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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob a orientação científica da Doutora Susana Patrícia Mendes Loureiro (Investigadora Auxiliar do Departamento de Biologia e CESAM da Universidade de Aveiro), e co-orientação da Doutora Sónia Alexandra Leite Velho Mendo Barroso (Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro).

o júri

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palavras-chave

Tributilestanho, toxicidade, *Porcellionides pruinosus*, *Folsomia candida*, *Aeromonas molluscorum* Av27, *Micrococcus luteus,* bioensaios, invertebrados e bioremediação.

resumo

Os compostos tributilestanho (TBT) foram amplamente usados como aditivos de tintas antivegetativas aplicadas em navios. Entre outros efeitos, o TBT é conhecido pela sua acção como disruptor endócrino. Em 2008, a International Maritime Organization - IMO proibiu o uso de tintas antivegetativas contendo TBT, mas devido à sua persistência e uso global durante os últimos 50 anos, este composto tornou-se ubíquo no ambiente. O TBT pode ser encontrado no compartimento terrestre, devido às suas diversas fontes de contaminação, e pode constituir um risco para a fauna do solo, nomeadamente para os invertebrados, e consequentemente para a função do solo. No entanto, a contaminação do solo por TBT, assim como os seus efeitos inerentes, têm recebido muito pouca atenção.

Actualmente, a degradação biológica é considerada a maior via de remoção do TBT do ambiente. Como exemplo, a bactéria *Aeromonas molluscorum* Av27 tem a capacidade de degradar o TBT em DBT (dibutilestanho) e MBT (monobutilestanho) em meio aquoso, pelo que, pode vir a ser aplicada no desenvolvimento de um biosensor para detectar TBT em solos e sedimentos, bem como em processos de bioremediação deste poluente.

Esta tese foi conduzida tendo em conta os seguintes objectivos: a) avaliar a toxicidade do TBT nos invertebrados *Porcellionides pruinosus* e *Folsomia candida*; b) usar bioensaios com *P. pruinosus* e *Micrococcus luteus* para avaliar o potencial da bactéria *Aeromonas molluscorum* Av27 degradar TBT em solo. Para avaliação da toxicidade do TBT, o isópode *P. pruinosus* foi exposto a alimento e solo contaminados com TBT, tendo sido avaliado o efeito da exposição pela inibição alimentar. Para além disso, foi efectuado um teste de evitamento com esta espécie. O colêmbolo *F. candida* foi exposto a solo contaminado por TBT e avaliou-se o seu sucesso reprodutivo. O sucesso do processo de bioremediação do solo por *A. molluscorum* Av27 foi avaliado através de bioensaios usando *M. luteus* como bactéria indicadora e um teste de inibição alimentar com *P. pruinosus*. O desenho experimental deste teste teve em conta todos os componentes de exposição e foi dividido em três sub-testes simultâneos: teste TBT-alimentação, com solo contaminado com TBT; teste TBT-meio crescimento, com solo contaminado com TBT em meio de cultura TSB; teste TBT-Av27, com solo contaminado com TBT, onde foi adicionada a bactéria *A. molluscorum* Av27 crescida em meio TSB.

Relativamente aos ensaios de toxicidade, os resultados da exposição dos isópodes indicam uma relação dose-resposta entre o decréscimo dos parâmetros de alimentação (rácios de consumo, assimilação e egestão) e a concentração de TBT.

Os resultados do teste de evitamento mostraram que esta espécie é capaz de detectar concentrações baixas de TBT e escapar do solo contaminado. O número de juvenis e a sobrevivência dos colêmbolos adultos também diminuiram com o aumento da concentração de TBT. *F. candida* mostrou ser mais sensível ao TBT que *P. pruinosus*, apresentando valores de EC_{50} e LC_{50} mais baixos.

Em relação aos ensaios de bioremediação, o bioensaio com *M. luteus* revelou que os solos em que *A. molluscorum* Av27 foi inoculada são menos tóxicos para este organismo, o que sugere uma redução da toxicidade do TBT pela sua degradação ou disponibilidade no meio. Relativamente ao bioensaio com isópodes, o teste TBT-meio crescimento revelou a maior toxicidade dos três testes e, comparando com o teste TBT-Av27, é possível que tenha ocorrido degradação do TBT por Av27. Apesar de apresentar um EC_{50} mais baixo que o EC_{50} do teste TBT-alimentação, os rácios de consumo do teste TBT-Av27 foram os maiores.

Como conclusão, os organismos teste revelaram-se espécies sensíveis à contaminação de solo por TBT, nas gamas de concentrações utilizadas, uma vez que este composto afectou o comportamento e alimentação dos isópodes e o sucesso reprodutivo dos colêmbolos. Os resultados da bioremediação necessitam de ser confirmados por análises químicas dos solos que demostrem se o TBT foi degradado em DMT e MBT, embora os resultados sugiram que *A. molluscorum* Av27 seja capaz de bioremediar TBT em solos. O bioensaio de alimentação com *P. pruinosus* e o bioensaio de inibição do crescimento da *M. luteus* demostraram ser potenciais ferramentas na avaliação da toxicidade de solos bioremediados anteriormente contaminados com TBT, embora o meio de cultura utilizado para crescimento da bactéria tenha alterado a toxicidade do composto químico. Neste âmbito, adaptações à metodologia poderão ser necessárias e novos estudos devem ser efectuados.

keywords

Tributyltin, toxicity, *Porcellionides pruinosus*, *Folsomia candida*, *Aeromonas molluscorum* Av27, *Micrococcus luteus*, bioassays, invertebrates and bioremediation.

abstract

Tributyltin (TBT) compounds were broadly used as ingredient of antifouling paints for ships. Among other effects, TBT is known to be associated with endocrine disruption. In 2008, the International Maritime Organization – IMO banned globally the use of TBT-based coatings, but due to their persistence and widespread use during the last 50 years, TBT became a ubiquitous compound in the environment. TBT reaches soil through various sources and constitutes a risk for soil fauna, namely the invertebrates, and consequently to soil function. However, soil contamination by TBT and its inherent effects have received very little attention.

Nowadays, biological degradation is considered to be the major pathway for TBT removal from the environment. As example, *Aeromonas molluscorum* Av27 is a bacterium that degrades TBT into DBT (dibutyltin) and MBT (monobutyltin) in aqueous solution and therefore has the potential to be used to develop a biosensor to detect TBT in soils and sediments and to bioremediate this pollutant.

This work was conducted considering the following goals: a) assess the toxicity of TBT on the invertebrates *Porcellionides pruinosus* and *Folsomia candida;* b) use bioassays with *P. pruinosus* and *Micrococcus luteus* to evaluate the potencial of TBT degradation in soil by the bacterium *Aeromonas molluscorum* Av27. For TBT toxicity evaluation, the isopod *P. pruinosus* was exposed to TBT contaminated food and soil and the exposure effects were assessed through feeding inhibition tests. An avoidance behaviour test was also carried out with this species. The collembolan *F. candida* was exposed to TBT contaminated soil and its reproduction success was evaluated. Soil bioremediation by *A. molluscorum* Av27 was assessed through bioassays using the bacterium *M. luteus* as indicator strain and a feeding inhibition test with *P. pruinosus*. The experiment took into account all components of exposure and was divided into three simultaneous sub-experiments: TBT-feeding test, with soil contaminated with TBT; TBT-growth media test, with soil contaminated with TBT in TSB growth medium; TBT-Av27 test, soil contaminated with TBT and inoculated with *A. molluscorum* Av27 in TSB medium.

Regarding the toxicity bioassays, the results of the isopod exposure indicate a dose related response between the decrease of feeding parameters (consumption, assimilation and egestion ratios) and toxicant exposure.

Results of the avoidance behaviour test showed that this species is able to detect very low concentrations of TBT and escape from the contaminated soil. The number of juveniles springtails and adult collembolan survival also showed a decrease with the increase of TBT concentration. *F. candida* revealed to be more sensitive to TBT than *P. pruinosus*, showing lower EC_{50} and LC_{50} values. Regarding bioremediation tests, the bioassay with *M. luteus* revealed that soils inoculated with *A. molluscorum* Av27 are less toxic to this organism, suggesting a decrease on TBT toxicity due to its degradation or its availability in the medium. From the bioassay with isopods, the TBT-growth medium test presented the highest toxicity of the three tests and its comparison with TBT-Av27 test may indicate TBT degradation by Av27. Besides the lower EC_{50} value of TBT-Av27 than the one obtained for TBT-feeding, it showed higher consumption ratios.

In conclusion, the tested organisms revealed to be sensitive to TBT contamination in soil, at the concentration ranges used, since TBT showed to affect the behaviour and feeding performance of isopods and the reproduction success of collembolan. Bioremediation results need to be confirmed by chemical analyses of soils to show that TBT was really degraded to DBT and MBT, although results obtained so far are suggestive that *A. mollucorum* Av27 is able to bioremediate TBT contaminated soils. Furthermore, feeding inhibition bioassay with *P. pruinosus* and inhibition growth bioassay with *M. luteus* are likely to be viable tools to assess toxicity from TBT bioremediated soils, although the culture medium used to grow the bacterium has changed the toxicity of the chemical compound. Therefore, adaptations to the methodologies may be needed and new studies should be performed as well.

Figure list

Table list

Chapter I: General Introduction

1. Soil contamination and Bioremediation

Soil environments play a central role in all terrestrial ecosystems. They have the potential to assimilate safely the chemical products of modern society, minimize adverse effects caused by human activities (Kloepper-Sams et al., 1996) and participate in many regulation services like climate regulation, flood control, detoxification and protection of plants against pests. They also mediate beneficial services (e.g. nutrient cycling, primary production and soil formation) that support most agro-sylvo-pastoral production systems (Lavelle et al., 2006). Soils contribute to cultural services, providing the physical substratum for virtually all human activities, such as buildings, agriculture, transport, resources for industrial use and waste management (Brussaard et al., 1997). This physical substratum has a great contribution to economy, for example, by providing physical support to shelter seeds and anchoring plant roots, which costs would be enormous without soil (Daily et al., 1997).

Soil contamination has become a serious problem in many industrialized countries. In regions with a high population density this issue gained a major proportion because of the intensively use of land (Van Straalen, 2002). The problematic of contaminated land is worldwide and it is recognized as a potential threat for both wildlife and human health, which led to international efforts to remedy those sites (Garbisu and Alkorta, 2000; Vidali, 2001).

There are several options to remediate soils, including incineration, cap and contain contaminated areas (Vidali, 2001), soil excavation and disposal to landfill sites (Christofi and Ivshina, 2002) and bioremediation (Virkutyte et al., 2002). Soil bioremediation can be a desirable cost-effective method for removing contaminants from soil-contaminated sites (Christofi and Ivshina, 2002).

Bioremediation can be defined as the use of living organisms, primarily microorganisms, to degrade environmental contaminants into less toxic forms from soil, water and gases (Vidali, 2001; Sarkar et al., 2005). It is an emerging technology and an option to conventional clean-up technologies, because is a low-cost and less complex method, and uses natural biological activity. The residues of treatment are expected to be generally harmless products (Vidali, 2001), offering a terminal solution and eliminates future liability costs (Timmis and Pieper, 1999; Alvarez and Illman, 2006).

Bioremediation utilizes the natural role of microorganisms, profiting from transformation or mineralization processes by directing those capabilities toward organic and inorganic environmental pollutants (Bollag et al., 1994). Fungi and plants are also used to degrade or detoxify hazardous substances through reactions that take place as a part of their metabolic processes (Vidali, 2001). It can occur by a natural process without human intervention – intrinsic bioremediation or natural attenuation (Mulligan and Yong, 2004; Ward and Singh, 2004). When natural attenuation does not remove/transform contaminants at a sufficient rate, it is required the construction of strategies to enhance natural detoxification – engineered bioremediation (Council, 1993).

Although, bioremediation has also its limitations, such as slow rate of degradation (Dua et al., 2002) and the requirement for extensive monitoring to ensure its success (Alvarez and Illman, 2006). In addition several contaminants, such as heavy metals, are not amenable to biodegradation (Boopathy, 2000), but can be immobilized by the decrease of its bioavailability and therefore their toxicity (Kamnev and Lelie, 2000; Gadd, 2004).

Soil bioremediation involve *in situ* and *ex situ* techniques (Scow and Hicks, 2005). *In situ* bioremediation generally involve the enhancement of indigenous microbial activity (Bollag et al., 1994) and are preferred techniques because they provide the treatment at the local, avoiding excavation and transportation of the contaminated soil, which reduces the exposure risks for clean-up personnel and for transportations accidents (Vidali, 2001). They have been successfully used to remediate fuel-contaminate soil (Hoeppe et al., 1991). *In situ* techniques are bioventing, biostimulation, biosparing (involving the supply of oxygen and nutrients) (McClure and Sleep, 1996; Kosaric, 2001; Kao et al., 2008) and bioaugmentation (inoculation of microorganisms of specific biotransforming abilities to the contaminated site in order to enhance biodegradation) (Vogel, 1996; Gentry et al., 2004).

Even though soil bioremediation research has focused on the role of microorganisms, there is an emerging *in situ* technique for soil bioremediation that uses terrestrial plants and their associated microbes for environmental clean-up from a variety of organic and inorganic pollutants. This technique is designed as phytoremediation (Knox et al., 1999; Pilon-Smits, 2005).

In *ex situ* bioremediation, soil is excavated and removed from the ground. Soil can be treated on or off site, which is a more rapid method. Landfarming, composting, biopiles and bioreactors are examples of those technologies (Vidali, 2001; Basharudin, 2008).

2. Tributyltin Compounds

2.1. Production, uses and restrictions

Tributyltin (TBT) compounds were first design to kill several species of freshwater snails. In the early 1960s, they became extensively used as components of marine antifouling paints to prevent the attachment and growth of fouling organisms, such tube worms and barnacles, on the hulls of vessels, improving the speed and economic efficiency of the ships (Champ and Seligman, 1996; Antizar-Ladislao, 2008). Their extremely efficiency and low economic costs led to the dominance of the market, being applied to more than 70% of the world's commercial shipping fleet. TBTs were used on ships and vessels, harbours structures, pleasure boats and aquaculture nets (Evans et al., 2000; Santillo et al., 2001; Thomaidis et al., 2007), therefore ending up in aquatic ecosystems, mainly accumulated in sediments.

TBT compounds are broadly used as biocides (e.g. insecticides, fungicides, bactericides, pesticides, slimicides) to control a wide spectrum of organisms. They are used as fungicides in the preservation of wood and are added to a variety of PVC-materials to protect against fungal and microbial attack. Thus, these compounds are still present in industrial water systems (cooling tower and refrigeration water systems), paper mill systems, wood pulp, breweries, windows frames, dispersion paints and a variety of household commodities and textiles, such clothes, furniture and food/beverage containers (Fent and Müller, 1991; Hoch, 2001; Marcic et al., 2006; Cruz et al., 2007; Antizar-Ladislao, 2008; Kannan and Tanabe, 2009).

Examples of commercial TBT-based pesticides products that are available are Lastanox (Pelikan, 1969), Sanitized Brand Bacteriostat RB-475, Ultrafresh 300 DD Nonionic, Sanitized Brand Bacteriostat OA-P, Pentox® Green Wood Preservative, Osmose End Cut Wood Preservative (Moss), Cuprinol Siding Stain & Preservative, bioMeT® TBTO (Health Canada, 2010) and bioMeT® 66 (Walser, 1980).

TBT is considered the organotin compound of most concern due to its persistence, bioaccumulation and high toxicity to non-target organisms (Thomaidis et al., 2007). In the early 1980s, TBT was recognized as the cause for shells malformations of the Pacific oyster (*Crassostrea gigas*) in France's Arcachon Bay, and for imposex (superimposition of

male sexual characteristics onto prosobranch females) in dogwhelks in the UK (Barroso and Moreira, 2002; Horiguchi et al., 2005; Kannan and Tanabe, 2009).

The decline in Pacific oyster production led France to be the first country banning the use of TBT-paints on small boats (< 25 m) in 1982 (Alzieu, 2000a). Similar legislations were adopted in most of European countries after 1989 and Portugal adopted the legislation in 1993 (Ruiz et al., 1996; Barroso et al., 2002; Díez et al., 2005; Galante-Oliveira et al., 2006). The European Union (EU) implemented in 2003 the Directive 2002/62/EC imposed by the International Marine Organization (IMO) that prohibits globally the application of TBT-based coatings on any boat in 2008 (Ruiz et al., 2008). However, no restrictions were applied on other uses of TBT.

2.2. Properties and physico-chemical characterization

Tributyltins are organotin compounds characterized by a central tin atom (Sn^{4+}) covalently bound to three organic substitutes, which are butyl groups (*n*-C_4H_9) (Fig. 1). They have the general formula R_3Sn-X, where R= butyls and X is a singly charged anion or anionic group (e.g. hydroxide, chloride or oxide) (Hoch, 2001; Fromme et al., 2005; Antizar-Ladislao, 2008). In general, the inorganic tin is non-toxic (Hoch, 2001) and the X group has little or no effect on the properties and biocide activity, unless if itself is a toxic component. The butyl radicals confer bactericide and fungicide properties to tin (Alzieu, 2000b). Generally, the toxicity of organotin compounds increases on the following order: R_4Sn < $RSnX_3$ < R_2SnX_2 < R_3SnX (Dubey and Roy, 2003). Therefore, trisubstitutes organotins, such TBT, are the most toxic, followed by diorganotins, monoorganotins and tetraorganotins (Ebdon et al., 1998). TBT derivate products are DBT (dibutyltin), then MBT (monobutyltin) and, ultimately, inorganic tin (Antizar-Ladislao, 2008).

In natural waters, TBT compounds are present predominantly as neutral form species (TBTOH) or as cationic species (TBT^+). In aqueous solution, the dissociation constant (pK_a) for TBT varies between 6.25 and 6.51 (Hoch et al., 2003). At pH < pK_a the cationic form (TBT^+) is the main specie, at pH > pK_a the neutral form (TBTOH) dominates and when pH = pK_a, [TBT^+] = [TBTOH] (Meador, 2000; Hoch et al., 2002; Hoch et al., 2003; Bangkedphol et al., 2009). Some studies suggest cation exchanges processes of TBT^+ to the negative charged surfaces as the main adsorption mechanism of TBT onto mineral surfaces (Hoch et al., 2002). On the other hand, neutral species tend to adsorb

onto organic matter in sediments (Weidenhaupt et al., 1997) Sorption behaviour of TBT is influenced by several factors, such as physicochemical properties of minerals, temperature, pH and salinity (Hoch et al., 2002; Fent, 2003).

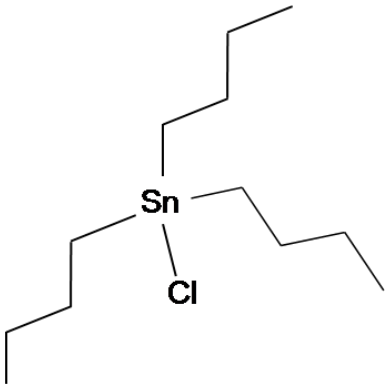

Fig. 1. Molecular structure of tributyltin chloride (from OECD, 2001).

Some studies showed that TBT presents a partition coefficient (K_d) value that also indicates hydrophobicity and higher affinity for sediments, but can be strongly influenced by salinity and pH (Langston and Pope, 1995; Meador, 2000). Highest adsorption is documented to occur at pH range 6-7 (Weidenhaupt et al., 1997; Hoch et al., 2002; Hoch and Weerasooriya, 2005) however information about the influence of salinity is divergent. For instance, Unger et al. (1988) reported a decline of TBT adsorption with increasing salinity, while Randall and Weber (1986) and Harris and Cleary (1987) stated the opposite trend (cited by Hoch (2004a)). In the study of Hoch (2004a) the highest adsorption was found at a salinity of 0%.

TBT presents low solubility (less than 10 mg/L at 20 °C and pH 7.0) and high specific gravity (around 1.2 Kg l^{-1} at 20 ºC). Therefore, when released from antifouling paints into water, TBT is quickly removed from the water column and adheres to bed sediments (Landmeyer et al., 2004; Antizar-Ladislao, 2008).

TBT presents moderately high octanol-water partitioning (K_{ow}), with determined log K_{ow} for seawater of 3.57 and 4.70 for TBTO and TBTCl, respectively (RPA, 2005; Murai et al., 2008). Those K_{ow} values contribute to its lipophilic properties and therefore to its high affinity to the lipids of tissues and membranes, with consequent uptake and bioaccumulation by marine organisms (Meador, 2000; Coelho et al., 2002).

2.3. TBT compounds in the environment: sources, fate and distribution

The widespread application of TBT compounds in a variety of anthropogenic activities caused the introduction of large amounts into the environment, reaching various ecosystems (Hoch, 2001).

The release of TBT from antifouling paints constitutes a direct input into the aquatic environment. Once in the water, TBT is easily adsorbed onto suspended particulate matter with subsequent sedimentation, which is a significant transport of TBT to bed sediments (Díez et al., 2005). TBT can also be removed from the water column by photo and biodegradation, biological uptake and flux (Adelman et al., 1990; Harino et al., 1997; Huang and Matzner, 2004).

The most common mode of TBT assimilation by aquatic organisms is generally via diet at the sediment-water interface, but TBT that remains in the water column is also uptaken by aquatic organisms (Eggleton and Thomas, 2004). The adsorption of TBT to suspended particles is reversible therefore, benthic sediments are regarded as the major sink for TBT in the environment (Hoch et al., 2002; Burton et al., 2004; Kannan and Tanabe, 2009) and a long-term source of dissolved-phase contamination to the water column (Antizar-Ladislao, 2008). Besides, TBT half-lives in sediments are higher (months, even years) (Ebdon et al., 1998; Harino et al., 1998) than in the water column (from several days to several weeks) (Watanabe et al., 1995; Dowson et al., 1996), which means that TBT is slowly degraded, and then more persistence, in sediments (Harino et al., 1998). Remobilization of contaminated bottom sediments by natural events, as tidal movement and storms, or human activities such as fishing and dredging (Eggleton and Thomas, 2004) may act as new contamination source in the overlaying water column (Díez et al., 2005). Several studies reported that after the legislative prohibitions on the usage TBT-based paints, TBT concentrations reduced in aquatic systems, registering the highest concentrations in coastal marine areas (ports and shipyards) due to stationary ships. Little or no reduction of TBT concentrations was observed in sediments (Kotrikla, 2009).

The illegal use of TBT-based antifouling in developing countries has to be taken into account as a continuous source to marine waters (Takahashi et al., 1999b). Dumping TBT polluted sediment into the sea was a concerning source, but the OSPAR convention release limited guidelines that reduced this issue (Novak and Trapp, 2005).

Dredging and disposal of harbour sediments on land constituted a major pathway of TBT into soil (Schaefer, 2005). It was developed an assessment framework for the re-use of organotin containing treated sediment on land as secondary granular building material (Cornelis et al., 2005; Cornelis et al., 2006). Another important source for TBT contamination in soil is its use as biocide in agriculture soils (Champ and Seligman, 1996), because the pulverization of those products can contaminate the surrounding soil, water and air, and can reach ground waters by leaching and runoff (Hoch, 2001). A recent study indicated that atmospheric deposition of TBT is a source for soil contamination to be also considered (Huang et al., 2004), as well as emissions from treated wood to air, water and mainly to soil (EUSES, 2002).

In wastewater treatment plants (WWTPs), TBT is transferred from wastewater to sewage sludge, due to its tendency to adsorbed onto particles (Díez et al., 2002). Thus, the disposal of sewage sludge to be used as soil amendment is a further important source of TBT contamination in the soil compartment, and its consequent leaching can contaminate ground waters (Fent, 1996b; Marcic et al., 2006). Nevertheless, the discharge of municipal and industrial wastewaters can contaminate freshwater systems and marine waters (Snoeij et al., 1987).

Due to lipophilic nature of TBT compounds they have the ability to bioconcentrate and bioaccumulate in aquatic organisms, especially by filter feeding, such bivalve molluscs and gastropods (Oberdörster et al., 1998). Laboratory studies with fish and molluscs determined bioconcentration factors (BCFs) for TBT up to 7000, and higher values have been reported in field studies (WHO-IPCS, 1999). A BCF of 25.000 was estimated for the oyster *Crassostrea gigas* (Shim et al., 1998). Therefore, TBT is not only present in water, sediments, air and soil compartments, but also into biota (Hoch, 2001), which potential leads to biomagnification along food webs.

Considerable levels were also measured in tissues and organs of marine mammals and higher trophic marine predators, such as dolphins, tuna, sharks (Kannan et al., 1996), porpoises (Iwata et al., 1995), pinnipeds (Tanabe, 1999), whales and also fish-eating birds and turtles (Iwata et al., 1997; Berge et al., 2004), suggesting a considerable bioconcentration potential (Fent and Hunn, 1991), bioaccumulation and consequent biomagnification through marine food webs (Murai et al., 2008).

Ingestion of contaminated seafood is thought to be the most important route of TBT exposure to humans. Leaching from food packers is another contamination source

for seafood (Kannan et al., 1995; Takahashi et al., 1999a). For this reason, studies of health risks have mainly focused on seafood (Belfroid et al., 2000; Rantakokko et al., 2006). A Tolerable Daily Intake (TDI) value for TBT of 0.25 µg/Kg body weight per day was determined (Belfroid et al., 2000). A market basket survey conducted in Shiga, Japan, estimated a TBT daily intake of 6.7 µg/day. This value does not exceed the NOEL of 0.25 µg/Kg body weight per day, but 95% of the total intake was from fish and shellfish sources (Tsuda et al., 1995; Takahashi et al., 1999a). The domestic use of wood preservatives and plastics containing TBT also constitutes a TBT exposure to humans, which can be contaminated by inhalation and absorption through the skin (Adeeko, 2003).

As it can be seen from Table 1, which indicates TBT concentrations in waters, sediments, wastewaters, sewage sludge and tissues/organs samples reported in several studies from different countries around the world, TBT represents a serious hazard for the environment and human health.

Table 1
Tributyltin concentrations in seawater, sediments, wastewater, sewage sludge and biological samples reported for several countries.

Location	Year	[TBT]	Units	References
Seawater				
South east coast, France	1993	<0.015–0.12	ng Sn l^{-1}	Michel and Averty (1999)
Coastal waters, Greece	1998-1999	n.d. - 70	ng Sn l^{-1}	Thomaidis et al. (2007)
North coast of Kyoto, Japan	2003	3.9-27	ng Sn l^{-1}	Ohji et al. (2007)
Harbours in Huelva coast, Spain	1993	>100	ng Sn l^{-1}	Gomez-Ariza et al. (1998)
Sado Estuary, Portugal	-	39-870	ng Sn l^{-1}	Hoch (2001)
Arcachon Bay, France	1977-1981	>100	ng Sn l^{-1}	Ruiz et al. (1996)
Arcachon Bay, France	late 1980s	~1	ng Sn l^{-1}	Ruiz et al. (1996)
Sediments				
Port of Osaka, Japan	1996	10-1200	ng Sn (g dw)$^{-1}$	Harino et al. (1998)
Marinas, Arcachon Bay, France	1990	>158	ng Sn g^{-1}	Saekadin et al. (1991)
Coast, Portugal	1999-2000	<3.8-12.4	ng Sn (g dw)$^{-1}$	Díez et al. (2005)
North-Western Sicilian coast, Italy	1999-2000	3-27	ng Sn (g dw)$^{-1}$	Chiavarini et al. (2003)
Southwest Spain	1994	<0.6-160	ng Sn g^{-1}	Gomez-Ariza et al. (2001)
Mumbai harbour, India	2000-2001	4.5-1193	ng.g^{-1} dw	Bhosle et al. (2006)
Bahía Blanca, Argentina	2004-2006	n.d.-3.288	ng Sn g^{-1}	Delucchi et al. (2006)
Burrard Inlet, Canada	1999	>5092	ng Sn (g dw)$^{-1}$	Maguire and Batchelor (2005)
Barcelona harbour, Spain	2002	98-4702	ng Sn g^{-1}	Díez et al. (2006)
Ria de Aveiro, NW Portugal	2005	2.7-1780	ng Sn (g dw)$^{-1}$	Sousa et al. (2007)
Wastewaters and sewage sludge				
Zürich, Switzerland (wastewater)	1988-1989	64-217	ng.l^{-1}	Fent and Müller (1991)
Zürich, Switzerland (sew. sludge)	1988-1989	0.28-1.51	mg/kg dw	Fent and Müller (1991)
Swiss (sewage sludge)	2001	18.6-648.5	µg.kg^{-1} dw	Plagellat et al. (2004)
Toronto, Canada (sewage sludge)	1999-2000	72-502	ng Sn g^{-1}	Lee et al. (2004)
Biological tissues/organs				
Porpoise[a], Norwegian	1998-2000	18-156	µg Sn kg^{-1} ww	Berge et al. (2004)
Common seal[b], Norwegian	1998-2000	<3	µg Sn kg^{-1} ww	Berge et al. (2004)
Human[a], Japan	1997-1998	<2	ng.g^{-1} ww	Takahashi et al. (1999a)
Raccon dog[a], Japan	1992-1994	5-7.4	ng.g^{-1} ww	Takahashi et al. (1999a)
Mussel, Chinhae Bay, Korea	1994	0.12-1.21	µg Sn g^{-1} dw	Hwang et al. (1999)
Oyster, Chinhae Bay, Korea	1994	0.10-1.80	µg Sn g^{-1} dw	Hwang et al. (1999)
Blue shark[c], Mediterranean Sea	1992	53-180	ng.g^{-1} ww	Kannan et al. (1996)
Bluefin tuna[d], Mediterranena Sea	1992	7.3-170	ng.g^{-1} ww	Kannan et al. (1996)
Bottlenose dolphin[a], U.S. Attlantic	1989-1994	5.8-770	ng.g^{-1} ww	Kannan et al. (1997)
Nassarius reticulatus, Ria de Aveiro	2005	15-73	ng Sn (g dw)$^{-1}$	Sousa et al. (2007)
Fish, The Netherlands	1993	9.2-67	ng.g^{-1} ww	Stäb et al. (1996)
Human blood, Michigan, U.S.A	1998	<1-85	ng.ml^{-1}	Kannan et al. (1999)

n.d.= non-detected
[a] liver
[b] blubber
[c] kidney
[d] muscle

2.4. Toxicity and biological effects

Despite legislation restrictions for TBT-based paints usage, this pollutant is still present in the aquatic environment at concentrations that can cause toxic effects on organisms (Bangkedphol et al., 2009). TBT is among the most toxic compounds ever introduced deliberately into the aquatic environment (Hwang et al., 1999).

TBT is a strong endocrine disruptor, causing imposex in marine invertebrates (Shimisaki et al., 2003). Several studies suggest that TBT acts as a competitive inhibitor of cytochrome P450-dependent aromatase, and aromatase inhibition results in a build-up of testosterone and consequently in imposex (Bettin et al., 1996; Matthiessen and Gibbs, 1998).

Molluscs are known to be the most sensitive species to TBT, for which the no observed effect concentrations (NOECs) are below 1 $ng.l^{-1}$ (Alzieu, 2000b) and concentrations below 1 $ng.l^{-1}$ cause imposex in gastropod females (Bettencourt et al., 1999). The large decline in dogwhelks (*Nucella lapillus*) populations in western Europe was a especially evidence of TBT-induced imposex (Oehlmann et al., 1996). Levels exceeding 1 $ng.l^{-1}$ limit also cell division and reproduction in some species of phytoplankton and zooplankton, respectively (Alzieu, 2000b). Concentrations above 2 $ng.l^{-1}$ provoke shell calcification anomalies in oysters and at 20 $ng.l^{-1}$ can induce disturbances in the reproduction of bivalve molluscs (Alzieu, 1998). Levels around 1-10 $\mu g.l^{-1}$ affect fish reproduction and between 1-1000 $\mu g.l^{-1}$ cause disturbances on fish behaviour (Alzieu, 2000b). Crustacean exuviations are affected at levels of 500 $ng.l^{-1}$ (Fent, 1996a; Alzieu, 1998; Oberdörster et al., 1998). It has been also demonstrated the masculinisation in fish when exposed to TBT (Shimasaki et al., 2003).

TBT is also a xenobiotic mitochondrial toxin (Stridh et al., 1999). It seems that the primary target of TBT is the immune system (Rice et al., 1995). TBT is known to be a immunosuppressive agent in mammals and their effects in rodents include thymic and splenic atrophy, reductions in thymic, circulating and splenic lymphocytes, suppression of T-cell-dependent immunity and suppression of tumoricidal activity (O'Halloran et al., 1998). In addition, TBT is able to pass through the placenta (Cornelis et al., 2005).

Several laboratory experiments with animals (such as rats, mice, dogs, rabbits) were carried out to extrapolate the toxic effects of TBT to humans (WHO-IPCS, 1999). These studies have demonstrated that TBT causes immunotoxicity, neurotoxicity,

teratogenicity, potential ocular and dermal toxicity, blood dyscrasias and possibly carcinogenic activity (Iwata et al., 1995; Iwata et al., 1997; Nakanishi, 2007).

Studies with soil invertebrates showed that TBT induces deleterious effects, in particular, to earthworms (Hund-Rinke and Simon, 2004; Römbke et al., 2007), showing high mortality rates and reduced reproduction (Schaefer, 2005). Hund-Rinke and Simon (2004) also suggest a possible endocrine effect on earthworms, because their results showed an EC_{50} value for earthworm reproduction far below the EC_{50} calculated for other test parameters. The reproduction success of collembolan (*Folsomia candida*) is also affected by TBT as well as the growth of the terrestrial plant *Brassica rapa*, however at higher concentrations (Hund-Rinke and Simon, 2004; Römbke et al., 2007).

A study with potatoes and beans demonstrated that TBT can be transferred and concentrated into plants after root uptake (Lespes et al., 2003). TBT has been reported to be non-toxic to willow trees and considered to be less toxic to higher plants (Trapp et al., 2004).

3. Bioassays with invertebrates for soil quality evaluation

Soil quality can be defined as “the capacity of a soil to function within “ecosystem boundaries” to sustain biological productivity, maintain environmental quality, and promote plant and animal health” (Kremer and Li, 2003). The ecotoxicology research for soil quality can be carried out by monitoring the status of communities in real ecosystems or through laboratory toxicity tests (bioassays) (Sochová et al., 2006).

Bioassays provide a more direct measure of relevant environmental toxicity of contaminated sites than chemical analyses, because organisms respond to biologically active components and the bioavailable fraction of the chemical, and results are an integration of all environmental variables and contaminants (Keddy et al., 1995). Bioassays with soil invertebrates are used as tools to evaluate contamination and toxicity in soils, determining whether the soil is in a natural state or the degree to which it is affected by pollution or other human activities (Ruf, 1998). Test organisms should be representative of edaphic functional roles and sensitive to the contaminants (Fountain and Hopkin, 2005; Fernandez and Tarazona, 2008). Soil invertebrates are good indicators of soil quality, because they are key organisms for the functioning of soil processes (Ruf,

1998). The most widely used soil invertebrates include earthworms, springtails (Collembola), enchytraeids and isopods because of their role in essential ecological processes of soil, such as decomposition, nutrient cycling and influence on soil core structure and texture (Blakely et al., 2002; Fernandez and Tarazona, 2008).

Bioassays are increasingly being incorporated in ecological assessment of soil remediation (Plaza et al., 2005). Chemical analyses are usually used to evaluate the removal efficiency of the pollutants (Abdel Migid et al., 2007) by measuring concentrations of target compounds and their degradation products however, in some cases, bioremediation may lead to the formation of potentially more toxic compounds and also increase their bioavailability (Mendonca and Picado, 2002). Hence, to monitor changes in toxicity during bioremediation, bioassays are often recommended to complement chemical analyses of contaminants and for evaluation of the effectiveness of soil remediation processes (Van Gestel et al., 2000; Abdel Migid et al., 2007).

Soil toxicity testing involves a wide range of methods with soil invertebrates, but most are poorly described (Kapanen and Itävaara, 2001). Standard laboratory tests currently available include: 1) acute toxicity tests, for assessing earthworm mortality (*Eisenia fetida*) (ISO, 1998a) and avoidance behaviour (*Eisenia fetida* and *Eisenia andrei*) (ISO, 2007) and collembolan (*Folsomia candida*) behaviour (ISO, 2008); 2) chronic tests to assess reproduction efforts in collembolan (*Folsomia candida*) (ISO, 1999), earthworms (*Eisenia fetida* and *Eisenia andrei*) (ISO, 1998b), enchytraeids (*Enchytraeus albidus*) (ISO, 2001) and predatory mites (*Hypoaspis (Geolaelaps) aculeifer*) (OECD, 2008).

Bioassays have also been performed to evaluate mortality of molluscs, beetles, predatory mites, isopods and spiders; reproduction of beetles, isopods and oribatid mites; behaviour of spiders, isopods and beetles and also survival, reproductive behaviour, dormant state and new shell growth with molluscs (Drobne, 1997; Løkke and Van Gestel, 1998; Loureiro et al., 2005).

The most widely used ecotoxicological endpoints are mortality, growth and reproduction. From an ecotoxicological point of view, mortality is a less sensitive parameter (Yasmin and D'Souza, 2010), because the chronic test, aiming at sublethal effects, is more sensitive, presenting a more realistic approach for the prediction of environmental effects, since the exposure concentrations of contaminants in the field are usually quite low (Römbke et al., 2007). Besides, the lethal effects of a chemical does not necessary result in intoxication, and sublethal effects may be produced (Yasmin and

D'Souza, 2010), such as effects on growth and fertility (Paoletti, 1999), and even small changes in these parameters can disturb the balance in a biocoenosis quite drastically (Schober and Lampert, 1977).

In nature most animals use chemical cues to move to best places (e.g. for feeding, reproduction) and to escape from deleterious environments (Aldaya et al., 2006). Avoidance behaviour is a relevant ecotoxicological endpoint because it is directly related with the energy budget of the individuals and indirectly with the soil structure (Amorim et al., 2005). Avoidance tests are inexpensive tools, easy to perform and sensitive to several contaminants. They also represent a strong endpoint to predict and assess environmental contamination effects in soil organisms (Santos et al., 2010), indicating the stress potential of a particular soil (Natal da Luz et al., 2004). The use of avoidance tests has been pointed as a good screening tool to initiate a proper assessment of soil toxicity (Aldaya et al., 2006).

In this dissertation bioassays were carried out with the soil invertebrates *Folsomia candida*, Willem 1902 (Collembola: Isotomidae) and *Porcellionides pruinosus*, Brandt 1833 (Isopoda: Oniscidea). Collembolans are among the most abundant soil arthropods in terrestrial ecosystems (Eaton, 2006). They are an integral part of soil ecosystems and vulnerable to the effects of soil contamination (Fountain and Hopkin, 2005). *Folsomia candida* is the most frequently used microarthropod for lethal and sublethal testing (Cortet et al., 1999), and have been successfully used as test organism to monitor soil remediation of contaminated soils (Fountain and Hopkin, 2005).

They are essential on decomposition processes and play important roles in food chains as prey for several invertebrates and small vertebrates (Nakamori et al., 2008), thus standardized protocols were established for the assessment of reproduction success (ISO, 1999) and avoidance behaviour (ISO, 2008) of this species. Besides, they are easy to culture in laboratory, have short generation time and a uniform genomic structure (reproduces by parthenogenesis), and are of easy manipulation during the experimental procedure (Markwiese et al., 2001). Reproduction tests with *Folsomia candida* are considered to be one of the most advanced ecotoxicological tests with soil arthropods (Paoletti, 1999).

Isopods are good candidates as standard tests species because they are wide spread and abundant in the soil upper layer, easy to handle and maintain in laboratory,

and respond quickly to environmental contamination (Markwiese et al., 2001; Loureiro et al., 2005). They are considered to be optimal indicators of metal pollution because they are able to accumulate large amounts of heavy metals from soil and food and persist in heavily polluted areas (Köhler et al., 1996). Isopods have chemoreceptors which allow them to detect and avoid contaminated soils. Therefore, they are also used as test species in avoidance behaviour assays (Loureiro et al., 2005). However, no standard tests are available for this group and reproduction success is difficult to evaluate due to its long reproductive cycle and the fact that females can retain sperm for long periods (Kapanen and Itävaara, 2001).

Porcellionides pruinosus is a synanthropic isopod and one of the most widely distributed species of terrestrial isopods (Michel-Salzat et al., 2001). Isopods feed on leaf litter, being crucial in the decomposition and nutrient cycling processes in soil ecosystems (Loureiro et al., 2002; Zimmer, 2002) and respond to contaminants in their food. This particular species play an important role in the decomposition of agriculture and cattle wastes (Loureiro et al., 2002). Ecological functions of isopods are extremely affected by food consumption rates (Drobne, 1997) thus, given the role of isopods in decomposition processes through litter fragmentation, feeding parameters are used as important toxicological endpoints, through feeding performance tests (Loureiro et al., 2006). Furthermore, differences in food quality have been shown to affect key populations parameters in several groups of decomposers (Rushton and Hassal, 1983).

4. Aims and dissertation structure

This dissertation was undertaken with the following aims:

1. Assess TBT toxicity on the invertebrate species *Porcellionides pruinosus* and *Folsomia candida*;

2. Taking the advantage of the potential of *Aeromonas molluscorum* Av27 to degrade TBT, employ bioassays with *Micrococcus luteus* and *Porcellionides pruinosus* to evaluate the capacity of the bacterium to bioremediate TBT contaminated soil.

In parallel to the second objective, it was also our aim to investigate whether bioassays with *Porcellionides pruinosus* are good ecotoxicological tools to assess the toxicity of bioremediated soils.

This dissertation is divided in four chapters. Chapter 1 contains a current General Introduction. Chapters two and three are structured in the form of scientific papers, reporting the results on the assessment of TBT toxicity on the test organisms (Chapter 2), the results of soil bioremediation by *A. molluscorum* Av 27 and the evaluation of bioremediation success by using feeding inhibition test with *P. pruinosus* and inhibition growth bioassay with *M. luteus* (Chapter 3). Chapter 4 consists on the General Discussion and Conclusion.

5. Relevance of the dissertation

The negative impact of TBT compounds on the aquatic environment is well documented and has led to restrictions on the use of TBT-based paints (Azenha and Vasconcelos, 2002). However, terrestrial environments have not received as much attention as waters and sediments (Marcic et al., 2006) and, to the best of our knowledge, there is no restrictions on TBT biocide usage beyond antifouling paints.

The disposal of TBT contaminated sediments from harbours and sewage sludge on land and agricultural soils are pathways of major concern. Although, the possible effects on terrestrial ecosystems and humans are much less studied (Cornelis et al., 2006).

Given the crucial role of terrestrial invertebrates on soil functions is important to understand the effects of TBT on those organisms. However, few studies have investigated the TBT effects on soil invertebrates (Hund-Rinke and Simon, 2004; Schaefer, 2005; Römbke et al., 2007). So far, TBT toxicity on the terrestrial isopod *Porcellionides pruinosus* has not been investigated.

Bioremediation is a promising clean-up technology and could be a significant method to remediated TBT contaminated soils. *Aeromonas molluscorum* Av27 is a bacterium that is able to degrade TBT into DBT and MBT in aqueous solutions and can potentially be used as a tool to remediate TBT contaminated environments (Cruz et al.,

2007). Therefore, it is of great relevance to investigate its ability to degrade TBT in soils as well as to evaluate the effects of the bioremediated soil on indicator species. Since bioassays are recommended for the assessment of soil quality during and after remediation processes herein bioassays with the isopod *Porcellionides pruinosus* and the bacterium *Micrococcus luteus* were employed to investigate TBT bioremediation by *A. molluscorum* Av27.

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Chapter II: Toxicity of Tributyltin (TBT) on the terrestrial invertebrates *Porcellionides pruinosus* and *Folsomia candida*

Abstract

The contamination of terrestrial environment by disposal of TBT contaminated harbour sediments, sewage sludge and biocide products has been raising concerns and it may pose a risk to soil invertebrates.

The aim of this study was to assess the toxicity of TBT on the terrestrial invertebrates *Porcellionides pruinosus* and *Folsomia candida*. For this, two feeding inhibition tests (with contaminated food and soil) and an avoidance behaviour assay were performed to evaluate TBT toxicity to isopods and the standard reproduction test with the collembolan *Folsomia candida* was also carried out. Results of the avoidance behaviour test showed that isopods are able to detect and avoid TBT contaminated soils at very low concentrations. Results from both feeding inhibition tests revealed a decreased of feeding parameters with the increase of toxicant exposure via food or soil. A dose-response relationship was also observed for the survival of isopods and adult collembolan and the number of juvenile springtails. *P. pruinosus* showed to be less sensitive than *F. candida*, with higher LC_{50} and EC_{50} values upon soil exposures. In conclusion, TBT proved to be toxic to the test organisms at the concentration ranges used, affecting the survival of both species, the behaviour of *P. puinosus* and reproduction success of *F. candida*.

Keywords: Tributyltin, *Porcellionides pruinosus*, *Folsomia candida*, invertebrates, toxicity.

1. Introduction

Tributyltin (TBT) compounds were widely used as antifouling agents on ships and aquaculture facilities since 1960s (Murai et al., 2005). Other uses include pesticides, fungicides, bactericides, wood preservatives, PVC stabilizers (Cruz et al., 2010a) and slime control in paper mills (Corsini et al., 1997).

TBT is worldwide recognized as an endocrine disruptor (Lintelmann et al., 2003), being the cause of imposex on female dogwhelks and shell thickening on oysters during the 1980s (de Mora and Pelletier, 1997; Evans and Nicholson, 2000; Morcillo and Porte, 2000). In vitro studies suggest that TBT may cause immunotoxicity, teratotoxicity and neurotoxicity in mammals, including humans (Snoeij et al., 1987; Whalen et al., 1999; Girard et al., 2000; Cooke, 2002; Tsunoda et al., 2006).

During the last three decades TBT aquatic contamination has been intensively investigated due to its adverse effects on marine organisms at very low concentrations (Svavarsson, 2000). Although, the terrestrial environment has received much less attention (Cornelis et al., 2006). The dredging of harbour sediments on land (Köthe, 2003) and the disposal of sewage sludge on agricultural soils (Heroult et al., 2008) constitutes the major pathways for TBT soil contamination (Schaefer, 2005). Pulverization of biocide products (Kannan and Lee, 1996), irrigation with contaminated water (Heroult et al., 2008) and atmospheric deposition (Huang and Matzner, 2004) are other sources of TBT pollution on the terrestrial compartment.

TBT-based compounds are very toxic, bioaccumulative and persistent (Maguire, 2000), with reported half-life values in soil ranging from 15 weeks to several years (Lespes et al., 2009). Hence, their presence in the soil compartment give rise to great concern and the risk of TBT reaching the soil fauna has to be considered.

Soil invertebrates represent a major component of all animal species in soils and play a crucial role in the functioning of the terrestrial ecosystem by enhancing soil structure and organic matter decomposition (Kammenga et al., 2000). Thus, the ecotoxicological impact of TBT on soil invertebrates is also a matter of concern and may reflect effects on soil function. However, few studies have reported the effects of TBT on soil invertebrates (Hund-Rinke and Simon, 2004; Schaefer, 2005; Römbke et al., 2007).

Isopods and collembolan have showed to be promising biomonitor invertebrates to evaluate the status of terrestrial ecosystems (Fernandez and Tarazona, 2008). *Folsomia candida* Willem (Collembola: Isotomidae) has a widespread distribution in Europe. It is a useful test species because it has short generation times, it is parthenogenetic and easy to maintain in laboratory cultures (Greenslade and Vaughan, 2003). They are critical to litter decomposition and nutrient cycling processes and therefore understanding the effect of soil contaminants on these organisms is important and provide useful information about how their populations levels can be affected (Eaton, 2006). Standardized protocols are available to evaluate contaminant exposure of *Folsomia candid*a, like survival and reproduction test (ISO, 1999) and the avoidance behaviour test (ISO, 2008).

The isopod *Porcellionides pruinosus* Brandt (Isopoda: Oniscidea) is a cosmopolitan species (Leistikow and Wägele, 1999) that plays an important role in nutrient cycling and decomposition processes (Zimmer, 2002; Loureiro et al., 2005; Ferreira et al., 2010), mainly by fragmenting dead plant material. Any changes in their feeding rates can affect decomposition processes, and subsequently matter and energy flux through ecosystems (Drobne, 1997). Taking into account their role in soil dynamics it is important to evaluate possible toxic effects of contaminants on this species. Although there is no standard protocols available, isopods have been proven to be sensitive to several chemical compounds (Loureiro et al., 2005) and important to maintain the soil/ecosystem function (Paoletti and Hassall, 1999).

The aim of this study was to evaluate the toxicity of TBT on the soil invertebrates *Porcellionides pruinosus* and *Folsomia candida*. The following tests conducted with *Porcellionides pruinosus* were carried out: an avoidance behaviour response test, to evaluate if *P. pruinosus* is able to detect TBT on soil; two feeding inhibition tests with two different routes of exposure (food and soil) in order to analyze if TBT contaminated food and soil affects feeding behaviour of this species. The standard reproduction test with *F. candida* was also performed. Although some information is available for the exposure of *F. candida* to TBT-based compounds, to our knowledge, studies have not yet been performed regarding the toxic effects of TBT on *P. pruinosus*.

2. Material and Methods

2.1. Test chemical and test soil

Tests were performed with tributyltin chloride ($C_{12}H_{27}ClSn$), obtained from Sigma-Aldrich (97% pure).

All tests were performed with an agricultural soil collected in the spring of 2011, from an agricultural field in the lower Mondego Valley (Portugal) that has been kept fallow during the last five years (Santos et al., 2011). The soil was sieved (< 5 mm) and air-dried. Soil properties included an organic matter content of 2.4 %, clay of 4.2 %, silt of 7.0%, sand of 88.7%, density (g/cm^3) of 2.4 (Santos et al., 2011), pH (H_2O) of 6.80 and a water holding capacity of 26%. Soil was adjusted to 40% and 60% of the WHC_{max}, for the isopods and collembolans tests, respectively.

2.2. Test organisms

The isopods (*Porcellionides pruinosus*) used in these experiments were previously collected from an unpolluted horse manure pill (Agriculture School of Coimbra, Portugal) and maintained in an in-house laboratory culture, at 25 ± 2 °C with a 16:8 h (light: dark) photoperiod. Once a week cultures were water sprayed and organisms were fed *ad libidum* with alder leaves (*Alnus glutinosa*). For all tests only adult males and non-gravid females (15-25 mg wet weight) with antenna were selected.

The collembolans were collected from a synchronized in-house laboratorial culture maintained at 19 ± 2 ºC with a 16:8 (light: dark) photoperiod. The springtails were cultured in plastic boxes containing a mixture of plaster of Paris and activated charcoal in a ratio of 9:1. Once a week, water and food (dry yeast) were added to the cultures. Only juvenile springtails with 10 to 12 days old were used in the experiment.

2.3. Experimental procedure

All experiments were maintained at 20 ± 2 ºC, with a 16:8 (light: dark) photoperiod, and at the beginning and end of each test soil pH was measured. Concentrations are expressed as µg TBT/mg leaf dw and mg TBT/Kg soil dw.

2.3.1. Feeding inhibition experiment – food as route of exposure

The experimental set up was performed according to the methodology proposed by Loureiro et al. (2006), consisting in two plastic boxes overlapping (Ø 80 mm; 45 mm high). The top box had a net bottom and the lower box had a plaster bottom (Fig. 2). The net bottom allows faeces to deposit in the plaster of the lower box for posterior collection, avoiding coprophagy. The plaster serves to maintain moisture.

Isopods were placed individually on the upper box with the contaminated alder leaves during the test period (14 days). Leaves were contaminated topically with an aqueous solution (containing TBT dissolved in ethanol and water) of the test chemical.

Food was not provided to animals one day before and after the test to allow them to empty their gut. Every other day, test containers were checked for dead organisms, the plaster was remoistened if needed, and faeces were collected to individual eppendorffs. Isopods were weighed after empty their gut at the beginning and end of the test period. Faeces and leaf disks were dried for one week, at 50 ºC, and then weighed. Five concentrations (1, 2, 4, 8 and 16 µg/mg leaf dw) and a water control were tested, with ten replicates per treatment and control.

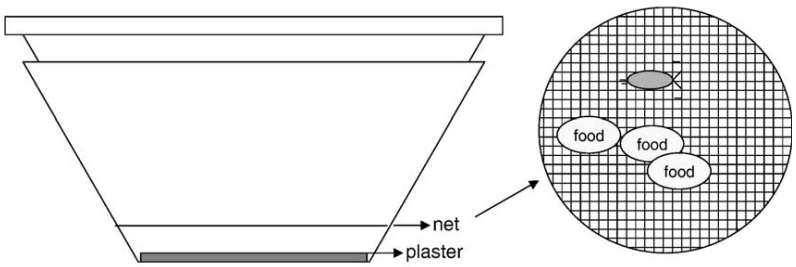

Fig. 2. Scheme of the experimental test boxes (from Loureiro et al., 2006)**.**

The parameters evaluated in this test were calculated by the following equations:

$C_R = (W_{Li} - W_{Lf}) / W_{isop}$

$A_R = [(W_{Li} - W_{Lf}) - F] / W_{isop}$

$E_R = F / W_{isop}$

where, C_R – consumption ratio (mg leaf/mg isopod); A_R – assimilation ratio (mg leaf/mg isopod); E_R – egestion ratio (mg faeces/mg isopod); W_{Li} – initial leaf weight (mg

dw); W_{Lf} – final leaf weight (mg dw); W_{isop} – initial isopod weight (mg dw) and dw – dry weight (Loureiro et al., 2006).

2.3.2. Feeding inhibition experiment – soil as route of exposure

Six concentrations (5.4, 17.3, 54.3, 173, 547 and 1732 mg/Kg soil dw), a solvent control and a water control were tested, and 10 replicates per treatment were performed.

Contaminated alder leaf disks and 50g of test soil were introduced into plastic boxes (Ø 80 mm; 45 mm high). The isopods were kept individually in the test containers during the 14 days of test period. Isopods and leaves were weighed at the beginning and end of the test. Leaves were dried at 50 ºC one week before and after the experiment. Isopod mortality was registered during the test. The consumption ratio was also calculated by the equation above described.

2.3.3. Avoidance behaviour response test

The avoidance behaviour test was carried out according to the procedure proposed by Loureiro et al. (2005). Rectangular plastic containers divided in two sections by a plastic split were used as test boxes. One section of the test box was filled up with control soil (water control) and the other with the test soil. The plastic split was removed and five isopods were introduced in the middle of each test chamber (Fig. 3). After the test period of 48h, the split was reintroduced and the number of isopods in each soil compartment was counted and mortality was registered. Missing animals were assumed to be dead due to test soil exposure and therefore counted as in the test soil. Animals found in the middle were considered as being in the soil to which the animal´s head was directed to.

Four concentrations were selected (0.2, 2, 20 and 200 mg/Kg soil dw) and tested against the control (water control soil), plus a control (water control soil vs water control soil) and a solvent control (water control soil vs solvent control soil), with three replicates each. A preliminary avoidance test was performed with selected ethanol concentrations (0.14 and 0.27 mL/Kg), in order to assess the sensibility of isopods to the solvent. Animals showed not to be sensitive to the highest ethanol concentration tested, the one used on the TBT avoidance experiment.

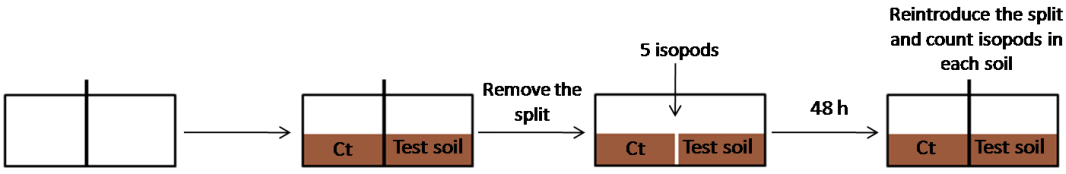

Fig. 3. Scheme of the avoidance behaviour test set up (adapted from Loureiro et al., 2005).

The percentage of avoidance was calculated by the equation $A = \frac{C-T}{N} * 100$, where *A* is the avoidance (%), *C* is the number of isopods in control soil, *T* is the number of isopods in test soil and *N* is the total number of organisms (ISO, 2007). To calculate avoidance between controls with water it was not made any distinction between sides, being one labelled as C and the other side as T in the equation.

2.3.4. Reproduction test

The experimental procedure for the reproduction test was performed according to the ISO 11267 protocol (ISO, 1999). Four concentrations of TBT (6, 12, 24 and 48 mg/Kg soil dw), a solvent control and a water control were tested. At the beginning of the test, 30g of soil and 10 springtails (10 to 12 days old) were introduced in individual glass jars. At the start of the test and after two weeks, 2 mg of dry yeast were added in all test jars and water was replenished.

After 28 days of test period, the content of each jar was transferred to larger glass vessels and filled up with water. The soil was stirred and the number of adult's survivors was counted. The content of vessels was photographed to allow juveniles automatic counting by the image analysis software provided by Sigma Scan.

2.3.5. Statistical analysis

All statistical analysis were performed using the software package SigmaPlot 11.0, provide by Systat Software Inc. To compare the two control types (control vs solvent control) for feeding inhibition experiment (exposure via soil), avoidance behaviour and reproduction tests, a Student's t test was performed.

For all tests, the statistical differences between control and TBT treatments were analysed through a one-way ANOVA, followed by the Dunnett's method when significant differences were found. EC_{50} values were calculated for the reproduction and feeding inhibition, using a sigmoidal logistic curve (3 parameters). The AC_{50} value for the avoidance behaviour and LC_{50} values for isopods and adult collembolan survival were calculated with the Probit Analysis, using the statistical package Minitab (Minitab, 2003).

3. Results and Discussion

Regarding pH measurements, no significant changes in pH values were observed in any test, ranging from 6.26 – 7.06, 6.46 – 6.71 and 6.26 – 6.88, for feeding inhibition (exposure via soil), avoidance behaviour and reproduction tests, respectively.

3.1. Feeding inhibition experiment – food as route of exposure

A significant decrease with the increase of TBT concentration was observed for the three ratios: consumption ratio (one-way ANOVA, $F_{5,25}$=5,722, P<0.05), assimilation ratio (one-way ANOVA, $F_{5,25}$=3.180, P<0.05) and egestion ratio (one-way ANOVA, $F_{5,25}$=3.625, P<0.05) (Fig. 4), with a NOEC of 1 µg/mg leaf dw and the LOEC of 2 µg/mg leaf dw.

Joosse (1981) refers that the decrease of food consumption at higher metal concentrations indicates an avoidance response (Drobne and Hopkin, 1995), so even though TBT is not a metal (but is an organometallic derivate), it could be possible that isopods exposed to food contaminated with higher TBT concentrations were avoiding those leaves. Although, this avoidance behaviour mechanism needs to be investigated (Zidar et al., 2005). This pattern of avoidance was observed on the avoidance behaviour test with soil (see below).

Several authors stated that isopods have the ability to discriminate between metal contaminated and uncontaminated food, rejecting the contaminated one (Zidar, 2004). In a study with *Porcellio scaber*, it is suggested that isopods might detect metal-contaminated food by contact-chemoreception (Weißenburg and Zimmer, 2003). Other study with *P. scaber* suggests different copper treatments to cause changes in microbial populations on food, and isopods may detect those differences by taste (Hassal and Rushton, 1982). Zidar (2004, 2005) proposed that the rejection of Co and Cd by *P. scaber*

can be due to adverse metabolic effects of the ingested substances. Although TBT is not a metal, may be possible that isopods in the present study could detect and reject TBT contaminated food based on taste or on adverse metabolic effects caused by TBT however those possible effects on isopods are still unknown or, at least, not understood.

For the egestion ratio, a significant decrease occurred on 2 and 8 µg/mg (Dunnett's method, $P<0.05$), decreasing from 0.10±0.08 in the control to 0.01±0.00 at 8 µg/mg and reaching a null excretion at 16 µg/mg. The decreased of consumption ratios can explain the decreased of the egestion ratios, because lower consumption of leaves result in less faecal production (Loureiro et al., 2006). The absence of values on the higher concentration probably means that isopods stop eating at a certain moment, which may be a consequence of adverse metabolic effects caused by TBT.

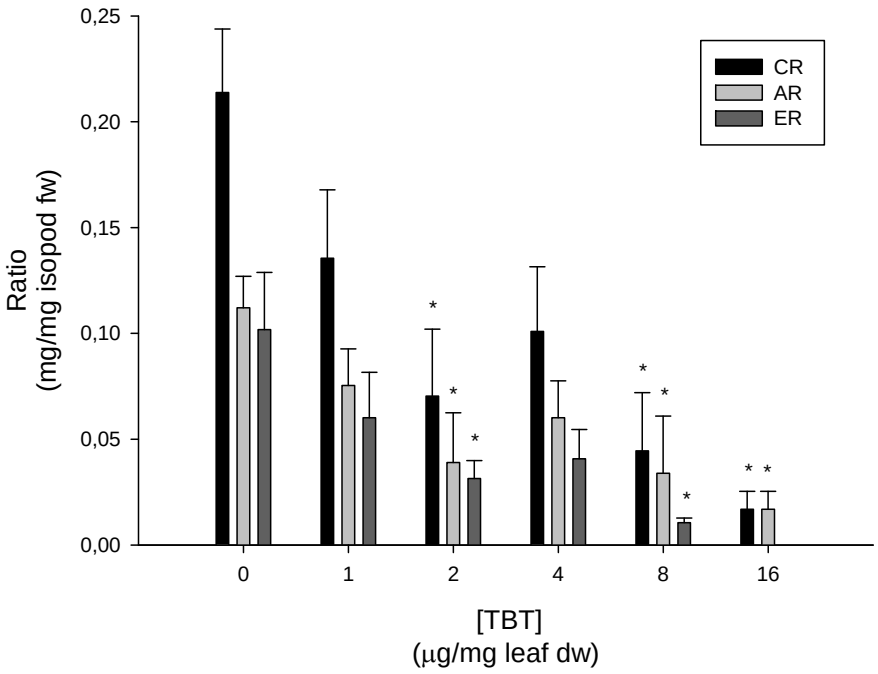

Fig. 4. Consumption (CR), assimilation (AR) and egestion (ER) ratios (mg/mg isopod fw) of *Porcellionides pruinosus* exposed to TBT via food. Data is expressed as mean values and standard error (* $P<0.05$, Dunnett's method).

Results showed a significant decreased on the assimilation ratios, mainly for animals exposed to the two highest concentrations (Dunnett's method, $P<0.05$), and therefore meaning that food might have stayed low periods of time on the gut, resulting on low nutrients assimilation. In all ratios, an increase occurred at 4 µg/mg, followed again by a reduction. No significant differences were observed at the lower concentration (1 µg/mg) (Dunnett's method, $P>0.05$). Mortality was observed in all treatments (one death in the control) during the 14 days of test period. A mortality of 50% was calculated for the highest concentration.

Loureiro et al. (2006) states that egestion is a parameter of ecological relevance, because faecal production occurs in the primary step of leaf decomposition, and it is a direct consequence of litter fragmentation, and also accelerates decomposition. Although no differences may exist on the EC_{50} values calculated, the egestion ratio was a sensitive parameter, showing the lowest EC_{50} value in this test (Table 2).

Table 2
EC_{50} values (µg/mg leaf dw) (with SE (standard error) values between brackets) of feeding parameters for *Porcellionides pruinosus* exposed to TBT via food (alder leaves) for 14 days.

Feeding Parameters	EC_{50}
Consumption ratio	1.51 (0.86)
Assimilation ratio	1.90 (1.53)
Egestion ratio	1.29 (0.83)

Novak and Trapp (2005) performed a study to investigate the feasibility of using land deposited harbour sludge for plant production. TBT concentrations were measured in several plants and in barley corn TBT was < 5 µg/Kg dw, in reed (whole plant) was 105 µg/Kg dw, and in clover/grass (whole plant) of 50 µg/Kg dw. The TBT uptake into stem and leaves was very low and not related to concentrations in soil (Novak and Trapp, 2005). In the study of Ciucani et al. (2003) willow trees were able to uptake TBT although, no or very low TBT was translocated to the upper stem and leaves. Plants growing outside on dumped sediments with TBT ranged concentrations of 170-590 µg/Kg (mean value of 490 µg/Kg) had taken up maximally 15 µg/Kg TBT into above-ground parts (Ciucani et al., 2003). The concentrations measured in plants are far below the concentrations used in this study therefore TBT contamination in plants may not constitute

a risk for isopods in terms of consumption of contaminated leaf litter, for short term exposures.

3.2. Feeding inhibition experiment – soil as route of exposure

Student's t test results found no significant differences between control and solvent control (P>0.05). No statistical differences were found between concentrations and the control (one-way ANOVA, $F_{3,32}$=2.419, P>0.05) (Fig. 5). Food consumption decreased from 0.27±0.12 (control) and 0.39±0.26 (5.4 mg/Kg) to 0.12±0.00 at the 173 mg/Kg concentration. At this concentration only one isopod survived, with low consumption ratio. Isopods also fed on soil particles (Shachak et al., 1976), thus the ingested TBT contaminated particles may induce stress and/or adverse metabolic effects on animals, causing the decrease of leaves consumption. Since the mortality reached 100% for the two highest concentrations, results were not considered in the measurements of consumption ratio. In this case it was possible to calculate a LC_{50} value of 99.23 mg/Kg (Table 3). No mortality was observed in the control. A mortality of 90% was observed at 173 mg/Kg, 20% at 5.4 mg/Kg and 10% at both 17.3 and 54.3 mg/Kg.

In a study with earthworms (*Eisenia fetida*) exposed to TBT contaminated soil (132 µg/Kg soil dw) it was reported a mortality of 42% (Schaefer, 2005). Although the mortality rate was different, the TBT concentration used was much lower than the one used in this study. Römbke et al. (2007) performed a study where the earthworm *Eisenia andrei*, the plant *Brassica rapa* and the collembolan *Folsomia candida* were exposed to different soil types contaminated with TBTO. For the exposure to three sandy soils, the LC_{50} values were of 8.5 and 15.3 mg/Kg (the LC_{50} was not determined for one soil) for *E. andrei*; 20.7, 91.9 and 127.1 mg/Kg for *F. candida*. The LC_{50} value of 91.9 mg/Kg registered for the collembolan was similar to the LC_{50} obtained in this test. Again, earthworms revealed lower LC_{50} values, and are considered to be the most sensitive species (Römbke et al., 2007).

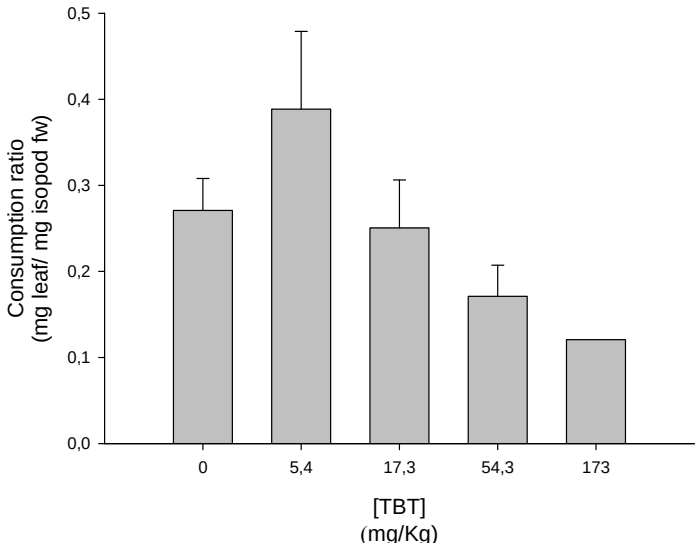

Fig. 5. Consumption ratio (mg leaf/mg isopod fw) of *Porcellionides pruinosus* exposed to TBT via soil. Data is expressed as mean values and standard error. On the 173 mg/Kg treatment, data shown refers only to one isopod.

A direct comparison of EC_{50} values between the two feeding inhibition tests cannot be made due to the different routes of exposure, with the data now available, although the different modes of TBT ingestion have to be considered. In this study the possible TBT uptake could be made by ingestion of soil particles and/or soil pore water. Isopods can intake water from soil through uropods (Drobne and Fajgelj, 1993), ingesting soil and absorption through the cuticle (Loureiro et al., 2005). Given the tendency of TBT to adsorb onto particles, TBT ingestion by water intake may not have been significant. Further conclusions may arise from data on TBT accumulation from isopods exposed to both food and soil routes (data not yet available).

This test may present a limitation because once in contact with soil, leaves can be degraded and fragments caused by deterioration of isopods can be missed and not included for measurement.

3.3. Avoidance behaviour response test

No mortality was observed in any of the treatments used, showing that they could escape from the TBT contaminated soils. Isopods avoidance behaviour was observed for 0.2, 2 and 20 mg/Kg, with *A*=60%. No avoidance was observed at the control (*A*=6.67%). At the highest concentration (200 mg/Kg) the isopods avoided 100% the contaminated soil, thus more than 80% of the isopods were found in the control soil, which is an indication of a loss in the habitat function or a "limited habitat function" of the soil (Hund-Rinke et al., 2003).

The AC_{50} value determined was lower than the lowest concentration used in the experiment (AC_{50}<0.2 mg/Kg). The results suggest that isopods are able to detect TBT in soil and able to escape at very low concentrations. No significant differences were found between controls by Student's t test (*P*>0.05), so is very unlike that solvent had some influence on the avoidance behaviour, mainly at 0.2, 2 and 20 mg/Kg, because the solvent concentrations used were much lower. The fact that isopods were able to escape from the contaminated soil indicates that animals probably were not affected in terms of orientation by those TBT levels, leastwise during 48h, which is one usual pattern when avoidance behaviour tests are used to test compounds that affect the central nervous system.

It is thought that isopods communicate by responding to an aggregation pheromone impregnated on the faeces and detected by the antennae (Takeda, 1980). Loureiro et al. (2005) states that the production of those pheromones can influence the recognition and evasion from chemicals in the avoidance behaviour tests, because isopods can escape from the contaminated soil only by following other isopods that have detected the contaminants. Thus, individual tests, as performed in the study of Loureiro et al. (2005), can help to reach a better conclusion about this behaviour.

3.4. Reproduction test

Student's t test showed no significant differences between reproduction effort under both controls conditions (control vs solvent control) (*P*>0.05). Regarding the reproduction results, it was observed a decrease in the number of juveniles with the increase of TBT concentrations (one-way ANOVA, $F_{4,25}$=11.166, *P*<0.05). Significant differences were found in the two highest concentrations (24 and 48 mg/Kg) when

compared with the control (Dunnett's method) (Fig. 6), decreasing from 455.60±137.17 (control) to 5.60±3.51 (48 mg/Kg). A mortality of 50% and 100% was observed for the 24 mg/Kg and 48 mg/Kg concentrations, respectively.

An EC_{50} value for the production of juveniles of 19.31 was calculated (Table 3), but regarding the mortality results, we cannot state clearly if the decrease on juveniles production was related to a direct effect on springtail reproduction or if it was due to the high mortality observed. In the study performed by Römbke et al. (2007), EC_{50} values of the reproduction test ranged from 23.4 to 177.8, accordingly to the soil type.

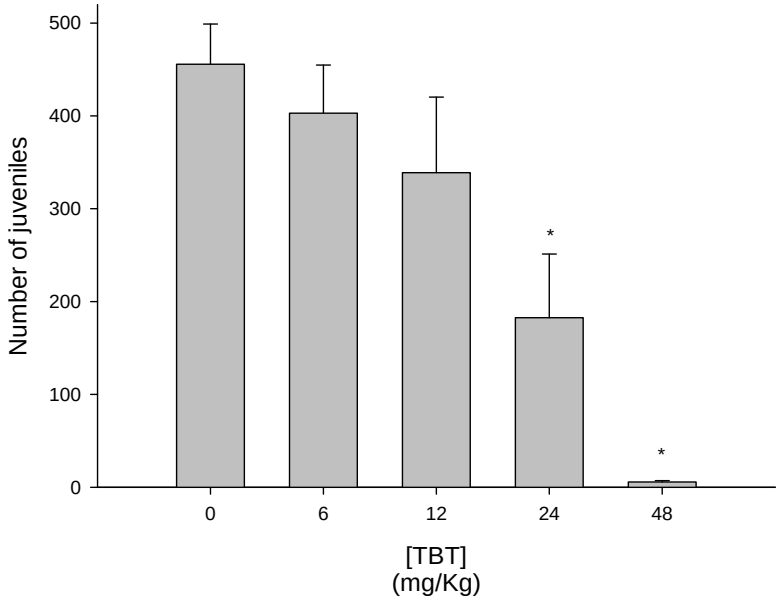

Fig. 6. Reproduction rate (number of juveniles) of *Folsomia candida* exposed to TBT. Data is expressed as mean values and standard error (* *P*<0.05, Dunnett's method).

For the three sandy soils - BWZ, Eco5, GGI (with the sand content similar to the one used in this study) - the EC_{50} values were of 26.0 (BWZ), 76.4 (ESo5) and 23.4 (GGI). The EC_{50} value of this test is close to the values of BWZ and GGi soils. The differences observed in the EC_{50} can be attributed to differences in soil properties (pH, organic C content and cation exchange capacity (CEC)), since they are considered to influence the availability of contaminants (Römbke et al., 2007).

The properties of those three soils differ, which could explain the differences between EC_{50} values. Other explanation could be attributed to TBT type, since in this study it was used TBTCl instead of TBTO used by Römbke et al (2007).

Hund-Rinke and Simon (2004) also performed a work to test the effects of TBTCl upon exposure on sandy, silty and loamy soils in *Eisenia fetida*, *Folsomia candida* and the plants *Brassica rapa* and *Avena sativa*. The EC_{50} values obtained for collembolan reproduction were of 22, 11 and 66 mg/Kg, for sandy, silty and loamy soils, respectively. Again, the EC_{50} value for the sandy soil was similar to the value obtained in this test. In both studies, earthworms showed to be high sensitive species when compared to collembolan and plants, with EC_{50} values of 2.0 (BWZ) and 0.5 (GGI) (Römbke et al., 2007), 1.3 (sandy soil), 3.0 (silty soil) and 2.7 (loamy soil) (Hund-Rinke and Simon, 2004)

Table 3
EC_{50} values (mg/Kg soil dw) (with SE (standard error) values between brackets), LC_{50} (95% confidence intervals (CI)), NOEC and LOEC for the test species *Porcellionides pruinosus* (feeding inhibition test – soil as route of exposure) and *Folsomia candida* exposed to TBT.

Test species	EC_{50}	LC_{50} (95% CI)	NOEC	LOEC
Porcellionides pruinosus	61.11 (32.17)	99.23 (60.91-137.55)	ND	ND
Folsomia candida	19.31 (3.75)	22.82 (20.09-25.54)	12	24

ND = Not determined

In the Römbke et al. (2007) study it was observed a correlation between C_{org} content and TBTO toxicity. The content of clay and humic material are dependent factors for the sorption of a chemical to soil (Hund-Rinke and Simon, 2004). Hund-Rinke and Simon (2004) determined that the sandy soil (with lowest clay (3.6%) and C_{org} content) showed the lowest EC_{50} values, indicating the general higher toxicity of TBT in that soil. The clay content of the test soil is also low (4.2%), which can be a reason for the low EC_{50} value.

The determined LC_{50} value was of 22.82 (Table 3), similar to one obtained by Römbke et al. (2007) for the BWZ soil (20.7). The LC_{50} values of that study ranged from 20.7 to 806.5 mg/Kg between all soil types. The sandy soils GGI and ESo5 showed LC_{50} values of 91.9 and 127.1, respectively. Once again, earthworms showed to be more sensitive to TBT, exhibiting LC_{50} values between 8.5 and 15.3 mg/Kg, depending on the soil type (Römbke et al., 2007).

Preliminary Remediation Goals (PRGs) attributed baseline values for TBT in soils at residential and industrial areas (US-EPA, Region 9) of 18 mg/Kg and 180 mg/Kg dm, respectively (Beuselinck and Valle, 2005). Considering the EC_{50} and LC_{50} values obtained in this study for isopod feeding inhibition test with contaminated soil and for the collembolan reproduction test (Table 3), as well as the avoidance behaviour of *P. pruinosus*, those baseline values may pose a risk for these invertebrates and thus lower values should be considered. Huang et al. (2004) determined contents of TBT < 0.01 ng Sn g-1 in wetland soils in Germany. Given the determined AC_{50} value, the isopod *P. pruinosus* may be able to detect those concentrations in soils. To our knowledge, no more data are available for TBT concentration in soils (Chau, 2005).

Plagellat et al. (2004) determined TBT concentrations in sewage sludge of 18.6-648.5 µg/Kg dw and Fent and Müller (1991) determined values of 0.28-1.51 mg/Kg dw. Fent (1996b) indicates TBT concentrations in sewage sludge from different urban and suburban areas in several countries ranging between 0.04-3.4 mg/Kg dw. Those concentrations are far below the concentrations used and the EC_{50} values calculated in this study however the isopod *P. pruinosus* might be able to detect such concentrations. Furthermore, due to the high persistence of TBT in soils (Lespes et al., 2009) the continuous dump of sewage sludge in crops for soil amendment cannot be ignored.

4. Conclusions

The results obtained showed a dose-response relationship between TBT concentration and the decrease on isopod feeding performance, on the number of juvenile springtails production and survival of isopods and adult collembolan. From the avoidance behaviour test it could be observed that isopods are able to detect the presence of TBT at very low concentrations and avoid contaminated soils. Feeding behaviour results suggest that TBT contamination affects and inhibits the feeding performance of terrestrial isopods using the concentration range used in this study.

Folsomia candida showed to be more sensitive to TBT contamination in soils than *Porcellionides pruinosus*. This reaffirms the differences of species sensitivity in ecotoxicology tests and therefore, bioassays with several invertebrate species can be useful to provide a better understanding of the toxicity of chemical compounds as well as the use of different soil types, since differences in soil properties showed to influence

toxicity of TBT. Further studies are needed to obtain a better knowledge and comprehension of TBT toxicity towards soil fauna.

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Chapter III: The use of bioassays to evaluate TBT bioremediation in soil

Abstract

The widespread distribution of tributyltin compounds is worldwide recognized as a pollution problem due to its high persistence, toxicity and bioaccumulation characteristics. Biological degradation is suggested to be the major pathway for TBT removal from the natural environment and the application of bioremediation technologies are environmental friendly and cost-effective approaches to clean-up this compound. This work aimed to assess the ability of the bacterium *Aeromonas molluscorum* Av27 to degrade TBT in contaminated soil through a feeding inhibition test with the terrestrial isopod *Porcellionides pruinosus*, and inhibition of growth of an indicator *Micrococcus luteus* strain using a simple bioassay. The feeding inhibition test consisted in three sub-experiments called TBT-feeding test (TBT contaminated soil exposure), TBT-growth medium test (soil contaminated with TBT + TSB medium) and TBT-Av27 test (soil contaminated with TBT + TSB medium + *Aeromonas molluscorum* Av27). The results from bioassays with *M. luteus* showed a decrease of the inhibition zone, suggesting TBT degradation by *A. molluscorum* Av27 or reduction of its toxicity. Results from the feeding bioassay showed a dose relationship between the TBT concentration and the decrease of food consumption in the three sub-experiments. TBT-growth medium test presented the highest toxicity. TBT-Av27 test showed higher consumption ratios but lower EC_{50} value than TBT-feeding test. Comparing TBT-growth medium with TBT-Av27 is observed a toxicity reduction, which is suggestive of degradation by Av27. Even though, chemical analyses are required to help results interpretation.

An overall analysis revealed that the feeding inhibition test with the terrestrial isopod and the growth inhibition of *Micrococcus luteus* seem to be good tools to assess TBT bioremediation in soil. Additionally, *A. molluscorum* Av27 seems to be able to bioremediate soils contaminated with TBT. Even though, more studies are needed to fully accomplish the aim of our study.

Keywords: *Aeromonas molluscorum* Av27, soil bioremediation, TBT, *Porcellionides pruinosus*, feeding inhibition, *Micrococcus luteus*.

1. Introduction

Tributyltin compounds exhibit strong biocide properties and therefore they have been extensively used in industry and agriculture as antifouling agents, fungicides, wood preservatives or polymer stabilizers, amongst others (Clark et al., 1988; Bancon-Montigny et al., 2002). The release of TBTs from maritime traffic caused a direct entry into the aquatic environment, leading to significant environmental problems and economic consequences (Caricchia et al., 1994). Toxic impacts have been reported on non-target organisms at very low concentrations (ranging values of ng l^{-1} of magnitude) (Reitsema and Spickett, 1999; Terlizzi et al., 2001; Sidharthan et al., 2002).

The negative impact of tributyltins has led to the inhibition of the use of TBT-based antifouling paints (Azenha and Vasconcelos, 2002), but many harbours remain highly contaminated, with TBT half-life reported to be up to several years (Carvalho et al., 2010). Some recent studies have also showed the presence of TBT in soils, sewage sludge, municipal wastewaters and ground waters (Behra et al., 2003; Stasinakis et al., 2005). TBT has reached the soil, mainly through the dredging of contaminated harbour sediments (Novak and Trapp, 2005) and application of pesticides and contaminated sewage sludge to agricultural fields (Hoch, 2001; Voulvoulis and Lester, 2006). Reported TBT half-lives in soils ranging from 15 weeks to several years (Lespes et al., 2009) indicates a slow degradation rate and high persistence in soils.

In order to try to solve this kind of contamination problems, biotic degradation is suggested to be the major process for TBT removal from the natural environment (Gadd, 2000; Bernat and Dlugonski, 2006; Sakultantimetha et al., 2011) and microorganisms are considered the key factor in biodegradation of TBT. The process involves progressive debutylation (or dealkylation), the breakage of the tin–carbon bond to produce less toxic and lipophilic metabolites dibutyltin (DBT) and monobutyltin (MBT) (Sakultantimetha et al., 2010b).

Several studies have suggested that TBT can be biodegraded by bacteria, fungi, microalgae and plants (Novak and Trapp, 2005; Bernat and Dlugonski, 2006; Luan et al., 2006; Taha et al., 2009; Jin et al., 2010; Sakultantimetha et al., 2010b). Bacteria play an important role on biogeochemical cycles. They can act as natural decontaminant agents and therefore they are important tools for bioremediation technologies to restore polluted environments (Cruz et al., 2007; Cruz et al., 2010a). Bioremediation techniques are more

advantageous than other removal methods because they convert toxic compounds into less or non-toxic products, being cheaper methods and reducing also ecological and health effects (Sarkar et al., 2005).

Cruz et al. (2007) isolated a bacterium, *Aeromonas molluscorum* Av27, that is highly resistant to TBT (up to 3 mM) and is able to degrade it into DBT and MBT in Marine Broth medium (MB). Additionally, this bacterium uses TBT as a sole carbon source. Given these abilities, this strain can be used to develop a biosensor to detect and also to remediate TBT in the environment (Cruz et al., 2007). To that end, it is important to test the ability of this bacterium to degrade TBT in soils, since TBT soil contamination is a problem of concern.

Other previous studies have also demonstrated TBT and/or DBT degradation by bacteria such as *Pseudomonas aeruginosa*, *P. chlororaphis*, *Alcaligenes faecalis*, *Streptomyces sp.* and *Enterobacter cloacae* (Yamaoka et al., 2002; Roy and Bhosle, 2006; Bernat and Długoński, 2009; Sakultantimetha et al., 2010a).

The assessment of soil bioremediation is usually carried out by chemical analysis. However, reduced target compound concentration does not always result in decrease of soil toxicity (Knoke et al., 1997). Chemical analyses usually are insufficient because they do not have into account the actual biological and ecological effects of pollutants, i.e. chemicals bioavailability. Bioassays do integrate exposure and effects (Bundy et al., 2001) and therefore they are recommended to evaluate the efficiency of soil remediation in terms of reduced toxicity (Udovic et al., 2009).

This study was performed taking into account the following aims: a) evaluate the potential of *A. molluscorum* Av27 to bioremediate TBT from contaminated soil; b) study if the feeding inhibition bioassay with the invertebrate species *Porcellionides pruinosus* can be used to assess the bioremediation of contaminated soil by *A. molluscorum* Av27; c) validate the bioassay with *Micrococcus luteus*.

2. Material and Methods

2.1. Test soil and test chemical

The test soil was collected from an agricultural field in the spring of 2011, situated in the lower Mondego Valley (Portugal) and has been kept fallow during the last five years. The soil showed a organic matter content = 2.4 %, clay = 4.2 %, silt = 7.0%, sand = 88.7%, density (g/cm^3) = 2.4 (Santos et al., 2011), a pH (H_2O) = 6.80 and a water holding capacity = 26%. The soil was sieved (< 5 mm) and air-dried.

Test soil was contaminated with tributyltin chloride ($C_{12}H_{27}ClSn$) (97% purity), purchased from Sigma-Aldrich.

2.2. Test organisms

The terrestrial isopod used in these experiments belong to the species *Porcellionides pruinosus* and were previously collected from an unpolluted horse manure pill (Agriculture School of Coimbra, Portugal). They were maintained in an in-house laboratory culture, at 25 ± 2 °C and with a photoperiod regime of 16:8 h (light: dark). Once a week cultures were water sprayed and food was provided (alder leaves - *Alnus glutinosa*). For all tests only adult males and non-gravid females (15-25 mg wet weight) with antenna were selected. Since isopods share the same weight range, it was assumed that they belong to the same age stage.

The indicator strain *Micrococcus luteus* ATCC 9341 was acquired from the American Type Culture Collection.

Aeromonas molluscorum Av27 is a Gram-negative bacterium isolated from sediments and water from Ria de Aveiro, Portugal (Cruz et al., 2007). This bacterium was used to bioremediate the TBT contaminated soil.

2.3. Experimental set up

Five concentrations (12.5, 25, 50, 100 and 200 mg/Kg soil dw), a water control and a solvent control were tested, with 10 replicates per treatment. Concentrations are expressed as mg TBT/Kg soil dw.

The experimental procedure consisted in three sub-experiments that were carried out simultaneously:

- TBT-feeding test: an ecotoxicity test, where soil was contaminated with TBT and exposure effects were evaluated in *P. pruinosus* and *M. luteus*;

- TBT-growth medium test: an ecotoxicity test where the soil was contaminated with TBT and the bacteria growth medium (Tryptic Soy Broth – TSB, 14 mL per container) including the control and solvent control; exposure effects of TBT + TSB growth medium were assessed on the test species *P. pruinosus* (after an incubation period of 21 days) and *M. luteus*;

- TBT-Av27 test: an ecotoxicity test where the test soil was contaminated with TBT (including the control and solvent control) and 14 mL of TSB growth medium inoculated with *Aeromonas molluscorum* Av27 (~10^8 mL^{-1}) per container; exposure effects to TBT + Av27 were evaluated on *P. pruinosus* (after an incubation period of 21 days) and *M. luteus*.

The soil was adjusted to 100% of the WHC_{max}, to provide better conditions for the bacteria to act. During the 21 days of incubation soil moisture decline was controlled in order to achieve a WHC_{max} of 40% for isopod exposure.

Some containers remained without animals and keep a WHC_{max} of 100% to enhance TBT degradation. During the 35 days (21 days without animals plus 14 days of isopod test period), soil samples were taken from those containers (each concentration from TBT-growth medium and TBT-Av27 tests), and frozen (-80º C) for 48h. This procedure killed all soil bacteria that previously existed so that samples could be assayed for TBT toxicity using the bacterium *M. luteus* with no interference from other bacteria. Samples were collected on day 1, 3, 6, 8, 15, 20, 27 and 35 (corresponding to the 14 day of the isopod test). The pH was measured at the beginning and end of the bioassays (Table 4).

2.3.1. Feeding inhibition experiment with Porcellionides pruinosus

Contaminated alder leaf disks and 50g of test soil were introduced into plastic boxes (Ø 80 mm; 45 mm high). The isopods were kept individually in the test containers during the 14 days of test period. Leaves were dried at 50 ºC one week before and after the experiment. Isopods and leaves were weighed at the beginning and end of the test and isopod mortality was registered during the test.

Consumption ratios were calculated by the equation $C_R = (W_{Li} - W_{Lf}) / W_{isop}$ where, C_R – consumption ratio (mg leaf/mg isopod), W_{Li} – initial leaf weight (mg dw); W_{Lf} – final leaf weight (mg dw); W_{isop} – initial isopod weight (mg dw) and dw – dry weight.

2.3.2. Evaluation of TBT toxicity with Micrococcus luteus

To assess TBT toxicity an experiment was carried out design as follows: TSB agar plates were seeded with the indicator strain *M. luteus* ATCC 9341 at a final concentration of 0.02 (optical density at 600 nm). Three wells (5 mm diameter) were made in each inoculated agar plate and filled to the top with the soil samples: one was filled with the TBT-growth medium sample, other with the TBT-Av27 sample and another with a control (Fig. 7). The control was performed using 100µL of a “freshly prepared” TSB contaminated with a given TBT concentration. The plates were incubated at 37º C overnight and after that period the inhibition zones were measured.

2.3.3. Statistical analysis

Statistical analyses were run using the software package SigmaPlot 11.0, provide by Systat Software Inc. A Student's t test was performed to compare the water control with the solvent control of the three sub-experiments, and when data did not present a normal distribution a non-parametric Mann-Whitney Rank Sum test was performed. A one-way ANOVA was performed to assess statistical differences between treatments (including control), followed by Dunnett's method when significant differences were found. Whenever data were not normally distributed and to evaluate the differences between groups, a Kruskal-Wallis One Way Analysis of Variance on Ranks was performed, followed by Dunn's method if significant differences were found. The EC_{50} value for the feeding

inhibition was assessed using a sigmoidal logistic 3 parameter curve. The LC_{50} value was calculated with the Probit method, through the statistical package Minitab (Minitab, 2003).

3. Results and Discussion

3.1. Evaluation of TBT toxicity with *Micrococcus luteus*

The results from the bioassay with *M. luteus* showed a decrease in the inhibition halo of the samples contaminated with 200 mg/Kg (Fig. 7). On day 1 (Fig. 7 A) the inhibition halos from TBT-growth medium and TBT-Av27 test samples were evident, indicating that in both samples TBT toxicity was inhibiting the microbial growth of *M. luteus*. On day 6 (Fig. 7 B), the inhibition halo decreased and was smaller than the halo from the sample without Av27, showing a decrease on toxicity. On day 15 (Fig. 7 C), the inhibition halo disappeared, but the one from the growth medium sample remained. On the last day of test (Fig. 7 D) the halo from TBT-growth medium sample was still present, indicating that TBT was not degraded by other factors (e.g. photodegradation), but the sample with Av27 still did not present inhibition halo, showing no toxicity.

Results from bioassays with lower TBT concentrations (< 200 mg/Kg) were not sufficiently clear for an interpretation as the inhibition halo could not be properly visualized, but considering the decrease of halos from the highest concentration, it is expected that the decrease in toxicity may also occur at lower levels.

It seems that moisture content was essential for TBT availability to the Av27 strain. A preliminary test with soil moister of 60% of WHC_{max} was performed in order to investigate if Av27 could degrade TBT in normal soil conditions (i.e. under soil ecotoxicological test conditions), but the results indicated no reduction of TBT toxicity (data not shown). For this reason, during 21 days the test soil was moistened with 100% of WHC_{max} and some containers maintained that moisture. On the other hand, TSB medium is reported to reduced TBT availability to bacteria, because TBT forms complexes with the organic matter of TSB (Cruz et al., 2010b). The pH also plays an important role on TBT availability because affects TBT speciation (Sakultantimetha et al., 2010a). TBT exhibits a pK_a (acid dissociation constant) ranging from 6.25-6.51 (Fent and Looser, 1995; Xiao et al., 2011) and, in aqueous solutions, at pH < pK_a the cationic form (TBT^+) is the

main specie and at pH > pK_a predominates the neutral form (Meador et al., 2002; Radke et al., 2003).

The octanol-water partition coefficient (K_{ow}) is another parameter that affects bioavailability of TBT and higher values indicate higher partitioning to the lipids of organisms (Meador, 2000). At pH 7-9 TBT presents a maximum constant log K_{ow}, which indicates that the neutral form provides a better uptake than the cationic form (Hunziker et al., 2001). TBT-Av27 test presented a pH > pK_a (Table 4), suggesting that the neutral form was the dominate one (Hoch, 2004a) however this specie has high tendency to adsorb onto the organic matter of soil particles (Bangkedphol et al., 2009), which is considered to be a much more significant sorption pathway than to mineral phases (Berg et al., 2001; Hoch, 2004b). The remaining TBT^+ tends to adsorb onto mineral surfaces or be retaining in the aqueous phase by complexation with deprotonated ligands of soluble organic matter (Arnold et al., 1998; Said-Pullicino and Vella, 2005). These facts hinders the interpretation about TBT availability to bacteria in this test, because given the pH range and the high organic content provided by TSB to soil particles TBT is likely to be mainly adsorbed on soil and the remaining cations forming complexes. Therefore, very few TBT is supposed to stay available for uptake at water phase, which is not supported by the obtained results. So, according to results, TBT seems to have been available for uptake by bacteria in this test.

The results suggested that TBT degradation by *A. molluscorum* Av27 has occurred or the toxicity was reduced to a point where it was no longer toxic to *M. luteus*, allowing the growth of the organism. Instead of degradation, TBT could have been taken up by bacteria remaining inside the cells without breakdown, into siderophore-like structures, or even bound to the cell membrane lipids (Cruz et al., 2007). Bacteria could also bioaccumulate TBT using metallothionein-like proteins, without degrading the compound (Dubey and Roy, 2003). Thus, TBT is not available and therefore non toxic to *M. luteus*, explaining the absence of inhibition zones.

Thence, future chemical analyses of the samples are needed to confirm TBT bioremediation by *A. molluscorum* Av27. Information about TBT resistance and degradation in Marine Broth medium by *A. mollucorum* Av27 are given in Cruz et al. (2007, 2010a, 2010b).

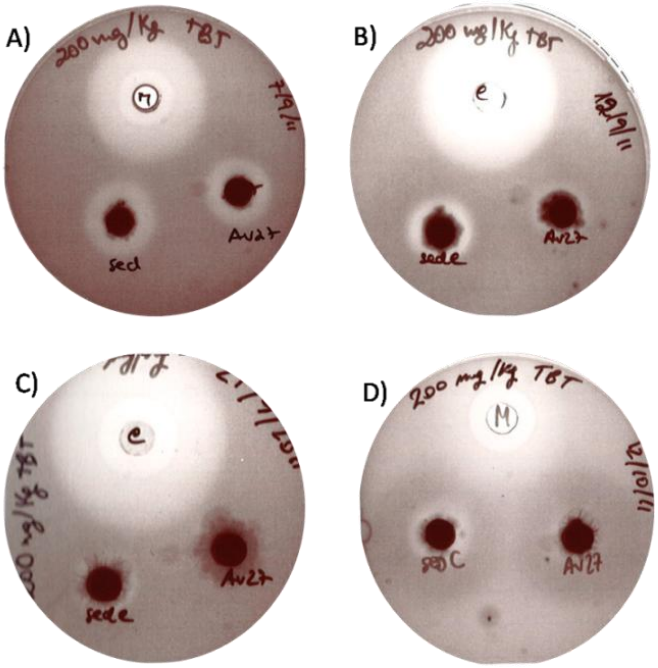

Fig. 7. TSB agar plates inoculated with *Micrococcus luteus* for the assessment of TBT toxicity. Samples were collected from the highest concentration (200 mg/Kg) on day 1 **(A)**, day 6 **(B)**, day 15 **(C)** and day 35 (last day of the test) **(D)**. The top wells correspond to the control, the left wells were filled with the TBT-growth medium test samples and the right wells were filled with the TBT-Av27 test samples.

It is noted that pH values of both TBT-growth medium and TBT-Av27 tests reached higher values (around 8), which suggests that TSB medium may have increased soil pH (Table 4).

Table 4
pH levels for all treatments of the three sub-experiments, measured at the beginning (left) and end (right) of the test period.

Sub-experiments	Control	Solvent control	12.5	25	50	100	200
TBT-feeding	7.01 – 6.71	7.03 – 6.81	6.94 – 6.71	6.88 – 6.58	6.90 – 6.70	6.88 – 6.60	6.81 – 6.95
TBT-growth medium	6.95 – 8.41	6.97 – 8.10	7.00 – 8.62	7.01 – 8.29	7.00 – 8.40	6.98 – 8.37	6.94 – 8.62
TBT-Av27	6.71 – 7.80	6.66 – 7.81	6.69 – 8.47	6.72 – 8.52	6.70 – 7.81	6.67 – 8.32	6.71 – 7.89

3.2. Feeding inhibition experiment with *Porcellionides pruinosus*

In a general analysis, food consumption was reduced in the three sub-experiments as a result of increasing TBT concentrations (Fig. 8). Student's t test results showed no significant differences between controls (water control vs solvent control) for the three sub-experiments (P>0.05). Significant differences were found between the control and concentrations for the TBT-feeding test (one-way ANOVA, $F_{5,41}$=5.071, Dunnett's method, P<0.05), TBT-growth medium test (Kruskal-Wallis One Way Analysis of Variance on Ranks, df=5, H=17.952, P<0.05, Dunn's method, P<0.05) and TBT-Av27 (Kruskal-Wallis One Way Analysis of Variance on Ranks, df=5, H=11.957, P<0.05) (Fig. 8).

A decrease of food consumption in higher concentrations is expected. In a feeding inhibition test with contaminated leaves, the decreased of consumption ratio could be a consequence of an avoidance response to contaminated food (Drobne and Hopkin, 1995). In this case leaves were not contaminated because soil was the exposure route. However, isopods also fed on soil particles (Kautz and Topp, 2000), and those contaminated particles can cause stress and then decreased food consumption.

Controls of the three sub-experiments were compared. No statistical differences were found for TBT-growth medium and TBT-Av27 tests (Kruskal-Wallis One Way Analysis of Variance on Ranks, df=2, H=8.440, Dunn's method, P>0.05), but both tests showed differences when compared to TBT-feeding control (Kruskal-Wallis One Way Analysis of Variance on Ranks, df=2, H=8.440, Dunn's method, P<0.05).

Soils from both TBT-growth medium and TBT-Av27 tests were moistened with TSB, which is a rich medium. Since leaves were in the soil they could be involved with TSB medium and acquire a better flavour for isopods, explaining the higher consumption ratios of their controls and the differences comparatively to the TBT-feeding.

The three tests were also compared in each concentration and no significant differences were found: 12.5 mg/Kg (one-way ANOVA, $F_{2,23}$=0.462, P>0.05), 25 mg/Kg (Kruskal-Wallis One Way Analysis of Variance on Ranks, df=2, H=1.844, P>0.05), 50 mg/Kg (Kruskal-Wallis One Way Analysis of Variance on Ranks, df=2, H=4.096, P>0.05), 100 mg/Kg (one-way ANOVA, $F_{2,12}$=0.780, P>0.05) and 200 mg/Kg (Kruskal-Wallis One Way Analysis of Variance on Ranks, df=2, H=0.179, P>0.05).

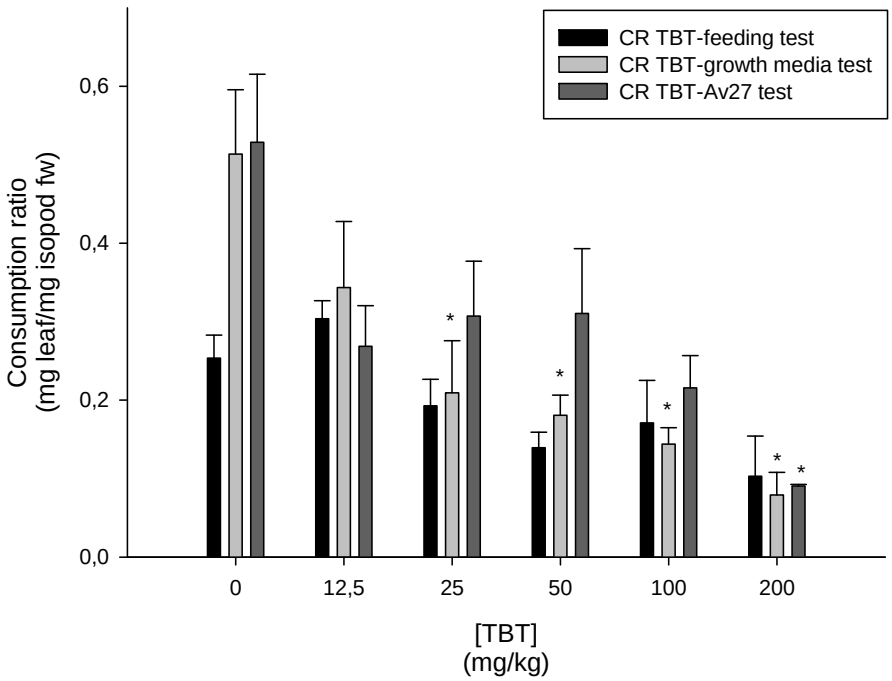

Fig. 8. Consumption ratio (mg leaf/mg isopod fw) of *Porcellionides pruinosus* exposed to TBT in the three sub-experiments: TBT-feeding, TBT-growth medium and TBT-Av27 tests. Data is expressed as mean values and standard error (* P<0.05, Dunn's method).

At 12.5 mg/Kg, the consumption of TBT-feeding test increased from (mean±SD) 0.25±0.09 (control) to 0.30±0.07, so apparently this concentration did not caused toxic effects on isopods from this test. The opposite occurred for TBT-growth medium and TBT-Av27, whose consumption decreased considerably relatively to control, from 0.51±0.25 to 0.34±0.22 and from 0.53±0.23 to 0.27±0.16, respectively. Possibly, the nutritive properties and flavour provided by TSB may have stimulated isopods to highly consume contaminated soil particles, causing stress and posterior decrease of consumption. This may explain the differences in terms of percentage of feeding inhibition in treatments of TBT-growth medium and TBT-Av27 when compared to control, which is not observed for the TBT-feeding test (Table 5). A LOEC of 25 mg/Kg was determined for TBT-growth medium test (Table 6).

Table 5
Feeding inhibition (%) of *Porcellionides pruinosus* of each treatment relatively to the control (mean±SD), when exposed to TBT in the three sub-experiments for 14 days.

Sub-experiments	12.5	25	50	100	200
TBT-feeding	119.56±27.17	75.84±42.29	54.88±24.62	67.32±47.64	40.58±34.86
TBT-growth medium	66.81±43.31	40.72±36.50	35.17±14.05	27.98±9.15	15.40±7.94
TBT-Av27	50.79±30.91	58.06±41.76	58.69±46.88	42.30±17.38	17.12±0.52

Considering the tendency of TBT^+ to form complexes with soluble organic matter of TSB, TBT uptake by isopods from these two tests may also have occurred, in a smaller part, by interstitial water intake through uropods (Drobne and Fajgelj, 1993), ingestion of particles and absorption through the cuticle (Loureiro et al., 2005). This evinces a higher exposure of isopods in these two tests. Soil of TBT-feeding test was moistened with distilled water, so possibly the organic content of the interstitial water may be considered negligible. TBT^+ was preferentially bound onto negative charged mineral surfaces and TBTCl to the organic content of soil particles. Therefore, TBT uptake by isopods from TBT-feeding is likely to have occurred mainly through ingestion of soil particles.

At 25, 50 and 100 mg/Kg TBT-Av27 test showed the highest ratios with means of 0.31±0.22, 0.31±0.25 and 0.22±0.09, respectively. Animals from TBT-feeding displayed lower ratios (0.19±0.11, 0.14±0.06 and 0.17±0.12). Reported means for TBT-growth medium test were of 0.21±0.19, 0.18±0.07 and 0.14±0.05. At 200 mg/Kg ratios were very low and similar for the three tests, due to higher mortality registered for this concentration (70% for TBT-feeding and 80% for TBT-growth medium and Av27 tests). High LC_{50} values for the three tests were determined, with TBT-growth medium presenting the lowest value and TBT-feeding presenting the highest one (Table 6), so it seems that these concentrations were not sufficient to cause high mortality, except for 200 mg/Kg.

An overall analysis of the results revealed that toxicity appeared to be higher in the TBT-growth medium test, presenting the lowest EC_{50} and LC_{50} values (Table 6). TBT-Av27 test presented higher consumption ratios, but lower EC_{50} and LC_{50} values than TBT-feeding test, and a NOEC at 100 mg/Kg (Table 6). Possibly the lower EC_{50} values of TBT-Av27 can be attributed to high control values rather than toxicity. Besides, the increase occurred at 100 mg/Kg for TBT-feeding test may have originated a higher EC_{50} value (Table 6). This test also displayed lower ratios, including the control, which is thought to

be due to the different moisture medium, so it is difficult to compare this test with the remaining.

As TSB seems to increase food consumption in isopods, results from animals exposed to soils from TBT-growth medium and TBT-Av27 tests may give a better approach to evaluate the possible decrease of toxicity of TBT. From the EC_{50} values calculated upon these two exposures, TBT-Av27 exposure seems to present less toxicity to isopods, but the SE (standard error) from EC_{50} values is high, which denotes uncertainty with this assumption (Table 6).

Table 6
EC_{50} values (mg/Kg soil dw) (with SE (standard error) values between brackets), NOEC and LOEC of consumption ratio and LC_{50} (95% confidence intervals (CI)) for *Porcellionides pruinosus* exposed to TBT in the three sub-experiments for 14 days.

Sub-experiments	EC_{50}	LC_{50} (95% CI)	NOEC	LOEC
TBT-feeding test	88.38 (36.92)	158.33 (122.34-194.32)	ND	ND
TBT-growth medium test	25.68 (12.40)	115.98 (64.83-167.12)	12.5	25
TBT-Av27 test	43.55 (62.46)	140.57 (109.27-171.86)	100	200

ND = Not determined

The results from the bioassays with *M. luteus* suggest degradation or reduction of TBT toxicity to that strain before the isopod feeding test started, so the toxicity to animals from TBT-Av27 test is expected to decrease. However, at 200 mg/Kg higher toxicity was observed when compared to the control and also to lower concentrations. The toxicity reduction observed for TBT-Av27 when compared with that of TBT-growth medium may suggest that degradation of TBT occurred, but DBT and MBT can still be toxic to isopods, but less toxic than TBT.

At least two possible scenarios can have occurred: a) *A. molluscorum* Av27 could have retained TBT into their cells rather than degraded the compound. Isopods feeding on soil particles may have ingested the bacteria along with the TBT retained, which is still toxic to isopods; b) *A. molluscorum* Av27 degraded TBT into DBT and MBT, which may still cause toxicity to animals. In the case of MBT it is very unlikely, but reported studies refer that MBT could be as toxic or even more toxic than TBT for some soil microorganisms (Paton et al., 2006; Heroult et al., 2008) and DBT can display higher

immunotoxicity than TBT in invertebrates and vertebrates (Bouchard et al., 1999; St-Jean et al., 2002; Nesci et al., 2011). TBT can also only have been degraded to DBT, which shows more toxicity than MBT.

4. Conclusions

In all sub-experiments the feeding performance of isopods was affected with TBT concentrations. The highest toxicity was noticed on TBT-growth medium test, with lowest EC_{50} and LC_{50} values.

TSB medium seems to affect food consumption, which difficult the interpretation of TBT toxicity from TBT-growth medium and TBT-Av27 tests when comparing with TBT-feeding test. Toxicity of TBT-Av27 was clearly lower than TBT-growth medium test, which can suggest a toxicity reduction. TBT-feeding test presented lower ratios but highest EC_{50} and LC_{50} values.

Several events can have occurred regarding TBT availability therefore chemical analyses will help on the interpretation of the obtained results. Organic content and pH probably are also influencing the bioavailability and toxicity of TBT in soil, hence future studies should include a battery of bioassays with different soil types and species, to provide different exposure scenarios and sensibilities.

TSB medium makes interpretation more confusing between tests and treatments of the same test. Therefore, this alerts for the use of a less rich growth medium in future assays.

Despite the need for chemical analyses to confirm the degradation of TBT, the bacterium *A. molluscorum* Av27 may be able to degrade TBT in soil. The bioassays performed are likely to be good tools to evaluate the toxicity of TBT bioremediated soils, although the TSB medium has changed the toxicity of the compound. Therefore, further studies are still required.

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Chapter IV: General Discussion and Conclusion

General Discussion and Conclusion

Feeding parameters such as consumption, assimilation and egestion ratios were assessed on *Porcellionides pruinosus* through a feeding inhibition test with contaminated food. In a general analysis, feeding parameters showed to decrease with the increase of toxicant exposure. Isopods seem to be able to discriminate between contaminated and uncontaminated food through their chemoreceptors, avoiding the contaminated leaves. TBT could also provide a less attractive flavour to leaves for isopods. On the other hand, TBT is a very toxic compound and may have caused adverse effects on isopods and interfere with their feeding processes.

Egestion ratio was likely to be the most sensitive parameter (lowest EC_{50} value), because faecal production can only occur as a result of food consumption. So, the decreased of egestion ratio can be pointed as a consequence of leave consumption reduction.

Regarding the results from the feeding inhibition test with contaminated soil as the exposure route, a dose response of consumption ratio with TBT concentrations was also observed. The ingestion of TBT contaminated soil particles may have caused stress on isopods with consequent decrease of food consumption. The determined LC_{50} value is higher than LC_{50} values obtained for *Eisenia andrei* and *Folsomia candida* exposed to TBT contaminated soil on previous studies (Römbke et al., 2007), suggesting that *Porcellionides pruinosus* is a less sensitive species to TBT.

Comparing the exposure routes of the two feeding inhibition tests, leaves were the only pathway for TBT uptake on the feeding assay with contaminated food. On the feeding test with contaminated soil, animals could have ingested TBT through soil particles and, in smaller quantities, by water intake through uropods and cuticle absorption. Further conclusions can be undertaken when body burdens for isopods exposed to both routes are compared in order to evaluate the most important route for TBT in isopods. This data is not available yet but it will be part of future studies and discussion.

TBT contaminated soil showed to affect the reproduction success and adult survival of the collembolan *Folsomia candida*. The comparison of EC_{50} and LC_{50} values between this test and other studies performed with the same organisms and earthworms exposed to several soil types indicate that soil properties have a large influence on the

availability and toxicity of TBT, resulting on a variety of EC_{50} and LC_{50} values for the same test species (Hund-Rinke and Simon, 2004; Römbke et al., 2007).

Concentrations of TBT determined in plants such as barley, reed, clover/grass (Novak and Trapp, 2005) and willow trees (Ciucani et al., 2003) are far below the concentrations used in the feeding inhibition test with contaminated leaves, which indicate that TBT contamination in plants may not pose a risk for isopods to consume contaminated leaf litter, for short term exposure. Regarding soil contamination, there is a lack of research of TBT concentrations in soil. Attributed baseline values (Beuselinck and Valle, 2005) may constitute a risk for *F. candida* and *P. pruinosus* if we considered the EC_{50} and LC_{50} values obtained for collembolan reproduction test and isopod feeding inhibition test with contaminated soil, and the AC_{50} value for the avoidance behaviour test. Concentrations determined in sewage sludge are also far below the concentration range used and the EC_{50} determined in this study, but we may not ignored the continuous use of sewage sludge as soil amendment as well as TBT high persistence in soil (Fent and Müller, 1991; Fent, 1996b; Plagellat et al., 2004; Voulvoulis and Lester, 2006). Moreover, the AC_{50} value determined indicates that *P. pruinosus* may be capable to detect such concentrations in soils.

Regarding the bioremediation tests, bioassays with *Micrococcus luteus* were performed to evaluate TBT toxicity to this bacterium. No inhibition zones were observed in the samples with 200 mg/Kg and Av27 inoculated after 15 days, which is suggestive of two possible scenarios: a) *A. molluscorum* A27 has degraded TBT in the soil; b) or, instead of degradation, a reduction of TBT toxicity to *M. luteus* could have occurred through accumulation/adhesion of TBT by Av27 (e.g. bounding to the lipids of cell membrane, siderophore-like structures) (Cruz et al., 2007). Chemical analyses are in course and will help elucidate if TBT was, in fact, degraded.

Concerning the results from the feeding inhibition bioassay with *P. pruinosus*, a dose-related response was observed between leaves consumption and TBT concentration, with an accentuated decrease in the higher concentrations. The ingestion of contaminated soil particles may have caused stress on animals and therefore decreased with their food consumption.

Consumption ratios of TBT-growth medium and TBT-Av27 tests were generally higher (mainly the controls) when compared to TBT-feeding test, which is thought to be due the TSB medium. This is a rich medium that can provide a better flavour or nutritive

characteristics to soil and leaves, leading to higher food consumptions. On the other hand, stimulation of soil particles consumption may have increased toxicity, mainly at TBT-growth medium test, which presented the highest toxic values (EC_{50} and LC_{50}).

It seems that TSB medium (and possibly the pH) had a great influence on the sorption behaviour of TBT. Since both TBT-growth medium and TBT-Av27 tests showed a similar pH range and probably a similar TBT sorption behaviour, the differences observed can be indication of a reduction of TBT toxicity in TBT-Av27 test. Isopods from these two tests probably were more exposed to TBT than animals from TBT-feeding test. TBT-Av27 presented the highest consumption ratios, but lower EC_{50} and LC_{50} values than TBT-feeding test. Maybe those differences can be attributed to high control values of TBT-Av27, but increase on toxicity may also be related to the increase on the amount of soil particles and leaves ingested and therefore uptake of TBT at higher levels.

A reduction of TBT toxicity or its degradation cannot be confirmed yet. For now, two routes of events can be hypothesized: a) TBT retention into the bacteria cells and its unavailability and consequently non toxicity to *M. luteus*, but toxic to isopods when they ingested soil particles along with the TBT retained in bacterial cells; b) if degradation did occur, DBT and MBT may be toxic to isopods (but less toxic than TBT) or DBT can be the only degradation product (or the predominant one), whereby more time for degradation by Av27 is needed. Chemical analyses are expected to draw appropriate conclusions about this work.

Despite those aspects, bioassays should always be used to evaluate soil bioremediation and to complement the chemical analyses, since they will provide information on chemical presence but also on its bioavailability. The bacterium *A. molluscorum* Av27 seems to be able to degrade TBT in soils, but this cannot be confirmed yet. The feeding inhibition test with *P. pruinosus* and the growth inhibition of *Micrococcus luteus* seem to be good tools to assess the toxicity of TBT bioremediated soils, although the bacteria growth medium used has changed the toxicity of TBT and therefore may not be the most appropriate.

As future considerations, in order to accomplish a better knowledge about TBT toxicity towards soil dwelling organisms it is necessary to perform assays with different soil types and natural soils aiming at obtaining a more realistic scenario and different exposure situations (Loureiro et al., 2009). Since sensibility differs between species, a larger battery of organisms should also be used for a more comprehensive study of TBT

toxic effects towards soil invertebrates and derive a threshold for advisable maximum levels for TBT in soils. The same should be considered for future bioremediation studies.

The strong endocrine disruptor properties of TBT should not be ignored as a risk for soil invertebrates hence future studies should be performed in order to investigate whether TBT is a potential endocrine disruptor to these organisms, as well as evaluate its neurotoxic and genotoxic effects.

In relation to bioremediation experiments, the use of a less rich medium (e.g. heavy metal MOPS medium) could be another option to be used on future bioassays, because, as stated before, TSB medium is likely to have an important role on TBT toxicity, influencing its sorption behaviour, isopod's food consumption and uptake by bacteria and isopods.

Moreover, it may also be important to carry out further investigation on the effects of TBT degradation products, DBT and MBT, on soil invertebrates. If chemical analyses confirm TBT bioremediation by *A. molluscorum* Av27 new tests should be carried out to determine more accurately the biodegradation time of TBT by this bacterium.

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