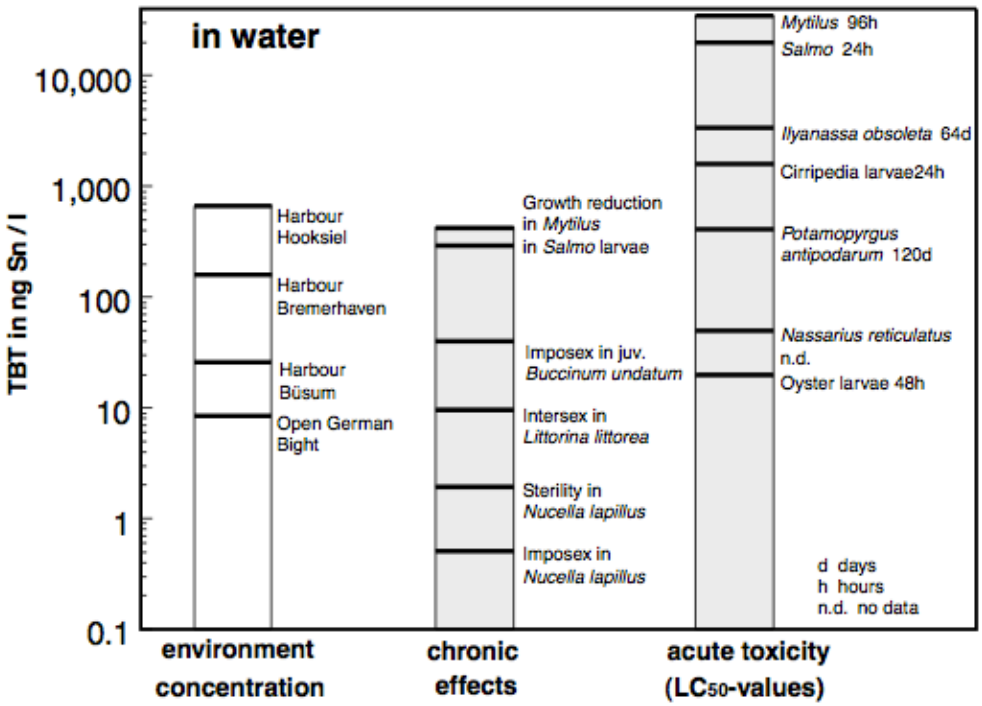

Figure 5.1 - Chronic effects and acute toxicity of tributyltin, and tributyltin concentrations in the water column on the German North Sea coast (original figure in Nehring, 2000).

Nevertheless, TBT affects other groups of marine organisms from crustaceans to fish. For example, the LC_{50} for the crustacean *Palaemon serratus* is 17.52 μg L^{-1} (48 h) (Bellas et al., 2005) and the 48 hour LC_{50} values for five gammarids ranged from 17.8 to 23.1 μg L^{-1} (Ohji et al., 2002), while on test fish Tilapia, a 96 hour LC50 of 3,800 μg L^{-1} was reported (Hongxia et al., 1998).

Most recent studies focus however on the chronic effects of TBT on gastropods, particularly imposex. Abidli et al. (2012) reported that the exposure of *Hexaplex trunculus* and *Bolinus brandaris* to 50 ng L^{-1} TBT for 2 months induces imposex. Similar results were obtained by Santos et al. (2005) on *Nucella lapillus* after 3 months of exposure to 50 ng L^{-1} TBT. Gooding et al. (2003) results revealed an increase of imposex incidence to 27% in *Ilyanassa obsoleta* at a concentration as low 1 ng L^{-1} TBT, an environmentally relevant concentration. Other laboratory studies indicated other effects of TBT exposure like shell abnormalities in oysters at concentrations as low as 2 ng L^{-1} (Chagot et al., 1990).

In this study, the concentration of butyltins in all water samples was below the quantification limit (< 50 ng L^{-1}). However there are some studies reporting the levels of organotins in Portuguese waters and sediments. During April 1999 to May 2000, Díez et al. (2005) collected samples from 46 stations along Portuguese coastal and continental waters (including samples near ports and harbors). The values of MBT, DBT and TBT vary between varied between >3.3 and 26 ng L^{-1}, >3.0 and 30 ng L^{-1} and >3.1 to 29 ng L^{-1}, respectively. In the same study, sediment samples from 14 stations revealed a total of butyltins ranging from 12 to 151 μg kg^{-1} dry weight, whilst TBT levels ranged between 3.8 and 12.4 μg kg^{-1} dry weight. Bettencourt et al. (1999) reported that values of TBT on samples collected Tagus estuary, Portugal, ranged from 1.13 to 21.13 ng L^{-1}. Also in Tagus estuary, Nogueira et al. (2003) reported that concentrations of TBT in sediment samples ranged from 5 to 35 μg kg^{-1} dry weight. These values are below the levels reported near ports and harbors in other regions of Europe. For example, in Gijon, Spain, Rodriguez-Gonzalez et al. (2006) detected concentrations of TBT in water samples collected near the marina up to 196.6 ng L^{-1}. In Saronikos, Greece, TBT levels in water were detected up to 70 ng L^{-1} (Thomaidis et al., 2007). Some of these reported concentrations, as seen above, are high enough to cause adverse effects on several organisms.

In this study, sediment samples were not collected. However, it is expected that the values of TBT in sediments are higher than in water, since butyltins have a strong affinity to sediments, where they persist (Antizar-Ladislao, 2008; Xiao et al., 2011). It is also expected that butyltin levels in coastal sediments to be high, due to boat traffic and shipyard activities (Díez et al., 2005). Plus, marinas end ports were already identified as hotspots of TBT contamination in the surface water and sediments (Dafforn et al., 2011). For example, in Gipuzkoa, Spain, TBT sediment concentrations were as high as 5480 μg kg^{-1} in superficial sediments (Arambarri et al., 2003). Field data suggests that TBT sediment concentrations between 300 and 1000 μg kg^{-1} have adverse effects on the bivalve *Scrobicularia plana* (Langston and Burt, 1991). Also, Meador and Rice (2001) observed a reduction in growth of the polychaete *Armandia brevis* at 100 μg kg^{-} TBT. Nevertheless, resuspension of sedimented TBT is possible through storms or dredging leading to an increase of organotin levels in the water column (Díez et al., 2005).

Regardless of the restrictions on TBT use, and consecutive bans, it is clear that the adverse effects to its exposure are still prominent, since it is a very persistent compound in the environment. Imposex, intersex and other adverse consequences are still being reported due to a chronic exposure to this toxicant. Chronic effects of TBT on *Gibbula umbilicalis* are yet to be studied. It might be important to assess the chronic effects on this organism, as it belongs to the most affected group by imposex. It is also important to study the mechanism of imposex development in prosobranch gastropods, as it is still not fully understood (Lima et al., 2011). Not less important is to develop tools to remove or reduce TBT levels from contaminated environments.

To select isolates for bioremediation assays and reduce more the number of potential candidates as a bioremediation tool (genomic fingerprinting only allowed to reduce 27% of isolates), selected microorganisms were exposed to a high concentration of TBT (6 mM) in TSA medium for 48 hours. A total of 66 isolates grew in these conditions and their growth curve was measured until stationary phase. In figure 4.3 is presented the growth curve of 5 of those bacteria – considered to be the most suitable candidates due to their higher growth in TBT medium. It is observed that the presence of TBT inhibits the growth of those isolates. It is also observed that there is not a dependence on the concentration between 1 mM, 3 mM and 6 mM of TBT, meaning that the growth of these isolates is similarly affected by all tested concentrations. To our knowledge there are no

reports of bacteria growing in the presence of 6 mM TBT. Cruz et al. (2007) reported that bacteria transferred to gradually higher TBT concentrations *in vitro*, results in an increased resistance to TBT, suggesting the existence of a "memory response" mechanism to TBT exposure. This mechanism could explain why some isolates grew in such high concentrations of TBT, however we couldn't confirm that hypothesis as we have no information if they were naturally resistant to 6 mM TBT because the highest concentration tested on the initial screening was 3 mM.

The five TBT-resistant previously selected isolates candidates were used in the bioremediation assays for 48h, and after their potential as bioremediation tools was evaluated through ecotoxicological tests. Different bioremediation periods were previously tested and results showed that this period was enough to find degradation (results not shown). After the potential remediation, the sea snails we exposed to the media, and except for the isolate "St11" all other treatments with the bacteria caused an increase in the LC_{50} after 72 and 96 hours when compared to the control, meaning a decrease of toxicity. The most promising bacterium was "S13", causing a great increase in the LC_{50} when compared to the control (>20% for S13 and 8.58% for control at 72 hours; 19.78% for S13 and 5.07% for control at 96 hours). An increase in LC_{50} values was also observed in "L31", "V11" and "F3". This suggests that there was bioremediation and the medium became less toxic. Although the ecotoxicological approach having its importance and showing the relevant effect on targeted species, this should be complemented with chemical analysis in order to assess and understand this occurrence. Chemical analysis will provide information if TBT has been degraded into DTB, MBT or inorganic tin, which are less toxic. It is important to take into account that these results were obtained after only after 48 hours of exposure. It is possible that a longer exposure to TBT will enhance the degradation of TBT, as shown by Sakultantimetha et al. (2011) where degradation of TBT by sediment microbial communities occurred in a seven-day period. Therefore, further testing should be performed with an increase in time of bioremediation tests to assess the increased biotechnological potential of longer remediation periods. It may be also interesting to change or optimize conditions for TBT removal. As demonstrated by Sakultantimetha et al. (2010), increasing temperature accelerates TBT degradation. Nevertheless, it is important to take into account that *in situ* degradation takes place at ambient temperature

where degradation is dependent on seasonal and geographical variations (Sakultantimetha et al., 2011).

It might be argued that in this experiment there was an abiotic degradation of TBT, but Voulvoulis and Lester (2006) demonstrated that anaerobic digestion of TBT is minimal, contrarily to aerobic degradation (Stasinakis, et al., 2005). Plus, TBT is not volatile (Sakultantimetha et al., 2011), as shown by Maguire et al. (1983) TBT dissolved in water does not volatize after 2 months in the dark at 20ºC. The treatments were maintained in the dark during bioremediation assays, so direct photolysis in surface water is not expected to have occurred. It might also be argued that TBT was adsorbed to the glass, however Maguire et al. (1983) experiments ruled out the possibility of TBT adsorbing to the glass. So any degradation of TBT from this experiment is expected to be carried out by bacteria.

As seen in table 4.4, the by-products resulting of the growth of two isolates (L31 and S13) are toxic to *Gibbula umbilicalis*. Nonetheless, at the highest dilution tested for bioremediation assays no mortalities were recorded. Interestingly, those two isolates had a promising result in bioremediation testing. It would be important in forthcoming studies to analyze and identify these by-products, if and how they interact with TBT and their influence on TBT degradation, taking in mind that this by-products may limit *in situ* application of these isolates. Some marine bacteria are known to cause mortality in gastropods, like *Vibrio*, for example *V. alginolyticus*, *V. harveyi* and *V. parahaemolyticus*, which have been isolated from diseased abalones (*Haliotis*) and are responsible by mass mortalities on that genus (Romalde and Barja, 2010). Moreover, Fukagawa et al. (1992) isolated a TBT-resistant *Vibrio* strain.

Ecotoxicological testing, with the ecologically relevant specie *Gibbula umbilicalis*, proved to be a strong tool to evaluate toxic effects of TBT and of potentially bioremediated waters. Nevertheless, chemical analysis of the potential remediated waters are still needed to assess TBT levels and possible degradation products. As mentioned above, some mechanisms of TBT degradation by bacteria have been proposed. TBT might be degraded through a dealkylation mechanism, might be metabolized as a carbon source or be accumulated into the cell of bacteria. Hence, it is important to also assess TBT by-products ecotoxicity and develop tools to remove them.

As previously discussed, the occurrence of TBT-resistant bacteria is usually not correlated with the occurrence of TBT. We here suggest that the occurrence of resistant bacteria in a TBT non-contaminated environment, might sustain that this environment was previously contaminated and these bacteria were able to restore it. But also it is possible that these contaminants have never been present in relevant concentrations, and the presence of these resistant bacteria is due to other contaminant resistance mechanisms. However, further studies are still needed to evaluate this. As shown here, naturally occurring marine bacteria from Portuguese ports are able to tolerate high concentrations of TBT and even degrade it. Plus, chemical analysis to near-sediment waters revealed that the levels of TBT in Portuguese ports with intense activity were below 50 ng L^{-1}, a much lower level than verified in other regions of the globe.

Although TBT levels have been decreasing in the last years, mainly due to restrictions in its use, it is still present at levels of concern. Unfortunately, present and future restrictions will not immediately remove TBT and its degradation products from the environment, since these compounds are retained in the sediments where they persist and can also be continuously ressuspended due to hydrodynamics.

Several marine invertebrates are extremely sensitive to TBT, and imposex can be observed in some prosobranch gastropods at concentrations usually found in the water column near ports and harbors. Mostly due to this effect, TBT has been considered as the most toxic substance ever introduced into the marine environment. Additionally, it is important to be aware that the effects of TBT contamination also have the potential to extend to higher organisms through consumption, making TBT a topic for human health concern.

This work presented a possible tool to accelerate TBT removal from contaminated waters: bioremediation using marine bacteria from Portuguese ports. It was demonstrated that some of these bacteria are able to tolerate extremely high concentrations of TBT and may be potential bioremediation tools of contaminated waters. Since resistance to heavy metals and antibiotics are common among TBT-resistant organisms, studying TBT-resistant might not only be vital for the restoration of TBT-contaminated environments, but to heavily contaminated environments in general.

Ecotoxicological testing using *Gibbula umbilicalis* provided information on acute toxicity of TBT and was also an efficient tool to assess the efficiency of bioremediation scenarios.

6. Future prospects

The use of natural microbial populations for bioremediation of TBT contaminated sites is far away from a large-scale application since little work has been done to explore the exact mechanism or the genes involved in the process.

In this work, a total of 111 different genetic profiles were detected among all 3 mM TBT-resistant bacteria. To our knowledge, there are not many identified TBT-resistant bacteria, especially to that high concentration. Therefore it would be important to identify the isolated bacteria in this study to extend the knowledge on TBT-resistant bacteria. Moreover, albeit the increasing studies on TBT resistance by bacteria, there is still limited work to understand the mechanisms behind that resistance. It has been proposed that TBT resistance may be plasmid mediated or coded in chromosomal DNA. Therefore identifying and cloning genes involved in degradation and in TBT resistance will be crucial for further studies.

Only 5 out of 111 different isolates were tested in bioremediation assays in this study, meaning that there are still a big number of TBT-resistant isolates from Portuguese ports to be tested as potential bioremediation tools for TBT. Ecotoxicological testing revealed to be useful to understand the effects of pollutants' toxicity on *Gibbula umbilicalis*, however chemical analysis will be essential on future studies to measure TBT and its degradation products. It would be also interesting in future studies to assess the growth of marine bacteria in the presence of environmental relevant concentrations of TBT, to evaluate how TBT affects the microbial community of a contaminated area and also to assess on possible synergisms between TBT degrading bacteria on the restoration of a contaminated environment.

Microorganisms adapted to extreme environments, are potential producers of enzymes with great practical importance for industrial applications (Ferrer et al, 2007). A simultaneous study with the same isolates aimed to detect enzymatic activity in TBT-resistant microorganisms has been performed (Lemos et al., 2011b). Results show these isolates have the capacity of producing enzymes with a large biotechnological potential, exhibiting extracellular activities such as proteolytic, lipolytic, and amylolytic activities.

Thus, further studies with these isolates should be explored in order to fully assess their biotechnological potential.

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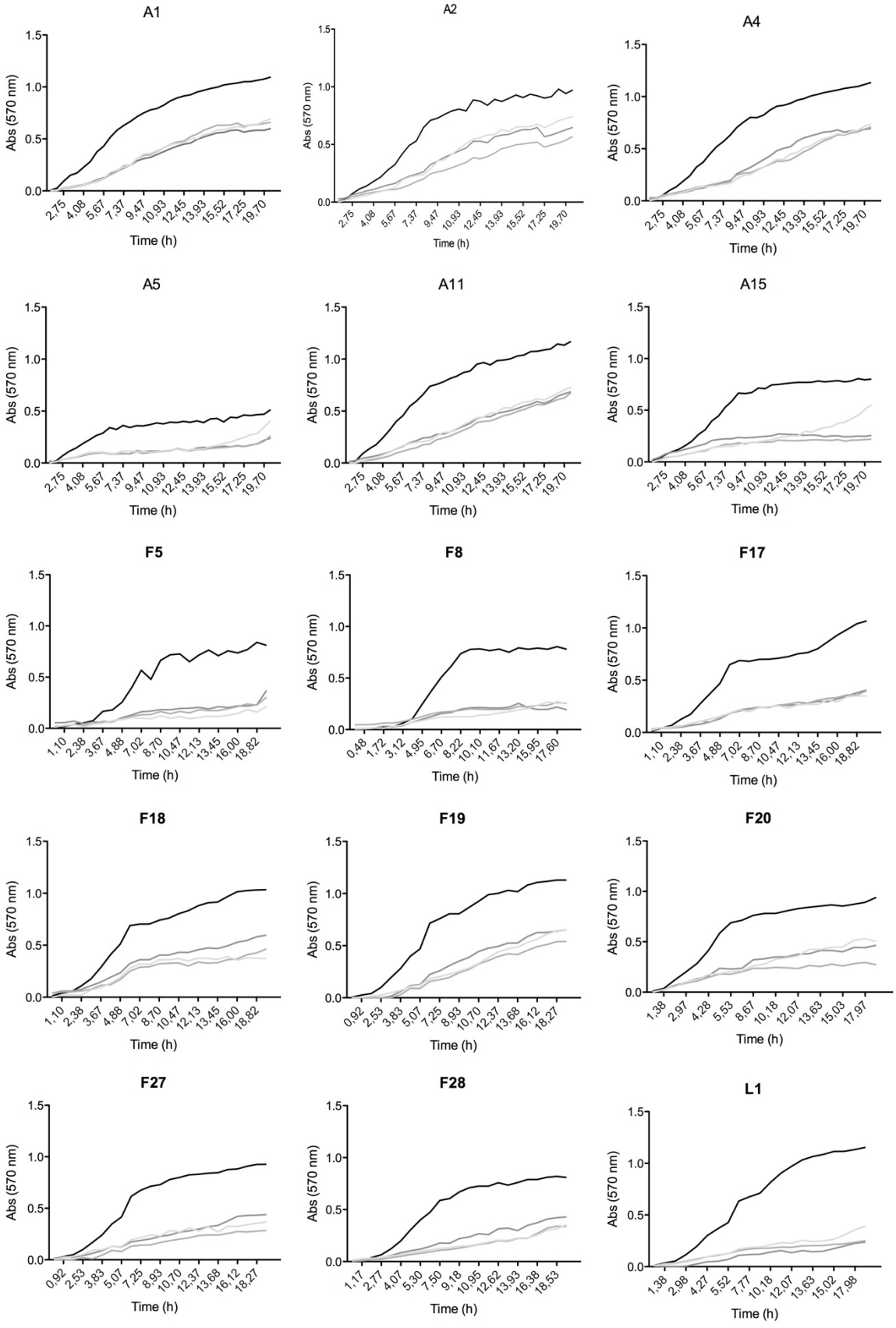
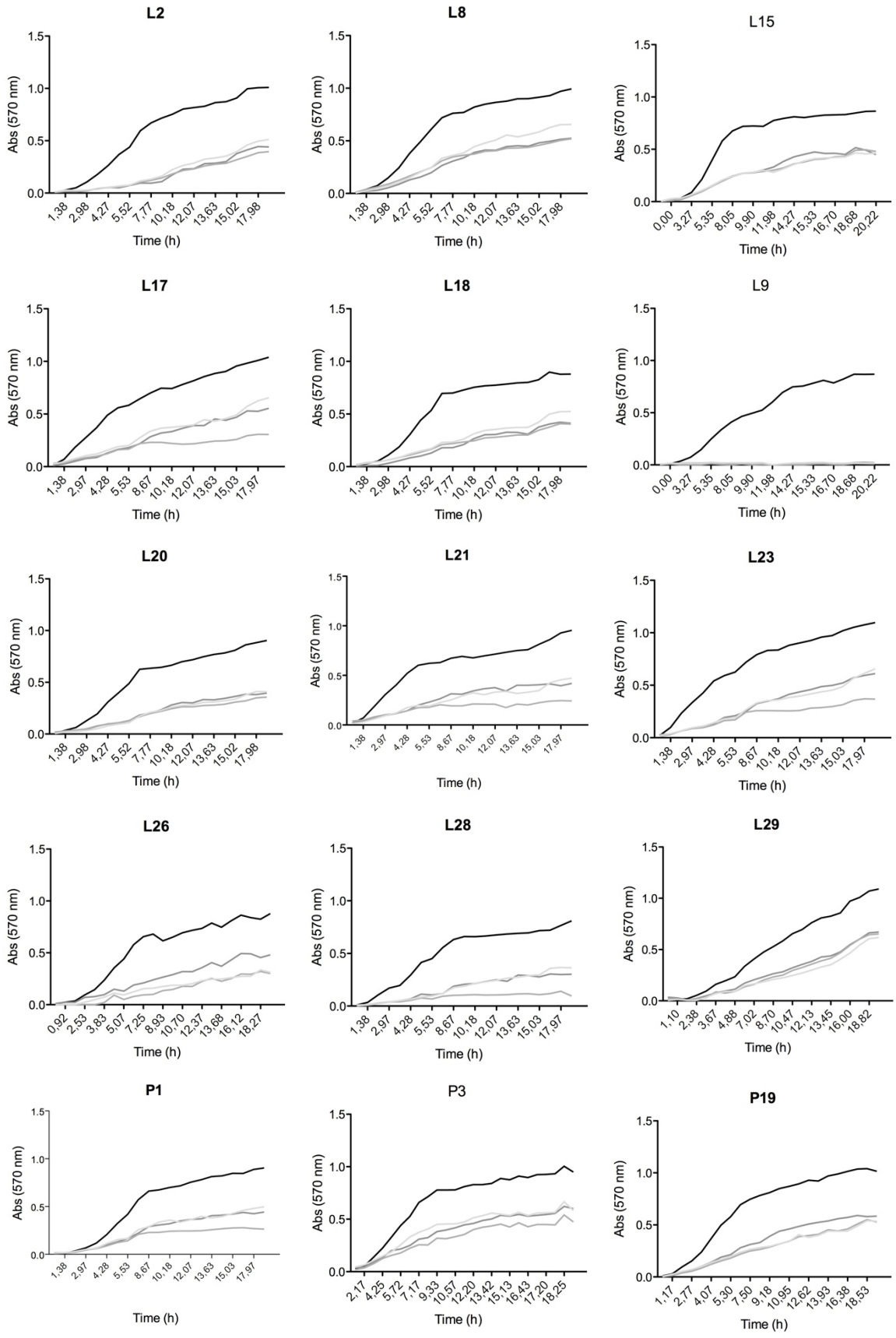
8. Annexes

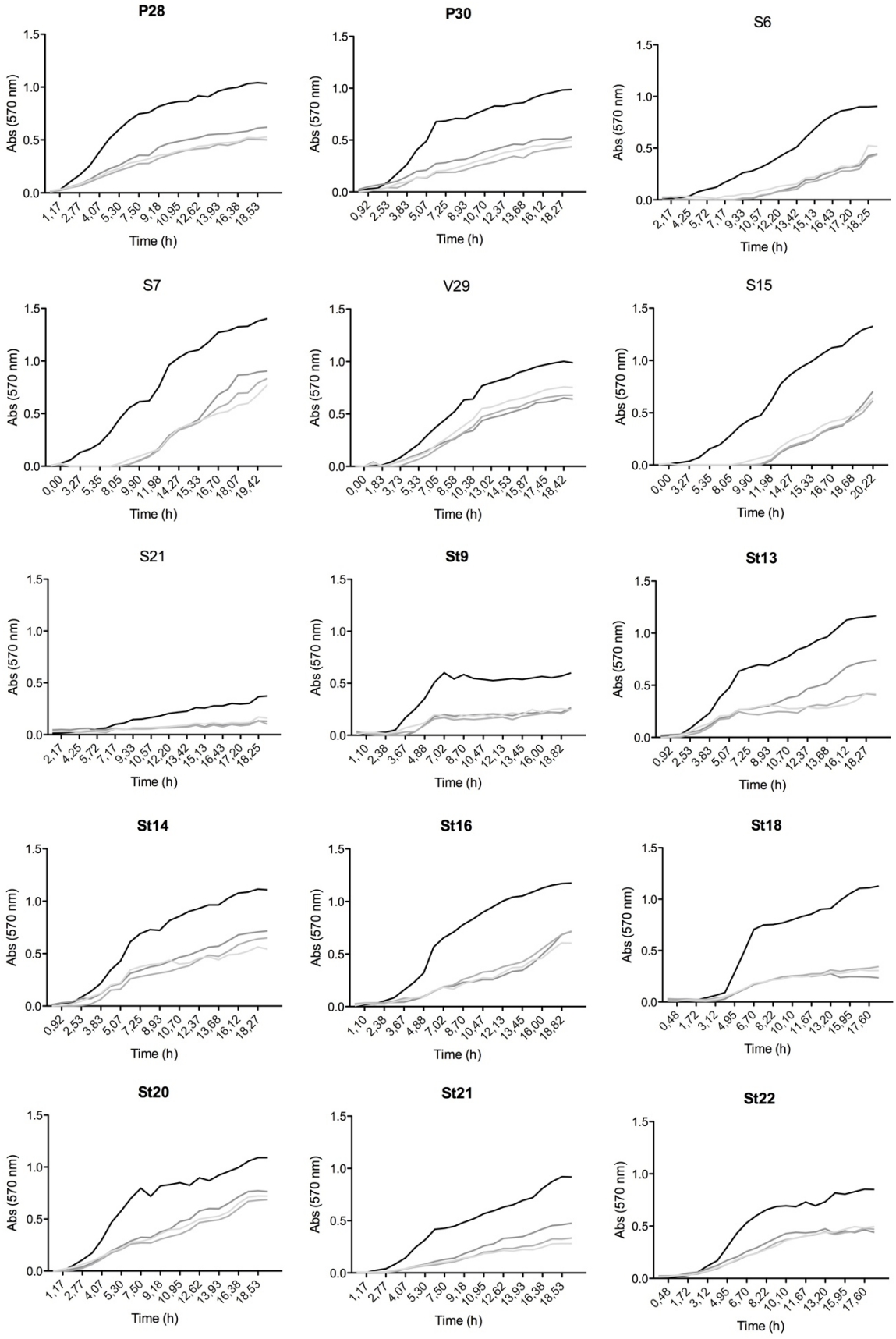
Table 8.1
Generation time of isolates with and without TBT

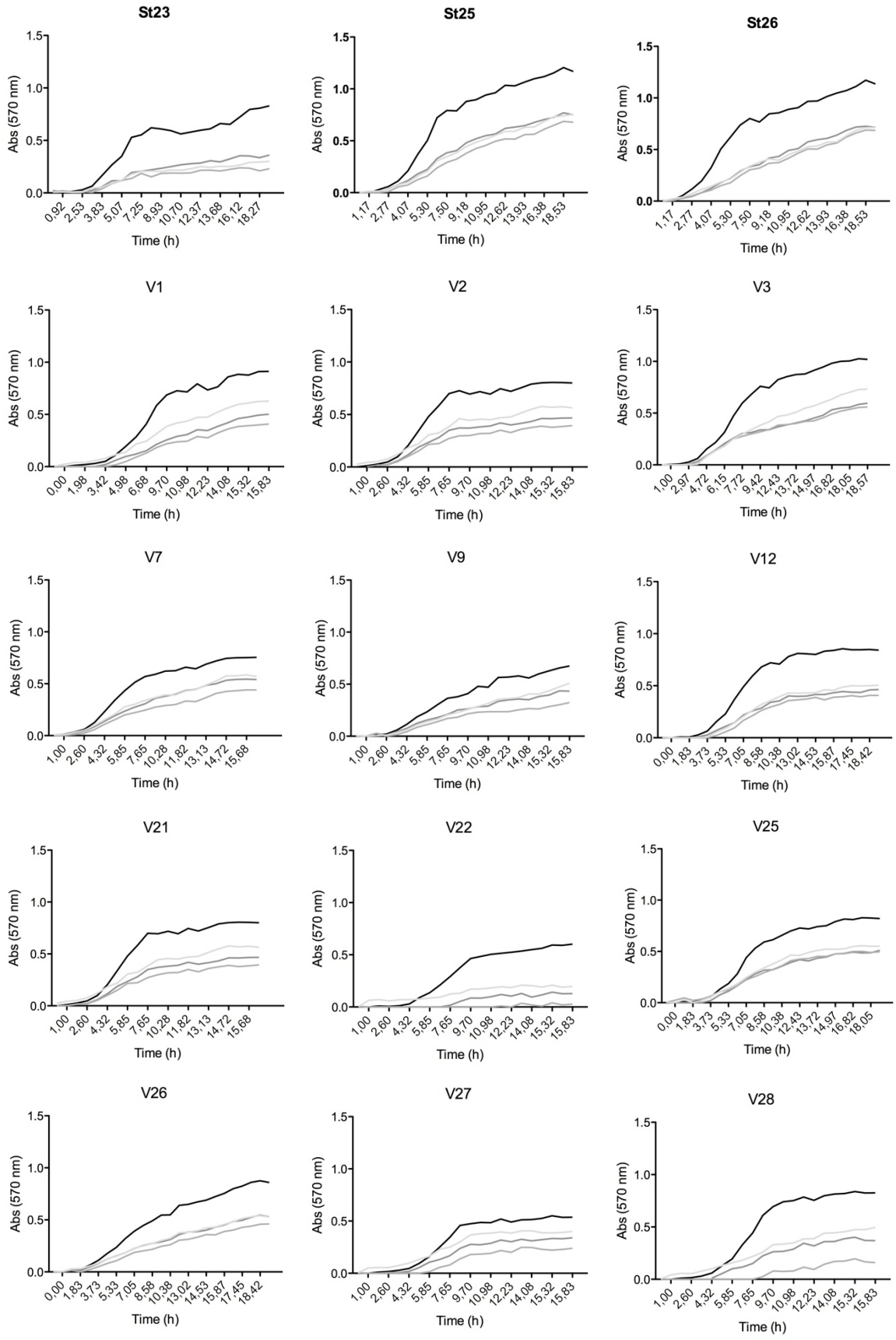
Isolate	Generation time (h)			
	Control	1 mM	3mM	6mM
A1	3.42	2.92	3.23	3.44
A11	2.68	n.d.	n.d.	n.d.
A15	2.49	n.d.	n.d.	n.d.
A2	2.03	2.32	2.17	1.94
A4	2.26	n.d.	n.d.	n.d.
A5	1.35	n.d.	n.d.	n.d.
F17	1.47	1.73	1.98	2.01
F18	1.34	1.46	1.39	1.26
F19	1.29	2.31	2.21	2.65
F20	2.5	n.d.	1.76	n.d.
F27	1.38	n.d.	n.d.	n.d.
F28	1.8	n.d.	n.d.	n.d.
F5	1.91	n.d.	n.d.	n.d.
F8	1.87	n.d.	n.d.	n.d.
L1	3.15	n.d.	n.d.	n.d.
L15	1.51	2.72	2.6	3.14
L17	n.d.	n.d.	n.d.	n.d.
L18	1.19	n.d.	n.d.	n.d.
L2	1.4	n.d.	n.d.	n.d.
L20	1.32	n.d.	n.d.	n.d.
L21	1.1	n.d.	n.d.	n.d.
L23	n.d.	n.d.	n.d.	n.d.
L26	1.43	n.d.	n.d.	n.d.
L28	1.65	n.d.	n.d.	n.d.
L29	3.82	3.25	3.26	2.33
L8	1.35	1.22	1.63	2.81
L9	n.d.	n.d.	n.d.	n.d.
P1	3.69	5.12	3.66	4.56
P19	1.53	n.d.	n.d.	n.d.
P28	1.8	2.17	2.19	2.23
P3	3.53	3.93	3.44	3.32
P30	1.06	n.d.	n.d.	n.d.
S15	n.d.	n.d.	n.d.	n.d.
S21	3.53	6.67	5.02	4.56
S6	2.76	2.21	2.31	2.56
S7	n.d.	n.d.	n.d.	n.d.
St13	3.67	3.4	1.1	1.67
St14	3.72	3.78	3.58	3.64

St16	2.76	4.1	4.31	4.73
St18	0.98	1.92	2.02	2.13
St20	1.58	2.91	2.86	2.91
St21	5.35	2.43	2.58	2.76
St22	2.27	2.68	2.43	2.49
St23	1.46	1.05	0.96	1.04
St25	1.93	3.89	3.54	3.85
St26	1.64	2.57	2.55	3.08
St9	3.66	0.81	0.87	1.57
V1	1.6	1.43	1.24	2.33
V12	2.34	2.65	2.29	2.74
V2	1.41	1.41	1.42	2.03
V22	2.01	n.d.	n.d.	n.d.
V25	1.61	2.76	3.31	3.16
V26	3.81	4.86	4.12	4.97
V27	1.69	1.4	1.07	2.66
V28	1.7	1.88	1.84	3.67
V29	3.74	3.34	2.85	3.62
V3	2.27	n.d.	n.d.	n.d.
V31	1.68	2.68	1.17	2.13
V7	1.43	2.15	1.82	2.26


A1
A2
A4
A5
A11
A15
F5
F8
F17
F18
F19
F20
F27
F28
L1
Abs (570 nm)
Time (h)


L2
L8
L15
L17
L18
L9
L20
L21
L23
L26
L28
L29
P1
P3
P19
Abs (570 nm)
Time (h)


P28
P30
S6
S7
V29
S15
S21
St9
St13
St14
St16
St18
St20
St21
St22
Abs (570 nm)
Time (h)


St23
St25
St26
V1
V2
V3
V7
V9
V12
V21
V22
V25
V26
V27
V28
Abs (570 nm)
Time (h)

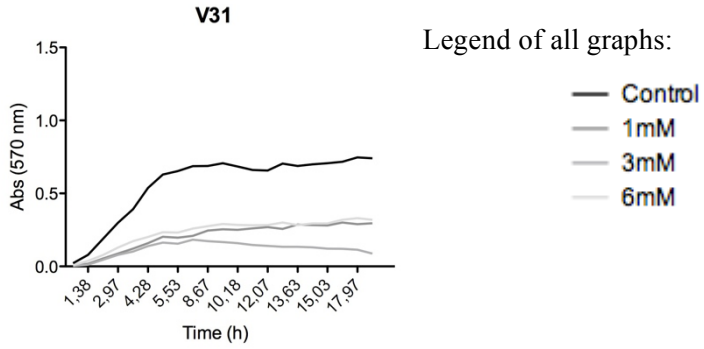


Figure 8.1 – Growth curves of total isolates (not selected for further use).