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# Evaluation of the effects of obesogenic compounds on microorganisms

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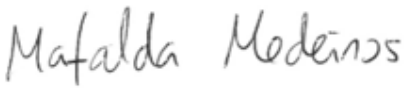

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# Resumo

Centenas de compostos químicos são gerados como resultado da atividade humana sendo que muitos destes compostos têm um grande potencial de acumulação tanto em vertebrados como em invertebrados. Alguns produtos químicos interferem na forma como o organismo armazena e processa os lipídios atuando como disruptores metabólicos, promovendo a adipogénese através por exemplo do aumento do tamanho e número de adipócitos, do aumento do tecido adiposo, interferindo no controlo hormonal do apetite e da saciedade. Estes compostos são denominados compostos obesogénicos e, têm sido sugeridos como possíveis causas para o aumento de números de casos de obesidade.

Inúmeros estudos foram realizados por forma a demonstrar o efeito dos compostos obesogénicos sobretudo ao nível dos vertebrados, porém para provar que o efeito destes compostos atravessa os diferentes níveis taxonómicos é importante adicionar evidências a nível dos organismos procariotas estudando diferentes tipos de bactérias. Por outro lado, é ainda importante estudar o impacto de compostos obesogénicos em bactérias que interagem com hospedeiros eucariotas. Nesse sentido torna-se essencial a escolha de um sistema bem estabelecido e fácil de trabalhar como a simbiose mutualista rizóbio-leguminosa.

Assim, o objetivo deste estudo é avaliar o efeito da exposição a um composto obesogénico, o tributilestanho (TBT), em bactérias modelo (*Escherichia coli* DSM 5698, *Bacillus subtilis* DSM 10$^{T}$, *Pseudomonas fluorescens* DSM 50090$^{T}$) e em diferentes espécies de rizóbio (*Mesorhizobium loti* MAFF303099, *Mesorhizobium mediterraneum* UPM-Ca36$^{T}$, *Mesorhizobium* sp. PMI-6-Portimão, *Mesorhizobium* sp. V-15b-Viseu, *Mesorhizobium* sp. BR15-Bragança, *Mesorhizobium* sp. LMS-1). Pretende-se avaliar a sensibilidade destas bactérias ao TBT e o seu efeito na acumulação lipídica, bem como o impacto deste composto na interação procariota-eucariota (nomeadamente, a interação rizóbio-leguminosa).

Os resultados demonstram que o tributilestanho afeta o crescimento das diferentes bactérias modelo estudadas bem como dos diferentes rizóbios avaliados, tendo sido possível estabelecer uma gama de concentrações subletais para o estudo da acumulação lipídica, nomeadamente 0.25 μM; 1.5 μM; 2.5 μM TBT.

Relativamente à acumulação lípica, avaliada em *Escherichia coli* DSM 5698 e *Mesorhizobium* sp. V-15b-Viseu foi possível verificar que a exposição a 2.5 µM TBT é responsável por um aumento significativo na quantidade de lípidos, demonstrando que o potencial obesogénico do TBT se estende a estes microrganismos.

Em relação à interação procariota-eucariota, foi analisada uma fase inicial desta interação em que ocorre uma modificação na morfologia dos pelos radiculares designada por *curling*. Foram observados poucos eventos de curling nas raízes de grão-de-bico inoculadas com a bactéria *Mesorhizobium* sp. V-15b-Viseu, não sendo assim possível concluir sobre o efeito que o TBT terá nesta interação. Será necessário efetuar mais estudos para concluir acerca dos eventuais efeitos do TBT nas interações procariota-eucariota.

# Palavras-chave

*Mesorizobium; Escherichia coli*; *Bacillus subtilis*; *Pseudomonas fluorescens;* Acumulação lipídica; Rizóbio; Interação procariota-eucariota

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# Abstract

Thousands of chemical compounds are generated as a result of human activity, many of which have a high potential for accumulation in both vertebrates and invertebrates. Some chemicals interfere with the way the body stores and processes lipids by acting as metabolic disruptors, promoting adipogenesis by, for example, increasing the size and number of adipocytes, increasing adipose tissue and interfering with the hormonal control of appetite and satiety. These compounds are called obesogenic compounds and have been suggested as possible causes for the increase in the number of cases of obesity.

Numerous studies have been carried out to demonstrate the effect of obesogenic compounds, mainly in vertebrates, but to prove that the effect of these compounds crosses different taxonomic levels, it is important to add evidence at the level of prokaryotic organisms by studying different types of bacteria. On the other hand, it is also important to study the impact of obesogenic compounds on bacteria that interact with eukaryotic hosts. In this sense, it is essential to choose a well-established system that is easy to work with, such as the rhizobium-legume mutualistic symbiosis.

The aim of this study is to evaluate the effect of exposure to an obesogenic compound, tributyltin (TBT), on model bacteria (*Escherichia coli* DSM 5698, *Bacillus subtilis* DSM 10$^{T}$, *Pseudomonas fluorescens* DSM 50090$^{T}$) and on different rhizobium species (*Mesorhizobium loti* MAFF303099, *Mesorhizobium mediterraneum* UPM-Ca36$^{T}$, *Mesorhizobium* sp. PMI-6-Portimão, *Mesorhizobium* sp. V-15b-Viseu, *Mesorhizobium* sp. BR15-Bragança, *Mesorhizobium* sp. LMS-1). The aim is to assess the sensitivity of these bacteria to TBT and its effect on lipid accumulation, as well as the impact of this compound on the prokaryote-eukaryote interaction (namely, the rhizobium-legume interaction).

The results show that tributyltin affects the growth of the different model bacteria studied as well as the different rhizobia evaluated, and it was possible to establish a range of sublethal concentrations for the study of lipid accumulation, namely 0.25 μM; 1.5 μM; 2.5 μM TBT.

Regarding lipid accumulation, evaluated in *Escherichia coli* DSM 5698 and *Mesorhizobium* sp. V-15b-Viseu it was possible to observe that exposure to 2.5 μM TBT was responsible for a significant increase in the amount of lipids, demonstrating that the obesogenic potential of TBT extends to these microorganisms.

Regarding the prokaryote-eukaryote interaction, curling, the initial phase of this interaction, in which a morphologic change in the root hairs occurs was analyzed. Few curling events were observed in the roots of chickpea inoculated with the bacterium *Mesorhizobium* sp. V-15b-Viseu, so it is not possible to evaluate the effect of TBT on this interaction. Further studies are necessary to understand the potential effects of TBT on the prokaryote-eukaryote interactions.


# Keywords

*Mesorhizobium*; *Escherichia coli*; *Bacillus subtilis*; *Pseudomonas fluorescens*; Lipid accumulation; Rhizobium; Procaryote-Eukaryote interaction

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# List of Abbreviations

| | |
|---|---|
| ABC transporters | ATP-Binding Cassette transporters |
| DBT | Dibutyltin |
| EDCs | Endocrine Disrupting Chemicals |
| LB | Luria-Bertani Broth or Agar |
| MBT | Monobutyltin |
| YMB/YMA | Yeast Mannitol Broth/ Yeast Mannitol Agar |
| MP | Microplastics |
| NB/NA | Nutrient Broth/Nutrient Agar |
| Ots | Organotins |
| PCB | Polychlorinated Biphenyls |
| PPARγ | Peroxisome Proliferator-Activated Receptor gamma |
| PVC | Polyvinyl Chloride |
| RXR | Retinol X Receptor |
| TBT | Tributyltin |
| TBTCl | Tributyltin Chloride |
| TSB | Tryptone Soy Broth |

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# I. Introduction

Over the last century a drastic change has been witnessed in the way humans live and relate with nature. The urge to meet the daily needs of individuals has led to the development and implementation of new solutions almost totally supported by the chemical industry. Not only the means of production used, but also the resulting products have led to the global proliferation of chemical agents at an unprecedented scale. Particular attention has been placed on the unidentified impacts that these chemicals may have on ecosystems in both short and long term. The recognition that man-made chemicals were affecting ecosystem's, including humans, and the consequent public awareness on this topic was raised more than 60 years ago by the book "Silent Spring" published in 1962 by Rachel Carson. In this landmark publication, Rachel Carson alerted to the risks of pesticides in the food chain and their consequences on ecosystems (Carson, 1962).

Thousands of chemical compounds are generated and discharged as a result of human activity, and many of these substances have a high potential for accumulation in both invertebrate and vertebrate species, including humans. Some of these substances also have the ability to mimic or antagonize the action of hormones, which can have negative effects on an organism's ability to function normally (Diamanti-Kandarakis et al., 2009). These substances, which are known as Endocrine Disrupting Chemicals (EDCs), or Endocrine Disruptors, are ubiquitous, being detected in diet, air, water, and soil samples. They also can be found in numerous items that consumers use daily, including food storage containers and personal care products (Bergman et al., 2013). This group of molecules is highly heterogeneous and includes, for example, synthetic chemicals used as industrial solvents/lubricants and their derivatives such as polychlorinated biphenyls (PCBs), plasticizers (phthalates and bisphenols), pesticides [dichlorodiphenyltricholoethane (DDT)], fungicides (vinclozolin) and pharmaceutical compounds (Gore et al., 2015).

Endocrine disruptors are associated with numerous adverse health effects ranging from metabolic disorders such as diabetes and obesity, to nervous system disorders (affecting behavior and neurodevelopment, for example) to effects on male and female reproduction (e.g., with increased endometrial cancer risk, ovarian failure, irregularities

in the menstrual cycle, decline in sperm quality thus reducing fertility rate) (Bergman et al., 2013).

Among the various classes of endocrine disruptors, organotin compounds (Ots) are quite relevant as they include tributyltin, which is considered the putative agent of the best example of endocrine disruption in the wildlife (see below, for full description).

Organotin compounds (Ots) are characterized by the presence of a tin atom (Sn) covalently attached to one or more substituent groups (Figure 1) (Hoch, 2001). The general formula of organotins is RSnX, in which R is an organic group (e.g, methyl, ethyl, propyl, butyl, phenyl) and X is an anionic group (e.g, chloride, fluorine, oxide, hydroxyl, carboxylate, and thiolate) (Hoch, 2001). These compounds are grouped into mono- $RSnX_3$, di- $R_2SnX_2$, tri- $R_3SnX$, and tetrasubstituted $R_4Sn$ compounds according to the number of organic compound groups (Hoch, 2001).

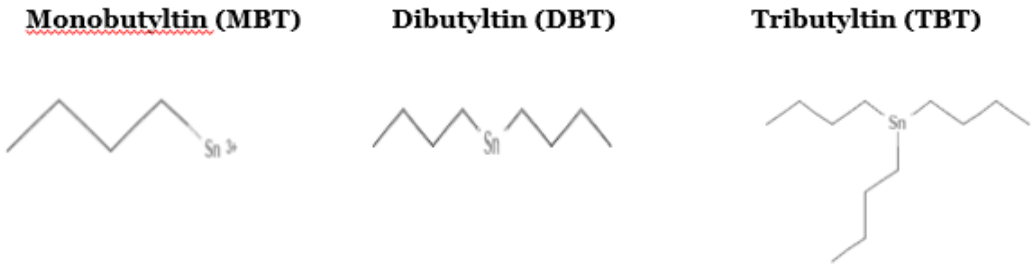



**Figure 1:** Chemical structures of butyltin compounds.

Usually, the number, type, and ligand of R groups affect the physical, chemical, and toxicity of Ots, with inorganic tin being practically nontoxic and trisubstituted compounds exhibiting the highest toxicity (Sousa et al., 2013; WHO, 1990). In addition to being utilized as fungicides and pesticides in agriculture and the marine activities, organotin compounds are additionally used in the manufacturing of silicone and PVC (polyvinyl chloride) in industry (Hoch, 2001; Sousa et al., 2014) (Figure 2).

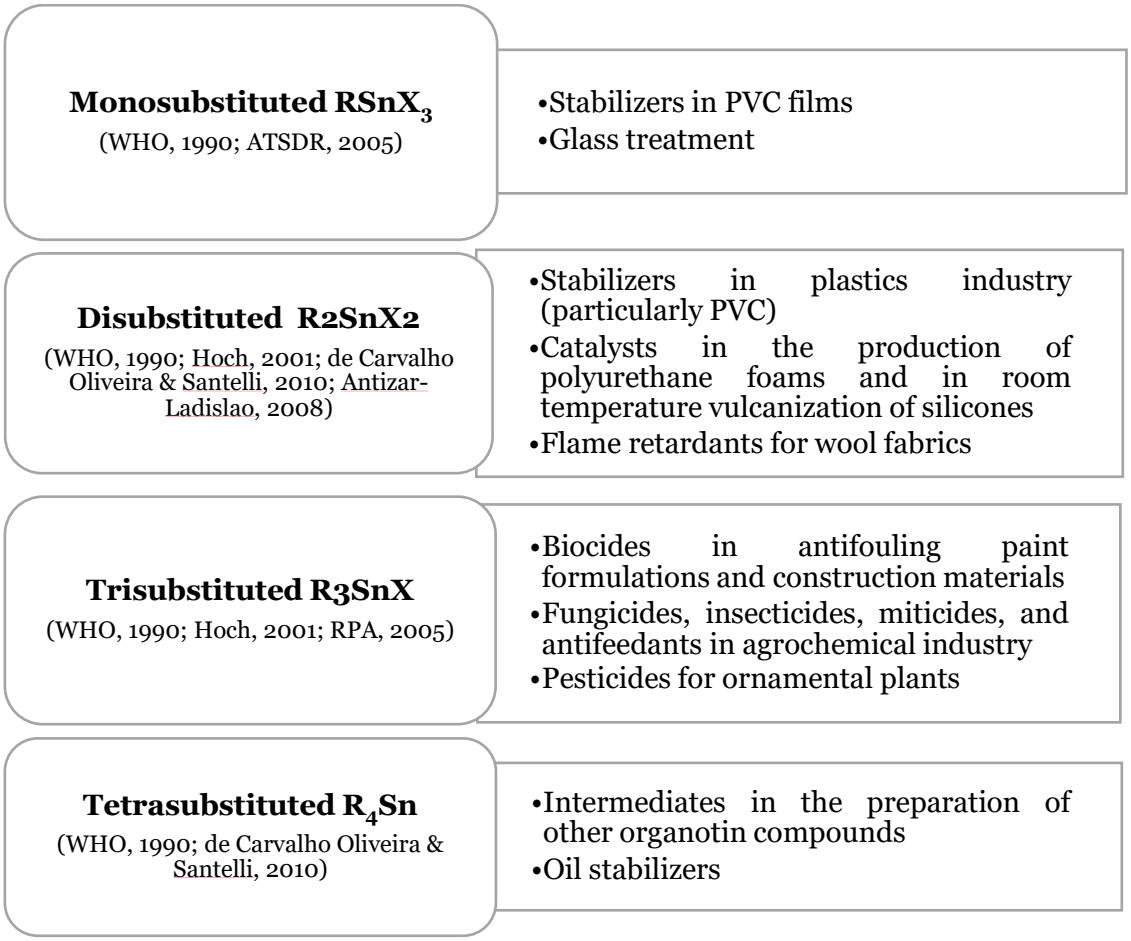



**Figure 2:** Summary of organotin compounds applications adapted from (Sousa et al., 2014).

Despite having numerous uses in consumer products and household items, such as stabilizers in PVC, insecticides, and oil stabilizers, tributyltin (TBT), is mostly known for its use as biocide in antifouling paint formulations. Due to its potent biocidal activity TBT was used in ships and submerged structures on a planetary scale however, TBT, was highly toxic towards non target organisms. One of these toxic effects was imposex, i.e., the development of male sexual characteristics in female gastropods, which is regarded as the best example of endocrine disruption in wildlife (Sousa et al., 2014).

Besides being responsible for imposex in gastropods, TBT was latter associated with adipocyte differentiation and weight gain in cells lines, amphibia and mice (Grün & Blumberg, 2006). Such observations on the ability of EDCs to induce adipogenesis in animal models (Grün & Blumberg, 2006) led to a new concept in which these

compounds, obesogens, act as metabolic disruptors, promoting adipogenesis by increasing energy storage in adipose tissue, and by interfering with the hormonal control of appetite and satiety (Rato & Sousa, 2021).

While the pathways in which obesogenic compounds act are not well defined, they are known to be related to peroxisome proliferator-activated receptor gamma (PPARγ) and retinoid X receptor (RXR) signaling pathways (Grün & Blumberg, 2006; le Maire et al., 2009). PPARγ is the key regulator that controls lipid proliferation, lipid differentiation, and also controls intracellular lipid flow (Grün & Blumberg, 2009). Additionally, when the formation of the PPARγ-RXR heterodimer occurs it serves as a ligand for hormones, fatty acids and metabolites, therefore promoting lipid biosynthesis and storage, leading to differentiation of existing pre-adipocytes, but also to the conversion of mesenchymal stem cells into pre-adipocytes leading to adipose tissue formation (Fang et al., 2015; Grün & Blumberg, 2006; Rato & Sousa, 2021).

Some environmental contaminants are known to be able to activate the PPARγ signaling pathway and positively regulate PPARγ expression, potentially increasing the risk of developing obesity (Fang et al., 2015; Rato & Sousa, 2021). These substances, include for example TBT, which is considered the model obesogen, as it was the first obesogenic substance reported and the most investigated to date (Grün & Blumberg, 2006). After the groundbreaking study identifying TBT as obesogen, many other compounds have been studied as possible obesogens. Currently, there are about 50 obesogenic compounds identified (Heindel & Blumberg, 2019). The first experiments to study obesogenic compounds are usually performed *in vitro* (with cell lines) and then *in vivo* (with animal models). The mouse pre-adipocyte cell line 3T3-L1 is one of the most used cell lines for testing potential obesogenic compounds (Kassotis et al., 2022).

With the increase in research and studies in this topic the obesogen hypothesis has become more accepted. The obesogen hypothesis proposes that exposure to environmental chemicals during critical developmental stages such as an early life stage or long-term (chronic) exposures may have an influence on the promotion of metabolic diseases such as obesity and obesity-related disorders (Grün, 2014; Grün & Blumberg, 2006).

## 1.1. Impact of Organotin Compounds in Microorganisms

Most of the published work on the effects of organotin compounds focuses on vertebrates, nevertheless there are some studies on the interaction of microorganisms with organotin compounds, mostly exploring the mechanisms by which TBT affects microalgae, fungi, and bacteria (Lustig et al., 2022).

Generally, OTs at higher concentrations, are extremely toxic to microalgae, being responsible for a decrease in the concentration of photosynthetic pigments leading to a deficit in growth, oxygen, and energy production (Touliabah et al., 2022;). In the study by Yoo and colleagues (Yoo et al., 2007) the effects of TBT were tested in the marine green algae *Tetraselimis suecica* for 96h. Overall, higher TBT concentrations had a stronger impact in cell growth and decreased culture viability after 4 days. Higher TBT concentrations also had a negative impact on the levels of chlorophyll a and b produced, whereas low TBT concentrations (0.0312 µg/ml) had a positive impact in the production of both types of chlorophyll. Yet, some microalgae can tolerate OTs and even degrade TBT into less toxic compounds. In fact, some microalgae like *Chlorella* Sp. or *Scenedesmus* Sp. are extremely important in degrading the TBT present in water, since they can absorb TBT and debutylate it intracellularly in less toxic compounds like DBT and MBT. For example, the degrading potential of cells of *Chlorella miniata, Chlorella sorokiniana, Scenedesmus dimorphus*, and *Scenedesmus platydiscus* was studied by Tam et al. (2002). The authors studied both the TBT removal potential by dead algae and the TBT removal and degradation potential by live algae. Overall, dead cells where able to efficiently remove TBT by simple biosorption and this biosorption mechanism was more important than the absorption and degradation processes. Nevertheless, live cells were able to efficiently remove TBT and degrade it into DBT and MBT over 14 days.

The fact that some microalgae can degrade TBT into less toxic compounds lead some researchers to test biobased solutions to address TBT pollution. For example, *Chlorella vulgaris* cells immobilized in alginate beads were compared to empty beads in terms of TBT degradation efficiency (Luan et al.2006). Both cell types were exposed to 0.01; 0.05 and 0.1 µg/mL TBT and the results suggested that immobilized cells in beads were more effective at degrading TBT and its derivatives, which according to the authors highlight the potential use of this biobased solution to tackle organotin pollution. Similar studies were conducted by other researchers (Zhang et al., 1998) used a different microalga, *Chlorella emersonii,* and exposed the free and immobilized algae to 1 µg/mL TBT. The results were similar with the ones from Zang et al (1998), with the immobilized cells

being more effective at degrading TBT as disclosed by the Bioconcentration Factors (BCFs).

Regarding fungi, *Cunninghamella echinulate* has been reported to be able to degrade concentrations of TBT up to 50 μg/mL with a reduction efficiency of 91% by (Soboń et al., 2016). Yet, TBT changed the protein and free amino acid profiles and increased makers of oxidative stress. Increased oxidative stress was also registered in the species *Cunninghamella elegans* (Bernat, Gajewska, et al., 2014). In this study, a lipidomic analysis was conducted and 49 lipid species were identified and quantified to understand the impacts of fungi exposed to TBT compared to a control group. The obtained results disclose that TBT reduced some lipids' levels and raised others. Amongst the ones that were increased was phosphatidic acid, which according to Huang et al. (2022) is inversely associated with obesity. Furthermore, exposure to TBT affected the cells' metabolism and exacerbated the production of reactive oxygen species which are responsible to attack the lipid membranes leading to cell death.

Regarding bacteria there are evidence that some species can degrade TBT. One of the most studied bacterial genus is *Pseudomonas* sp. These microorganisms can live in a variety of environments, such as the rhizosphere and endosphere of various plants, and therefore exhibits a flexible metabolic capability and a wide range of possibilities for environmental adaptation (Mukhtar et al., 2021). Some compounds can be broken down by these bacteria, and there is evidence that *Pseudomonas* sp. can also break down TBT. It has been shown that *Pseudomonas* sp. have the capability to use TBT as its sole carbon source even though the mechanism is not yet known. Bernat, Siewiera, et al., 2014 studied the organotin degradation ability, and the composition of the membrane lipid and cellular proteins composition in the presence of TBT. They concluded that changes in the phospholipids species were responsible for membrane modifications in a way that could protect the cell from TBT, partly explaining the resistance of *Pseudomonas* sp. to TBT (Anayo et al., n.d.; Jun et al., 2016; Polrot et al., 2022).

Another well studied microorganism is the gram-negative marine bacterium *Aeromonas molluscorum Av27*. Cruz et al. (2015) conducted a study with the objective of understanding the molecular level effects of TBT exposure in this bacterium. Bacteria were exposed to two different TBT concentrations (5 μM and 50 μM) and the results were compared with non-exposed bacteria. The gene ontology results disclosed that exposure to lower TBT concentrations resulted in higher numbers of repressed genes whereas exposure to higher TBT concentrations resulted in an increased number of over-

expressed genes. Furthermore, it was possible to conclude that at both TBT concentrations, higher variations in gene expression were found in the functional categories associated with enzymatic activities, transport/binding, and oxidation-reduction. Additionally, the authors by using the STRING tool were able to identify several proteins with unknown function associated with other involved in organotin degradation processes.

Besides the environmental microbiology studies, there is increasing interest on the effects of contaminants, including TBT, on the human gut microbiome. The gut microbiome plays a crucial role in the pathogenesis of obesity including an increase in the number of bacteria that can ferment carbohydrates, increasing the biosynthesis of short-chain fatty acids, contributing to an additional source of energy to the host, ultimately stored as lipids or glucose (Muscogiuri et al., 2019). For instance, the loss of bacterial diversity leading to a lower number of beneficial bacterial and an increase in harmful bacteria (dysbiosis) is associated to reduced lipid degradation rate (Guo et al., 2018; Requena et al., 2013; Zhan et al., 2020). In the study of Jiang et al. (2021) the effects of TBT were studied on bile acids and gut microbiome in mice. This study focused on testing the effects of microplastics and TBT in the microbiome and for the TBT exposure group, rats were exposed to 100 ng/Kg TBT for 33 days. The results demonstrated that TBT alone increased inflammation markers and the authors suggested that TBT could cause gut microbiome dysbiosis and consequently alter bile acids profiles.

There are few studies on the impact of organotin compounds on prokaryote-eukaryote interactions. The rhizobium-legume mutualistic symbioses, represent an example of plant-microbe interactions, which has been extensively studied due to its importance in natural systems, but also in agroecosystems. Rhizobia are soil bacteria capable of converting atmospheric nitrogen into nitrogenous compounds that can be used by plant (Hawkins & Oresnik, 2022). This interaction between rhizobia and legumes is characterized by the formation of nodules, usually in the roots of compatible host plant. The nodule formation results from a successful root colonization by rhizobia, which initiates with a molecular dialog between the two partners that allows mutual recognition (Yang et al., 2022). This process typically involves the production of flavonoids by the legume, which will induce the nod genes expression in rhizobia, leading to the secretion of signaling molecules (Nod factors), which will induce the growth of the radicular hair in the plants' roots and triggers the early stage of root infection: root hair curling (Hawkins & Oresnik, 2022; Yang et al., 2022). Curling causes the entrapment of rhizobia

and leads to the formation of the infection thread, which allows rhizobium entrance on the host tissue and initiation of nodule organogenesis (Laranjo et al., 2014).

Although studies have linked specific microorganisms, such as fungi and bacteria, with effects of organotin compounds, there is still much to understand about how these chemicals impact the metabolism of microorganisms as well as other species, such as soil bacteria and prokaryote-eukaryote interaction. There is still no evident relationship between TBT exposure and its impact in bacterial lipids apart from the membrane lipids, which makes this study relevant and important.

# II. Aims of the dissertation

The theory of obesogenesis suggests that environmental contaminants contribute to adipocyte differentiation, metabolic disorders, and obesity. It was initially proposed for tributyltin (TBT), a potent endocrine disruptor from the organotin family. Multiple studies support this theory, identifying over 50 obesogens with effects observed in various models, from cell lines to mammals. However, to prove that this phenomenon is transversal and that it occurs at different taxonomic levels, it is essential to add evidence at the level of prokaryotes. The study of different types of bacteria is particularly relevant due to their pivotal role in the human and environmental microbiome. Nevertheless, there is limited information about the impact of these compounds on bacteria that interact with eukaryotic hosts. In this context, the well-established system of rhizobium-legume symbiosis will be used as model.

To address this objective, the following specific aims were outlined:

- To evaluate TBT sensitivity of model bacteria and different strains of *Mesorhizobium*.

- To evaluate the impact of TBT exposure on lipid accumulation in model bacteria and *Mesorhizobium*.

- To evaluate the impact of TBT exposure on the interaction of prokaryotic-eukaryotic organisms, using the rhizobium-legume symbiosis.

# III. Materials and Methods

## 3.1. Bacteria

In this study, three model bacterial species and six strains of *Mesorhizobium* were studied. The model bacteria *Escherichia coli* DSM 5698 (the same as strain K12), *Bacillus subtilis* DSM 10$^T$ and *Pseudomonas fluorescens* DSM 50090$^T$ were used, representing bacteria with very different taxonomy and lifestyles (for example, *E. coli* and *P. fluorescens* are a gram-negative species belonging to Gammaproteobacteria class, while *B. subtilis* is a gram-positive belonging to Firmicutes phylum and Bacilli class), were acquired from Leibniz-Institute DSMZ from the lot number 0421, 0321 and 0320, respectively. Six strains of *Mesorhizobium* were used in this study, four of them were obtained from different locations of Portugal namely *Mesorhizobium* sp. PMI-6-Portimão, *Mesorhizobium* sp. V-15b-Viseu, *Mesorhizobium* sp. BR15-Bragança, *Mesorhizobium* sp. LMS-1 (Alexandre et al., 2009); the remaining two species used in this study were *Mesorhizobium loti* MAFF303099 (Kaneko, 2000) and *Mesorhizobium mediterraneum* UPM-Ca36$^T$ (Nour et al., 1995) All these mesorhizobia are symbionts of chickpea (*Cicer arietinum*), with the exception of *M. loti* MAFF30399, which nodulates *Lotus* species. All bacteria were kept in glycerol stocks at -80°C.

## 3.2. TBT solutions

TBT chloride with a purity of 96%, CAS number 1461-22-9 and a molecular weight of 325.51 was obtained from Sigma Aldrich. A stock solution with a final volume of 10mL and a concentration of 600 mM was prepared by diluting the TBT Chloride with ethanol. Later, three new stock solutions were prepared using serial dilutions with a dilution factor of 10 to attain the concentrations of 60 mM, 6 mM and 0.6 mM, respectively.

## 3.3. Nile red solutions

Nile red stain, CAS number 7385-67-3, was obtained from Sigma Aldrich. Two stock solutions were prepared, stock solution A (500 mg/mL) and stock solution B (5 µg/mL). Solution A was prepared by weighting 5 mg of Nile red into an aluminum foil piece and transferred to a 10 ml volumetric flask filled up with acetone. Solution B was prepared by diluting Solution A: 100 µL of the solution A to a 10 mL volumetric flask filled up with distilled water. Since Nile red is degraded when exposed to light, both solutions were stored and manipulated in the dark conditions.

## 3.4. Evaluation of TBT tolerance

Before each assay, the bacteria were grown overnight at 180 rpm at 28ºC in TSB medium for *Pseudomonas fluorescens*, at 28 ºC in NB medium for *Bacillus subtilis*, at 37ºC in LB medium for *Escherichia coli*, and at 28ºC in YMB medium for the rhizobium strains (See Appendix 1 for medium information). Before incubation with TBT, the bacteria were adjusted to an initial optical density of 0.10 at 540 nm (OD540 nm). The initial TBT concentrations tested were based on the study of Cruz et al. (2007). The concentration range initially tested was too high and several assays were performed until a suitable range of sub-lethal concentrations was found for all species (Data provided in Appendix 2).

For each species, the following sub-lethal TBT concentrations were tested: 0.25 µM; 1.5 µM; 2.5 µM, alongside with a positive and several negative controls. The positive control consisted of the culture medium with bacteria (with no TBT) whereas in the negative controls no bacteria was added (one set of negative control to each concentration of TBT tested, including a set with no TBT).

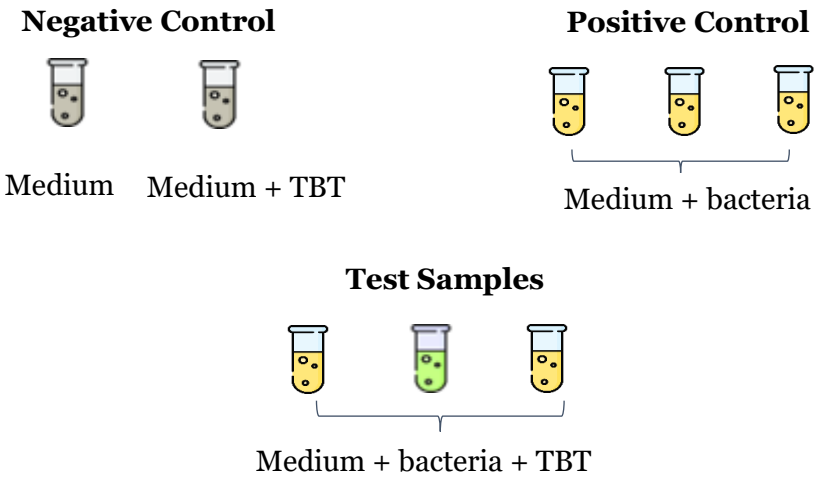



**Figure 3:** Schematic diagram of TBT tolerance assay.

To study the bacterial growth, culture samples were taken in multiple time points until the stationary phase was reached, with last sample taken at 55 hours. The optical density

was measured at 540nm. The results were analyzed by the construction of bacterial growth curves, and the evaluation of the tolerance of the bacteria to the presence of TBT was performed by comparing the bacterial growth curves with and without TBT. All assays were composed of 3 technical replicates.

## 3.5. Evaluation of the impact of TBT on lipid accumulation

Lipid accumulation was evaluated in the bacteria *Escherichia coli* and *Mesorhizobium mediterraneum* UPM-Ca36$^T$ after 24 h exposure to 2.5 μM TBT. The exposure procedure was the same as described in Section 3.4.

Lipid accumulation was evaluated using confocal fluorescence microscopy after staining with the fluorochrome Nile red, by adapting a previously described protocol (Idris et al., 2022), with some adjustments. Firstly, the bacteria were adjusted to an optical density of 1.0 in a final volume of 1.5 mL. Then, the bacteria were centrifuged at 5000 x g for 5 minutes and resuspended in 1 mL of saline solution (85% NaCl). This process was repeated twice. Since Nile red degrades when exposed to light, the protocol was performed with the minimum light exposure possible. 100 μL of Nile red solution (5 μg/mL) and 500 μL of distilled water was added, and vortex mixed. The suspension was kept at 37 ºC for 2 h and then centrifuged at 5000× g for 5 min. The supernatant was discarded, and the pellet was washed twice with distilled water. A volume of 500 μL of distilled water was added to the pellet and vortex mixed. Finally, 20 μL of the stained cell suspension was placed into a glass slide and covered with a glass slip and observed using a Leica TCS SPE Confocal Microscopy System with the Software Leica LAS AF with a laser of 488 nm. Two biological replicas were performed. For the control and the TBT treatments a minimum of 5 technical replicates were analyzed.

Fluorescence quantification was carried out on the images captured with the Leica software mentioned previously using Fiji Software. Fluorescence was quantified in a predefined and identical area for all images (height of 1024 pixels and width of 1024 pixels). The data was then analyzed in Fiji software and the fluorescence was obtained by measuring the integrated density obtained for each cell using multiple cells per image. The individual results for all technical replicates were combined for each biological replicate and the average of the two biological replicates were subsequently used. Basic statistical analysis, including T-test to compare between group were performed using GraphPad Prism software. A significance level of 0.05 was used.

## 3.6. Evaluation of the impact of TBT on prokaryotic-eucaryotic interaction

To understand the impact of TBT on the rhizobium-legume symbiosis, the chickpea seeds and *Mesorhizobium* sp. V-15b-Viseu (from now on designated by V-15b) were used and the effects of exposing the pre-germinated chickpea seedlings to the bacteria previously treated with 0.25 µM and 2.5 µM of TBT were observed after 48 and 72 hours. The methodology was adapted from (Paço et al., 2016) . Chickpea seeds were disinfected in a solution of calcium hypochlorite (65% active chlorine, see Appendix 1) during 25 min and washed 25 times with sterile distilled water, in the laminar flow cabinet. The seeds were then incubated in sterile distilled water at 4ºC for 30 minutes; after this time, the water was changed, and the seeds incubated again for 30 minutes at 4ºC. Afterwards, the seeds were re-washed 5 times with sterile distilled water to completely remove the calcium hypochlorite and transferred to water-agar (75% of agar) plates with sterile forceps. In due course, the seeds were incubated for 48h at 28ºC, in the dark.

In the following two days, after a preliminary overnight growth, the bacteria were exposed to TBT for 24 hours following the same methodology described in Section 3.4.

In the last day, five chickpea seeds with pre-germinated roots with a length of approximately 10 mm were transferred to a new plate with water-agar and filter paper. The seedlings were inoculated by adding 100 µL of a bacteria suspension (OD540 of 0.4) with the following treatments: Control (V15b without TBT), TBT 2.5 µM (V15b exposed to 2.5 µM TBT) and TBT 0.25 µM (V15b exposed to 0.25 µM TBT). Each agar plate had 5 seedlings and was kept at room temperature and involved in tin foil at the seed level, in order decrease the light exposure (simulating the soil conditions).

Three replicates were performed in order to make a total of fifteen seeds per treatment. Two biological replicas were made. The differentiation zone of the roots was observed using a Leica DM6000B microscope and images were captured using a Leica LMD SS7000 digital camara during the 4 days following the last step of the experimental design.

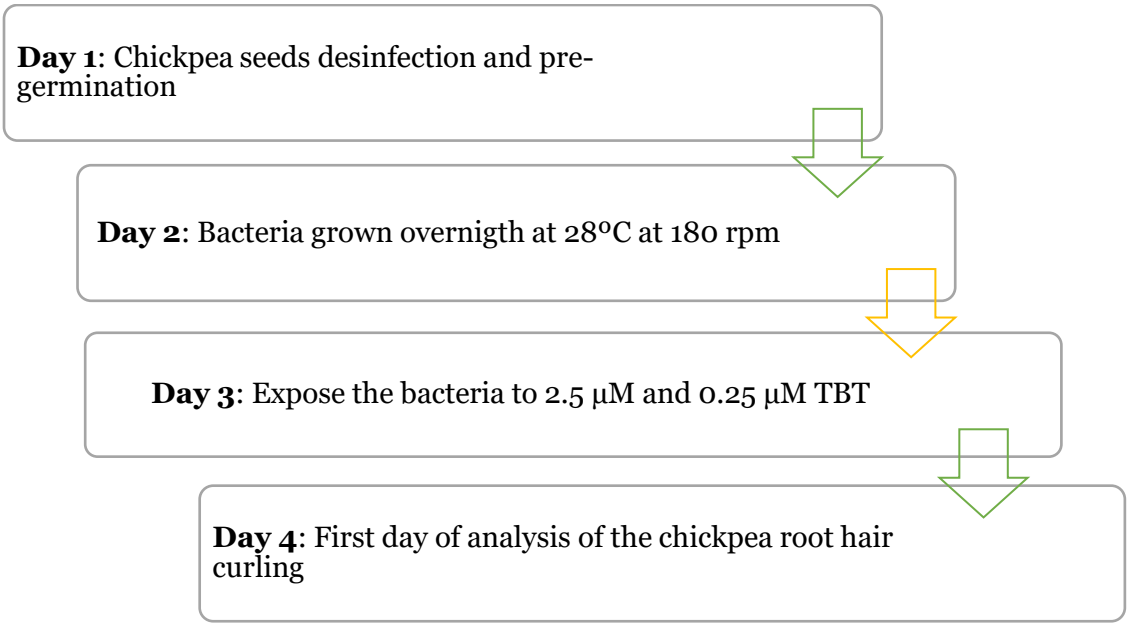



**Figure 4:** Schematic diagram of the procedure performed to evaluate the impact of TBT on prokaryotic-eucaryotic interaction.

# IV. Results and Discussion

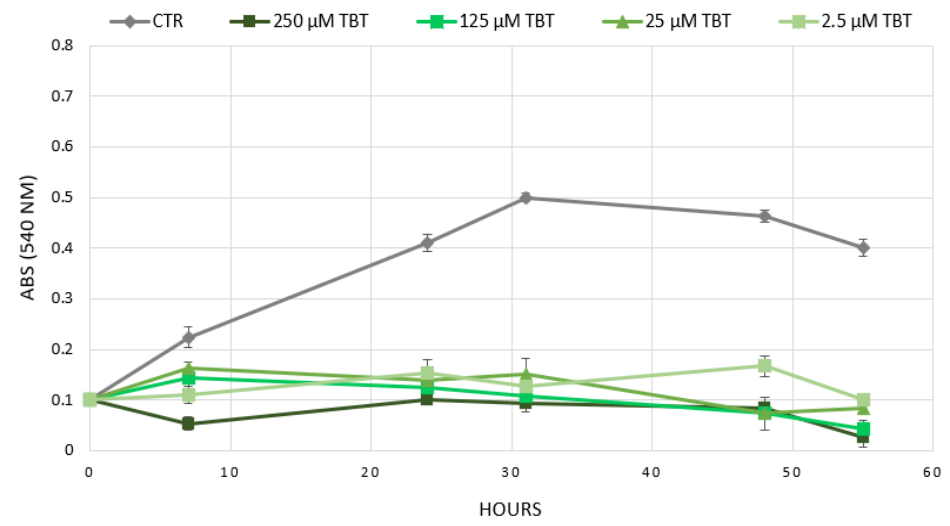
## 4.1. Evaluation of OTs tolerance

### 4.1.1. *Mesorhizobium* strains

The effects of TBT exposure on the six strains of *Mesorhizobium* were evaluated using the initial concentrations of 3000; 1500 and 750µM suggest by Cruz et al. (2007). However, these concentrations were responsible for a high growth inhibition on the six rhizobium strains (Figure A5-A10, appendix 2). Since the focus of this study was to evaluate the effects of TBT exposure on lipid accumulation, sublethal concentrations were mandatory. Therefore, a series of range finding experiments were performed, namely: i) 750; 500; 250 µM; ii) 250; 125; 25; 2.5 µM; iii) 2.5 µM; 1.5 µM and 0.25 µM as shown in figures A5-A10 of appendix 2. Herein, only the range ii) and iii) assays will be presented and discussed.

Regarding ***Mesorhizobium loti*** **MAFF303099**, the assay with the TBT concentrations of 250; 125; 25 and 2.5 µM (figure 5a, green dose-response curves) demonstrated that these concentrations were still too high, with a clear inhibition in the growth as depicted by the reduction of 63% in the absorbance value at 24 h and a reduction of 75% at 55 h at the lowest concentration of 2.5 µM of TBT. Hence, a new set of concentrations was tested, starting with 2.5; followed 1.25 and 0.25 µM TBT (Figure 5b, yellow dose-response curves). From the analysis of the obtained results, it is clear that these concentrations had a lower impact on the growth of the bacterium, with all TBT concentrations exhibiting no negative impacts on growth after 55 h. Nevertheless, when comparing both assays it is obvious a different behavior of the bacteria for the TBT concentration of 2.5 µM. In the first assay, the concentration of 2.5 µM was responsible for a growth inhibition of 63% after 24 h and 75% after 55 h, whereas the same concentration in the second assay was responsible for much lower growth inhibitions of 56% after 24 h and no inhibition at 55 h. Such differences amongst different assays for the same conditions are a problem as they compromise the reproducibility of the results. Therefore, at first, the assays were repeated several times (data not shown), and similar

results were obtained. Afterwards a new experiment to elucidate this phenomenon was performed.

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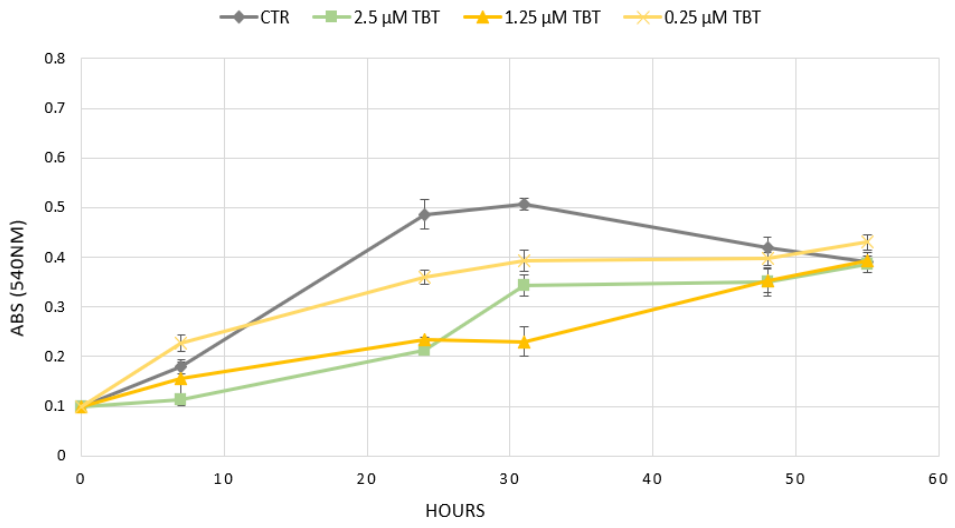


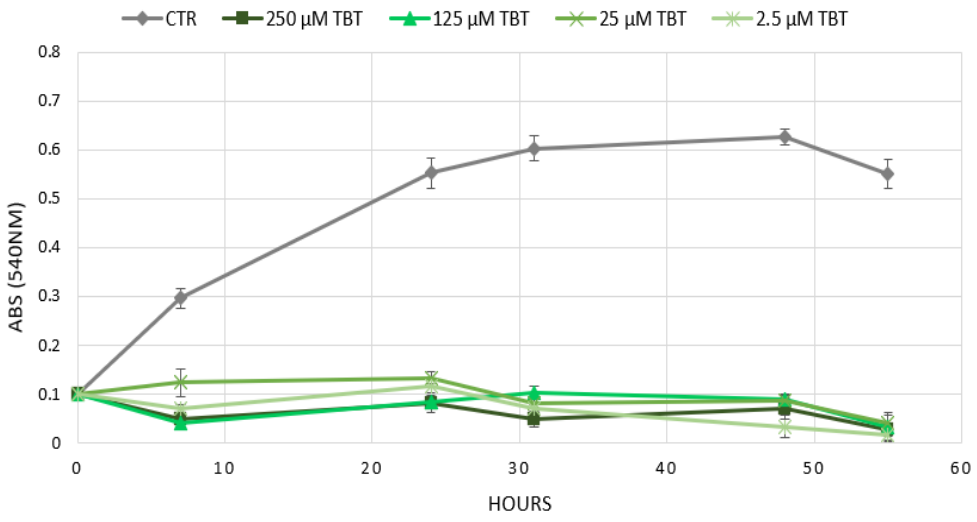

b)

**Figure 5:** Grown curves of *Mesorhizobium loti* MAFF303099 when exposed to a) 250; 125; 25; 2.5 µM of TBT (top, green curves) and b) exposed to 2.5;1.25;0.25 µM of TBT (bottom, green and yellow curves). ABS – absorvance; CTR – control (no TBT)

Our hypothesis was that as TBT is semi volatile, in the first assay, the real concentration tested in the 2.5 µM condition was higher than the nominal concentration (2.5 µM) due to the carryover from the highly concentrated solutions in the same assay (250 and 125 µM). Whereas the nominal 2.5 µM concentration in the second assay was probably

similar to the real one as this was the highest TBT concentration tested in the second assay. This type of transfer was potentiated by the fact that all assays were fully wrapped with aluminium foil, since TBT is degraded by light exposure. To test our hypothesis, two different types of tubes were tested simultaneously, using 250 and 0.25 µM TBT. These concentrations were prepared using the normal glass tubes for bacterial growth, which have cap-o caps and are not completely sealed and repeated (using the same bacteria batch) using fully sealed falcon tubes. The experimental design and the obtained results are detailed in figures A11 and A12 of appendix 3. These results showed that the growth inhibition of the bacteria exposed to 2.5 µM in non-sealed test tubes was higher than the one observed in the falcon tubes (sealed and preventing volatile entrance), particularly at the end of the assay, which corroborates our hypothesis. Such results highlight the need to carefully design the experimental protocol in order to avoid such carry over effects, which is particularly relevant for compounds for which chemical analysis to determine the real concentrations are not possible.

In the case of ***Mesorhizobium* sp. V-15b-Viseu**, the tests with the first group of TBT concentrations (250; 125; 25 and 2.5 µM in figure 6a, green dose-response curves) showed that these concentrations were still too high, with a clear inhibition in growth as depicted by the reduction of 80% in the absorbance value at 24 h and a reduction of 96% at 55 h at the lower concentration of 2.5 µM of TBT . The second set of concentrations was then tested, starting with 2.5; followed 1.25 and 0.25 µM TBT (Figure 6b, yellow dose-response curves). The obtained results disclose that the lowest concentrations tested (0.25 µM) had almost no impacts on growth after 55 h.

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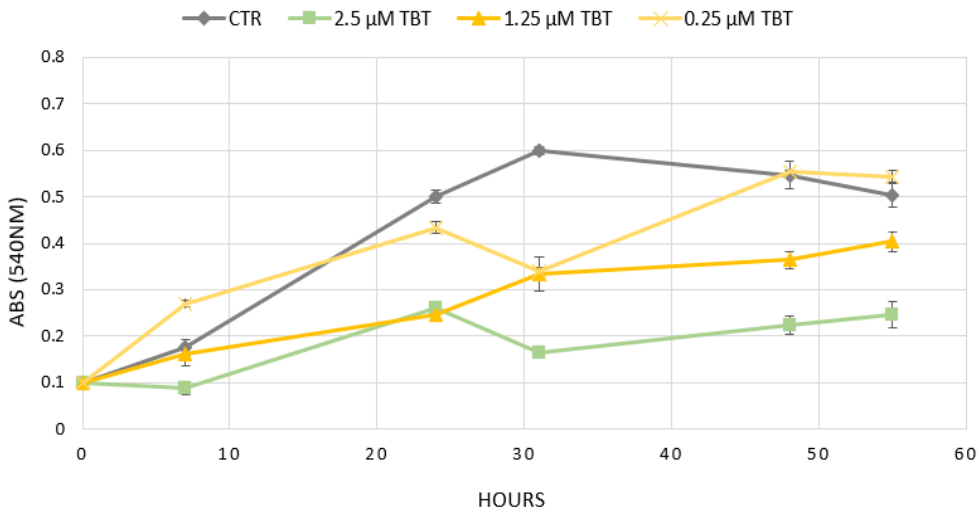


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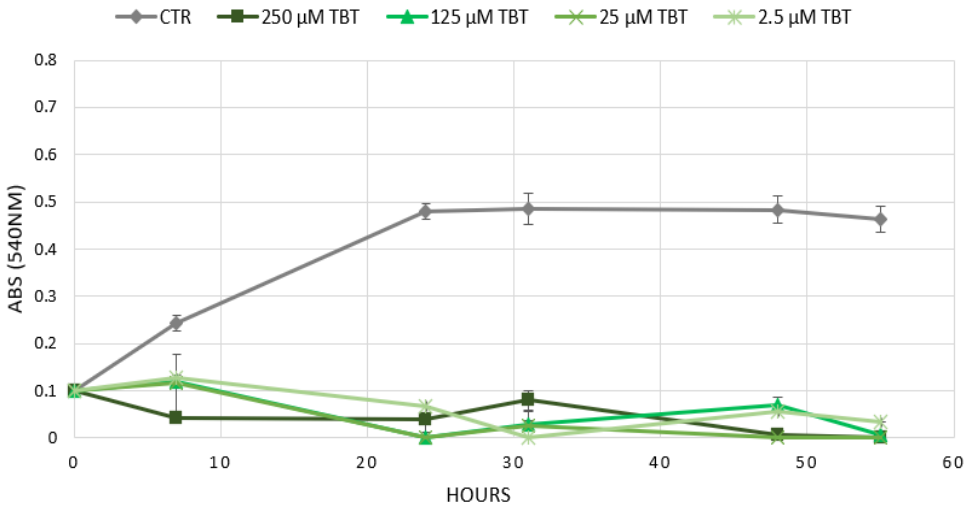
**Figure 6:** Grown curves of *Mesorhizobium* sp. V-15b-Viseu when exposed to a) 250; 125; 25; 2.5 µM of TBT (top, green curves) and exposed to b) 2.5;1.25;0.25 µM of TBT (bottom, green and yellow curves). ABS – absorvance; CTR – control (no TBT)

Similar results were obtained for ***Mesorhizobium* sp. PMI-6-Portimão**, with TBT concentrations of 250; 125; 25 and 2.5 µM being too high (figure 7a, green dose-response curves), with a clear inhibition in growth as depicted by the reduction of 88% in the absorbance value at 24 h and a reduction of 94% at 55 h for the concentration of 2.5 µM of TBT. The concentrations tested in the second experiment (2.5; 1.25 and 0.25 µM TBT in Figure 6b, yellow dose-response curves) had no negative impacts on the growth of the bacterium, in fact an increase of about 36% in the optical density for all TBT treatments was observed after 55 h of exposure.

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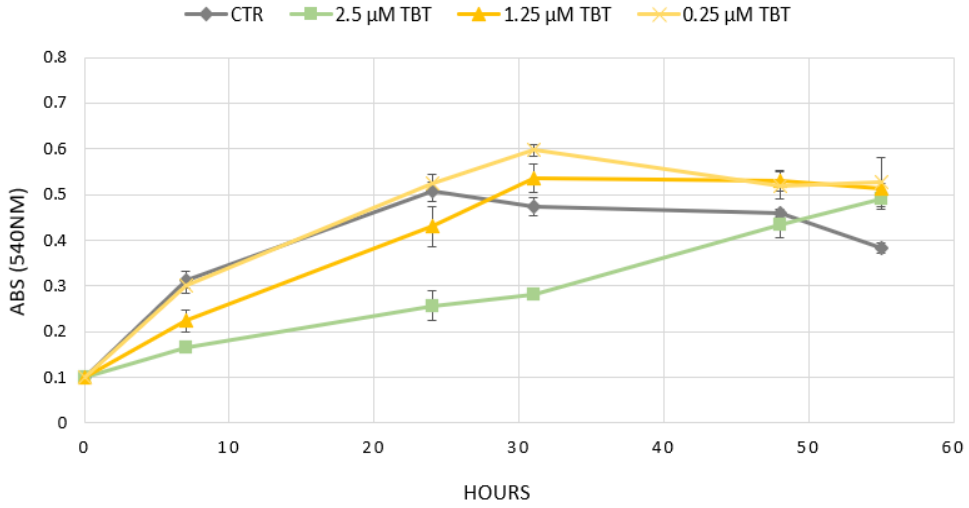


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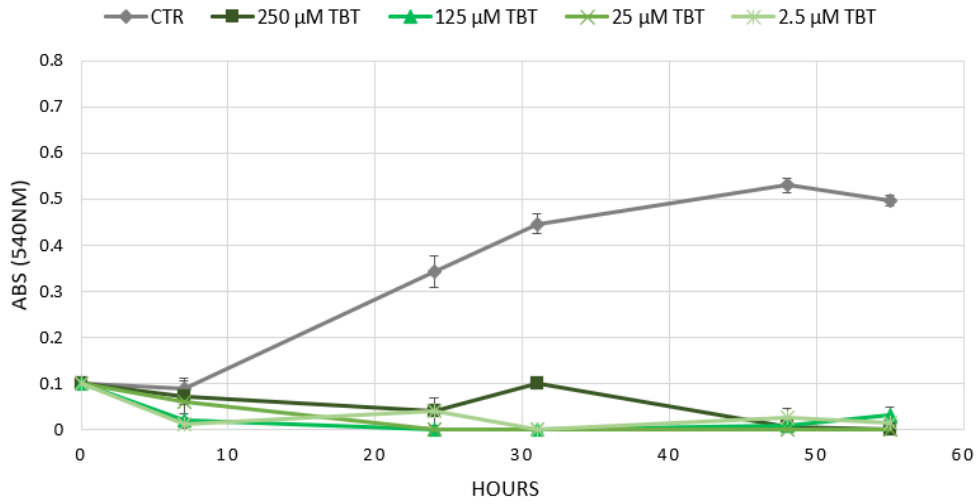
**Figure 7:** Grown curves of *Mesorhizobium* sp. PMI-6-Portimão when exposed to a) 250; 125; 25; 2.5 µM of TBT (top, green curves) and exposed to b) 2.5;1.25;0.25 µM of TBT (bottom, green and yellow curves). ABS – absorvance; CTR – control (no TBT)

In the assays with ***Mesorhizobium* sp. BR15-Bragança**, the tests with the first group of TBT concentrations (250; 125; 25 and 2.5 µM in figure 8a, green dose-response curves) showed once again that these concentrations were still too high, with a clear inhibition in growth as depicted by the reduction of 88% in the absorbance value at 24 h and a reduction of 96% at 55 h at the lower concentration of 2.5 µM of TBT . The second group of concentrations had similar effects to the ones observed in the previous strains (Figure 8b, yellow dose-response curves). These concentrations had a lower impact on the

growth of the bacterium, with the lowest concentrations tested having no impacts on growth after 55 h.

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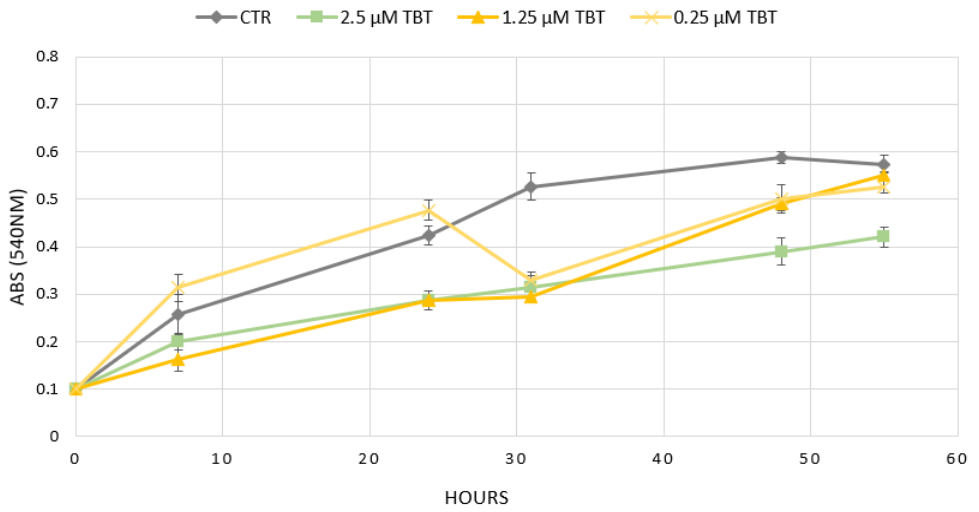
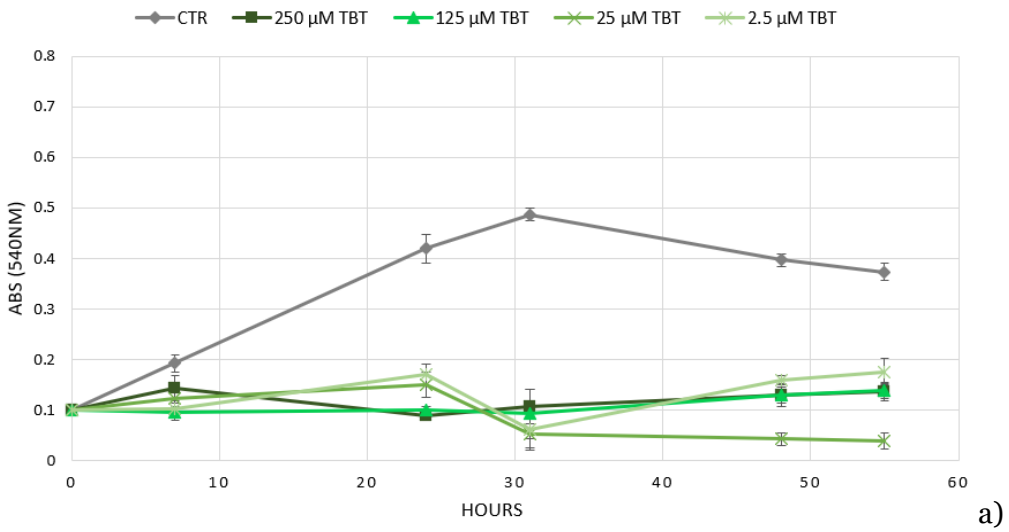



b)

**Figure 8**: Grown curves of *Mesorhizobium* sp. BR15-Bragança when exposed to a) 250; 125; 25; 2.5 µM of TBT (top, green curves) and exposed to b) 2.5;1.25;0.25 µM of TBT (bottom, green and yellow curves). ABS – absorvance; CTR – control (no TBT)

Regarding ***Mesorhizobium mediterraneum*** **UPM-Ca36$^T$**, the assays with the first group of TBT concentrations (250; 125; 25 and 2.5 µM in figure 9a, green dose-response curves) showed that these concentrations, similarly to the results with the other rhizobia were still too high, nevertheless the inhibition of growth when cells were exposed to 2.5 µM of TBT, was less prominent than in the previous strains, with a reduction of 60% in the absorbance value at 24 h and a reduction of 54% at 55 h. The second set of concentrations was then tested (Figure 9b, yellow dose-response curves) and the results showed that TBT exposure had higher impact than in previous strains, with a reduction of 51% and 29% in the absorvance at 24 h and 55 h, respectively.

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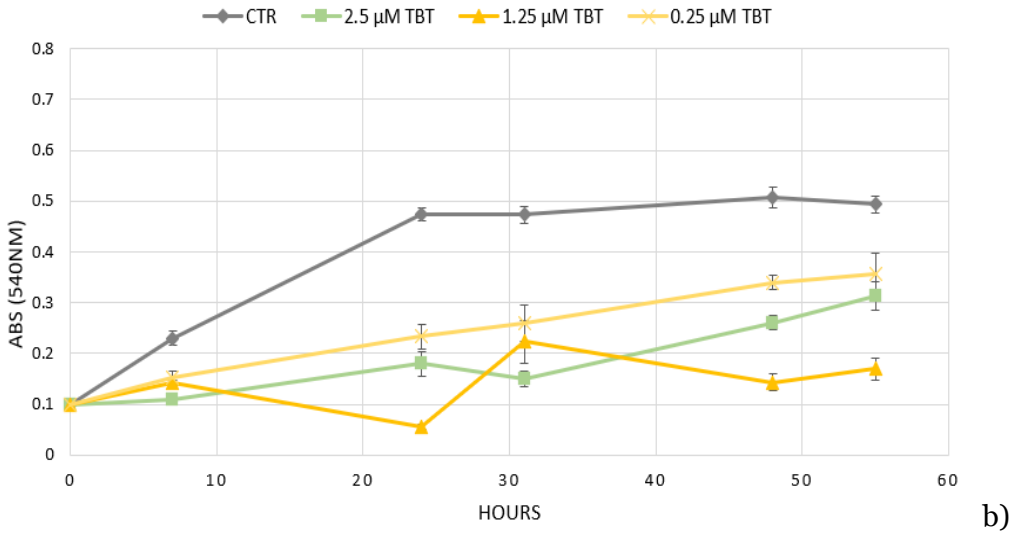
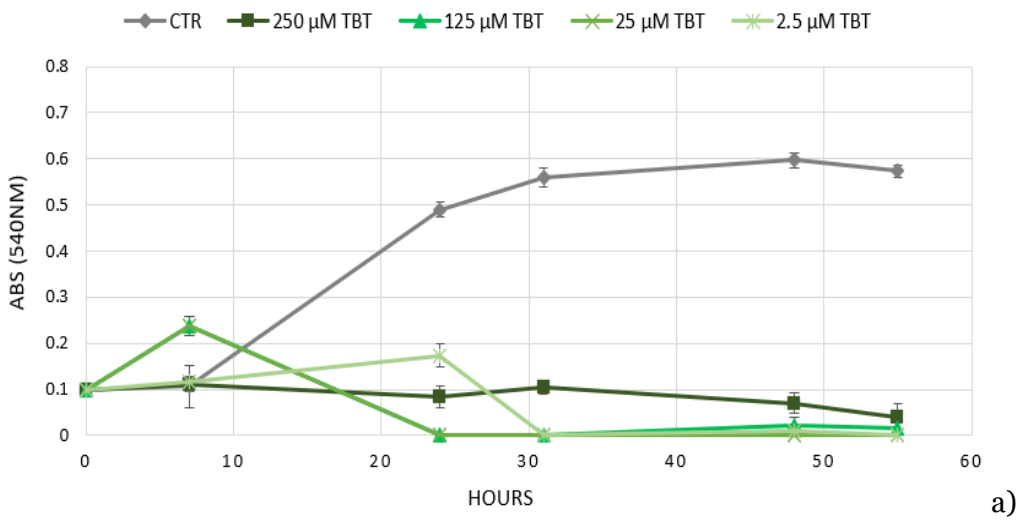



b)

**Figure 9:** Grown curves of *Mesorhizobium mediterraneum* UPM-Ca36$^T$ when exposed to a) 250; 125; 25; 2.5 µM of TBT (top, green curves) and exposed to b) 2.5;1.25;0.25 µM of TBT (bottom, green and yellow curves). ABS – absorvance; CTR – control (no TBT)

Lastly, regarding ***Mesorhizobium* sp. LMS-1**, the assay with the first group of TBT concentrations (250; 125; 25 and 2.5 µM in figure 10a, green dose-response curves) showed that these concentrations were still too high, with a clear inhibition in growth as depicted by the reduction of 65% in the absorbance value at 24 h and a reduction of 100% at 55 h, even for the lowest concentration of 2.5 µM. The second set of concentrations was then tested, starting with 2.5; followed 1.25 and 0.025 µM TBT (Figure 10b, yellow dose-response curves). These concentrations had a lower impact on the growth of the bacterium, with the lowest concentrations tested having no impacts on growth after 55 h.

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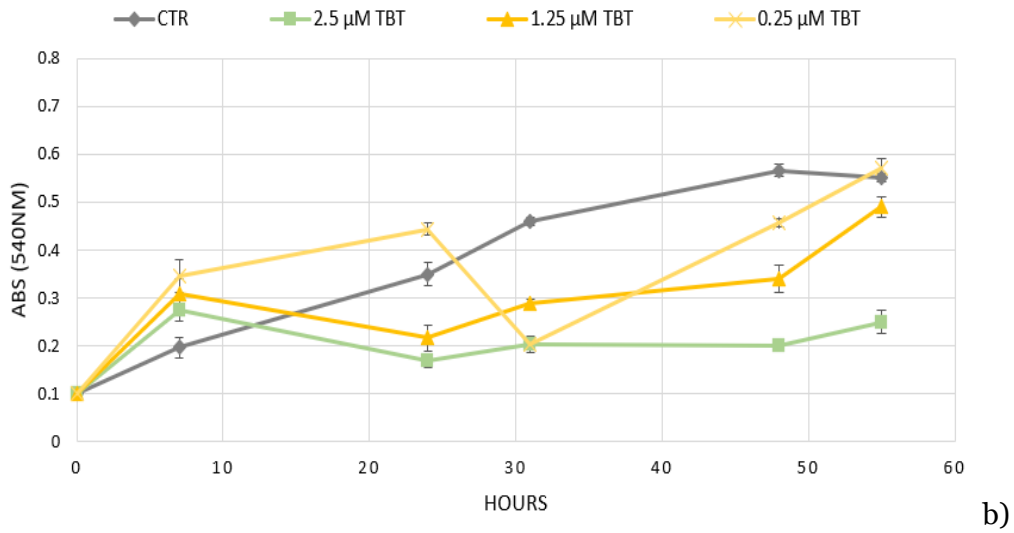


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**Figure 10:** Grown curves of *Mesorhizobium* sp. LMS-1 when exposed to a) 250; 125; 25; 2.5 µM of TBT (top, green curves) and exposed to b) 2.5;1.25;0.25 µM of TBT (bottom, green and yellow curves). ABS – absorvance; CTR – control (no TBT)

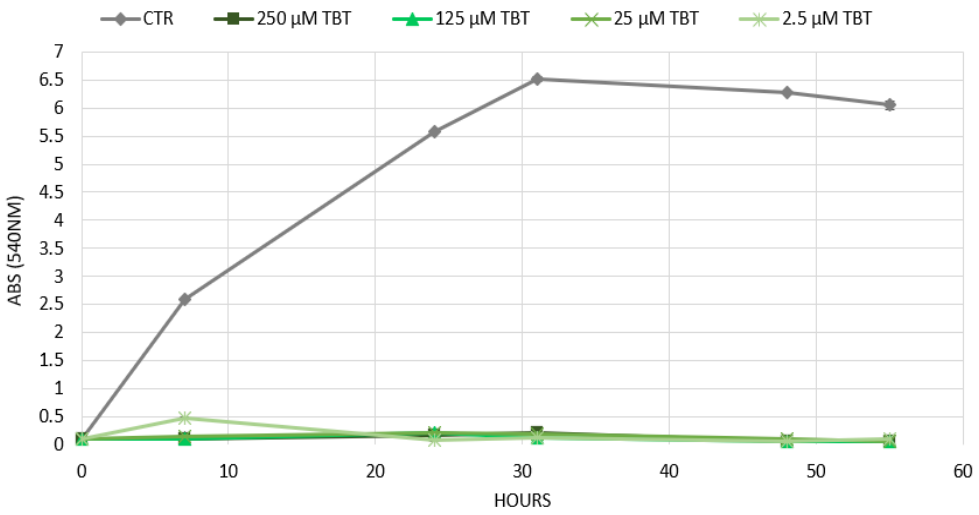
Overall, these results showed that all the *Mesorhizobium* strains tested have a high sensitivity to TBT, with clear growth reductions when exposed to concentrations higher than 2.5 μM TBT. When exposed to sublethal concentrations the impacts on growth showed some variation among strains, with *M. loti* MAFF303099 and *Mesorhizobium* sp. PMI-6-Portimão, which nodulate different hosts, showing a higher tolerance to TBT and *M. mediterraneum* UPM-Ca36$^T$ showing more sensitivity to the tested concentrations. Interestingly, the growth response of bacterial strains to TBT didn't always show a dose-dependent pattern. For example, *M. mediterraneum* UPM-Ca36$^T$ showed a higher growth at 2.5 μM TBT than at 1.25 μM TBT, in several timepoints (including at 55 h). There are few reports on the effects of TBT on rhizobia, nevertheless there are reports of rhizobium being able to use TBT as carbon source (Hassan et al., 2019), so that might be the case for the strains that showed a higher growth in the presence of TBT, when compared to control. Even in the case of high tolerance, we cannot assume that there are no intracellular impacts on the bacteria and further studies are required to evaluate these impacts. Since all the rhizobia strains tested belong to the *Mesorhizobium* genus, a higher diversity of tolerance phenotypes could be found in further studies with other rhizobia genera.

### 4.1.2. Model bacteria

The effects of TBT exposure on the model bacteria *Escherichia coli*, *Bacillus subtilis* and *Pseudomonas fluorescens* were evaluated following the same protocol as the one used for mesorhizobia in the section 4.1.1. The model bacteria were exposed to the same two sets of TBT concentrations: i) 250; 125; 25 and 2.5 μM TBT and ii) 2.5; 1.5 and 0.25 μM TBT.

Regarding, ***Escherichia coli***, a gram negative, facultative anaerobic coliform bacterium, the obtained results disclose that the first set of concentrations tested (250; 125; 25; 2.5 μM TBT, figure 11a, top panel, green dose-response curves) reduced the growth to basically zero even the lowest concentration tested (2.5 μM). Yet, when evaluating the second set of concentrations (2.5; 1.25; 0.25 μM TBT, figure 11b, bottom panel, green and yellow dose-response curves) it is possible to verify that all the concentrations tested are sublethal. This was valid even for 2.5 μM, which in this second

assay exhibited a much-reduced effect relatively to the one observed in the first assay. Such results can, once again, be explained by the carryover effects of TBT in the first set of concentrations.

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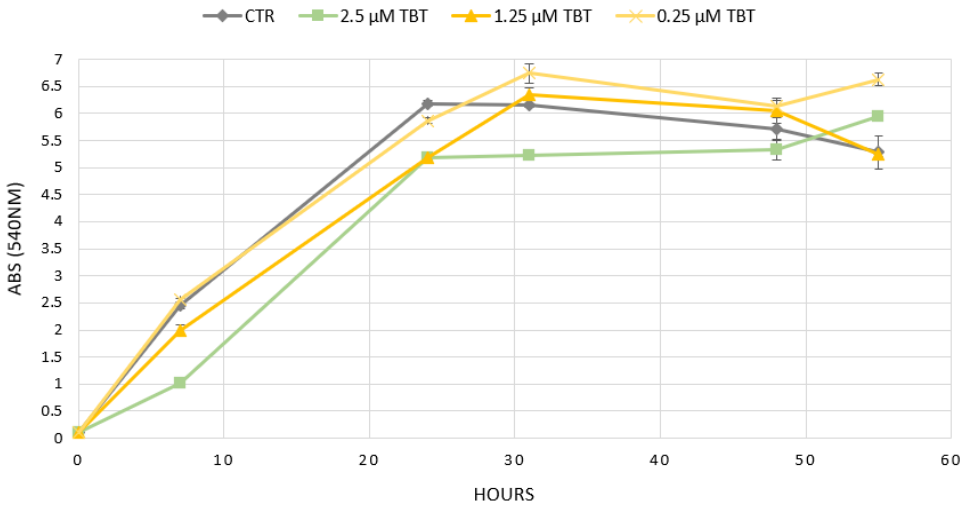
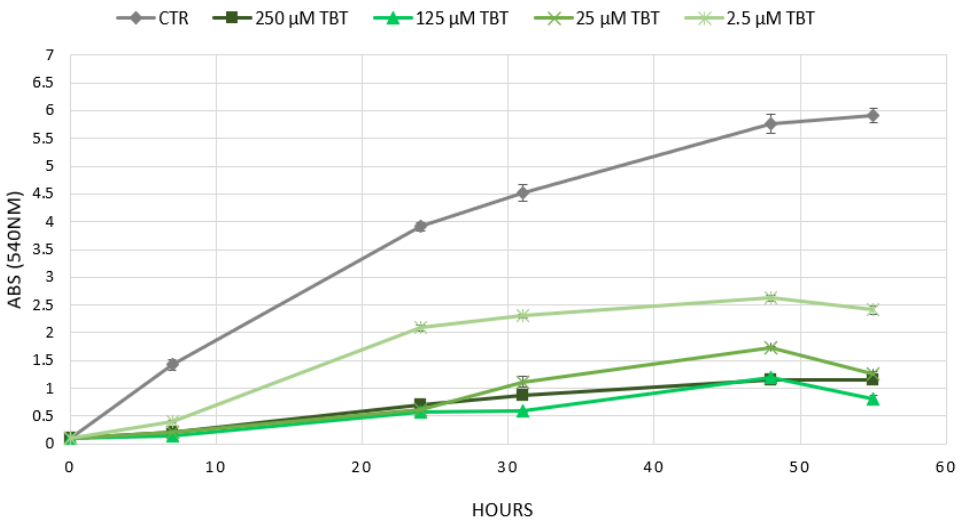



b)

**Figure 11:** Grown curves of *Escherichia coli* when exposed to a) 250; 125; 25; 2.5 µM TBT (top panel, green curves) and b) 2.5; 1.25; 0.25 µM TBT (bottom panel, green and yellow curves). ABS – absorvance; CTR – control (no TBT)

The effects of TBT exposure on ***Pseudomonas fluorescens***, a gram negative, aerobic, were evaluated using the same two concentrations groups previously mentioned. In the first group of concentrations (250; 125; 25; 2.5 µM TBT, figure 12a, top panel, green dose-response curves) it is possible to observe that *P. fluorescens* can tolerate the lowest concentration, although with an important growth reduction. In the second group of concentration (2.5; 1.25; 0.25 µM TBT, figure 12b, bottom panel, green and yellow dose-response curves) it is possible to observe that the TBT exposure had a much lower impact on the growth. It is noteworthy that the concentration of 0.25 µM TBT has basically no effect and is similar to the control curve which suggests that *Pseudomonas fluorescens* is capable of degrading that concentration of TBT but to prove this hypothesis more studies would be necessary in order to quantify TBT levels at different points during this assay. This supposition can be supported by the study of (Bernat, Siewiera, et al., 2014) that suggest this may due to its capability to tolerate TBT since some of its proteins and phospholipids adapt when exposed to it. Once again, there were differences between the growth responses of this bacteria when exposed to 2.5 µM in the two assays. Such results can, once again, be explained by the carryover effects of TBT in the first set of concentrations.

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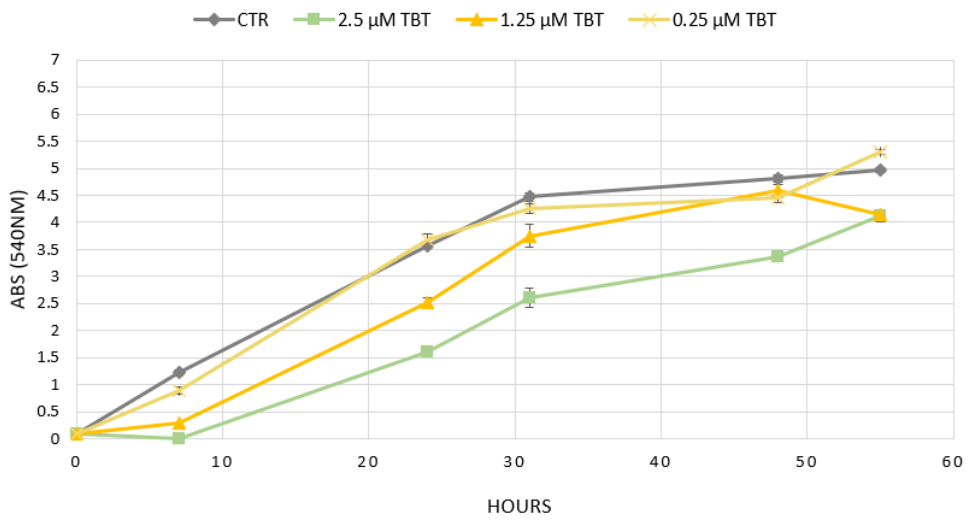
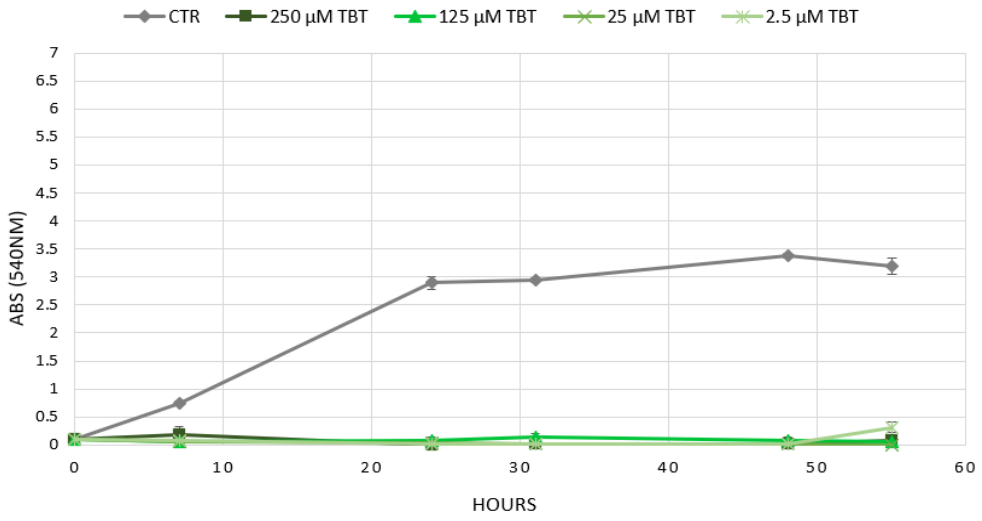


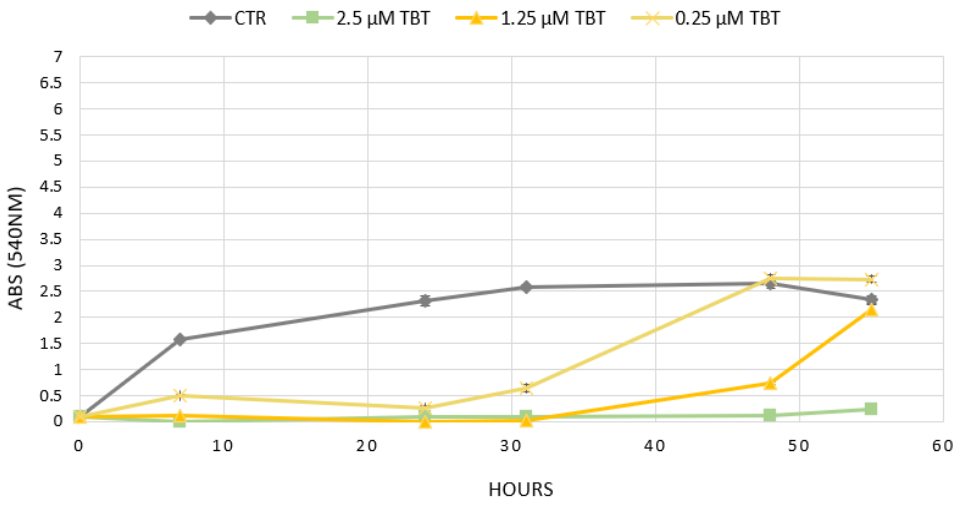

b)

**Figure 12:** Grown curves of *Pseudomonas fluorescens* when exposed to a) 250;125; 25; 2.5 µM TBT (top panel, green curves) and b) 2.5; 1.25; 0.25 µM TBT (bottom panel, green and yellow curves). ABS – absorvance; CTR – control (no TBT)

In the case of ***Bacillus subtilis***, a gram positive, facultative anaerobic, the exposure to higher TBT concentrations resulted in a near total inhibition of the growth (250; 125; 25; 2.5 µM TBT, figure 13a, top panel, green dose-response curves) whereas lower concentrations had visible impact until 30h of exposure, yet at the end of the experiment the lowest concentrations tested (1.25; 0.25 µM TBT) had almost no impact on the growth (figure 13b, bottom panel, green and yellow dose-response curves).




a)




b)

**Figure 13:** Grown curves of *Bacillus subtilis* when exposed to a) 250; 125; 25; 2.5 µM TBT (top panel, green curves) and b) 2.5; 1.25; 0.25 µM TBT (bottom panel, green and yellow curves). ABS – absorvance; CTR – control (no TBT)

These results demonstrate that the three model bacteria have different responses when exposed to TBT. Higher concentrations of TBT have a higher impact in the bacterial growth *of Escherichia coli* and *Bacillus subtilis,* while *Pseudomonas fluorescens* exhibited a much higher resistance to TBT since the growth inhibition was far less pronounced (59% after 55 h when exposed to 2.5 µM TBT versus 100% reduction in *E. coli* and 90% reduction in *B. subtilis* for the same conditions). The resistance presented in *Pseudomonas fluorescens* corroborates what was previously described in the literature (Anayo et al., n.d.; Bernat, Siewiera, et al., 2014; Jun et al., 2016; Polrot et al., 2022). This resistance may be due to the changes in the phospholipids of the membrane as suggested by Bernat, Siewiera, et al. (2014) for *Pseudomonas* sp. or can be due to some changes in the protein profile and gene expression as reported for the *Aeromonas molluscorum* Av27 (Cruz et al., 2015).

## 4.2. Evaluation of the impact of TBT on lipid accumulation

The impact of TBT exposure on lipid accumulation was tested in *Mesorhizobium* sp. V-15b-Viseu, as this strain presented a very clear dose dependent growth rate when exposed to sub-lethal TBT concentrations. In addition, the model bacteria *Escherichia coli* was also evaluated. Based on the tolerance results, the assays were performed for both strains with the sublethal TBT concentration of 2.5 µM. Bacteria were exposed for 24 h to TBT and lipid accumulation was compared between exposed and non-exposed (control) *Mesorhizobium* sp. V-15b-Viseu and *Escherichia coli* after staining with Nile red. The results (figures 15-18) show that TBT exposure significantly increased (t-test, $p<0.001$) the amount of lipids in both species. This increase was more pronounced in the model bacteria *E. coli* (151%) than in the *Mesorhizobium* sp. (63%) (figures 15 and 17).

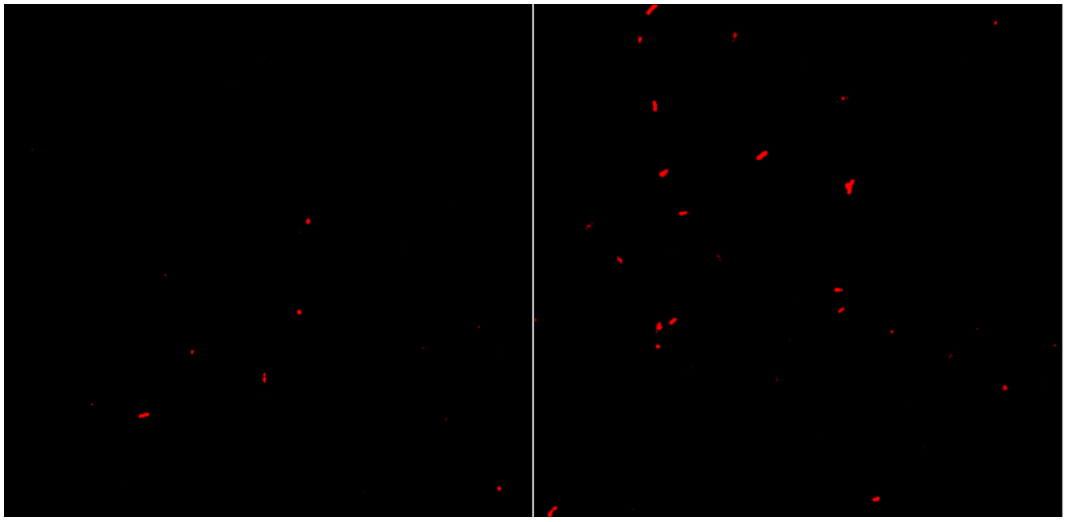


**Figure 14:** *Mesorhizobium* sp. V-15b-Viseu lipid accumulation visualized with confocal microscopy (magnification of 630x) after Nile red staining in the control treatment (left) and after exposure to 2.5 µM TBT (right).

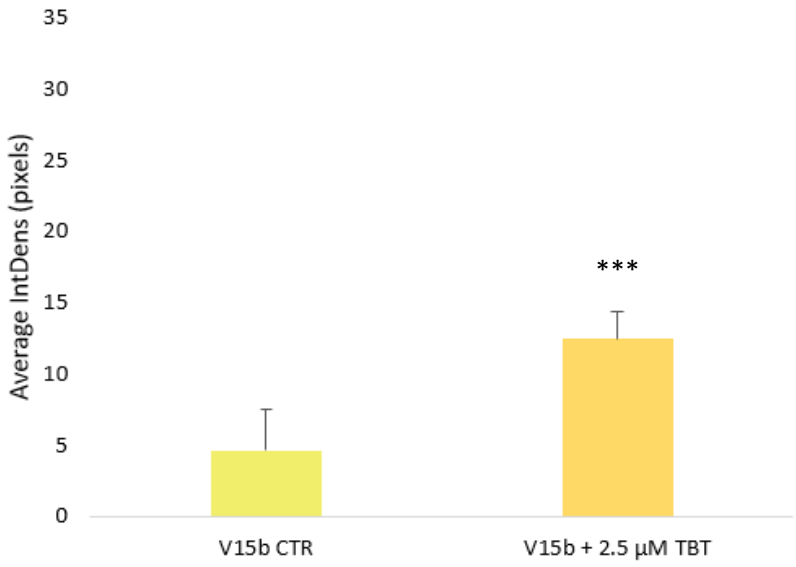



**Figure 15:** *Mesorhizobium* sp. V-15b-Viseu lipid accumulation measured as mean integrated density in pixels. The average number of cells in the control was 98 and in TBT treatment was 338 (*** $p<0.001$).

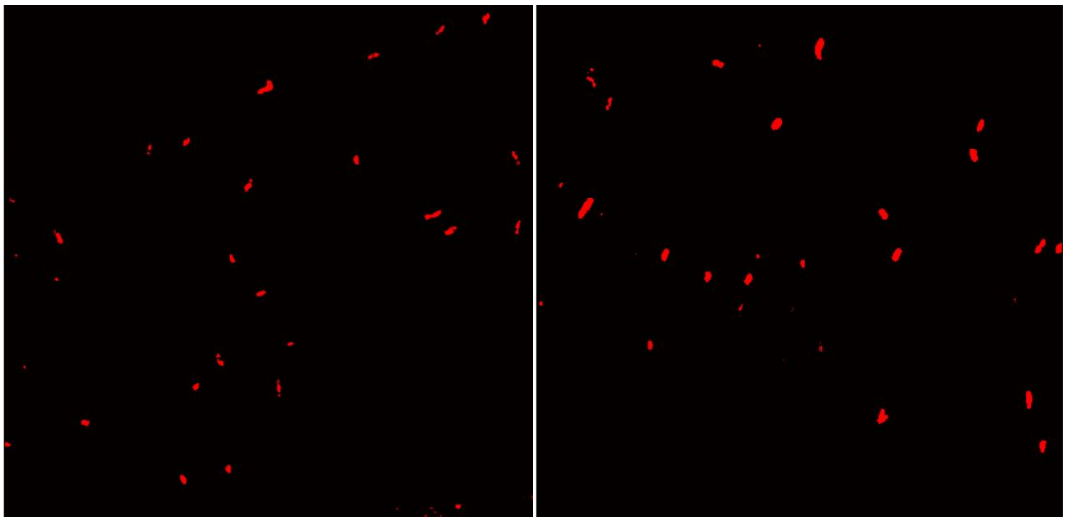


**Figure 16:** *Escherichia coli* lipid accumulation visualized with confocal microscopy (magnification of 630x) after Nile red staining in the control treatment (left) and after exposure to 2.5 µM TBT (right).

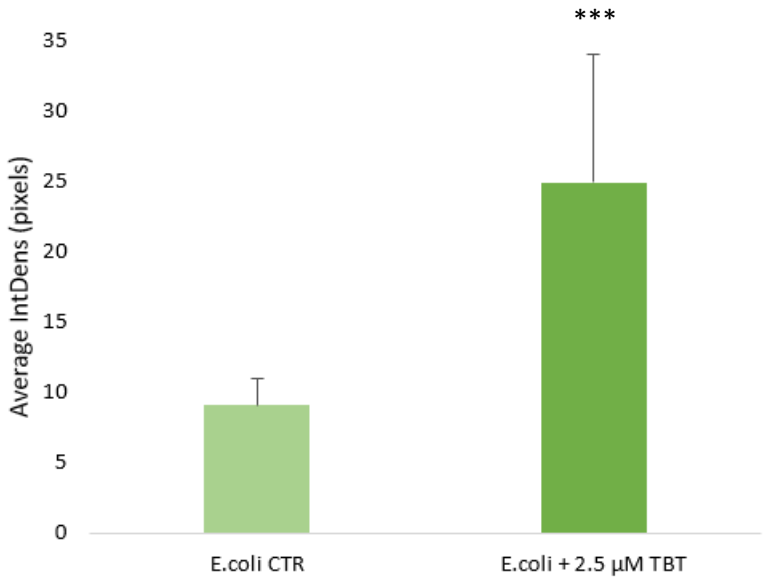



**Figure 17:** *Escherichia coli* lipid accumulation measured as mean integrated density in pixels. The average number of cells in the control was 134 and in TBT treatment was 284 (*** $p<0.001$).

## 4.1. Evaluation of the impact of TBT on prokaryote-eucaryote interaction

In order to evaluate if TBT affects the interaction between prokaryotes and eucaryotes, an early stage of this interaction was analyzed. After a first stage of molecular dialogue of the two symbiotic partners, a successful rhizobia colonization of the host root will lead to the root hair curling. Following inoculation with rhizobia previously exposed to TBT, chickpea roots were observed for four days and compared to the control treatment (no TBT). In all the timepoints there were few curling events in the control and in the two TBT treatments applied, 2.5 μM and 0.25 μM TBT. Since there were little curling events in the control, there's no solid baseline to compare the effects of TBT in this stage of the interaction. In figure 18 there is an example of the root hairs observed in all the assays conducted in the two biological replicates.

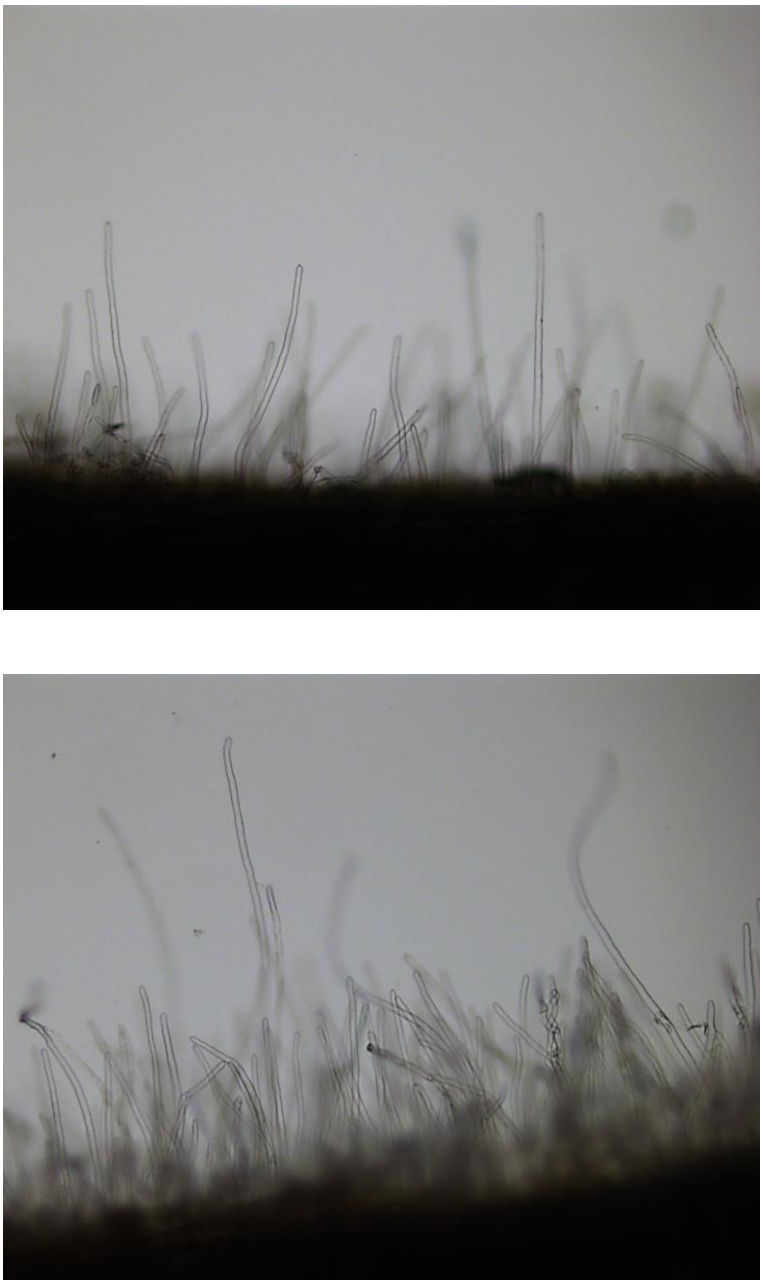


**Figure 18:** Pictures obtained with the electronic microscope (magnification of 100x) showing the root hair curling observed on chickpea seeds inoculated with *Mesorhizobium* sp. V-15b-Viseu. In the top image, the bacteria were not exposed to TBT (control), on the middle one the bacteria were exposed to 2.5 μM TBT and in the bottom one was exposed to 0.25 μM TBT.

There were few events of curling observed in all the treatments, which can be due to various factors being the more plausible that an optimization of the inoculation process

is needed (for example, adjusting cell density) or extend the time of analysis. Nevertheless, both previous factors were not expected to differ from what was described by Paço et al. (2016). Extending the analysis for more than 4 days would require a different plant growth system, as for example hydroponic conditions. The agar plate setup has limitations, since longer times would lead to root dehydration and consequent symbiosis inhibition.

# V. Conclusion

While the obesogenesis theory is supported by multiple studies and there is evidence that obesogenic compounds have effects in different taxonomic groups there is still a need to add more data at the prokaryote level and in the interaction between prokaryote-eukaryote.

By testing the effects of TBT on six strains of *Mesorhizobium* and in three model bacteria (*Escherichia coli, Bacillus subtilis* and *Pseudomonas fluorescens*), this study established that TBT exposure has a negative impact on the growth of all the bacteria analyzed. Overall, the six strains of *Mesorhizobium* and *Escherichia coli* had a similar tolerance to TBT, whereas *Pseudomonas fluorescens* demonstrated to be less sensitive, which corroborates the information present in the literature that suggests that *P. fluorescens* has higher tolerance to TBT.

The growth curves of the different bacteria allowed to determine the sub-lethal concentrations of TBT that were situated in between 2.5 and 0.25 μM for all the bacteria tested apart from *Pseudomonas fluorescens* that supported concentrations as high as 250 μM. The low levels of tolerance found in rhizobia, *E. coli* and *B. subtilis* were not previously reported in the literature and constitute an important contribution to future studies. Another important finding of this study was the collateral effect of testing simultaneously TBT concentrations that differ by several orders of magnitude. The present results indicated that the carry-over of the volatile portion of TBT may strongly influence the results.

Exposure to TBT was responsible for a significant increase in the accumulation of lipids as demonstrated by the analysis of the images of both *E. coli* and *Mesorhizobium* sp. after staining with Nile red. Such significant increases demonstrated the TBT obesogenic potential in these bacteria, adding important new data to corroborate the obesogenesis hypothesis. Further studies to understand these phenomena are necessary and should include metabolomic and lipidomic analysis.

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# VII. Appendix 1

Preparation of the calcium hypochlorite with 65% active chlorine solution

Dissolve 7.92 g of calcium hypochlorite (65% active chlorine) (Roth) in 100 mL of distilled water.

Filter twice using a 9-cm disk paper filter.

Culture medium preparation

- LB medium preparation for 2 L:

20 g of tryptone (Biochem, CAS number 91079-40-2), 10 g of yeast extract (Biochem, CAS number 8013-01-2), 10 g of sodium chloride >= 99% (Fisher Chemical, CAS number 10035-04-8) and 2 L of distilled water. After homogenization, correct the pH to 7.5. For solid medium add 15 g/L of agar. Autoclave at 121°C for 20 minutes.

- NB/NA medium preparation for 2L:

6 g of meat extract (Scharlau, CAS number 07-075-500), 20 g of peptone (Scharlau, CAS number 07-154-500) and 2 L of distilled water. After homogenization, correct the pH to values between 7 and 7.2. For solid medium add 15 g/L of agar. Autoclave at 121°C for 20 minutes.

- TSB medium preparation for 2L:

A pre-prepared medium was used (Lote 092016502, Liofilchem) and prepared according to the manufacturer's instructions. Dilute 60g in 2 L of distilled water. Autoclave at 121°C for 20 minutes.

- YMB/YMA medium preparation for 2L:

Add first 1.35 g of potassium dihydrogen phosphate (Panreac, CAS 231-834-5) since it is the hardest to dissolve. After it is totally homogenized, add 0.4 g of magnesium sulfate

heptahydrate (VWR, CAS number 10034-99-8), 20g of mannitol (Merck, number 1.05982.0500), 0.2 g of sodium chloride 99,2% (JMGS, CAS number 7647-14-5), 1 g of yeast extract (Biochem, CAS number 8013-01-2) and 2 L of distilled water. After homogenization, correct the pH to values between 7 and 7.2. For solid medium add 15 g/L of agar. Autoclave at 121°C for 20 minutes.

# VIII. **Appendix 2**

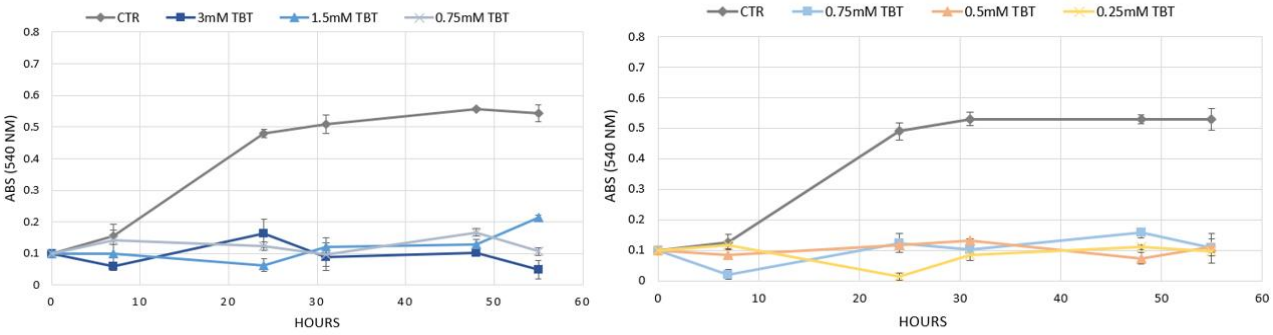


**Figure A5**: Grown curves of *Mesorhizobium loti* MAFF303099 when exposed to the TBT concentrations of 3; 1.5; 0.75 mM (left panel) and 0.75; 0.5; 0.25 mM (right panel).

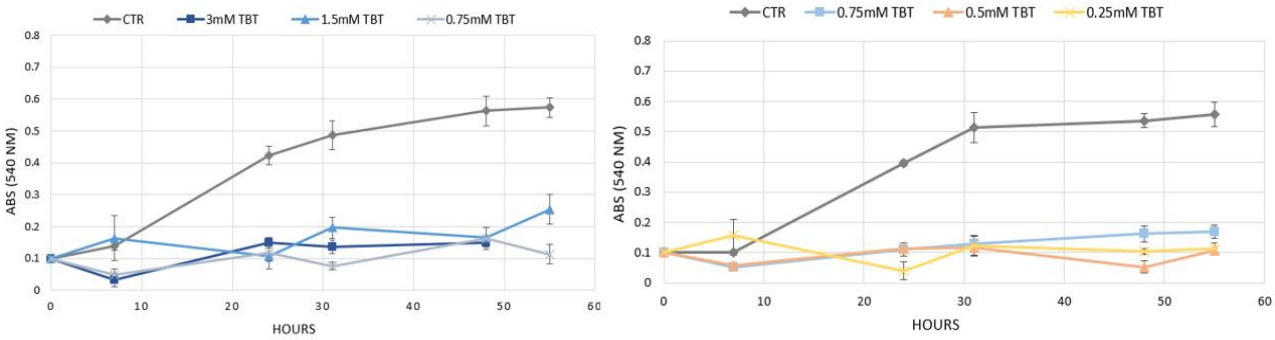


**Figure A6**: Grown curves of *Mesorhizobium* sp. V-15b-Viseu when exposed to the TBT concentrations of 3; 1.5; 0.75 mM (left panel) and 0.75; 0.5; 0.25 mM (right panel).

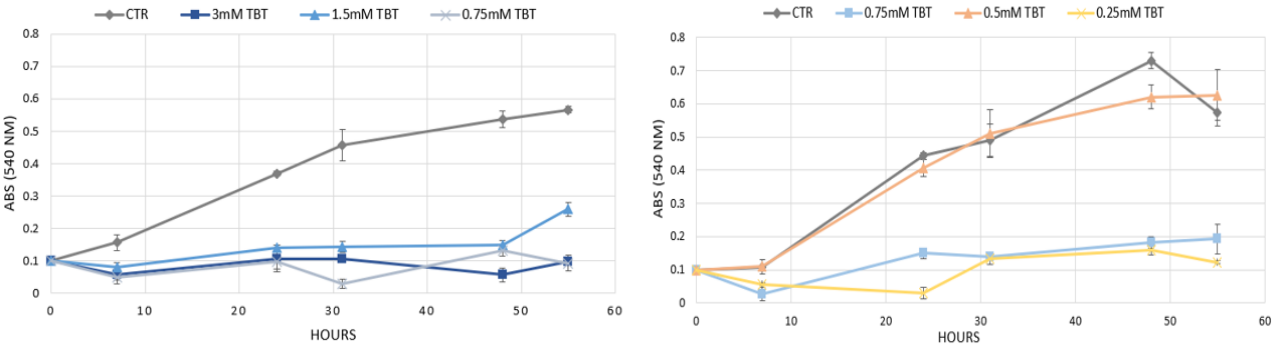


**Figure A7**: Grown curves of *Mesorhizobium* sp. PMI-6-Portimão when exposed to the TBT concentrations of 3; 1.5; 0.75 mM (left panel) and 0.75; 0.5; 0.25 mM (right panel).

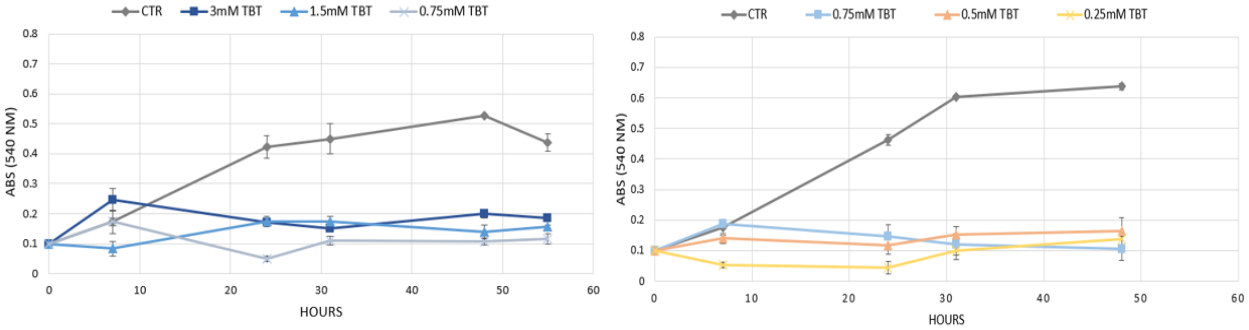


**Figure A8**: Grown curves of *Mesorhizobium* sp. BR15-Bragança when exposed to the TBT concentrations of 3; 1.5; 0.75 mM (left panel) and 0.75; 0.5; 0.25 mM (right panel).

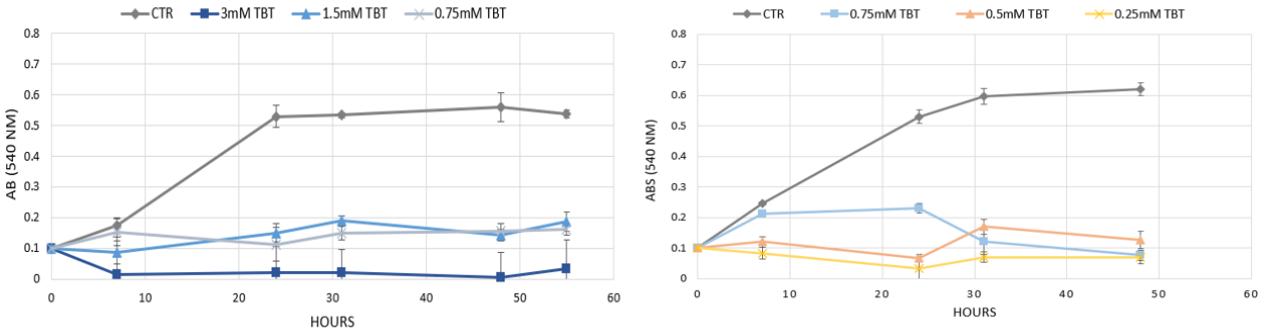


**Figure A9**: Grown curves of *Mesorhizobium mediterraneum* UPM-Ca36 (type strain) when exposed to the TBT concentrations of 3; 1.5; 0.75 mM (left panel) and 0.75; 0.5; 0.25 mM (right panel).

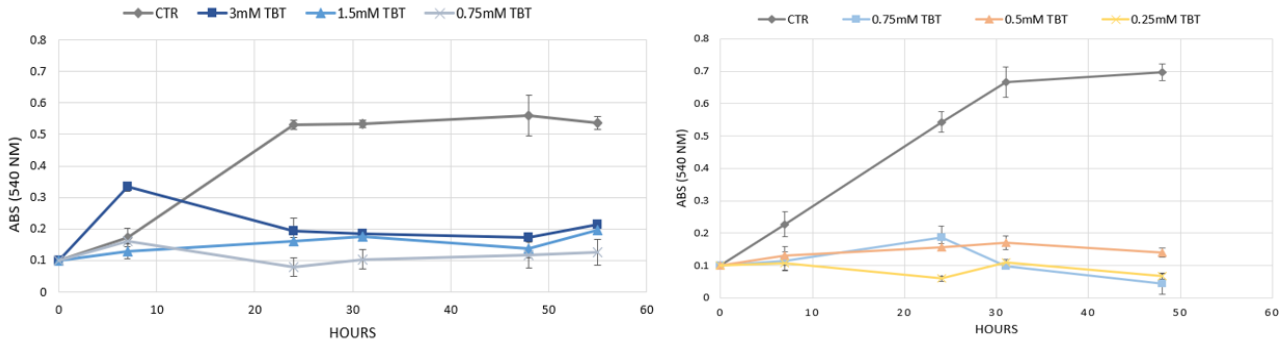


**Figure A10**: Grown curves of *Mesorhizobium* sp. LMS-1 when exposed to the TBT concentrations of 3; 1.5; 0.75 mM (left panel) and 0.75; 0.5; 0.25 mM (right panel).

# IX. Appendix 3

Evaluation of the impact of TBT volatilization on the growth curves of *Mesorhizobium loti* MAFF303099

Before the assay, *M. loti* MAFF30399 was grown overnight at 180 rpm at 28ºC in liquid YMB medium. Before incubation with TBT, the bacteria were adjusted to an initial optical density of 0.10 at 540 nm (OD540 nm). The following concentrations of TBT were tested: 250 and 0.25 μM (these concentrations were chosen for the volatilization test, because were the highest and lowest concentrations of the concentrations previously tested), alongside with a positive and negative control. The positive control consisted of the culture medium with bacteria (with no TBT) whereas in the negative control TBT was added to the culture medium without bacteria (figure A11, appendix 3).

To follow the bacterial growth, culture samples were analyzed by measuring its absorvance at OD540nm at several timepoints until the stationary phase was reached, and a last sample was taken at 55 hours. The results were analyzed by the construction of OD540 nm growth curves as a function of time, and the evaluation of the tolerance of the bacteria to the presence of TBT was performed by comparing the bacterial growth curves.

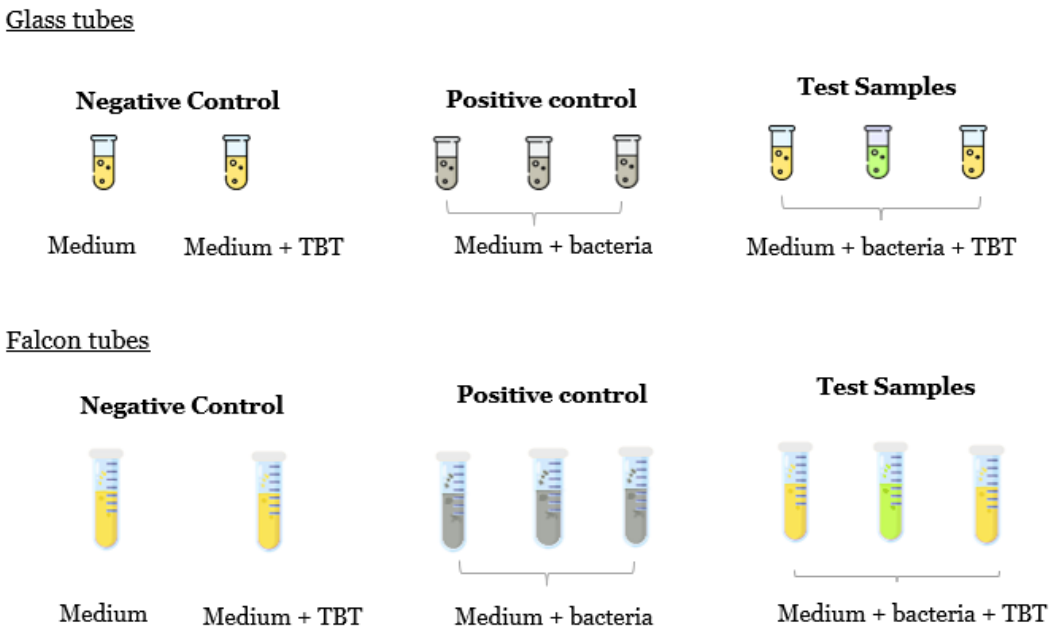


**Figure A11**: Schematic diagram of the evaluation of the impact of TBT volatilization on the growth curves of *Mesorhizobium loti* MAFF303099.

By observing the growth curves of *M. loti* MAFF303099 (figure A12, appendix 3), it is possible to observe differences (particularly in the end of the assay) between the same concentrations when comparing glass tubes (not totally sealed) and sealed falcon tubes. As explained in section 4.4.1, this difference may be due to the semi-volatility of TBT, and with this test it was possible to corroborate this hypothesis.

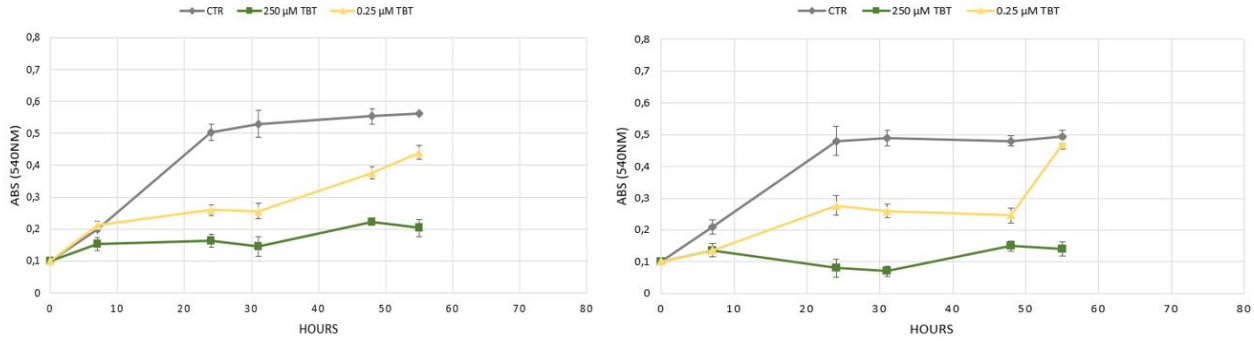


**Figure A12**: Grown curves of *Mesorhizobium loti* MAFF303099 when exposed to the TBT concentrations of 250 and 0.25 µM realized in glass tubes (left panel) and falcon tubes (right panel).