Universidade de Aveiro Departamento de Biologia

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TOXICITY AND BIOREMEDIATION EVALUATION OF TBT: BIOASSAYS WITH PLANTS

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Dissertação apresentada à Universidade de Aveiro para cumprimentos dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada – Ramo de Toxicologia e Ecotoxicologia, realizada sob a orientação científica da Professora Doutora Susana Loureiro, Investigadora auxiliar, do Departamento de Biologia e CESAM da Universidade de Aveiro, e co-orientação da Professora Doutora Sónia Mendo, professora auxiliar do Departamento de Biologia da Universidade de Aveiro.

O júri

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palavras-chave

Tributilestanho, *Brassica rapa*, *Triticum aestivum*, *Aeromonas molluscorum* Av27, biorremediação

resumo

A poluição ambiental é um problema que afecta todo o ecossistema. Os compostos orgânicos de estanho, e em particular o tributilestanho (TBT), estão amplamente dispersos, por terem uma variedade de aplicações, sendo utilizados como estabilizadores de cloreto de polivinila (PVC), em canalizações de água, como catalisadores, conservantes da madeira e em vários productos de consumo doméstico, como líquidos de limpeza, esponjas e papel. Devido à acção biocida do TBT, este composto era principalmente utilizado em tintas antivegetativas. A sua crescente libertação para o ambiente contribui para a sua presença em quase todos os compartimentos, desde água, sedimentos, solos e até organismos.

O coeficiente de partição octanol/água atinge um valor de 3.85, e por isso, o TBT tende a acumular-se na matéria orgânica e nos organismos. Devido à sua persistência e potencial de bioacumulação os compostos orgânicos de estanho tri-substituidos, como o TBT, são considerados extremamente tóxicos para organismos não-alvo, causando disrupção endócrina, incluindo impossexo em fêmeas de gastrópodes.

O tempo de meia-vida do TBT nos sedimentos chega a atingir vários anos. Sendo o compartimento sedimentar um reservatório destes compostos. Actividades como a dragagem de sedimentos contaminados com TBT e posterior deposição nos solos, juntamente com a deposição atmosférica e pulverização directa de pesticidas, constituem fontes de contaminação destes compostos no solo. Além disso, a propagação de esgotos contaminados e águas de irrigação contaminadas podem levar a uma contaminação dos solos e das águas subterrâneas.

A biorremediação pode acelerar a biodegradação natural dos contaminantes, sendo importante na remoção do TBT. A bactéria *Aeromonas molluscorum* Av27 tem a capacidade de degradar o TBT nos seus produtos de degradação, dibutilestanho e monobutilestanho, em meio de cultura, *Marine Broth*. Sendo, deste modo, uma potencial ferramenta para diminuir a contaminação do TBT no ambiente.

Este estudo tem como objectivo investigar o efeito de um solo contaminado com tributilestanho em duas espécies de plantas, o nabo *Brassica rapa* e o trigo comum *Triticum aestivum.* Foram realizados bioensaios com plantas, onde se avaliou a emergência das sementes e parâmetros de crescimento (comprimento da parte aérea da planta e produção de biomassa). Na segunda parte do estudo, a bactéria *Aeromonas molluscorum* Av 27 foi adicionada ao solo previamente contaminado para avaliar a sua capacidade para biorremediar TBT. Esta avalição foi realizada com o mesmo bioensaio usando uma espécie teste de ciclo rápido, *Brassica rapa.*

As plantas, quando expostas ao TBT, mostraram um atraso e diminuição na germinação das sementes juntamente com uma dimuição nos parâmetros de

crescimento, apresentando um comprimento menor, bem como uma produção de biomassa baixa quando comparadas com as réplicas do controlo (sem TBT). No teste com a bactéria *A. molluscorum* Av27 e no teste com meio de cultura, no final do ensaio, observou-se um menor número total das plantas germinadas quando comparadas com o teste controlo, no entanto, as plantas eram maiores e consequente possuiam uma maior produção de biomassa (peso fresco). Em conclusão, são necessários mais estudos para avaliar a capacidade da *Aeromonas molluscorum* Av27 remediar solos contaminados com TBT, uma vez que a toxicidade do TBT no solo não diminuiu num dos parâmetros avaliados (germinação das sementes).

keywords

Tributyltin, *Brassica rapa*, *Triticum aestivum*, *Aeromonas molluscorum* Av27*,* bioremediation

abstract

Environmental pollution is a real problem that affects the entire ecosystem. Organotin compounds, particularly tributyltin (TBT), are widespread contaminants that are applied as polyvinyl chloride (PVC) stabilizers, water pipes, catalysts, wood preservatives and in several consumer products such as washing liquids, sponges and paper. TBT was mainly used in antifouling paints, because of its biocide action. Its increasing discharge into the environment leads to its inclusion in almost all compartments, such as water, sediments, soils and organisms.

The octanol/water partition coefficient of TBT reaches 3.85, thus it tends to accumulate in organic matter or organisms. Due to is persistence and bioaccumulation potential trisubstituted organotins, like TBT, are considered to be extremely toxic, even to non-target species, causing endocrine disruption, including imposex in female gastropods.

TBT half-life in sediments is estimated to reach several years. The sedimentary compartment acts as a sink for these compounds. Some activities where dredging of sediments contaminated with TBT will follow a disposal in soil, jointly with atmospheric deposition and direct pulverization of pesticide products are sources of tributyltin contamination in soils. Moreover, spreading of contaminated sewage sludge and irrigation with contaminated water may lead to soils and groundwater contamination.

Bioremediation processes can accelerate the natural biodegradation of contaminants, playing an important role in TBT removal. *Aeromonas molluscorum* Av27 has the ability to degrade TBT in its less toxic byproducts, dibutyltin and monobutyltin, in the liquid culture medium, Marine Broth. Therefore it can be used as a powerful tool to diminish contamination of TBT in the environment.

The present study aims to investigate the effect of tributyltin contaminated soil in two plants species, *Brassica rapa* and *Triticum aestivum.* Plant bioassays were carried out by measuring seed germination and growth parameters (shoot length and biomass production). In the second part of the study, *Aeromonas molluscorum* Av 27 was added to the previously contaminated soil to evaluate its capability to bioremediate TBT. This evaluation was carried out with the same plant bioassays using as test-species the rapid cycle turnip *Brassica rapa.*

When exposed to tributyltin, plant species showed a delay and diminish in seed germination and a decrease on growth parameters, with smaller plants and lower biomass production when compared to the control replicates. In the Av27 test and test with culture medium a lower value of total number of germinated seeds at the end of the assay was observed when compared to the replicates of the control test, but plants were bigger and consequently had a higher biomass production (fresh weight). In conclusion, further studies are needed to clearly

evaluate the ability to remediate TBT in soils by *Aeromonas molluscorum* Av27 because the toxicity of TBT in soil did not decrease in one of the parameters measured (seed germination).

Index

List of figures and tables

List of figures

List of tables

Chapter 1

General introduction

1. General Introduction

1.1. Soil contamination

Soil is a multi-functional medium with extremely environmental importance. It is formed by a porous matrix in which water, air and biota interact, being the basis of some of human food. Thus, soil has important ecological and socio-economical functions (Agency EE 2000).

Soil contamination is one of the major problems that interfere with the environmental quality. Emission of various chemical compounds to air, water and land, originated from several industries, atmospheric deposition, agriculture practices or municipal wastewater, contribute to soil contamination (Agency EE 2000). One chemical of concern is tributyltin (TBT); this compound may lead to soil contamination through several origins like direct pulverization of pesticide products (Marcic, Le Hecho *et al.* 2006), and possible disposal of contaminated sediments on soils. Harbor areas are hot spots of TBT contaminated sediments, because TBT was exceedingly used as a biocide in antifouling paints (White, Tobin *et al.* 1999; Rudel 2003).

Contaminated soils might lead to groundwater contamination and biomagnifications of these compounds along the food chain, and occasionally might have an effect on human health (Loureiro, Soares *et al.* 2005).

1.2. Tributyltin (TBT)

1.2.1. Properties and uses

Since 1980s tributyltin have been considered an environmental problem. Tributyltin was used as biocide in antifouling paints to prevent the attachment of fouling organisms (Carter, Turoczy *et al.* 1989). Organotin compounds (OTCs) are widely used for wood preservation as fungicide, polyvinyl chloride (PVC) stabilizers, as biocides in textiles, washing liquids, sponge and also in the paper industry as slimicide (Lespes, Marcic *et al.*

2009). In addition the use of mono- and di- substituted OTCs in a variety of consumer products is increasing public concern, because it has been found in food packaging, and water pipes (Fent 1996).

Fungicide, disinfectant, algicide and microbiocide are different types of pesticides that have TBT. They are used, as referred above, as wood preservatives, in water cooling waters, and also in materials preservatives in textiles, carpet backing, foam, fiberfill, rope and building materials (in joint compound or grout) (EPA 2008). Fungitrol is a trade name for a biocide that has TBT in its composition (Ash and Ash 2004).

TBT, an hydrophobic substance, has an octanol/water partition coefficient (log K_{ow}) varying with pH and salinity conditions. This coefficient represents the distribution of a compound between water and octanol, where octanol symbolizes organisms or organic matter. High pH values will lead to an increase in log K_{ow} value. Values published in literature indicate that the partition coefficient ranges from 3.21 to 3.85 for TBTCl at pH values of 5.8 and 7.8, respectively (Fent 1996). Regarding to salinity, some authors reported a decrease (Unger, Macintyre *et al.* 1988) while others reported an increase in TBT adsorption with elevated salinity (Harris and Cleary 1987). Some authors verified that at low to intermediate salinities the partitioning coefficients, including octanol/water coefficient, had lower values. This might be explained by the increasing stabilization of charged species with high chlorinity, as with metal cations, and consequently partition coefficients decreased (Langston and Pope 1995). Bioavailability of the compound to the organisms depends on octanol/water partition coefficient, the higher the log K_{ow} value, more it bioaccumulates in organisms, thence being more toxic. At pH around 6 TBT is predominant in cation form - $TBT^{+,}$ whereas at higher pH, around 8, it predominates as a neutral form - TBTOH or TBTCl. At pH 8 it is expected an increase on bioconcentration, bioavailability, and consequently toxicity of TBT than at pH 6. This may be explained by the fact that TBTCl can penetrate biological membranes more easily than the cation TBT^{+}. Chemical speciation and partitioning are affected depending on the interaction of TBT with the humic acids. Increasing concentrations of humic acids leads to a decrease in bioavailability of TBT (Fent and Looser 1995).

Dibutyltin (DBT) and monobutyltin (MBT) are degradation byproducts of tributyltin and they are present in the coastal environment, in water, sediments and living organisms. Due to the characteristics of these compounds, they are adsorbed onto sediments, being marine sediments a reservoir of these compounds. In harbor areas, contamination is high, reaching average concentrations that generally range between 1 and 2 mg kg^{-1} dry weight. Consequently, mobility of organotin compounds seems reduced (Alzieu 1998). This is explained by the octanol/water partition coefficient, as referred before, and when this value is high, means that tributyltin tends to accumulate more in sediments (Fent 1996) remaining there. Usually organotin compounds tend to associate with the clay fraction of particulate matter (Hoch 2001). Even after the ban of TBT based paints, there are still occurring in surface sediments significant levels of butyltin compounds and the degradation process of these compounds in sediments is much slower than in overlying waters (Sarradin, Astruc *et al.* 1991).

Toxicity of organotin compounds depends mostly on the number of organic groups bounded to the tin atom. According to that number, organotins are distributed in mono-, di- and tri- substituted compounds, being the triorganotins the most toxic to organisms (Hoch 2001).

These compounds enter into the environment through several routes, affecting the aquatic and terrestrial compartment. The main route, which enters directly to the aquatic system, is from the compounds released from antifouling paints (remarkable in harbors). (Rudel 2003). Both compartments are affected, directly or indirectly, by TBT through the following sources: urban sources, including house paints (Cornelissen, Pettersen *et al.* 2008); spills of timber treatment facility (De Mora and Phillips 1997); the self-polishing copolymers and contaminated effluents from industrial plants, municipal wastewater and spreading of sewage sludge (Rudel 2003).

In January 2003 the International Maritime Organization banned the use of TBT-based paints and in 2008 it was established its effective prohibition in the European Union (Cornelis, Bierkens *et al.* 2006). These initiatives were based on many studies where TBT was found to accumulate in sediments and induce disruption of the endocrine

system, malformations and the impairment of the immune system of marine shellfish (Fent 1996; Alzieu 1998; Morcillo and Porte 2000).

Organotin compounds degradation depends on abiotic and biotic factors. In aquatic and terrestrial ecosystems, abiotic factors such as UV and chemical cleavage are the most significant. In soils, freshwater and estuarine environments the most important factors of TBT degradation are biotic processes. Microorganisms play an important role in the degradation of this compound (Dubey and Roy 2003). Biodegradation is influenced by environmental conditions, as temperature, pH, presence of dissolved organic matter, oxygenation, mineral elements and the nature and adaptation capacity of the microflora. Abiotic and biotic degradation of TBT corresponds to a breakdown of this compound by progressive oxidative debutylization - splitting of the carbon-tin bond (WHO 1990). Higher temperature and aerobic conditions are ideal to increase biological degradation of TBT (Brandsch, Nowak *et al.* 2001).

TBT has been classified as a persistent organic pollutant (Bangkedphol, Keenan *et al.* 2009) and it depends on biotic and abiotic degradation processes and processes of adsorption/desorption (Rudel 2003). Bioaccumulation potential in organisms is high due to its lipophilicity nature (WHO 1990).

Bioavailability and consequently toxicity of tributyltin in soils depends on the soil properties, such as pH, organic carbon content or cation exchange capacity (CEC). High CEC in soil, generally, represents a lower toxicity of the compound (Rudel 2003; Römbke, Jänsch *et al.* 2007). Tributyltin toxicity depends on time of exposure to the compound and its concentration, bioavailability and the sensitivity of the species (Rudel 2003). Therefore, bioassays should be used to complement chemical analyses, because chemical analyses do not demonstrate the toxicological effects in biota as they not provide information on chemical bioavailability to living organisms (Fent 2003; Loureiro 2004).

It is described that TBT degradation in sediments is slower than in freshwater (Gadd 2000), and in soils its half-life is in a range of several weeks to years (Huang and Matzner 2004), therefore being considered highly persistent. This information is supported by a chemical fate model developed in 2008 (Yamamoto, Yonezawa *et al.* 2009).

Due to high affinity of TBT to suspended particles, harbors' sediments can play an important role as sinks of these compounds, becoming highly contaminated areas. Natural disturbance or dredging of these contaminated sediments and possible disposal in soil (White, Tobin *et al.* 1999) jointly with pulverization of pesticide products (Marcic, Le Hecho *et al.* 2006) are point sources of TBT pollution. Organotin compounds used in agriculture, for example, direct application of pesticides and irrigation with contaminated water are other sources of soil contamination (Fent 1996). Diffuse sources of OTCs pollution, for instance, atmosphere deposition of contaminated precipitation and fog may also lead to soil contamination, consequently affecting fauna and flora. Atmosphere depositions into forest ecosystems, jointly with the high half-life time of tributyltin that reaches several years, due to slow degradation, may lead to OTCs concentrations up to 20-100 µg(Sn)Kg^{-1} in soils (Huang, Schwesig *et al.* 2004). In sediment/soil corers in flood-plains in a Germany river, triorganotins were detected at levels between 23 to 764 ng OTC g^{-1} dry weight (Götz, Bauer *et al.* 2007). TBT concentration in soils in industrial areas can reach 180 mg kg^{-1} dry matter (Beuselinck and Valle 2005) and in sewage sludge may reach 18 mg kg^{-1} dry weight (Voulvoulis and Lester 2006). Thus, it is of extremely importance to study effects on the terrestrial environment. This widespread contamination leads to significant OTCs concentrations in several compartments (Table 1.1).

		OTCs concentration	**Reference**
Water	River water	3 - 30 ng Sn L^{-1}	(Díez, Lacorte *et al.* 2005)
	Estuarine water	0.5-31 ng Sn L^{-1}	(Gomez-Ariza, Giraldez *et al.* 2001)
	Sea water	<5-164 ng TBT L^{-1}	(Choi, Choi *et al.* 2008)
Sediment	Marine sediment	4 - 12 µg Sn kg^{-1}	(Díez, Lacorte *et al.* 2005)
	Estuarine sediment	1-520 µg Sn kg^{-1}	(Díez, Lacorte *et al.* 2005)
	Sewage sludge	18 mg TBT kg^{-1}	(Voulvoulis and Lester 2006)
Soil	Industrial areas	180 mg TBT kg^{-1}	(Beuselinck and Valle 2005)
	Forested soils	20-100 µg Sn kg^{-1}	(Huang, Schwesig *et al.* 2004)

Table 1.1. OTCs concentration in water, sediment and soil.

1.2.2. Biological effects

It is known that TBT releases easily from materials where it has been incorporated, resulting in the contamination of the environment (Morcillo and Porte 2000). The wide range of TBT effects begin at the prokaryotic level, in bacteria. A study performed with two bacteria, *Bacillus stearothermophilus* and *Bacillus subtilis*, have shown that TBT produces alterations in growth, lipid membranes and respiratory activity with an oxygen consumption decrease with the increasing TBT concentrations (Martins, Jurado *et al.* 2005).

Marine microalgae, *Nannochloropsis oculata,* have shown high sensitivity to TBT and growth characteristics and photosynthetic pigment content were shown to be affected (Sidharthan, Young *et al.* 2002). The freshwater algae, *Scenedesmus quadricauda*, exposed to tributyltin oxide (TBTO) had a decrease in growth accompanied with a small, loss of color (pigment) and disintegrated cells (Fargasová and Kizkink 1996).

Daphnia magna exposed to concentrations above 2.5 µg TBT L^{-1} showed mortality and an enhancement in testosterone metabolism, supporting the fact that TBT acts as an endocrine disruptor (Oberdorster, Rittschof *et al*. 1998). When compared to fish, daphnia seems to have a lower bioconcentration of these compounds, this may be explained by the species physiology, where fish has more lipids than daphnia, and as explained above organotin compounds have high affinity with lipids (Fent and Looser 1995).

Some species of molluscs are notably sensitive to TBT as, for example, oysters, where it is noticed shell malformation upon exposure. A major concern is that TBT is considered an endocrinal disrupter in some species, for instance to clams (Morcillo and Porte 2000). TBT induces masculinization - imposex - by increasing testosterone levels in female gastropods (Fent 1996), which will lead to population changes.

Some harbor sediments are polluted with organotin compounds, and in some cases they will end up disposed in soils. To assess if contaminated sediments might be a risk to the soil fauna, and thus a potential risk to human health, a study with earthworms was performed. The results showed that in TBT contaminated sediment there was 94%

mortality when compare to the control soil. Beyond TBT, salinity might affect also the results, increasing mortality (Schaefer 2005).

Even birds are affected by organotin pollution. These xenobiotics can enter through the food chain by ingestion of lower trophic level organisms that are contaminated. In birds tributyltin accumulates in liver, kidneys, muscles and feathers (Kannan, Senthilkumar *et al.* 1998). In addition and due to similarities in fish consumption, although at different levels, birds can be indicative species for humans exposure though the ingestion of fish.

In mammalian there are effects on several groups, for example seals, dolphins, and mice. In seals, butyltin compounds tend to accumulate in liver with concentrations from 2 to 99 ng g^{-1} wet weight (Kajiwara, Kannan *et al.* 2001). Dolphins organs are also affected by butyltin compounds, like kidney, muscle, liver, lung, bladder and heart, being liver the organ with the highest bioaccumulation of tributyltin (Harino, Ohji *et al.* 2008). Male mice exposed to low levels of TBT from puberty and during 45 days were affected by this compound. Increases in plasma insulin levels, body weight and hepatic steatosis (abnormal retention of lipids) were also observed (Zuo, Chen *et al.* 2011). Other effects are atrophy in thymus and spleen, suppression of humoral and cellular immune responses and immunotoxicity which is associated with thymocyte apoptosis (Chen, Zhang *et al.* 2011).

There are different sources of human exposure to organotin compounds, such as food consumption, ingestion of contaminated soil, inhalation or dermal absorption, but the main source is via food (EFSA 2004).

Several studies reported that fish consumption is the main route of organotin compounds to humans. In a population of Finland, the major contributors to the OTCs intake were domestic perch, imported salmon and rainbow trout. But it is important to refer that the total average daily intake in this population was below the Tolerable Daily Intake (Airaksinen, Rantakokko *et al.* 2010). This value was established by European Food Safety Authority (EFSA) and it is 0.025 mg kg^{-1} body weight per day for the sum of dibutyltin, tributyltin, triphenyltin and dioctyltin (EFSA 2004).

In Portugal, butyltin compounds were detected in fish, crustaceans, cephalopods and bivalves. The highest levels of these compounds were found in bivalves, including mussels, clams and cockles, but the majority of the samples showed levels below the tolerable daily intake, thus an average consumer is not exposed to significantly OTCs levels (Santos, Enes *et al.* 2009).

Samples of outdoor settled dust from several sites of the island of Malta, where shipyards and maintenance activities in dry docks occurs, had organotin compounds including TBT, DBT and MBT (Decelis and Vella 2007). Even at home we might be exposed to organotin compounds. In 2004 a study revealed that house dust contains considerable levels of these compounds, including TBT and its degradation byproducts. Thus, babies and young children who crawl on the floor may be exposed (Fromme, Mattulat *et al.* 2005).

TBT exposure will result in irritation of the upper respiratory tract, skin and eyes. If the skin is exposed to high tributyltin concentration, skin burns may occur (WHO 1990). Studies in human blood and liver, revealed the presence of organotin compounds, but generally human risk assessment is based on extrapolations of studies on animal experiments. Numerous experiments suggested effects such as immunosuppressive, neurotoxic action and dermal, cardiovascular, pulmonary, respiratory, gastrointestinal, kidney and liver problems. Other harms are blood dyscrasias, reproductive, teratogenic and a probable carcinogenic activity. The hazard of these compounds to humans depends on the solubilization and the possibility that they may degrade during ingestion. TBT blocks the absorption of oxygen in the mitochondria, disturbing its function (Antizar-Ladislao 2008).

1.3. Bioremediation

The importance of studies about bioremediation is undeniable. Bioremediation accelerates the natural biodegradation of contaminants and usually tends to transform the contaminants into less harmful products (Margesin and Schinner 2001).

Biodegradation plays an important role in TBT removal. This is helped with aeration of the soil, which provides oxygen to the system. Consequently, degradation under aerobic conditions is faster than anaerobic. TBT can be degraded in water four times more than in sediment, this is related to bioavailability of the compound (Brandsch, Nowak *et al.* 2001).

One possible way to remove some organotin pollutants from the environment is by immobilization in microalgal beads; beads with algae species, like *Chlorella miniata* and *Chlorella vulgaris*, which are pollutant-resistant, can remove several compounds by biosorption and biodegradation, including organometallic compounds, such as TBT. TBT enters the cell of these unicellular algae and is degraded into dibutyltin, monobutyltin and inorganic tin (Tam, Wong *et al.* 2009). In addition, fungi, like *Cunninghamella elegans,* showed the ability to biotransform tributyltin into less toxic compounds (Bernat and Dlugonski 2007).

Other studies revealed that nutrient addition and inoculation with *Enterobacter cloacae* in TBT contaminated sediments increase degradation efficiency and consequently, result in half-life reduction of this compound. Aeration of sediments and adjustments of temperature to 28^{o}C resulted in a better degradation process (Sakultantimetha, Keenan *et al.* 2010; Sakultantimetha, Keenan *et al.* 2011).

Some bacteria, such as *Aeromonas molluscorum* Av27, showed the ability to remove TBT in laboratory, in liquid culture medium - Marine Broth Agar. A study performed with the same bacterium isolated from a TBT contaminated site in Ria de Aveiro, Portugal, revealed a high resistance to TBT and a memory response when bacteria were exposed to increasingly TBT concentrations. *A. molluscorum* Av27 uses TBT as a carbon source and degrades TBT in its major breakdown products, DBT and MBT. Hence, being a potentially bioreporter system to monitor TBT contaminated areas, and might also be used as a remediator of TBT polluted areas (Cruz, Caetano *et al.* 2007; Cruz, Henriques *et al.* 2010).

After remediation of organotin contaminated sediments, an assessment framework for re-used sediments was performed, and a soil remediation value for residential land-

use for TBT of 0.51 mg/kg dry matter was obtained. This value was calculated taking into account exposure via vegetables (Cornelis, Bierkens *et al.* 2006).

However, even with several methodologies showing that TBT can be remediated, it is not possible to remove all the TBT from sediments (Sakultantimetha, Keenan *et al.* 2011). In addition, and although TBT is transformed by remediation processes in other compounds, their toxicity should always be checked.

Several studies have been developed to evaluate or establish bioremediation in water and sediments but to our knowledge no studies have been carried out to evaluate potential remediation TBT in soils.

1.4. Plant bioassays to assess soil quality

The use of natural soils to assess toxicity of compounds reproduces better the exposure conditions in the field (Römbke, Jänsch *et al.* 2007) when comparing to tests performed in artificial soils like the OECD soil (OECD 2003 (draft version)).

One of the bioassays that is part of the Guidance on the ecotoxicological characterization of soils and soil materials (ISO 2003) is the ISO 11269-2 guideline on the emergence and growth of soil flora (ISO 1995). Bioassays with plants are essential ecotoxicological tools to verify the effect of exposure to chemical compounds, e.g. TBT, present in soils, or other stressors (e.g. changes in water regime) (Lima, Soares *et al.* 2011) as well to verify the achievement of remediation processes of contaminated soils (ISO 1995).

On the guidelines for plant bioassays (ISO 1995; ISO 2003), seed emergence and growth parameters, measured as shoot length, fresh and dry weight, or root elongation are evaluated advisably on plant species, one monocotyledonous and one dicotyledonous.

Sensitivity to TBT is variable depending on the plant species tested (EPA 2003). To higher plants toxicity of TBT can be considered low in some cases, being one possible

explanation the good aeration available under vegetation soil, preventing the leaching of toxic compounds and stimulating the microbial action. Thus, a faster degradation of TBT might occur (Novak and Trapp 2005).

In various vascular plants TBTCl influences the morphology and physiology of vegetable cells, by occurring alterations of the chromosomes and inhibition of mitotic spindle. With increasing concentrations of TBTCl, oxygen and chlorophyll produced by cells decreases and therefore there is a decrease in respiration and growth of plants. It has been also shown that TBTCl has a high capacity to bioaccumulate in some plants, like garlic (*Alliym cepa*), potatoes (*Solanun tuberosum*) and eggplant (*Solanum melongena*) (Caratozzolo, Bellini *et al.* 2007), usually also on the base of human consumption.

Higher marine plants, such as the seagrass, *Ruppia maritima,* exposed to TBT showed a decrease in the photosynthetic activity, accompanied with a decrease on their growth rate. The decrease in the photosynthetic activity was probably caused by uncoupling the photophosphorylation in the chloroplasts, and therefore oxygen and ATP production was inhibited. Consequently, less energy was available to the plant growth and plants were smaller when exposed to TBT (Jensen, Holmer *et al.* 2004).

Studies performed in lettuce, revealed that there is an uptake of TBT by the plant, mainly in roots, but might be also transferred into its shoots (Lespes, Marcic *et al.* 2009). Thus, this is an indication of a possible entrance in the food chain, and a possible risk to reach humans.

Potatoes and French beans cultivated in a sandy soil spiked with organotin solutions, including TBT, and sludge soil revealed an organotin transfer from contaminated soil to plants. These compounds are taken up by plant roots and transferred to the aerial plant parts. Tributyltin concentrations in different parts of French beans and potato tubers cultivated in contaminated soil varies, respectively, between 10-73 μg (Sn) kg^{-1} and 460 μg (Sn) kg^{-1}. Distribution in plant parts of organotin compounds depends on the organotin nature and source, but also on the plant species (Lespes, Marcic *et al.* 2003). Translocation of organotin compounds, including TBT, DBT and MBT occurs into leaves and stems in reed, clover and grass (Novak and Trapp 2005).

In soil-plant systems there is a debutylation produced by bacteria and other organisms in soil, favoring the degradation of tributyltin (Lespes, Marcic *et al.* 2003), which might turn TBT less toxic to plants.

1.5. Aim

This study has two main goals:

1) To evaluate the effects of TBT exposure in two plant species *Brassica rapa* and *Triticum aestivum* (Fig. 1.1);
2) To evaluate the ability of the bacterium *Aeromonas molluscorum* Av27 to bioremediate soil contaminated with TBT; this evaluation will be carried out using a plant bioassay with *Brassica rapa*, considering that it is expected a decrease of toxicity after chemical remediation.

Fig. 1.1. *Brassica rapa* and *Triticum aestivum* at the end of the 14 days bioassay.

To fulfill both of our aims we used plant bioassays where seed emergence and growth parameters, measured as shoot length and biomass production (fresh weight),

were evaluated. A scheme for the setup pots in the plant bioassay is represented in Fig. 1.2.

To evaluate TBT toxicity in soil, a rapid-cycling variety of turnip rape and dicotyledonous plant species, *Brassica rapa*, and common wheat *Triticum aestivum,* a monocotyledonous plant species, were used in the plant bioassays as recommended by ISO and OECD guideline (ISO 1995; OECD 2003 (draft version)).

Along the bioassay, and in addition to the endpoints pointed out above, we performed a visual assessment, checking for any damage to the plant species, for example, chlorosis (yellow leaves), stunting, necrosis (brown/dark color leaves) or other symptoms. In the plant growth room, temperature, humidity and light conditions were controlled to support germination and growth of the species (ISO 1995).

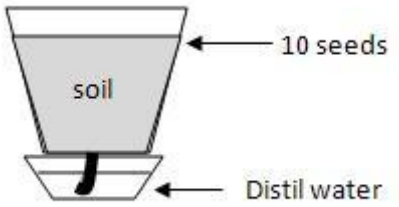

Fig. 1.2. Scheme for the setup pots in the plant bioassay.

1.6. Relevance of the study

A significant part of the Portuguese coast is affected by organotin compounds, mainly tributyltin (Sousa, Ikemoto *et al.* 2009). The majorities of the studies focuses on the aquatic system and do not examine the effects on soil or terrestrial organisms.

Generally, TBT pollution in sediments is higher than in the water phase (White, Tobin *et al.* 1999), and the degradation of these compounds in sediments and soils is slower than in freshwater (Gadd 2000; Huang and Matzner 2004). Contamination of soils by organotin compounds is a real scenario that can occur. Possible sources may be via disposal of contaminated sediments in soil, pulverization of pesticide products (Marcic, Le Hecho *et al.* 2006) and/or atmosphere deposition (Huang and Matzner 2004) as mentioned previously.

Currently, few studies have been carried out on the toxicity of TBT to soil flora or organisms. Some were performed with earthworms, *Eisenia fetida* (Hund-Rinke, Lindemann *et al.* 2005), others with turnip, *Brassica rapa* (Hund-Rinke and Simon 2004; Römbke, Jänsch *et al.* 2007), but to our knowledge there are no published studies with effects of TBT in an important species such as common wheat, *Triticum aestivum*.

In a World with an increasing concern about pollution, studies about techniques of bioremediation are of extremely importance. Already in 1997 a study about organotin degradation (including TBT) was performed with bacterial populations in river water. Degradation of TBT in DBT was observed (Harino, Fukushima *et al.* 1997). Over the years, other studies about TBT degradation in water, sediments, or culture medium, using for instance bacteria, were performed (Brandsch, Nowak *et al.* 2001; Cruz, Caetano *et al.* 2007; Sakultantimetha, Keenan *et al.* 2010), but as far as we are aware no studies about bioremediation of TBT in soils were performed.

1.7. Organization of the thesis

The present thesis is organized in four chapters. The second and third chapters are structured as scientific papers, describing some results.

Chapter 1. Describes soil contamination; the organotin compound - tributyltin, its properties, sources, adverse biological effects; plant bioassay to assess soil quality; bioremediation processes and the main aims and relevance of the study.

Chapter 2. Evaluation of the toxicity of Tributyltin Chloride (TBTCl) in two plant species, *Brassica rapa* and *Triticum aestivum*. Using bioassays where seed emergence and growth parameters were evaluated.

Chapter 3. Bioassays with plants to assess bioremediation process on a TBT contaminated soil using *Aeromonas molluscorum* Av27 and its effect on the plant species *Brassica rapa*.

Chapter 4. Provides a general discussion and conclusions of this study.

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Chapter 2

Assessment toxicity of Tributyltin Chloride (TBTCl) in *Brassica rapa* and *Triticum aestivum*

2. Assessment toxicity of Tributyltin Chloride (TBTCl) in *Brassica rapa* and *Triticum aestivum*

2.1. Abstract

Organotin compounds, including tributyltin (TBT), are widely spread in the environment, because of their variety of applications, as biocides, wood preservatives, PVC stabilizers and food packaging materials. Various sources of TBT can contaminate soil, such as dredging of contaminated sediments disposed in soil and direct application of pesticides. In laboratory, a natural agriculture soil from the Centre of Portugal was contaminated with TBT. Bioassays with two plant species, *Brassica rapa* and *Triticum aestivum*, were performed to assess the effects caused by TBT in soil. *B. rapa* and *T. aestivum* when exposed to TBT showed a delay on seed germination and a decrease on growth parameters, including a decrease in plants length and biomass production (fresh weight).

Keywords: Tributytiln chloride (TBTCl), *Brassica rapa*, *Triticum aestivum*, toxicity

2.2. Introduction

Organotin compounds (OTCs) have been used in numerous industrial, agricultural and household application as fungicides, bactericides, insecticides, wood preservatives or PVC stabilizers leading to a diffuse contamination in environment (Hoch 2001). Mono- and di- substituted OTCs are used in a variety of consumer products and water pipes (Fent 1996). Nowadays, the European Community has listed OTCs as priority water pollutants and they are also considered endocrine disrupters (Marcic, Le Hecho *et al.* 2006).

This compound is one of the most toxic organotin compounds for aquatic organisms. The main use of tributyltin was as a biocide in antifouling paints to prevent the attachment of fouling organisms (Carter, Turoczy *et al.* 1989). In different species of animals, such as female gastropods, TBT induces masculinization (imposex) by increasing testosterone levels and acts as an endocrine disrupter in some animals (Fent 1996).

Tributyltin (TBT) is a trisubstituted organotin that in aqueous solution forms an equilibrium between the TBT^+ ionic form and the hydro- and chloro-species (Alzieu 1998). TBT is an hydrophobic substance with an octanol/water partition coefficient (log K_{ow}) varying with salinity and pH. Values of log K_{ow} are higher at high pH values. The values published in the literature indicate that the partition coefficient ranges from 3.21 to 3.85 for TBTCl at pH values of 5.8 and 7.8, respectively. TBT, as an hydrophobic compound, in water tends to adsorb to organic matter or sediment, where it accumulates (Fent 1996). Therefore, it is potentially bioavailable for some filtrators and benthic organisms (Rudel 2003). In literature there are different conclusions about the effects of salinity on TBT's toxicity, where some authors reported a decrease (Unger, Macintyre *et al.* 1988) and others an increase in TBT adsorption with elevated salinity (Harris and Cleary 1987). Owing to the persistent nature and bioaccumulation potential, TBT has been classified as a persistent organic pollutant (Bangkedphol, Keenan *et al.* 2009). It bioaccumulates in organisms due to its solubility in fat (high lipophilicity) (WHO 1990). Thence, in January 2003 the International Maritime Organization banned the use of TBT-based paints and in 2008 its effective prohibition in the European Union was established (Cornelis, Bierkens *et al.* 2006). There is also some risk to humans as revealed by a study in 2004 showing that house dust contains considerable levels of these compounds, including TBT and its degradation byproducts. Thus, babies and young children who crawl on the floor are one of the groups that might be more exposed (Fromme, Mattulat *et al.* 2005).

Data compiled since the 1980s reveal that TBT and its degradation byproducts, such as dibutyltin (DBT) and monobutyltin (MBT), are present in all compartments of the coastal environment: water, sediments, living organisms, including large mammals (Alzieu 1998). Attention has mainly been given to TBT pollution in water and sediments because of its high toxic effect to aquatic life even at low concentrations (Hoch 2001).

Due to the great stability of the TBT, it is adsorbed to the sediments, thence contamination is sometimes found to reach high levels, reflecting the long-term storage capacity of this compartment. In harbor areas, average concentrations generally range between 1 and 2 mg kg^{-1} dry weight (Alzieu 1998). In Ria de Aveiro, Portugal, TBT levels in

sediments vary from 6 to 88 ng Sn g^{-1} dry weight (Barroso, Moreira *et al.* 2000). In general, TBT concentration in sediments is higher than in the water phase (White, Tobin *et al.* 1999). It is described that the degradation of this compounds in sediments is slower than in freshwater (Gadd 2000), having in soils half-life times that are in range of several weeks to years (Huang and Matzner 2004), thus it may affect the terrestrial compartment.

Soil contamination is possible via dredging of contaminated sediments and later disposal in soils, pulverization of pesticide products (Marcic, Le Hecho *et al.* 2006), atmosphere deposition (Huang and Matzner 2004), contaminated municipal wastewater and sewage sludge (Fent 1996). Currently, nearly all published studies focus on the aquatic system and not on the soil and as far as we know no study has provided information about the toxicity of TBT in *Triticum aestivum*. This species is very important in terms of economy and social impacts as it is widely used as human food source. Furthermore a study with a mussel species revealed that the Portuguese coast is affected with organotin compounds, where tributyltin is the dominant butyltin present (Sousa, Ikemoto *et al.* 2009).

The aim of this study was to assess the toxicity of soil contaminated with TBTCl to the plant species, *Brassica rapa* and *Triticum aestivum* and verify if plant bioassays are good ecotoxicological tools.

2.3. Material and Methods

Plant species and soil

Two species were selected, the monocotyledonous *Tritucum aestivum* and the dicotyledonous *Brassica rapa* a rapid-cycling variety of turnip rape, based on the species list presented in the ISO guideline 11269-2 (ISO 1995). Wheat seeds were acquired from an agricultural store in Esmoriz, Portugal, whereas turnip seed were obtained from Carolina Biological Supply Company.

An agricultural soil collected in the central region of Portugal, Coimbra, was used with these pedological characteristics: pH= 7.48, organic matter content = 2.4%, clay = 4.2%, silt = 7.0%, sand = 88.7%, density (g/cm^3) = 2.4 and water holding capacity = 70% (Santos, Ferreira *et al.* 2011). Soil samples were air dried and sieved (5 mm mesh size). The soil has not been treated with pesticide in the last five years (Lemos 2010).

Test chemical

Tributyltin chloride ($[CH_3(CH_2)_3)]_3Sn$ Cl); 97% purity) with a molar mass of 325.49 g/mol was obtained from Sigma Aldrich.

Plant Growth Bioassays

The methodology of bioassays used to evaluate the toxicity of the TBT in soil was adapted from the standard protocol from ISO 11269-2. A 14-day bioassay to evaluate the effect of TBT in soil on *Triticum aestivum* and *Brassica rapa* was performed (ISO 1995).

All of the bioassays had four replicates per treatment and controls. Each replicate consists in a plastic pot (100 mm Ø, 9 mm height) with 450 g of soil (controls or soils with TBT), where 10 seeds were placed at a maximum depth of 1 cm from the soil surface. The seeds were not treated with any fungicide. A hole was made in the bottom of the plastic pots where a fiberglass wick (between 5-10mm Ø) was placed; pots were placed inside a plastic bowl with water to maintain the soil moisture, by capillarity. Bioassays were carried out in a plant growth room at 20^{o}C ± 2 ^{o}C, with a illumination of 7,000 lx, in a 16:8 (light:dark) photoperiod.

Four concentrations were selected based on literature: 12.5, 25, 50 and 75 mg TBT/kg, plus a control and a control with solvent (ethanol); the water-holding capacity (WHC) was adjusted to 70% in the beginning of the test. For soil contamination, a stock solution of ethanol and TBT was prepared (absolute ethanol obtained from VWR); then

aliquots of this stock solution were mixed with distilled water regarding each concentration needed (to obtain 70% WHC) and finally it was mixed with the soil.

After inserting the seeds, the test begun after 50% of the control seeds had emerged, and lasted for 14 days. The total number of germinated seeds (cumulative germination) was recorded daily and observations were done regularly to check for any change in plant color, other symptoms or death. At the end of the test, all plants were harvested (cut above the soil surface) and growth, measured as shoot length, fresh and dry weight was recorded. In *Brassica rapa* the number of flower buds were also recorded. For both species the hydric content (HC) was calculated using the following equation:

$$HC = \frac{FW - DW}{FW} \text{ x } 100$$

Where FW is the plant fresh weight and DW is the plant dry weight.

The pH was measured according to the ISO standard procedure, adding 1:5 volume of water to the soil sample (ISO 1994).

Statistical Analysis and Parameters Calculation

Differences on plants exposed to the control soil and plants exposed to the control solvent were checked using a t-test (SPSS 2008). When differences were observed between controls, all TBT related effects were compared with the control solvent situation.

The comparison between the control and TBT concentrations was made using a One Way ANOVA. If data were not normally distributed and data transformation did not correct for normality, a Kruskal-Wallis One Way Analysis of Variance on Ranks was performed. Whenever significant differences occurred the Dunn's Method or the Holm-Sidak Method were carried out to discriminate statistical differences between treatments (SPSS 2008).

The 50% effective concentration (EC_{50}) values were calculated using a nonlinear regression with a sigmoidal function, using always the one that showed better adjustment.

2.4. Results and Discussion

Comparison between control and control solvent exposure

The exposures to control and control solvent situations were compared using a t-test. In *Brassica rapa* significant differences were found for the flower buds ($p<0.05$) and hydric content ($p<0.05$). On the other hand, no differences were observed for length ($p>0.05$), fresh weight ($p>0.05$) and dry weight ($p>0.05$). In *Triticum aestivum* significant differences were also found for hydric content ($p<0.05$). On the other hand, no differences were observed for length ($p>0.05$), fresh weight ($p>0.05$) and dry weight ($p>0.05$). So, all the TBT treatments were compared with the control solvent situation.

Seed emergence and growth parameters in Brassica rapa

In control replicates seed emergence was reported for *Brassica rapa* after three days. Apparently, tributyltin concentrations higher than 50 mg TBT/kg not only caused a delay on germination of the turnip grains but also an inexistence of seed germination in some replicates. There was a dose response pattern for germination of plants exposed to TBT.

Plants' growth showed a significant decrease upon TBT exposure when compared to the control solvent (Kruskal-Wallis one-way ANOVA, $H=67.703$, $df=4$, $p<0.001$, Dunn's method, $p<0.05$). Different concentrations of TBT in *Brassica rapa* produced significant effects on biomass production (fresh weight) when compared to the control replicates (Fig. 2.1), with a NOEC lower than 12.5 mg TBT/kg and a LOEC of 12.5 mg TBT/kg (Kruskal-Wallis one-way ANOVA, $H=63.045$, $df=4$, $p<0.001$, Dunn's method, $p<0.05$). For dry weight these effects were also observed (Kruskal-Wallis one-way ANOVA, $H=50.152$, $df=4$, $p<0.001$, Dunn's method, $p<0.05$) and also for the hydric content (Kruskal-Wallis one-way ANOVA, $H=10.671$, $df=4$, $p=0.031$, Dunn's method, $p<0.05$). For these parameters it was possible to observe a dose-response relationship, therefore EC_{50} values were calculated (Table 2.1).

In the *B. rapa* replicates exposed to 25 mg TBT/kg it was observed smaller plants than in the control but with more flower buds. At the end of the bioassay, there was a significant difference in the number of flower buds between treatments (Kruskal-Wallis one-Way ANOVA, H= 22.993, df= 4, p<0.001), where at concentrations higher than 25 mg TBT/kg, there was a decrease on their number.

At the end of the 14 days, some plants of the highest concentration were very small, had chlorosis (yellow leaves) and some showed some signs of necrosis (brown/dark color leaves). It can be assumed that tributyltin is toxic to these species, with consequences in seed germination and in growth parameters.

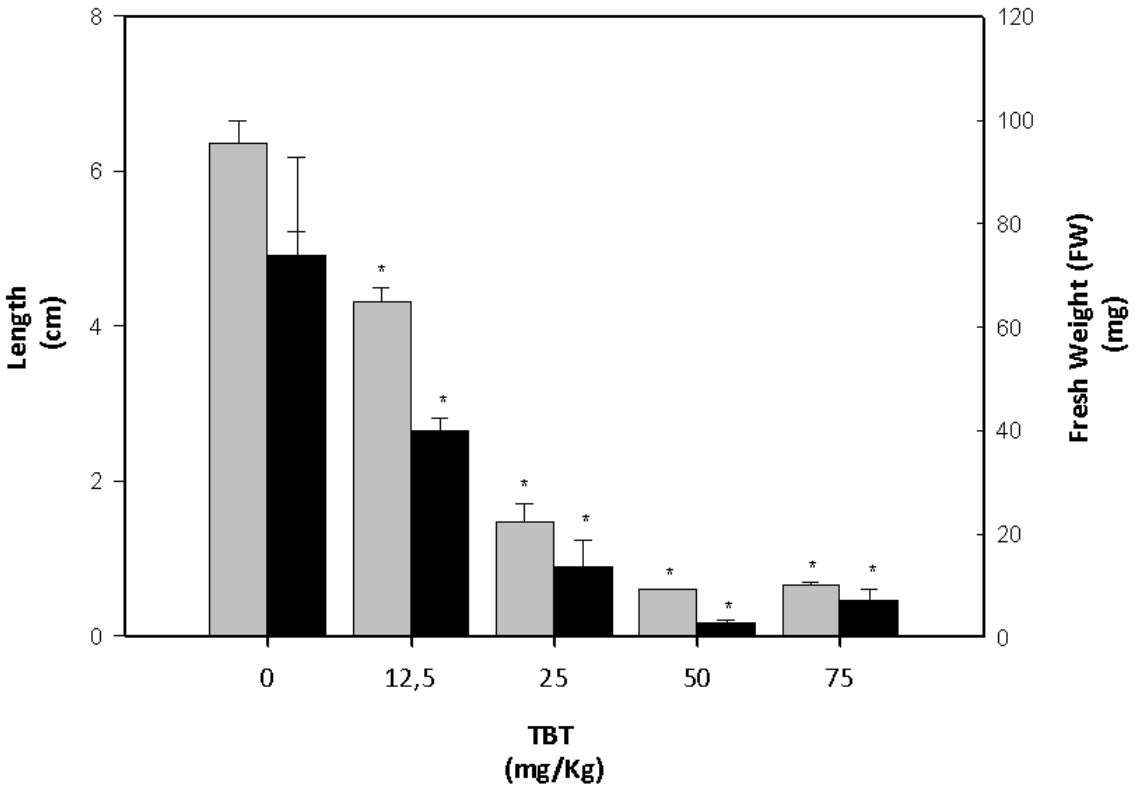

Fig. 2.1. Length and biomass production (fresh weight) of *Brassica rapa* exposed to different concentrations of TBT [*significantly different from the control soil (Dunn's test, p<0.05)]; grey bars are for data on plant's length and black bars refer to fresh weight data.

Table 2.1. EC_{50} (as mg TBT/kg), NOEC (as mg TBT/kg) and LOEC (as mg TBT/kg) values for length, fresh and dry weight obtained from the exposure of *Brassica rapa* to TBT.

Parameter	EC_{50} value	Standard Error	r^2	NOEC	LOEC
Length	14.81	1.66	0.66	<12.5	12.5
Fresh weight	11.83	1.64	0.49	<12.5	12.5
Dry weight	12.33	2.63	0.40	<12.5	12.5

Seed emergence and growth parameters in Triticum aestivum

Seed emergence for *Triticum aestivum* was observed in control replicates after five days. Tributyltin concentrations higher than 50 mg TBT/kg caused a delay in germination of wheat grains. In some plants on the highest concentration (75 mg TBT/kg) abnormal germination was observed on wheat caryopsis. In some plants the caryopsis coat did not release from the shoot and jeopardizing the plant growth.

A significant decrease on the growth (expressed as length) of plants exposed to TBT was observed when compared to the control replicates (Kruskal-Wallis one-way ANOVA, $H=67.898$, $df=4$, $p<0.001$, Dunn's method, $p<0.05$). Thus, biomass production (fresh weight) was also affected, showing a significant decrease too (Fig. 2.2) (Kruskal-Wallis one-way ANOVA, $H=62.004$, $df=4$, $p<0.001$, Dunn's method, $p<0.05$). Significant differences were observed also for the hydric content (Kruskal-Wallis one-way ANOVA, $H=10.671$, $df=4$, $p=0.031$, Dunn's method, $p<0.05$).

The results from the exposure to 25 mg TBT/kg in *Triticum aestivum* (Fig. 2.2) might be explained by the fact that in some concentrations TBT might be favorable for plants, as TBT has a biocide action, being toxic for instance for some insects, soil invertebrates and fungi that act against plants (Novak and Trapp 2005). In our assay, it was observed that fungi were present on the soil surface and might affected some replicates.

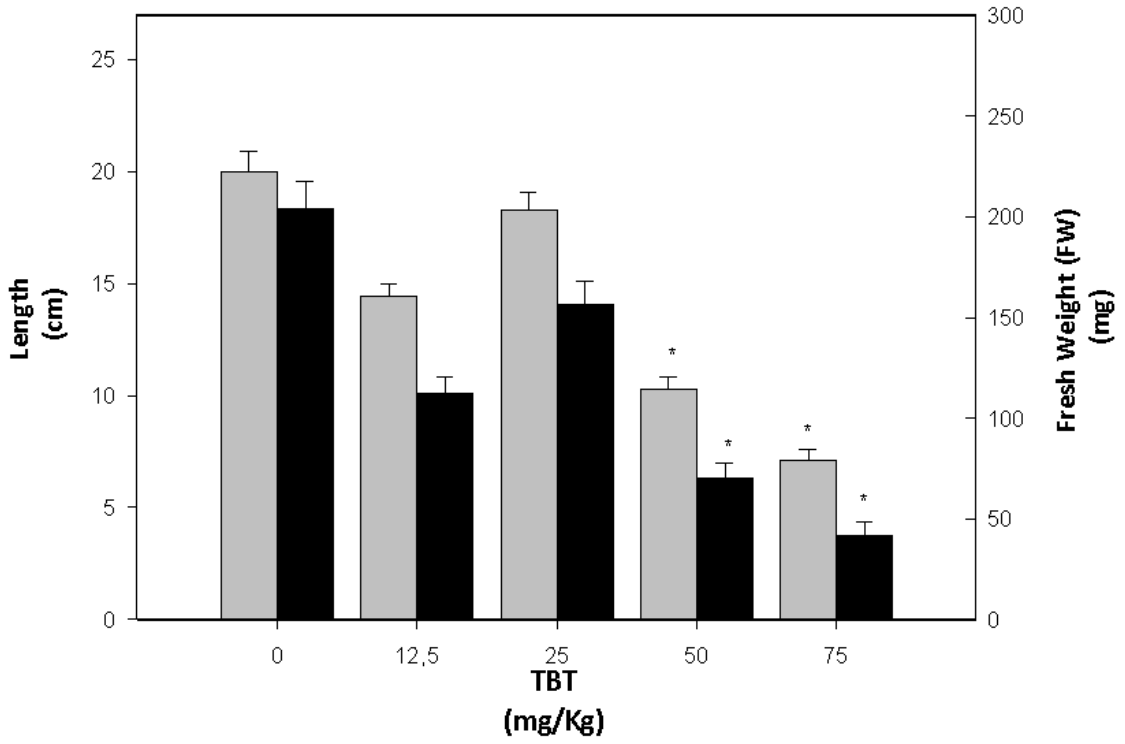

Fig. 2.2. Length and biomass production (fresh weight) of *Triticum aestivum* exposed to different concentrations of TBT [*significantly different from the control soil (Dunn's test, $p<0.05$)]; grey bars are for data on plant's length and black bars refer to fresh weight data.

Table 2.2. EC_{50} (as mg TBT/kg), NOEC (as mg TBT/kg) and LOEC (as mg TBT/kg) values for length, fresh and dry weight obtained from the exposure of *Triticum aestivum* to TBT.

Parameter	EC_{50} value	Standard Error	r^2	NOEC	LOEC
Length	33.82	35.12	0,48	25	50
Fresh weight	28.40	6.41	0,45	25	50
Dry weight	52.80	6.90	0,36	n.d.	n.d.

n.d. = data not determined

The objective of this study was to assess the toxicity of soil contaminated with TBT to the plant species, *Brassica rapa* and *Triticum aestivum*. Tributyltin presented toxic effects to both plant species. It seems that *B. rapa* is slightly more sensitive when exposed to tributyltin comparing with *T. aestivum,* showing an EC_{50} value (for length) two times lower than the EC_{50} value for *T. aestivum* (Table 2.1 and Table 2.2).

Other signs of toxicity of TBT in plants are the observation of chlorosis. This suggests that TBT might act in the photosynthetic apparatus, and probably the protein, rubisco

(ribulose biphosphate carboxylase oxygenase), is also affected. Biomass production also depends on proteins. If they are affected, some biochemical processes are also affected, therefore growth and plant biomass production are conditioned (Desimone, Henke *et al.* 1996; Loureiro 2006). Therefore, in future studies to evaluate photosynthetic efficiency might also be useful to clarify the mode of action of TBT in plants.

It is known that different characteristics of soils, affect chemical compounds toxicity (Römbke, Jänsch *et al.* 2007). TBT sorption is pH dependent and the persistence of TBT is higher when the pH is high (Marcic, Le Hecho *et al.* 2006). Organotins are also more bioavailable in sandy soils, than in loamy soils (Novak and Trapp 2005) and our studied soil had an high percentage of sand, 88.7%. In our results, the EC_{50} value for biomass production of 11.83 mg TBT/kg to *B. rapa*, was similar to the Hund-Rinke study (25 mg TBT/kg). This may be explained by pedological characteristics of the soil, with a similar percentage of sand, around 70-80%, and an organic matter content around 2% (Hund-Rinke and Simon 2004).

In *Brassica rapa* bioassay we obtained a pH value around 7.94 in the beginning of the assay, and at the end was 7.95. In *Triticum aestivum* bioassay pH value was initially 8.12 and decreased to 7.92 at the end of the assay.

In a natural soil with the following characteristics, pH= 5.8, organic matter content= 3.37%, sand= 27.0%, silt= 47.1%, clay= 25.9%, for *Brassica rapa* an EC_{50} value of 189.2 mg TBTO/kg was determined (Römbke, Jänsch *et al.* 2007). These differences, comparing to our obtained EC_{50} values, are probably due to differences in characteristics of soil but also might also be related to the TBT formulation used (TBTCl vs TBTO). In an artificial soil like the OECD soil (pH= 6.0, organic matter content= 4.7%, sand= 75.4%, silt= 16.6%, clay= 8.04%) the EC_{50} value was 535.5 mg TBTO/kg (Römbke, Jänsch *et al.* 2007). Comparing with our results, this suggests that the use of natural soils like the one used in this study show more realistic scenarios than the use of artificial soils.

In a sandy soil, the earthworm, *Eisenia fetida*, exposed to tributyltin chloride seemed to be slightly more sensitive than the two plant species used in our bioassay, with an EC_{50} value of 1.3 mg TBT/kg. The collembolan, *Folsomia candida*, demonstrated to have similar sensitivity to TBT, comparing to our tested species, with an EC_{50} value of 22

mg TBT/kg. The higher plant oat, *Avena sativa*, has shown to be less sensitive to tributyltin than the other species, with an EC_{50} value of 452 mg TBT/kg (Hund-Rinke and Simon 2004). In tests performed with Bis (tri-n-butyltin) oxide (TBTO) a LC_{50} of 178.4 mg TBTO/kg was observed to *Folsomia candida* (Römbke, Jänsch *et al.* 2007). This means that also depending on the butyltin type, TBTCl or TBTO, different effects on species are observed. In addition, several species are advised to be used when studying the toxicity of chemical compounds as different species exhibit different trends of toxicity.

2.5. Conclusions

From this study we can witness that tributyltin affects the germination and growth of the two plant species, *B. rapa* and *T. aestivum*. A diminish in seed germination and in growth parameters was observed in replicates with TBT.

Additional information could be important to understand the mode of action of organotin compounds, by using, for example, biochemical parameters or others to check if the plant photosynthetic apparatus is affected. More studies should be also carried out to assess the uptake of TBT on plants, especially those used for human consumption.

In conclusion, investigations on potential human effects, dietary intake via contaminated vegetables or seafood, and strategies to remove TBT compounds are needed.

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Chapter 3

Bioassays with *Brassica rapa* to evaluate bioremediation in soil after *Aeromonas molluscorum* Av27 inoculation

3. Bioassays with *Brassica rapa* to evaluate bioremediation in soil after *Aeromonas molluscorum* Av27 inoculation

3.1. Abstract

Tributyltin (TBT) is an organotin compound used in antifouling paints, wood preservatives, PVC stabilizers and in various consumer products. Due to its persistence and bioaccumulation potential it remains a worldwide pollution problem. Thence, bioremediation techniques to remove this compound are extremely important. Bioremediation accelerates the natural biodegradation of contaminants, playing an important role on TBT removal. The aim of the study was to test the ability of *Aeromonas molluscorum* Av27 to bioremediate soil contaminated with TBT and to carry this evaluation using plant bioassays with *Brassica rapa. Aeromonas molluscorum* Av27, a TBT resistant bacterium, is already known to bioremediate water contaminated with TBT and in this study it was added to TBT contaminated soil. Plant bioassays were performed after two weeks of bacteria inoculation, and seed germination and growth parameters were evaluated. The results showed that, in the conditions tested, this bacterium induces a decrease in the number of germinated seeds but stimulates plant growth. Therefore more tests are needed to conclude whether *A. molluscorum* Av27 is able or not to diminish the toxicity of TBT in soil.

Keywords: Tributytiln chloride (TBTCl), toxicity, *Brassica rapa*, *Aeromonas molluscorum* Av27, bioremediation

3.2. Introduction

Tributyltin (TBT) is an organotin compound used as biocide in antifouling paints to prevent the attachment of fouling organisms (Carter, Turoczy *et al.* 1989). This trisubstituted organotin compound has several applications, being used as wood preservatives, disinfectant, in pulp and paper mills, leather processing and as a PVC stabilizer (WHO 1990).

TBT is an hydrophobic compound, with an octanol/water partition coefficient (log K_{ow}) around 3.21 and 3.85, depending on the pH. Thence, in water tributyltin tends to absorb to organic matter or sediments (Fent 1996). Disposal of dredge spoils from these contaminated sediments, pulverization of pesticide products (Marcic, Le Hecho *et al.*

2006), atmosphere deposition (Huang and Matzner 2004), contaminated municipal wastewater and sewage sludge spreading (Fent 1996) can lead to TBT contamination of soils. Therefore, sometimes TBT contamination reaches sites far away from the initial source (White, Tobin *et al.* 1999).

Toxicological studies revealed that TBT is a threat to non-target organisms. TBT is considered an endocrinal disruptor (Morcillo and Porte 2000) and in gastropods it is known to cause imposex - masculinization of female gastropods (Fent 1996)

Organotin compounds degradation depends on abiotic and biotic factors, but in soils the most significant factor regards biotic processes (degradation by microorganisms) (Dubey and Roy 2003). TBT is degraded, by sequential removal of organic groups from the tin atom, in dibutytin (DBT), monobutyltin (MBT) and finally in inorganic tin. These compounds can be present in several environmental compartments, from water, sediments to living organisms (Alzieu 1998).

Bioremediation is a cleanup technology that is non-destructive and treatment effective, and that accelerates the natural biodegradation of contaminants (Margesin and Schinner 2001). Biodegradation processes can play an important role in TBT removal, which is helped by aerobic conditions in soils (Brandsch, Nowak *et al.* 2001). There are several species reported to bioremediate TBT in waters, such as bacteria, *Enterobacter cloacae* (Sakultantimetha, Keenan *et al.* 2010), algae, *Chlorella* species (Tam, Wong *et al.* 2009), and fungi, *Cunninghamella elegans* (Bernat and Dlugonski 2007).

Aeromonas molluscorum Av27 isolated from TBT contaminated sediment in Ria de Aveiro, Portugal, is a TBT resistant bacterium with a memory response to this compound. Gram negative bacteria, such as Av27, are reported to have more tolerance to TBT than the Gram positive bacteria. In liquid culture medium, Marine Broth Agar (MBA), TBT is degraded to its degradation byproducts, DBT and MBT and it is used in metabolic activities, for instance as carbon source by *A. molluscorum* Av27 (Cruz, Caetano *et al.* 2007).

The aim of the study was to assess the ability of *Aeromonas molluscorum* Av27 to bioremediate soil contaminated with TBT using bioassays with plants and to evaluate its

effect on the plant species, *Brassica rapa*. For that, seed emergence and growth parameters (measured as shoot length and fresh weight) were evaluated.

Bioassays with plants are important tools to verify the toxicity of chemical compounds but also to achieve effectiveness of remediation processes of contaminated soils (ISO 1995). Transformation of chemicals into their metabolites can induce, for example a transformation into a more toxic degradation byproduct, e.g. TBT into MBT, or increase its bioavailability; therefore it is expected that bioassays can provide information in terms of toxicity and bioavailability of compounds present in matrices (Paton, Cheewasedtham *et al.* 2006).

Tributyltin is known to be extremely toxic and it may be accumulated along the food chain, being a problem to many organisms. Fungal remediation techniques are being studied to remove organic compounds from soil (Steffen and Tuomela 2010), but there are few studies examining processes of bioremediation in TBT contaminated sites (Gadd 2000). To the best of our knowledge, there are no published studies assessing the capability of *Aeromonas molluscorum* Av27 to bioremediate a soil contaminated with TBT.

The results showed that, apparently, *Aeromonas molluscorum* Av27 was not able to bioremediate the soil contaminated with TBT, since the total number of germinated seeds in replicates that were placed in soils that were previously inoculated with Av27 was lower than in the control situation.

3.3. Material and Methods

Plant species, soil and chemical compound

A dicotyledonous plant species, the rapid-cycling variety of turnip rape *Brassica rapa*, was used in this study and seeds were obtained from the Carolina Biological Supply Company.

An agricultural soil with pH= 7.48, organic matter content = 2.4%, clay = 4.2%, silt = 7.0%, sand = 88.7%, density (g/cm^3) = 2.4 and water holding capacity = 70% collected in the central region of Portugal, Coimbra, was used and soil samples were air dried and

sieved (5 mm mesh size) (Santos, Ferreira *et al.* 2011). The soil has not been treated with pesticide in the last five years (Lemos 2010).

Tributyltin chloride ($[CH_3(CH_2)_3)]_3Sn$ Cl); 97% purity) with a molar mass of 325.49 g/mol was obtained from Sigma Aldrich.

Preparation of Aeromonas molluscorum Av27 inoculum

Aeromonas molluscorum Av27 was isolated from TBT contaminated sediments from Ria de Aveiro, Portugal (Cruz, Caetano et al. 2007). Cells were grown overnight, in an orbital shaker at 26^{o}C in organic-reach culture medium, Tryptic Soya Broth - TSB, (Merck, Germany). Optical density (OD_{600nm}) was measured (OD=1.85).

Plant Growth Bioassay

A 14-day plant bioassay with *Brassica rapa* based and adapted from the standard protocol ISO 11269-2 was performed to evaluate the effect of the TBT alone and after Av27 inoculation in soil (ISO 1995).

Therefore the experimental setup considered three sub-experiments that were carried out simultaneously:

- A TBT contaminated soil experiment - a regular ecotoxicity test, where TBT exposure effects were evaluated in *Brassica rapa;* here a control and control solvent was included; from now called TBT-ISO test;
- A TBT contaminated soil experiment with the bacteria growth media - a regular ecotoxicity test, where TBT plus growth media (Tryptic Soya Broth - TSB, 100 mL per pot) effects were evaluated in *Brassica rapa;* here a control with bacteria growth media was also added; from now on called TBT-Growth media test;
- A TBT contaminated soil, previously inoculated with *Aeromonas molluscorum* Av27 - a regular ecotoxicity test, where soil with TBT was inoculated for 14 days,

and afterwards, exposure effects were evaluated in *Brassica rapa*; a control with Av27 bacteria (and growth media) was added; in this setup, 100 mL of TSB with *Aeromonas molluscorum* Av27 per pot, corresponding to approximately 5.95 x 10^8 cells, were used; from now on called TBT-Av27 test;

All bioassays had three replicates per treatment and controls. Each replicate consists in a plastic pot (100 mm Ø, 9 mm height) with 450 g of soil. A hole was made in the bottom of the plastic pots where a fiberglass wick (between 5-10mm Ø) was placed to maintain soil moisture. This maintenance was made by water capillarity through the fiberglass wick, between the pot and a plastic bowl with water. Bioassays were carried out in a plant growth room at 20°C ± 2°C, with a illumination of 7,000 lx, in a 16:8 (light:dark) photoperiod regime.

Three concentrations were selected based on the study presented on Chapter 2: 10, 20 and 40 mg TBT/kg plus a control and a control with solvent (ethanol); water-holding capacity (WHC) was adjusted to 70%. For soil contamination, a stock solution of ethanol and TBT was prepared (absolute ethanol obtained from VWR); then aliquots of this stock solution were mixed with distilled water, growth media or growth media containing the strain Av27 (depending on each sub-experiment), regarding each concentration needed (to obtain 70% WHC) and finally it was mixed with the soil.

After contamination or contamination plus inoculation, the soil was distributed into pots in the growth plant room (including controls). After 2 weeks, 10 seeds were placed in each pot at a maximum depth of 1 cm from the soil surface.

The total number of germinated seeds (cumulative germination) was recorded daily and observations were done regularly to check changes in plant color, other symptoms or death. All tests started when 50% of seeds had germinated in the control replicates of the TBT-ISO test and lasted for 14 days. All plants were harvested (cut above the soil surface) and growth measured as shoot length, fresh and dry weight.

The pH was measured according to the ISO standard procedure, adding 1:5 volume of water to the soil sample for all treatments (ISO 1994).

Microccocus luteus: a bioindicator species of TBT toxicity

Microccocus luteus is a bacterium exhibiting low TBT resistance (Cruz, Caetano et al. 2007). Thus it has been used to detect the presence of TBT in water samples. In our study we tried to evaluate if these bacteria could also be used as an indicator for the presence/toxicity of TBT in soil. These cells were grown overnight in TSB medium at 37ºC, in an orbital shaker (100 rpm) until an optical density (OD_{600nm}) of 1 was reached. 250 µL of the *M. luteus* culture was incorporated in 32.5 mL of Tryptic Soy Agar (TSA) (Merck, Germany), mixed and poured into Petri dishes. After solidification, two wells were made on the agar plate; one well, was filled with TBT contaminated soil (one was also left without TBT to be used as control), and the other, for comparison purposes, was filled with TSB´s solution with TBT in the same concentration as the soil sample. A control only with the soil was also performed. Finally Petri dishes were incubated overnight at 37ºC. The presence of TBT was detected if an inhibition halo of bacterial growth was observed.

A preliminary test was carried out where soil previously contaminated with TBT (30g of soil with TBT concentrations of 2, 8, 16, 32 and 160 mg TBT/kg) was firstly frozen (-80ºC, for 48h) to eradicate all the endemic bacteria to avoid competition with *M. luteus*; afterwards soils moisture was adjusted to 80%.

48h, 7 days and 14 days after the inoculation with Av27, soil corers of 2g were collected from pots where plant bioassays would be carried out. In this case, moisture was adjusted to 70%, water holding capacity, because 80% could be too moisty for plants efficient growth. During the bioassay, despite the use of a fiberglass wick to maintain moisture, it was observed that soil was becoming drier.

Statistical Analysis and Parameters Calculation

SigmaPlot v11.0 software was used to perform the statistical analysis (SPSS 2008). To check if there was any effect of the solvent (ethanol), control was compared versus control with solvent with a t-test. When differences were observed between controls, all TBT related effects were compared with the control solvent situation.

The comparison between different concentrations and treatments was made using One Way ANOVA. If data were not normally distributed and data transformation did not correct for normality, a Kruskal-Wallis One Way Analysis of Variance on Ranks was the performed. Whenever significant differences occurred the Dunn's Method or the Holm-Sidak Method were carried out to discriminate statistical differences between treatments (SPSS 2008).

The 50% effective concentration (EC_{50}) values were calculated using a nonlinear regression with a sigmoidal function using always the one with better adjustment.

3.4. Results and Discussion

Seed emergence and growth parameters in Brassica rapa

Along the 14 days of the test period some plants in the TBT-ISO test, TBT-Growth media test and TBT-Av27 test, at concentrations higher than 20 mg TBT/kg, showed signs of chlorosis (yellow leaves) and necrosis (brown/dark color leaves).

In the TBT-ISO test, for plants in control replicates, seed emergence was reported after three days (when at least 50% of seeds had germinated). Tributyltin concentrations higher than 20 mg TBT/kg caused a delay on the germination of turnip grains and also, in some replicates, no germination was observed. Decrease in the germination with the increasing concentrations was also observed. When exposed to concentrations higher than 40 mg TBT/kg on the TBT-Growth media test and TBT-Av27 test no seeds germinated. In general, germination was affected by the culture medium and by *A. molluscorum* Av27, being the total number of germinated plants lower than in the TBT-ISO test (Table 3.1).

Table 3.1. Total number of germinated plants at the end of the bioassay.

	Control solvent	10 mg TBT /kg	20 mg TBT /kg	40 mg TBT /kg
TBT-ISO test	30	28	27	11
TBT-Growth media test	16	19	14	0
TBT-Av 27 test	13	4	4	0

Although the bacteria growth media and the presence of Av27 affected negatively seed germination, plants in the TBT-Growth media test and TBT-Av27 test, were bigger than those on the TBT-ISO test, and consequently had higher biomass production (Fig.3.1 and 3.2). This may be explained by the acquisition of nutrients that are present in the culture medium (TSB) improving the plant's growth. The increase of microbial activity has been referred as a factor that may improve the nutrients uptake from soil by plants (Tinker 1984).

In control replicates significant differences were observed on the growth (expressed as length) of plants exposed in the TBT-ISO test, TBT-Growth media test and TBT-Av27 test (Kruskal-Wallis one-way ANOVA, $H=73.078$, $df=3$, $p<0.001$, Dunn's method, $p<0.05$). The same was verified on the biomass production (fresh weight) in control replicates between the three treatments (Kruskal-Wallis one-way ANOVA, $H=73.970$, $df=3$, $p<0.001$, Dunn's method, $p<0.05$). For 10 mg TBT/kg concentration there were significant differences on length between the three test types (Kruskal-Wallis one-way ANOVA, $H=53.992$, $df=3$, $p<0.001$, Dunn's method, $p<0.05$) and also for 20 mg TBT/kg concentration (Kruskal-Wallis one-way ANOVA, $H=58.463$, $df=3$, $p<0.001$, Dunn's method, $p<0.05$).

For the biomass production parameter, for 10 mg TBT/kg concentration there were significant differences between the three test types (Kruskal-Wallis one-way ANOVA, $H=59.143$, $df=3$, $p<0.001$, Dunn's method, $p<0.05$); for 20 mg TBT/kg concentration significant differences were observed as well (Kruskal-Wallis one-way ANOVA, $H=33.180$, $df=3$, $p<0.001$, Dunn's method, $p<0.05$).

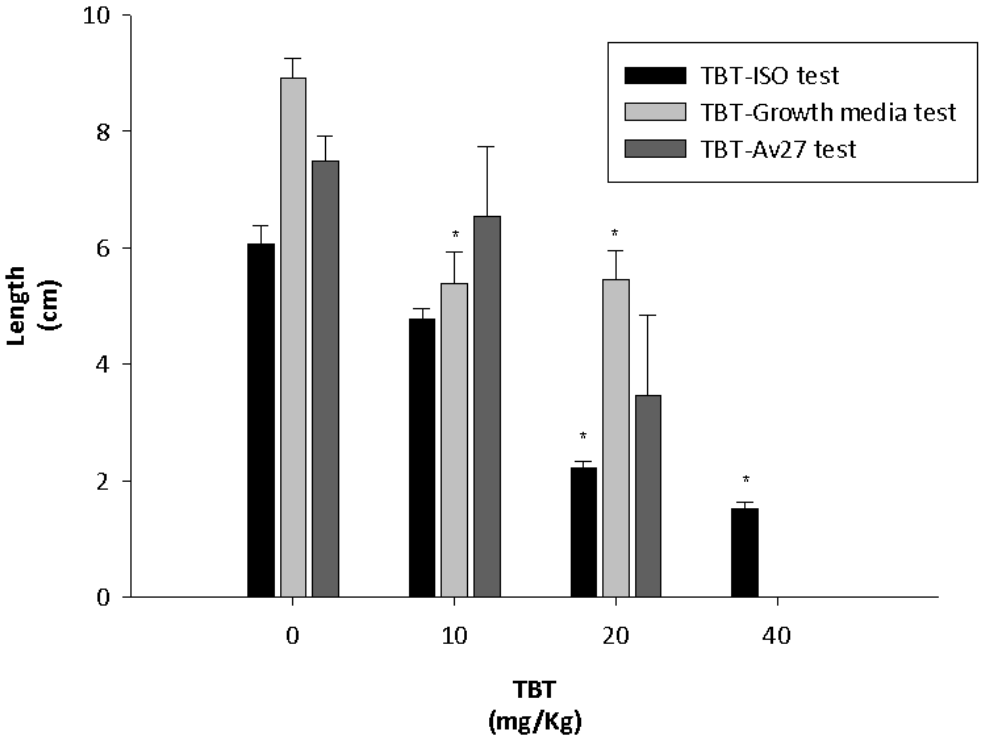

Fig. 3.1. Length of *Brassica rapa* in the TBT-ISO test, TBT-Growth media test and TBT-Av27 test; control solvent is represented by 0 mg TBT/kg. Data expressed as means ± st. error; [*significantly different from the control soil (Dunn's test, $p<0.05$)].

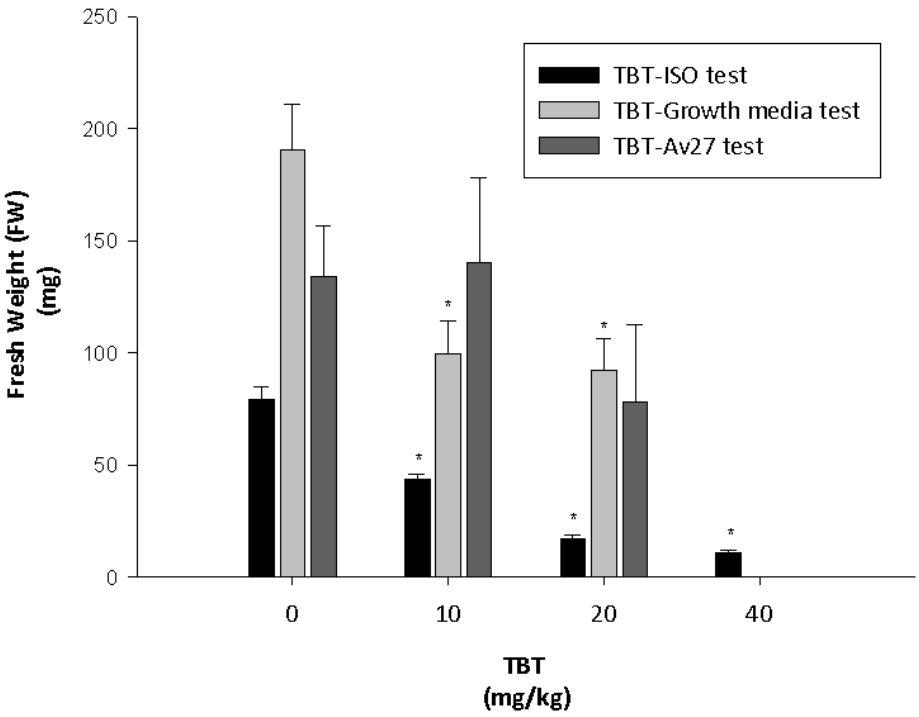

Fig. 3.2. Biomass production (fresh weight) of *Brassica rapa* in the TBT-ISO test, TBT-Growth media test and TBT-Av27 test; control solvent is represented by 0 mg TBT/kg. Data expressed as means ± st. error; [*significantly different from the control soil (Dunn's test, $p<0.05$)].

In general, plants in TBT-Growth media test and TBT-Av27 test had more flower buds than plants on the TBT-ISO test (Fig. 3.3). In control replicates significant differences were observed on the number of flower buds comparing the TBT-ISO test, TBT-Growth media test and TBT-Av27 test (Kruskal-Wallis one-way ANOVA, $H=40.138$, $df=3$, $p<0.001$, Dunn's method, $p<0.05$). In replicates of 10 mg TBT/kg differences were observed as well (Kruskal-Wallis one-way ANOVA, $H=62.454$, $df=3$, $p<0.001$, Dunn's method, $p<0.05$), and the same occurred with 20 mg TBT/kg (Kruskal-Wallis one-way ANOVA, $H=56.359$, $df=3$, $p<0.001$, Dunn's method, $p<0.05$).

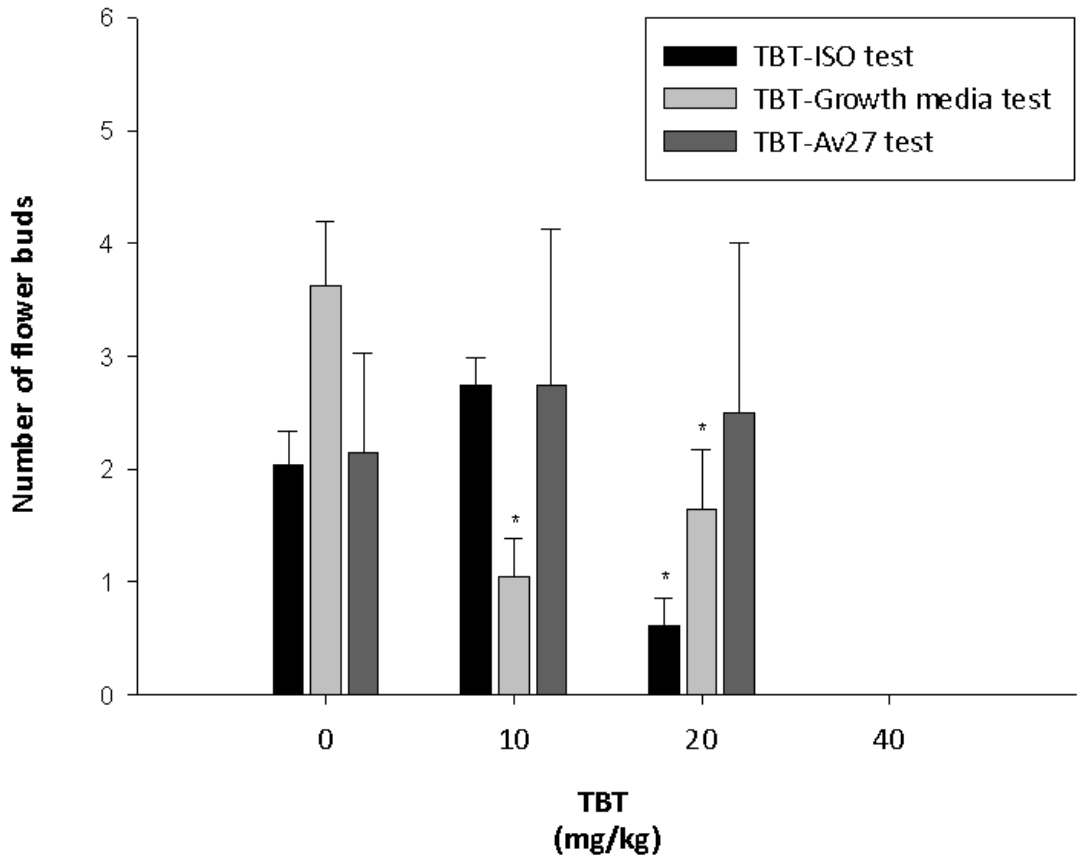

Fig. 3.3. Number of flower buds of *Brassica rapa* in the TBT-ISO test, TBT-Growth media test and TBT-Av27 test; control solvent is represented by 0 mg TBT/kg. Data expressed as means ± st. error; [*significantly different from the control soil (Dunn's test, $p<0.05$)].

Despite the closest EC_{50} values in the different tests (Table 3.2), effects on the plant species were different; differences on the germination, length and biomass production were observed.

Table 3.2. EC_{50} (as mg TBT/kg), NOEC (as mg TBT/kg) and LOEC (as mg TBT/kg) values obtained from the *Brassica rapa* bioassay in the TBT-ISO test, TBT-Growth media test and TBT-Av27 test.

	Parameter	EC_{50}	NOEC	LOEC
TBT-ISO test	**Length**	13.13	10	20
	Fresh weight	8.27	<10	10
	Flower buds	19.19	<10	10
TBT-Growth media test	**Length**	n.d.	<10	10
	Fresh weight	n.d.	<10	10
	Flower buds	n.d.	<10	10
TBT-Av27 test	**Length**	19.06	10	20
	Fresh weight	n.d.	n.d.	n.d.
	Flower buds	n.d.	n.d.	n.d.

n.d. = data not determined

In TBT-ISO test we obtained a pH value of 8.7 (mean). In TBT-Growth media test and TBT-Av27 pH value decreased to a mean of 7.8. Differences in pH, with a lower value in the TBT-Growth media test and TBT-Av27 test, might have affected the results, because it is know that at pH 8 it is expected an increase on bioavailability, and consequently toxicity of TBT (Fent and Looser 1995).

Some authors reported that with organic pollutants, seed germination measurement endpoint is less sensitive than shoot biomass measurement. Seed germination is less sensitive to pollutants because it depends on the energy reserved in cotyledons (Gong 2001). Therefore, the fact that the total number of germinated plants in TBT-Av27 test and TBT-Growth media test were lower than in TBT-ISO test, may not be so significant as the other parameters measured, such as shoot length and biomass production (fresh weight). Nevertheless this cannot be disregarded, as the presence of the growth media or bacteria Av27 induced significant low germination rates.

In bioremediation, interactions between soil, contaminant, nutrients and bacteria are also known to occur. In this case, these interactions may influence the toxicity of tributyltin. To enhance bioremediation some soil conditions, for instance moisture content, should be changed. A low moisture content seems to imply an inactivity of microorganisms, thus decreasing bioremediation rates (Liebeg and Cutright 1999).

Our results revealed that toxicity of TBT in soil using *Aeromonas molluscorum* Av27 was not decreased in one of the parameters measured, seed germination. Hence, further studies with these bacteria are needed and in the future we could extend the Av27 inoculation time.

3.5. Conclusion

This study showed two contradictory results upon the presence of the bacterium *A. molluscorum* Av27 and also its growth media. In one hand, their presence induced low seed germination but, nonetheless, plants from seeds that could germinate had higher biomass production (fresh weight) and length.

Additional studies must be carried out to further clarify these results and to help understanding the effects of the growth media and the bacterium in plants. For instance, TBT analyses in plants, to determine not only the uptake of tributyltin but also dibutyltin/monobutyltin (degradation products) by the plant could provide useful information to understand results. In addition, the presence of the Av27 bacterium inside the plants might also be studied to try to understand its mode of action and what is happening during this process.

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Chapter 4

General Discussion and Conclusions

4. General Discussion and Conclusions

4.1. General Discussion and Conclusions

Tributyltin is a global problem affecting the aquatic and terrestrial ecosystem (Hoch 2001). It is known that currently TBT, as well as its degradation products, DBT and MBT, are present in the ecosystem due to TBT persistence (Dubascoux, Lespes *et al.* 2008).

All the Portuguese coast is affected by TBT pollution (Barroso, Mendo *et al.* 2004) and the main cause seems to be due to the high naval traffic in commercial ports. These tributyltin levels may be preoccupant, not only near coast, where dockyards exist, but even in zones far away from the coast (Rato 2009). As referred previously, TBT may contaminate soils. Concentration in soils may reach 0.05 to 0.24 mg TBT Kg^{-1} (Huang, Schwesig *et al.* 2004) and in soils at industrial areas, TBT concentrations may reach 180 mg kg^{-1} dry matter (Beuselinck and Valle 2005). TBT concentrations in sewage sludge are approximately 18 mg kg^{-1} dry weight; these biosolids are sometimes used in agricultural land, thence being a potential risk to environment and health (Voulvoulis and Lester 2006). There are various studies regarding TBT, most of them devoted to aquatic system, leaving just a few papers related to TBT in soils.

The present study aimed to assess the effect of tributyltin contaminated soil in two plant species, *Brassica rapa* and *Triticum aestivum*. A natural agricultural soil from the Centre of Portugal was used. Moreover, it intended to investigate the ability and efficiency of the bacterium *Aeromonas molluscorum* Av27 to remove TBT from soil, by using bioassays with plants.

The use of a natural soil, despite the advantages described in the general introduction it has the disadvantage of increasing variability and confounding factors for the interpretation of the results (OECD 2003 (draft version)). Some studies are performed with artificial soils, making it difficult to compare results with others or even with real scenarios.

Terrestrial plants are important monitors of environmental pollutants. They have a sedentary existence, so they are continuously exposed to the contaminants. They are primary producers, being the basis of food chains. Some plant species have an high sensitivity to certain contaminants when compared to animals. They are inexpensive and demonstrate an effect on a living material, which may demonstrate chemical bioavailability. And it is easy to monitor the state of plants with physical observations, checking for example for signs of chlorosis or necrosis (Pfleeger, Ratsch *et al.* 1993).

Different plant species have shown different sensitivity to tributyltin. In higher plants tributyltin chloride seems to interfere in the electron transport in the photosystem I and in the photosystem II (Krugh and Miles 1996). And in some plants, such as French beans, phytoaccumulation of tributyltin can be high (Simon, Bueno *et al.* 2002). Some plants such as barley, *Hordeum vulgare*, grew well in a TBT contaminated sediment and demonstrated ability to degrade TBT much faster than in unvegetated sediments (Novak and Trapp 2005). Three halophytes, sea purslane, *Halimione portulacoides*, small cordgrass, *Spartina maritima*, and shrubby swampfire, *Sarcocornia fruticosa*, not only were able to grow in TBT contaminated sediment but also showed the ability to enhance remediation of TBT. This may be explained by the enhancement of bacterial growth, among roots, which may include bacteria with ability to degrade butyltins (Carvalho, Basto *et al.* 2010). Our results revealed toxicity of tributyltin to the two plant species, *Brassica rapa* and *Triticum aestivum*. Plants exposed to TBT were smaller and had lowest biomass production. Hence, plant bioassays with this two species demonstrated to be useful ecotoxicological tools to evaluate the environmental risk and the achievement of remediation processes of this compound in a soil.

Additionally the use of more than two species is recommended, because it is known that different species have different sensitivity to TBT. TBT uptake in more and different plant species should be also regarded, because TBT represents a potential risk to human health, for example, via dietary intake, by consuming contaminated vegetables or fruits.

Further studies evaluating effects at different species and species representative of different trophic levels should be performed (Loureiro 2004).

Other remediation techniques have been performed along years. Bacterial strain isolated from a TBT polluted river in Osaka City, and inoculated to autoclaved estuarine water, revealed the capacity to degrade TBT. TBT degradation was helped by the addition of organic nutrient broth and occurred in 24h on the 4-20 μg Sn L^{-1} concentration (Kawai, Kurokawa *et al.* 1998). Other bacterium, *Aeromonas molluscorum* Av27, showed the ability to remove TBT from liquid culture medium - Marine Broth with TBTCl, in laboratory conditions (Cruz, Caetano *et al.* 2007). In our bioassay *A. molluscorum* Av27 was unable to diminish TBT toxicity in soil as evaluated by one of the parameters measured – seed germination in *Brassica rapa*. Even though, to plants that could germinate under exposure to Av27, an increase on biomass production was observed (in length and weight).

Complementary chemical analyses to plants should be performed to evaluate TBT uptake. It is important to know if plants taken up TBT and/or the bacterium and if the degradation byproducts of TBT, DBT and/or MBT, are present in plants. Chemical analyses to the soil of our bioassay should also be performed, to assess the presence of TBT or its major metabolites. Since, as referred by some authors, some degradation byproducts of TBT, such as MBT, may be more toxic than the parent compound TBT (Paton, Cheewasedtham *et al.* 2006).

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