# Pollution-Induced Tolerance of Soil Bacterial Communities to Oxytetracycline-Spiked Manure

**Leire Jauregi**
  NEIKER-Basque Institute for Agricultural Research and Development

**Maddi Artamendi**
  NEIKER-Basque Institute for Agricultural Research and Development

**Lur Epelde** ( ✉ lepelde@neiker.eus )
  NEIKER-Basque Institute for Agricultural Research and Development   https://orcid.org/0000-0002-4624-4946

**Fernando Blanco**
  NEIKER-Basque Institute for Agricultural Research and Development

**Carlos Garbisu**
  NEIKER-Basque Institute for Agricultural Research and Development

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# Abstract

The use of manure as a fertilizer is a common agricultural practice that can improve soil physicochemical and biological properties. However, antibiotics and their metabolites are often present, leading to the adaptation of soil bacterial communities to their presence. The aim of this study was to assess the effects of the extensively used, broad-spectrum antibiotic oxytetracycline on soil microbial community adaptation using a pollution-induced community tolerance assay. Manure-amended soil was spiked with oxytetracycline (0, 2, 20, 60, 150, and 500 mg kg$^{-1}$) three times every ten days in the selection phase. The detection phase was conducted in Biolog EcoPlates with a second oxytetracycline exposure (0, 5, 20, 40, 60, and 100 mg L$^{-1}$). All treatments demonstrated decreased metabolic activity after exposure to $\geq$ 5 mg L$^{-1}$ oxytetracycline during the detection phase. Meanwhile, a significant increase in tolerance was observed following exposure to $\geq$ 20 mg oxytetracycline per kg soil during the selection phase. Therefore, the pollution-induced community tolerance approach with Biolog EcoPlates was a useful system for the detection of antibiotic selection pressures on soil bacterial communities. It is important to properly manage animal waste before their application to the soil to reduce the occurrence of antibiotic-resistance in the environment.

# 1. Introduction

Livestock production has increased to meet the rising demand for food, with animal biomass for feed far exceeding human biomass (Van Boeckel et al. 2015). Meat production worldwide has increased by 400% from 65 to 279 million tons in the last 50 years (Marques et al. 2018). Under these circumstances, 70% of the antimicrobials used worldwide are administered to animals for veterinary or food production (Van Boeckel et al. 2017). In 2018, tetracyclines (31%), penicillins (29%), and sulfonamides (8%) accounted for 68% of the total antimicrobial sales for use in food-producing animals in 31 European countries (EMA/24309/2020). Antibiotics are poorly metabolized with approximately 30–90% excreted in urine and feces (Sarmah et al. 2006). For example, Winckler and Grafe (2001) recovered 72% of orally administered tetracyclines in swine manure within two days of application. Additionally, the following oxytetracycline (OTC) concentrations were found in various animal manures: between 0.41 and 354 mg kg$^{-1}$ in swine manure (Campagnolo et al. 2002; Chen et al. 2012), 10 mg kg$^{-1}$ in calf manure (De Liguoro et al. 2003), and between 0.5 and 200 mg kg$^{-1}$ in cow manure (Ince et al. 2013).

The application of animal organic wastes to agricultural soils can enhance soil quality and crop yield (Epelde et al. 2018; Urra et al. 2019; Urra et al. 2020). However, this common agricultural practice can also lead to the spread of antibiotics, antibiotic-resistant bacteria (ARB), and/or antibiotic resistance genes (ARGs) in the soil (Chee-Sanford et al. 2009; Berendonk et al. 2015; Jauregi et al. 2021).

Assessment of the potential risks of pollutants released into the environment is of utmost importance, particularly emerging contaminants such as antibiotics. Pollution-induced community tolerance (PICT) is an ecotoxicological tool that is used to determine the selective pressure of a pollutant and exerts a direct effect on the community (Schmitt et al. 2005). A PICT assay comprises of the following two phases: (i) a

selection phase where the soil bacterial community is exposed to a concentration gradient of the studied pollutant, and (ii) a detection phase where the soils are subjected to a second pollutant gradient for the evaluation of tolerance levels. The increase in tolerance could be due to: (i) the replacement of sensitive species by more tolerant species, (ii) physiological changes that make the organisms less sensitive, or (iii) genetic changes through horizontal gene transfer (HGT) to acquire mobile genetic elements (MGEs) encoding for enhanced resistance (Schmitt et al. 2005). Cause-effect relationships between several pollutants and microbial communities using PICT are established for antibiotics (Schmitt et al. 2004; Brandt et al. 2009; Fang et al. 2018), herbicides (Zabaloy et al. 2010), metals (Stefanowicz et al. 2009; Aliasgharzad et al. 2011), and metals that confer co- or cross-tolerance to other pollutants (Wakelin et al. 2014; Li et al. 2015; Santás-Miguel et al. 2020).

The main objective of this study was to evaluate the effects of OTC exposure on soil bacterial communities in a PICT assay using Biolog EcoPlates. The effects of OTC exposure on soil bacterial metabolic activity, the number of utilized substrates, and the Shannon diversity index were analyzed. The OTC concentrations at which the metabolic activity decreased by half ($EC_{50}$) and the tolerance of soil bacterial communities was also assessed. The novel aspect of this study could be highlighted by the fact that we performed simulation of a repeated application of antibiotics into soils through manure application under controlled conditions. It is hypothesized that soils exposed to higher OTC concentrations demonstrate increased tolerance to OTC.

## 2. Material And Methods

## 2.1. Collection and characterization of soil and manure

Soil sample for the experiment was collected from the upper 30-cm layer of a semi-natural grassland field located in Derio, northern Spain, which has never been amended with inorganic or organic fertilizer to our knowledge. The soil was air-dried at 30°C for 48 h, following which it was sieved to < 4 mm and was subjected to physicochemical characterization according to standard methods (MAPA 1994). It was characterized as a clay loam with a pH of 6.2, 6.3% organic matter (OM), 0.32% total N content, with Olsen P and $K^+$ content at 3.4 and 395 mg $kg^{-1}$ dry weight (DW) soil, respectively.

The aged manure (~two years old) from heifers was kindly provided by an organic dairy farm located in the province of Biscay, Spain, without a known history of antibiotic treatment. It was collected in polyethylene bags, following which it was immediately transferred to the laboratory and stored at 4°C; then, it was sieved to < 4 mm and subjected to physicochemical characterization (MAPA 1994). It had a pH of 8.7, 66% OM, 0.77% total N content, with Olsen P and $K^+$ content of 9.9 and 25 mg $kg^{-1}$ DW soil, respectively. The following metal concentrations were determined following aqua regia digestion (McGrath and Cunliffe 1985): 0.93, 24, 25, 13, 61, and 303 mg $kg^{-1}$ DW soil for Cd, Cr, Cu, Ni, Pb, and Zn, respectively.

## 2.2. Experimental design of the selection phase

Aged manure was manually incorporated into pots with one kg of soil and was thoroughly mixed to obtain an equivalent of 100 kg N ha$^{-1}$ except for the control treatment (OTC0-M, no OTC and no manure). The pots were incubated at room temperature in the dark and allowed to exchange air for 55 days. During incubation, distilled water was added every week to maintain constant soil moisture.

Soil communities were exposed to a gradient concentration of OTC (CAS 2058-46-0, ≥95% purity, Merck) in the selection phase of the PICT assay. Each pot was contaminated by spiking with 60 mL OTC solution to obtain final concentrations of 2, 20, 60, 150, and 500 mg OTC per kg soil (OTC2, OTC20, OTC60, OTC150, and OTC500, respectively). This spiking procedure was repeated three times on days 0, 10, and 20. OTC0-M and the other control pot containing manure with no OTC (OTC0) received an equivalent amount of distilled water. Each treatment was performed in triplicate.

## 2.3. Detection phase with Biolog EcoPlates

A second OTC gradient was established in the detection phase to reveal differences in community tolerance using 96-well Biolog EcoPlates. The plates contained a triplicate set of 31 relevant carbon sources for environmental samples (Insam 1997). Fresh soil equivalent to 5 g DW was added to 50 mL of autoclaved Milli-Q water, following which the mixture was agitated for 1 h in an orbital shaker (220 rpm) and then allowed to settle for 5 min. Subsequently, 450 μL of the liquid was mixed with 30 mL of Milli-Q water. This solution (100 μL) was mixed with OTC (20 μL), and then the mixture was aliquoted into each well to obtain the following six final OTC concentrations: 0, 5, 20, 40, 60, and 100 mg L$^{-1}$. The plates were incubated at 30°C for 14 days (336 h), followed by color development and absorbance measurement at 595 nm using a microplate reader (Anthos Zenyth 3100, Anthos Labtec Instruments GmbH, Salzburg, Austria).

## 2.4. Data processing

Average well color development (AWCD) was determined by calculating the mean absorbance values of each treatment at each time point. The absorbance value for each well was corrected by subtracting the zero hour time point and the blank control for each reading time (Epelde et al. 2008). The values corresponding to the incubation time of the midpoint of the exponential portion of the curve representing the highest microbial growth rate were selected for further calculations. The number of utilized substrates (NUS) was calculated when the absorbance value was > 0.1 (Epelde et al. 2008). Similarly, Shannon's diversity (H') index was determined by considering the absorbance values at each well as equivalent to species abundance. Nonlinear curve fitting based on the Gompertz function was performed to yield the kinetic parameters (Lindstrom et al. 1998):

$$y = OD_{595nm} = \frac{K}{(1 + e^{-r(t-s)})}$$

where $K$ represents the asymptote that the absorbance curve approaches, $r$ represents the exponential rate of absorbance changes, $t$ represents the time following microplate inoculation, and $s$ represents the time to the midpoint of the exponential portion of the curve when y = K/2. OTC concentrations that

reduced the color formation to 50% of the maximum ($EC_{50}$ values) were determined. Bacterial community tolerance was quantified using an adapted tolerance index (TI) (Brandt et al. 2009):

$$TI = \frac{AUC_{OTC}}{AUC_{Control}} - 1$$

where $AUC_{OTC}$ equals the average area under the curve of AWCD in triplicate samples amended with OTC, and $AUC_{Control}$ represents the corresponding average area in triplicate control treatments (OTC0-M). The integral of the Gompertz function based on the trapezoidal method was used to calculate the area under the curve using the pracma R package (Borchers 2021).

Statistically significant differences between treatments ($p < 0.05$) in AWCD, NUS, H', $EC_{50}$, and TI were established using ANOVA and Tukey's post-hoc test using the agricolae R package (de Mendiburu 2020).

# 3. Results

Dose-response curves showed that the application of $\geq$ 5 mg $L^{-1}$ OTC in the detection phase inhibited the average metabolic activity of the soil bacterial communities in all treatments (Figure 1). The most considerable reduction in activity was observed in the OTC0-M, OTC0, OTC2, OTC20, and OTC60 treatments when the OTC concentration was between 5 and 20 mg $L^{-1}$. The most substantial reduction in metabolic activity with the OTC150 and OTC500 treatments was observed when the OTC concentration was between 20 and 40 mg $L^{-1}$. Both treatments presented significantly higher AWCD values than the OTC0-M, OTC0, and OTC2 treatments. Overall, the dose-response curves of the treatments with a lower OTC concentration in the selection phase showed a faster reduction in the AWCD values compared to higher OTC concentration treatments.

Dose-response curves for the NUS by the bacterial communities were obtained at different OTC concentrations in the detection phase (Figure 2). In the OTC0-M, OTC0, and OTC2 treatments, the NUS decreased by approximately half at 20 mg $L^{-1}$ OTC. Meanwhile, the NUS decreased by approximately half in the OTC150 and OTC500 treatments at 60 mg $L^{-1}$ OTC. OTC150 and OTC500 exhibited a significantly higher NUS than the OTC0-M, OTC0, and OTC2 treatments. Overall, the NUS decreased as the OTC concentration in the detection phase increased.

Dose-response curves of each treatment with respect to the Shannon diversity index against different antibiotic concentrations in the detection phase are shown in Figure 3. When both OTC0-M and OTC0 treatments were exposed to 20 mg OTC $L^{-1}$, a notable decrease in H' was observed. The H' curve was similar in the OTC20, OTC60, OTC150, and OTC500 treatments, with each of these treatments having a significantly higher H' than the OTC0-M and OTC0 treatments. In summary, OTC exerted an inhibitory effect on H' and decreased as the antibiotic concentration in the detection phase increased.

The following average $EC_{50}$ values represent the OTC concentrations required to reduce the microbial activity by half in the tested treatments: 15.6, 17.1, 24.5, 30.2, 32.1, 38.9 and 49.0 mg $L^{-1}$ for OTC0-M, OTC0, OTC2, OTC20, OTC60, OTC150, and OTC500, respectively (Figure 4). OTC150 and OTC500 treatments presented significantly higher $EC_{50}$ values than the OTC0-M treatment. In summary, higher OTC concentration in the selection phase resulted in the requirement of greater OTC concentrations (higher $EC_{50}$) in the detection phase to reduce the activity of soil bacterial communities by half.

The following average TI values were found: 0, 0.04, 0.13, 0.18, 0.19, 0.29, and 0.38 for OTC0-M, OTC0, OTC2, OTC20, OTC60, OTC150, and OTC500, respectively (Figure 5). Furthermore, OTC150 and OTC500 presented significantly higher TIs than OTC0-M, OTC0, and OTC2 treatments. Therefore, the TI values increased as the OTC concentration in the selection phase increased.

# 4. Discussion

Soil microorganisms play essential roles in soil function, such as organic matter decomposition, nutrient cycling, and formation of soil structure (Loreau 2001; Nannipieri et al. 2003; Bronick and Lal 2005), and are extremely sensitive to slight environmental fluctuations. Thus, soil microbial parameters are deemed suitable indicators for determining disturbances in soil quality (Epelde et al. 2010; Garbisu et al. 2011). Changes in the absorbance values of Biolog EcoPlates are indicators of the overall metabolic activity of culturable heterotrophic bacteria possessing the ability to utilize different carbon substrates (Ma et al. 2016). Although only a limited proportion of the total community exhibits responses to this growth-dependent method (Smalla et al. 1998), it can help detect toxicant effects on soil communities (Epelde et al. 2008). In this study, the PICT assay and EcoBiolog approach were considered to assess whether OTC addition to soils exerted impact on microbial communities. In fact, the assessment of tolerance to a chemical helps infer causal relationships between exposure and effects (Tlili et al. 2015).

Antibiotics may be subjected to various processes in the soil environment, including sorption by soil components, transformation, photodegradation, and plant uptake and transport (Kumar et al. 2005; Sarmah et al. 2006; Kuchta et al. 2009; Kong et al. 2012; Reichel et al. 2013). Tetracyclines are particularly susceptible to adsorption by the soil matrix (Loke et al. 2002; Schmitt et al. 2005; Sarmah et al. 2006), resulting in low bioavailability. Antibiotic half-lives range from hours to several months depending on their molecular structure and soil physicochemical properties (Sarmah et al. 2006; Walters et al. 2010; Braschi et al. 2013). OTC half-life ranges from 8 to 79 days in manure or slurry (Kay et al. 2004; Wang and Yates 2008) and between 18 and 56 days in soils (Kay et al. 2004; Wang and Yates 2008; Li et al. 2010). It is a broad-spectrum antibiotic that inhibits bacterial protein synthesis by binding to the 30S ribosomal subunit (Brodersen et al. 2000). However, microorganisms can also use antibiotics as a carbon source, resulting in increased microbial biomass (Thiele-Bruhn and Beck 2005) and activity (Liu et al. 2005). For example, Jiang et al. (2018) reported that the bacterial population increased in soil polluted with 5 mg $kg^{-1}$ of OTC. In this study, OTC inhibited metabolic activity in all treatments. However, treatments exposed to increasing OTC in the selection phase required higher OTC concentrations in the

detection phase to reduce their metabolic activity. The same response was observed with the NUS and the H' index. A concentration of 5 mg L$^{-1}$ of OTC resulted in an overall reduction of metabolic activity, while 20 mg L$^{-1}$ of OTC was necessary for reductions in the NUS and the H' index. This indicated that although the metabolic activity of certain microorganisms was affected by exposure to 5 mg L$^{-1}$ of OTC, the soil microbial community utilized substrates in a similar way until 20 mg L$^{-1}$ of OTC. The soils in this study presented higher H' values than those reported in soils amended with pig manure contaminated with up to 200 mg kg$^{-1}$ of OTC (Liu et al. 2012).

The EC$_{50}$ helps estimate the concentration of a toxicant that causes a 50% reduction of test populations against a specific endpoint under particular conditions (Rozman et al. 2010). An EC$_{50}$ of 79 mg sulfachloropyridazine kg$^{-1}$ soil amended with pig slurry was found in a PICT assay with Biolog EcoPlates (Schmitt et al. 2005). The EC$_{50}$ values in this study ranged from 15.6 to 49.0 mg L$^{-1}$ for the non-OTC-exposed soil in the selection phase to the soil treated with the highest OTC concentration (500 mg kg$^{-1}$), respectively. This indicated that soil exposed to higher OTC concentration in the selection phase required higher OTC concentrations in the detection phase to reduce their microbial metabolic activity by half.

In this study, the tolerance of soil microbial communities to OTC increased with higher OTC concentrations in the selection phase which correlated with findings reported in previous studies conducted using antibiotics such as sulfachloropyridazine, tylosin, sulfadiazine, carbendazim and ciprofloxacin (Schmitt et al. 2004; Demoling and Bååth 2008; Fang et al. 2014; Fang et al. 2016; Han et al. 2019; Santás-Miguel et al. 2020). Thus, repeated OTC spiking of a microbial community that is sensitive to antibiotic results in increased tolerance. In culture-based studies, antibiotic resistance is considered when soil bacteria grow at 20 mg L$^{-1}$ (D'Costa et al. 2006; Bhullar et al. 2012). In our case, a significant increase in tolerance was observed, beginning from 20 mg kg$^{-1}$ OTC exposure during the selection phase. These results indicated that repeated OTC application over time in the selection phase promoted the adaptation of soil microbial communities due to selective pressure (Fang et al. 2014). Ppb levels of tetracycline concentrations statistically increase the prevalence of HGT from *E. coli* to activated sludge (Kim et al. 2014). Similarly, pretreatment of sewage effluent populations with a very low 10 μg L$^{-1}$ of tetracycline increases the prevalence of HGT by four-fold compared with untreated populations (Jutkina et al. 2016). Therefore, exposure to non-lethal antibiotic concentrations reported in this study and previous studies may have induced the survival of tolerant bacteria through HGT (Andersson and Hughes 2012; Liu et al. 2017).

In another respect, differences between the treatment amended with aged manure and the unamended treatment were expected since the presence of metals in aged manure might promote the spread of antibiotic resistance. This occurs via the following co-selection mechanisms: (i) co-resistance, when different resistance systems are present in the same genetic element, and (ii) cross-resistance, when one resistance system confers resistance to both a metal and an antibiotic (Baker-Austin et al. 2006). However, statistically significant differences were not found between the treatments.

# 5. Conclusions

Repeated short-term contamination of soils by manure amended with OTC promoted antibiotic tolerance in microbial communities with possible adverse effects exerted on the environment. The repeated application of organic amendments containing antibiotics to soils is a common agricultural practice. Its use should be limited well below 20 mg OTC per kg soil to avoid the dissemination of antibiotic-resistance. To this end, it is important to effectively manage animal waste and to decrease the amount of antibiotics present in manure before applying them to the soil.

Although PICT is a sensitive method used for detecting changes in soil microbial communities due to OTC exposure, it is difficult to determine the cause of this tolerance. A metagenomic analysis may provide insights into the structure of microbial communities and their adaptation processes along the OTC gradient.

# Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Availability of data

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

### Competing interests

The authors declare that they have no competing interests.

### Funding

### Authors' contributions

LJ: Formal analysis, Investigation, Writing-original draft. MA: Formal analysis, Investigation. LE: Conceptualization, Writing: Review & Editing. FB: Methodology. CG: Conceptualization, Writing: Review &

Editing.

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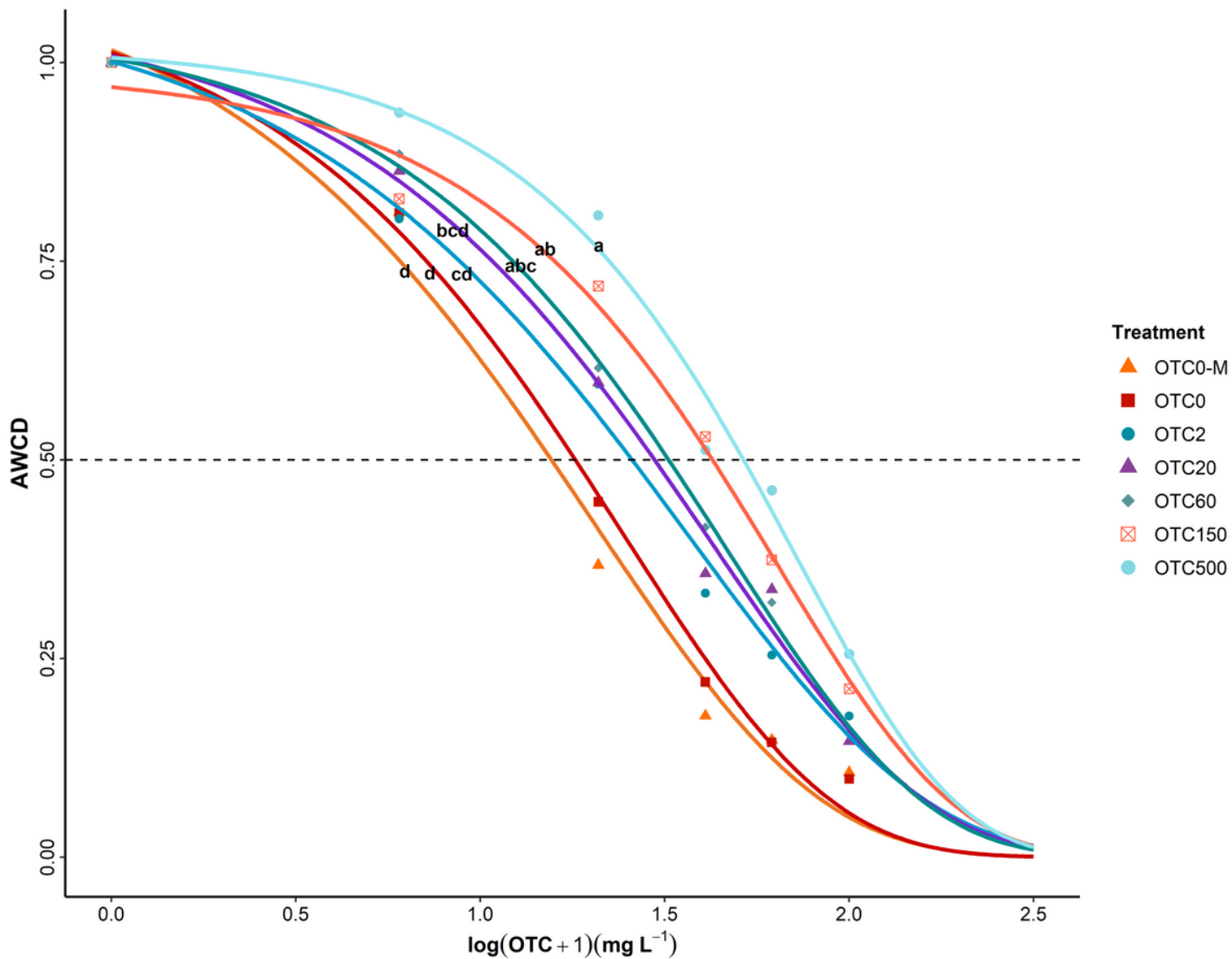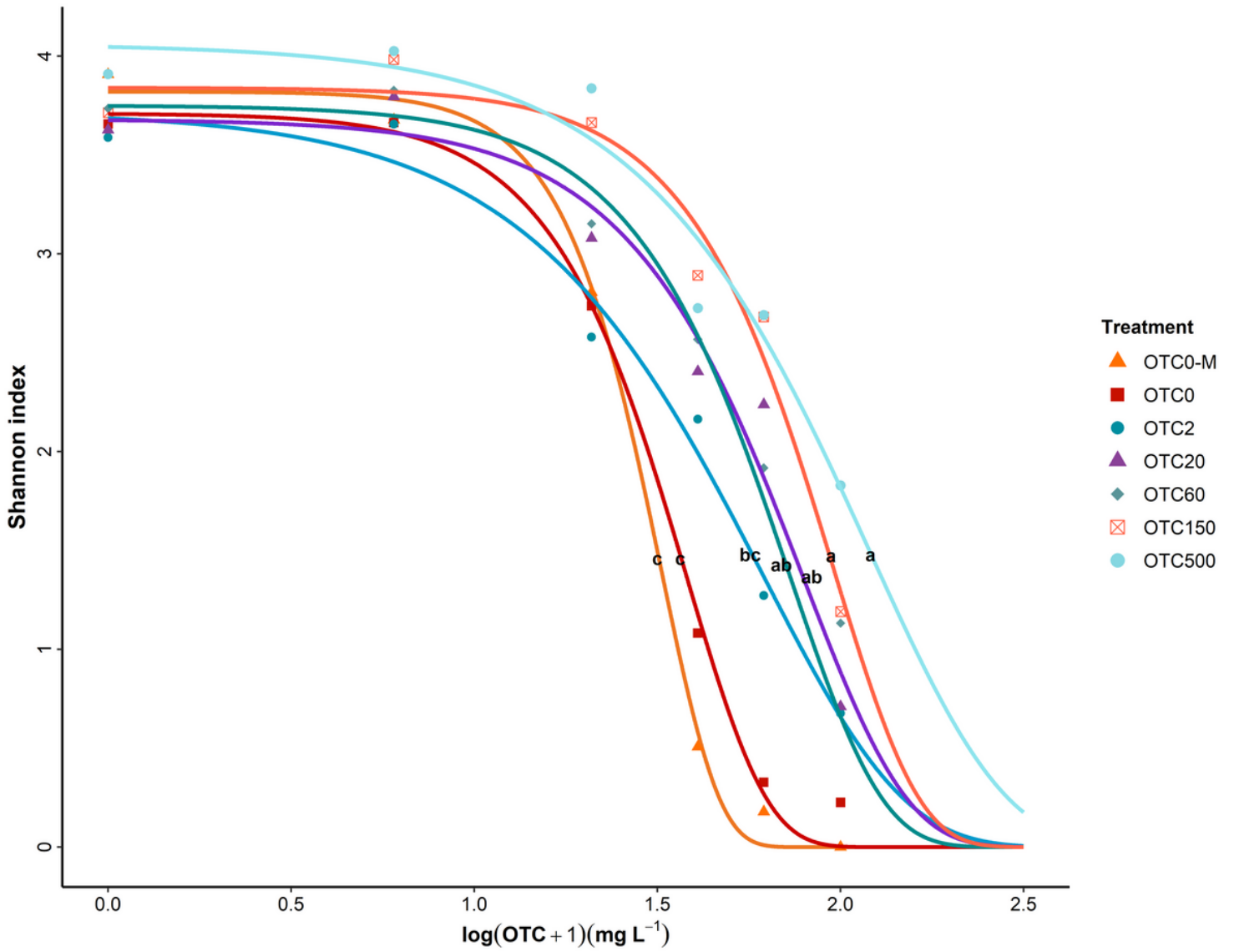
# Figures

**Figure 1**

Dose-response curves showing OTC inhibition of the metabolic activity of soil bacterial communities in the detection phase fitted with the Gompertz function ($R^2 > 0.9$). Data have been expressed as average AWCD values (n=3). Different letters represent significant differences ($p < 0.05$) according to Tukey's post hoc test.
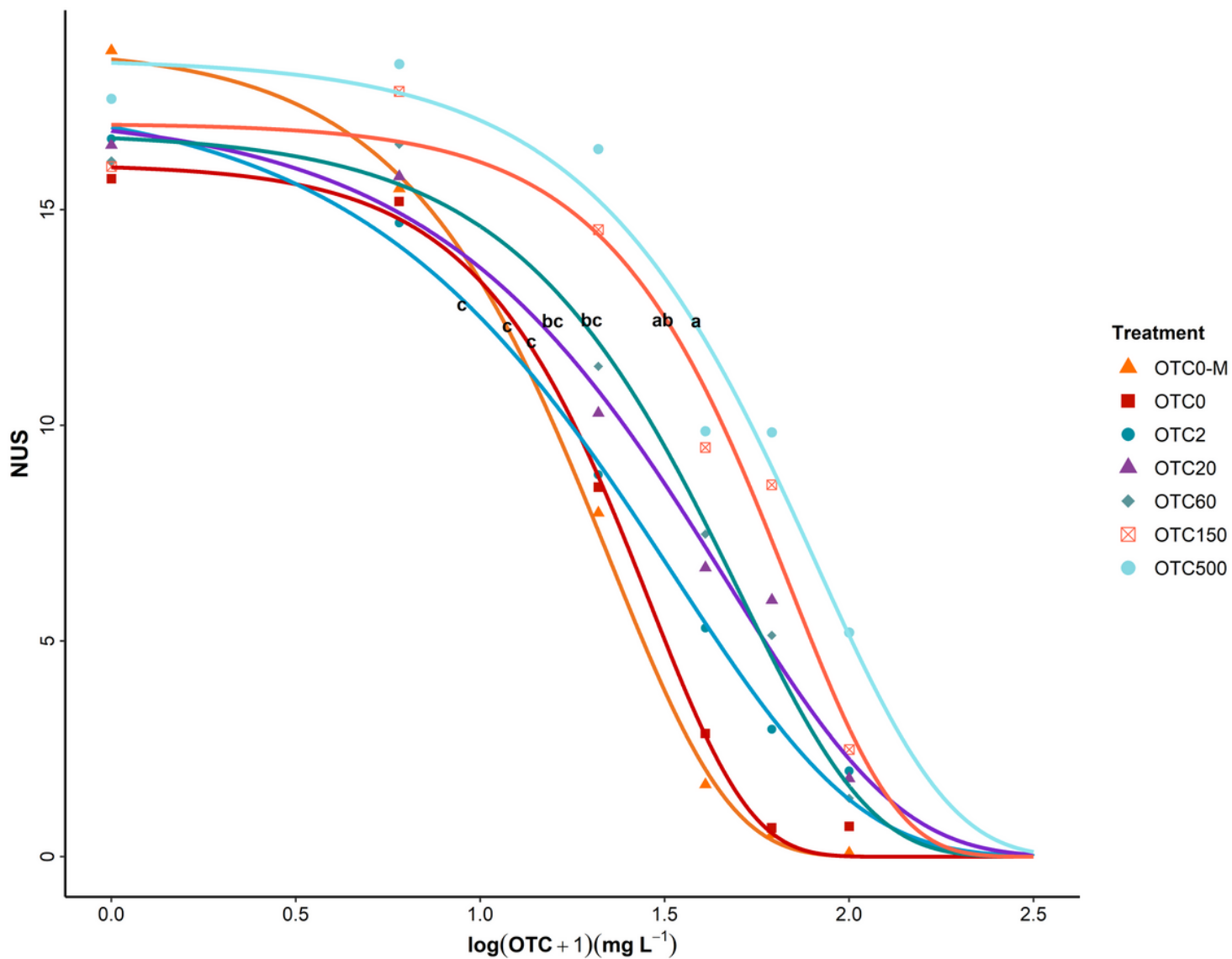
**Figure 2**

Dose-response curves showing OTC inhibition on the average number of utilized substrates (NUS) in the detection phase fitted with the Gompertz function (n=3). Different letters represent significant differences (p < 0.05) according to Tukey's post hoc test.
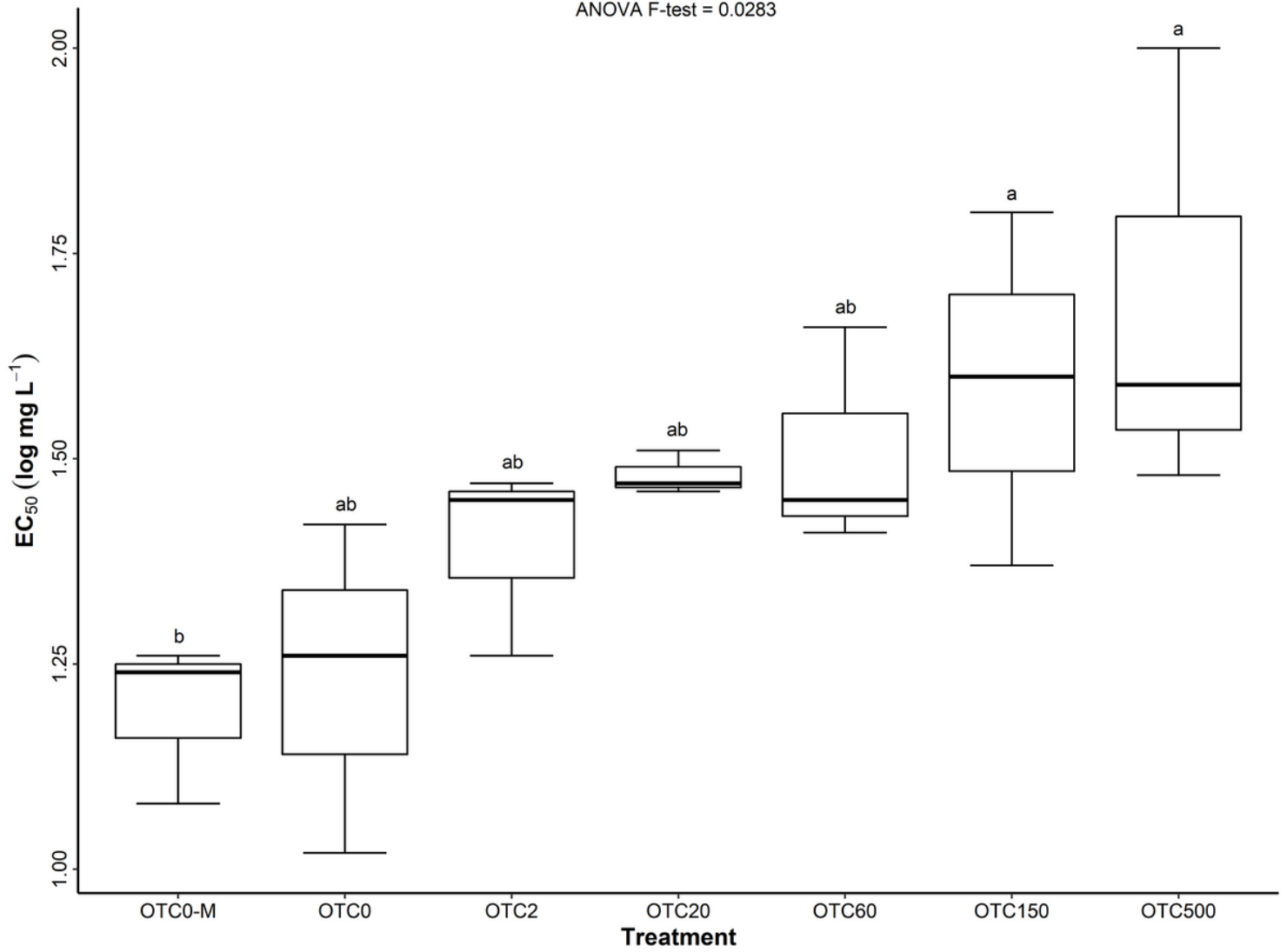
**Figure 3**

Dose-response curves of OTC inhibition on the average Shannon diversity index value (n=3) in the detection phase fitted with the Gompertz function. Different letters represent significant differences (p < 0.05) according to Tukey's post hoc test.
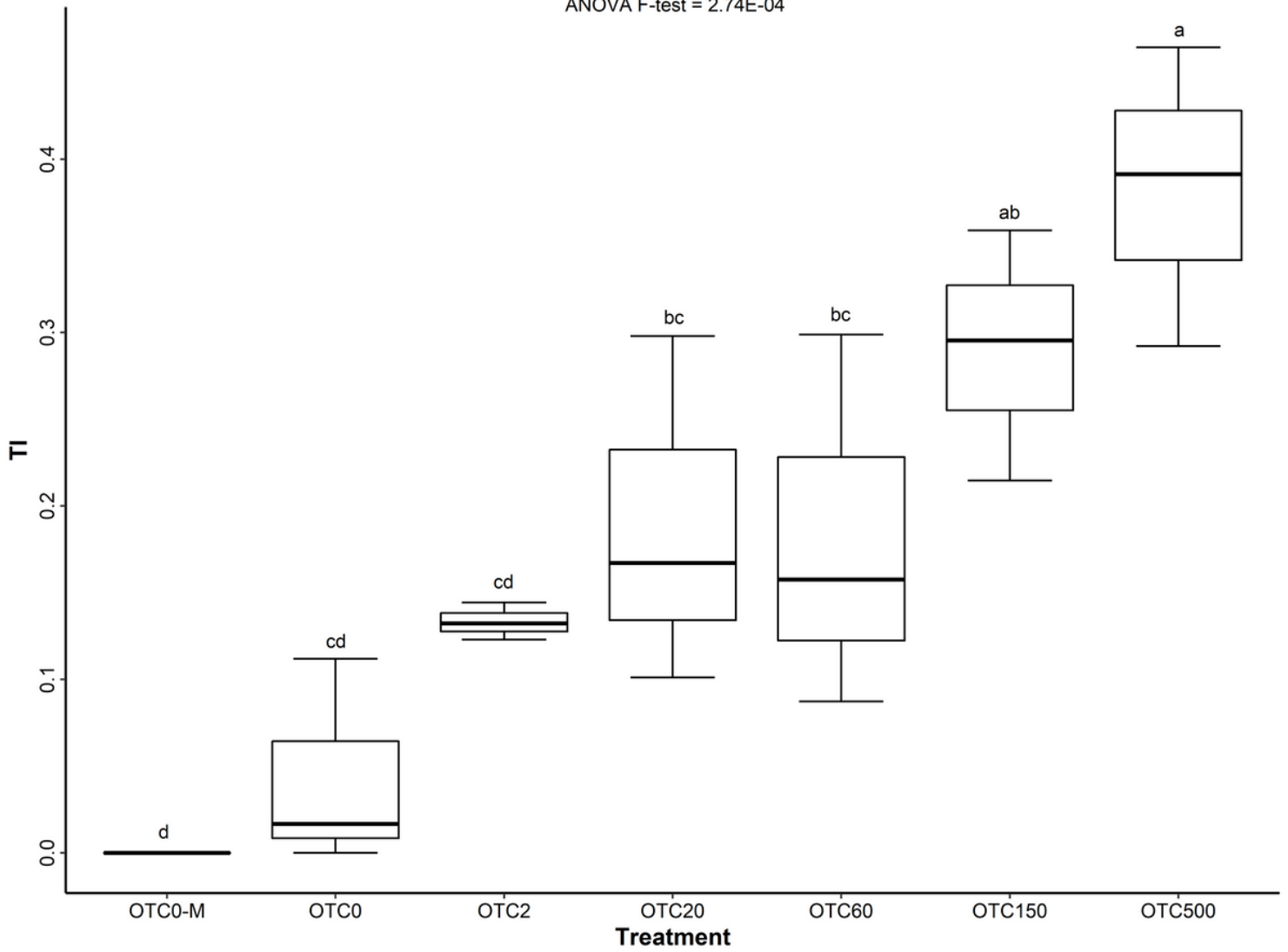
**Figure 4**

OTC concentrations that reduced the AWCD to 50% of the maximum AWCD (EC50 values) have been fitted with the Gompertz function(n =3). Different letters represent significant differences ($p < 0.05$) according to Tukey's post hoc test.

**Figure 5**

Bacterial community tolerance index (TI) to OTC (n=3). Different letters indicate statistically significant differences (p < 0.05) according to Tukey's post hoc test.