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Article

The analysis of tetracycline impact on aquatic systems by in vitro ecotoxicity tests

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Abstract
Tetracycline is one of the most worldwide used antimicrobial agents. Unfortunately, it is partially metabolized by human body and a significant amount is excreted unchanged into wastewater. In addition, its resistance to biodegradation during wastewater treatment leads to a persistent occurrence in aquatic environments. There, tetracycline exerts a selective pressure on microbial communities, promoting the emergence and spread of antibiotic resistance genes such as tetA and tetM. Tetracycline concentrations in surface waters could reach up to 20 µg/L. This concentration level has been sufficient to induce sublethal effects in sensitive bacteria and to promote adaptive mechanisms within aquatic microbial populations. The aim of this study was to explore microbial adaptation mechanisms, especially of Vibrio fischeri incubated in presence of 0.3÷5 mg/L tetracycline. Tested tetracycline concentrations were higher than those typically found in the environment, but they were selected to define adaptation limits and clearly observe toxic effects under controlled laboratory conditions such as Vibrio fischeri bioluminescence and optical density (OD_{600}). Results revealed significant reductions in both bioluminescence and viability at concentrations as low as 0.6 mg/L and a complete inhibition at 5 mg/L. The Relative Light Units (RLU) / OD_{600}, ratio indicated changes in bacterial metabolic efficiency across the tested range. The dose–response patterns were aligned with ecotoxicity thresholds and supported the integration of V. fischeri-based assays into environmental risk assessment frameworks. Chronic exposure results further suggested that sublethal antibiotic levels may influence microbial community structure and resistance development in aquatic ecosystems.

Keywords: *tetracycline, Vibrio fischeri, bioluminescence inhibition, antibiotic resistance, ecotoxicological assessment*

INTRODUCTION

Tetracycline (TET) is a broad-spectrum antibiotic extensively used in human and veterinary medicine. Its widespread application and incomplete biodegradation during metabolic processes result in its release into wastewater via bodily excretion, eventually reaching surface waters, sediments, and even groundwater. The persistence of TET in these ecosystems has raised ecotoxicological concerns due to its potential effects on non-target organisms across trophic levels [1]. In order to evaluate its presence and environmental risk, several parameters are commonly used: the effective concentration at 50% (EC_{50}), the lethal concentration at 50% (LC_{50}), the no observed effect concentration (NOEC), and the lowest observed effect concentration (LOEC), all of which are essential for environmental risk assessment. EC_{50} refers to the concentration producing 50% of the maximal observed effect (e.g., bioluminescence inhibition in *V. fischeri*) and is used to assess sub-lethal potency. LC_{50} indicates the concentration at which 50% of exposed organisms die, providing a measure of acute toxicity. NOEC is the highest concentration tested that causes no statistically significant effect compared to the control, while LOEC is the lowest concentration at which statistically significant adverse effects are first observed. These parameters enable a nuanced

evaluation of immediate and long-term biological impacts of emerging contaminants, including antibiotics. Reporting EC_{50} together with NOEC/LOEC is recommended because response and sensitivity differ between acute and longer exposures [2]. Phytoplankton, particularly green algae and cyanobacteria, have shown notable sensitivity to TET with cyanobacteria frequently exhibiting greater susceptibility [3, 4]. Various other biological models have shown sensitivity to TET. *Chlorella pyrenoidosa* exposed to 0.5÷10 mg/L TET developed oxidative stress, significantly inhibiting pigment production (up to 62%) and growth (up to 49%) [5]. Algal toxicity has been reported at EC_{50} values between 0.0018 and 20.6 mg/L [6, 7], while cyanobacteria was identified as particularly sensitive [8]. Invertebrates such as *Daphnia magna* exhibit chronic sensitivity, with NOEC values as low as 0.01 mg/L. Exposure of *Daphnia magna* to 0.1÷0.5 mg/L reduced growth rate by 30÷60%, while 29.4 mg/L over 21 days caused population collapse [9]. Acute LC_{50} values for Danio rerio exposed to tetracycline generally range from 84 to over 500 mg/L [6], while sublethal developmental and endocrine effects, including embryotoxicity, apoptosis, and thyroid disruption, have been reported at concentrations as low as 0.4 mg/L [10, 11]. These findings indicate that TET effects can occur well below acute toxicity thresholds, emphasizing the need for chronic and sub-lethal exposure assessments. Regulatory agencies such as the European Medicines Agency and Joint Research Centre report standardized EC_{50} values ranging from 0.0018÷20.6 mg/L in algae, 0.22÷120 mg/L in invertebrates, and 84÷1000 mg/L in fish. Predicted no-effect concentrations (PNECs) are often below 1 µg/L, suggesting ecological risk even at environmentally relevant levels. The marine bacterium *V. fischeri*, a Gram-negative, rod-shaped, non-pathogenic organism known for its natural bioluminescence regulated via quorum sensing, is a widely used bioindicator in ecotoxicological research. Its bioluminescence inhibition forms the basis of standardized assay Microtox® test [12], offering high sensitivity, short duration, low cost, and ease of use [13]. Its luminescence is tightly linked to cellular metabolism, with disturbances in respiratory activity or protein synthesis rapidly reflected in reduced light emission [14]. Across classes (metals, PAHs, pesticides, pharmaceuticals, nanomaterials), *V. fischeri* bioluminescence inhibition tests often provide early and highly sensitive indicators of contaminant toxicity—sometimes detecting sub-lethal effects long before other bioassays [15]. However, short exposure times (acute teste) may underestimate pollutants that impact long-term physiological processes [16]. Because acute-to-chronic relationships in *V. fischeri* depend on chemical mode of action, it was considered both exposure durations [17]. Recent studies integrate chronic exposure protocols, enabling assessment of microbial adaptation and resistance mechanisms under prolonged stress [18]. Thus, combining acute and chronic tests, *V. fischeri* offers a more accurate representation of ecological stress and adaptation potential under prolonged TET exposure. This study aimed to characterize *V. fischeri* sensitivity to varying TET concentrations by measuring bioluminescence and OD_{600} and exploring adaptation mechanisms under chronic conditions.

Despite the extensive ecotoxicological research on tetracycline, several important knowledge gaps remain unresolved. Most available studies focus either on acute toxicity or on single-endpoint measurements, providing limited insight into how bacterial systems respond to prolonged, sublethal antibiotic exposure. In particular, comparative assessments of *V. fischeri* under acute versus chronic tetracycline stress are scarce, leaving uncertainties regarding the transition between short-term metabolic inhibition and long-term adaptive responses. The mechanisms by which *V. fischeri* modulates bioluminescence and viability at sublethal tetracycline concentrations also remain poorly characterized. Furthermore, the metabolic efficiency indicator RLU/OD_{600}—highly relevant for distinguishing between growth impairment and metabolic disruption—has rarely been used to evaluate antibiotic-induced stress [19]. Additionally, tetracycline's mode of action, involving binding to the 30S ribosomal subunit and inhibition of aminoacyl-tRNA entry [20, 21], suggests potential impacts on the lux bioluminescent pathway.

Addressing these gaps, this study integrates acute and chronic assays with metabolic normalization to evaluate sublethal stress and adaptation thresholds in *V. fisch*eri. To our knowledge, this is one of the few studies comparing acute and chronic effects of tetracycline on *V. fischeri* using the RLU/OD_{600} metabolic ratio to investigate early adaptation mechanisms.

MATERIALS AND METHOD

Bacterial strain and culture conditions

The bioluminescent strain *V. fischeri* (ATCC 7744) was reactivated following ISO 11348-3 [22] using a kit produced by Modern Water. Lyophilized bacteria were reconstituted in 1 mL of bacterial activation solution – AZF686016, serving as the stock culture.

Preparation of tetracycline solutions

A tetracycline stock solution (1 g/L; 98.0÷102.0% purity, HPLC; Sigma-Aldrich, Germany) was prepared in sterile water, sterilized by filtration through a 0.22 µm membrane, and stored at 4°C in dark glass bottles.

Ecotoxicological acute and chronic tests

Although the tetracycline concentrations selected for this study exceed those typically reported in natural aquatic environments, their inclusion is essential for defining the physiological and adaptive limits of *Vibrio fischeri* under controlled laboratory conditions. Higher concentrations allow the identification of inflection points between sublethal stress, metabolic compensation, and complete functional inhibition—transitions that cannot be fully captured at environmentally relevant levels alone. Furthermore, localized contamination events, such as effluent discharges from wastewater treatment plants or pharmaceutical production sites, may temporarily expose microbial communities to elevated antibiotic levels, making upper-range testing ecologically meaningful. By encompassing both environmental and supra-environmental concentrations, the study provides a more comprehensive understanding of bacterial resilience, stress thresholds, and potential adaptation mechanisms relevant to real-world exposure scenarios.

In acute toxicity tests, *V. fischeri* was exposed to tetracycline (1 mg/L÷1 g/L) for up to 30 min. This upper range was selected to ensure a full inhibition profile for accurate EC_{50} determination.

During all bioassays, pH values were maintained within the recommended range for *V. fischeri* (6.0÷8.5, optimum 7.0÷7.5), and salinity was adjusted to 2% NaCl (within the acceptable range of 1.8÷2.2%) according to ISO 11348-3 [22] and Microtox® testing standards. Chronic tests were carried out up to 48 h, in presence of 0.3, 0.6, 1.25, 2.5, and 5 mg/L TET. Incubations were carried out at 25°C in the dark, with continuous shaking at 160 rpm. The 25°C incubation temperature was selected to optimize bacterial metabolic activity during prolonged exposure. Negative controls contained LB broth (Lennox) from Chemsolute, without TET. Positive controls consisted of 3, 5-dichlorophenol (3, 5-DCP; Sigma-Aldrich, Germany), used as a reference toxicant according to ISO 11348-3 [22] guidelines, to verify the sensitivity of *V. fischeri*.

Bioluminescence and optical density measurements

Bioluminescence of *V. fischeri* cultures exposed to various TET concentrations was measured using a Microtox® Model 500 Analyzer (Modern Water Inc., USA). The emitted light was recorded as Relative Light Units (RLU). Bioluminescence inhibition (%) was calculated by comparing the luminescence of *V. fischeri* exposed to TET with that of the untreated control, using the formula (1):

$$\text{Inhibition (\%)} = (1\text{-}RLU_{exposed} / RLU_{control}) \times 100 \quad (1)$$

Optical density at a wavelength of 600 nm (OD_{600}) was recorded to monitor bacterial growth and was acquired with a SHIMADZU spectrophotometer (Kyoto, Japan) [23]. The EC_{50} was estimated from the inhibition curve using non-linear regression analysis. NOEC was observed compared to the control, while LOEC was identified as the lowest concentration producing a statistically significant effect. The RLU/OD_{600} ratio monitor the biomass-related variability and assess the metabolic activity per cell density.

Statistical analyses

All experiments were performed in triplicate. Bioluminescence and OD_{600} were analyzed separately by one-way ANOVA. Normality (Shapiro–Wilk) [24] and homogeneity of variances were tested; Tukey's HSD was used for $p < 0.05$. The concentration–response for bioluminescence inhibition was fitted using a four-parameter logistic (4PL) model [25] in Microsoft Excel (GRG Nonlinear Solver). EC_{50} and Hill slope were estimated; fit quality was evaluated with R^2.

RESULTS AND DISCUSSION

Acute tests

Exposing *V. fischeri* to concentrations of 1 mg/L to 1 g/L TET using acute Microtox assay, it was determinate $EC_{50} \approx 5$ mg/L.

Chronic tests and adaptation

Results showed a significant decrease in bioluminescence at concentration higher than 0.6 mg/L TET, with luminescence completely suppressed at 5 mg/L TET. OD_{600} followed a similar decreasing trend. The metabolic activity per unit biomass (RLU/OD_{600}) had a relatively higher metabolic efficiency at 0.3 mg/L TET, suggesting a potential bacterial adaptation to sublethal stress (Table 1).

Statistical interpretation: ANOVA revealed highly significant differences among treatments for both bioluminescence and OD_{600} ($p < 0.001$ for each). Tukey's HSD statistical analyses tests identified a LOEC for bioluminescence at 0.3 mg/L and a NOEC below 0.3 mg/L. The OD_{600} analyses showed a LOEC at 0.6 mg/L and a NOEC at 0.3 mg/L. The 4PL regression model provided an EC_{50} estimate of 0.6 mg/L, with an excellent goodness-of-fit ($R^2 > 0.98$), confirming the robustness of the concentration–response relationship (Table 2). These values reflect short-term metabolic inhibition and do not capture adaptation effects observed in chronic exposure. These results indicated that *V. fischeri* bioluminescence was more sensitive than growth measurements to TET exposure. This divergence between acute and chronic sensitivity aligns with mode of action-dependent patterns reported for *V. fischeri* [15].

Table 1. Bioluminescence, OD_{600}, and normalized RLU/OD_{600} for *V. fischeri* under chronic exposure (48 h)

TET (mg/L)	Bioluminescence (Mean ± SD)	OD_{600} (Mean ± SD)	RLU/OD_{600}*	Inhibition (%)**
0.00	200± 2.00	0.225 ± 0.001	889	0.00
0.30	160 ± 2.00	0.139 ± 0.001	1151	20.0
0.60	96.0 ± 2.00	0.034 ± 0.001	2824	52.0
1.25	23.0 ± 1.00	0.023 ± 0.001	1000	88.5
2.50	1.00 ± 0.00	0.021 ± 0.001	47.6	99.5
5.00	0.00 ± 0.00	0.020 ± 0.001	0.00	100

*RLU/OD_{600} values were calculated to assess metabolic activity per cell density. Deviations across concentrations may reflect differential stress responses or survival dynamics

Table 2. Statistical analyses for ecotoxicological parameters of *V. fischeri* exposed to TET

Parameter	Statitical Values
ANOVA p-value (Bioluminescence)	< 0.001
ANOVA p-value (OD_{600})	< 0.001
NOEC (Bioluminescence)	< 0.3 mg/L
LOEC (Bioluminescence)	0.3 mg/L
NOEC (OD_{600})	0.3 mg/L
LOEC (OD_{600})	0.6 mg/L
EC_{50} (Bioluminescence)	0.58 mg/L
R^2 (4PL Fit)	> 0.98

Notes: NOEC (No Observed Effect Concentration) and LOEC (Lowest Observed Effect Concentration) were determined using one-way ANOVA followed by Tukey's HSD test ($p < 0.05$). EC_{50} was derived from a 4PL model fitted to the bioluminescence inhibition data. The coefficient of determination (R^2) indicates the goodness-of-fit of the model.

Statistical analysis revealed that *V. fischeri* exhibited greater sensitivity in bioluminescence responses than in OD_{600}-based growth measurements when exposed to low tetracycline concentrations. This finding aligns with previous observations that bioluminescence inhibition often precedes detectable growth inhibition [26], and that metabolic readouts such as luminescence are more responsive than growth metrics under stress conditions [27]. The 4PL model confirmed that half-maximal inhibition occured at ~0.58 mg/L, which was within the concentrations range known to induce sublethal stress in aquatic microorganisms. The difference in LOEC values between bioluminescence (0.3 mg/L) and OD_{600} (0.6 mg/L) suggested that metabolic impairment preceded measurable growth inhibition, making bioluminescence a more sensitive early-warning endpoint. The high R^2 of the model (>0.98) supported the robustness of the dose–response relationship, and the steep slope indicated that toxicity rapidly escalates once a threshold was reached (fig.1). NOEC values determined using one-way ANOVA followed by Tukey's HSD test ($p < 0.05$). The NOEC for bioluminescence was <0.3 mg/L; for graphical purposes, it is represented as 0 mg/L (fig.2).

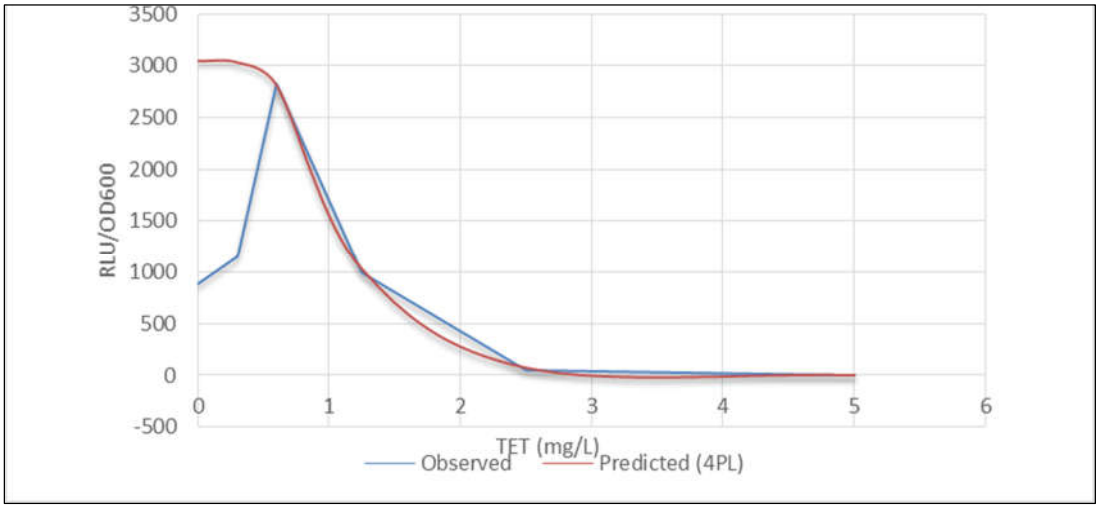

Fig.1 Tetracycline dose–response of *V. fischeri* (RLU/OD_{600}). Blue dots show mean values (±SD), red line shows the fitted 4PL curve

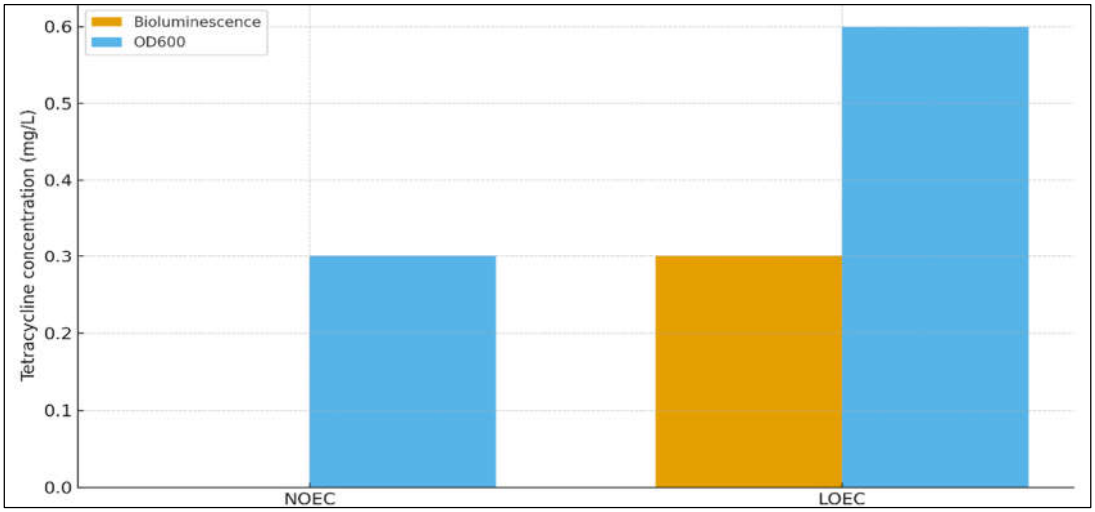

Fig. 2. Comparison of NOEC and LOEC values for bioluminescence and optical density (OD_{600}) in *V. fischeri* chronically exposed to TET

Building on these statistical findings, a deeper mechanistic interpretation helps clarify why V. fischeri exhibited such differential sensitivity between bioluminescence and OD_{600}-based growth measurements. As previous work has shown, luminescence inhibition typically precedes detectable growth effects because the lux bioluminescent system is tightly coupled to cellular metabolic status and respiratory activity [14]. Thus, the lower LOEC observed for bioluminescence reflects the fact that even mild perturbations in energy metabolism are rapidly translated into decreased light emission, making this endpoint a more sensitive and rapid indicator of antibiotic-induced stress. The increase in the RLU/OD_{600} ratio at 0.3÷0.6 mg/L TET further supports this interpretation, suggesting a transient hormetic stimulation characterized by metabolic overcompensation under mild stress. Such sublethal activation is consistent with hormesis theory and may reflect the early engagement of stress-response pathways and metabolic reconfiguration mechanisms previously described in bacteria exposed to sub-inhibitory antibiotic levels [28, 29]. At higher concentrations, however, the sharp decline in luminescence indicates substantial metabolic impairment. This effect is consistent with tetracycline's mode of action—binding to the 30S ribosomal subunit and blocking aminoacyl-tRNA entry—which disrupts protein synthesis and may directly suppress luxA/luxB expression, thereby diminishing luciferase activity. Because bacterial luminescence is also closely linked to respiratory chain efficiency, disturbances in electron transport could further amplify the observed inhibitory effects. The substantial difference between acute and chronic EC_{50} values (5 mg/L vs. 0.6 mg/L) reinforces the importance of long-term toxicity assays. Acute tests alone may underestimate ecological risks, as chronic exposures reveal cumulative or delayed physiological disruptions that do not manifest in short-term assays. This acute–chronic divergence, widely recognized in environmental toxicology [30], underscores the necessity of integrative, multi-endpoint approaches when assessing the impact of emerging contaminants such as antibiotics. Collectively, these results demonstrate that tetracycline exerts both sublethal and inhibitory effects on *V. fisch*eri, with bioluminescence providing a highly sensitive measure of early physiological stress. The hormetic response at low concentrations suggests transient metabolic adaptation, whereas higher concentrations overwhelm cellular defenses, leading to pronounced translational and metabolic inhibition. These findings highlight the ecological relevance of sublethal antibiotic exposures and confirm the value of *V. fischeri* as a bioindicator for assessing microbial stress, adaptation potential, and antibiotic-driven ecological changes in aquatic environments.

CONCLUSIONS

Overall, these findings demonstrate that *V. fischeri* provides a sensitive and reliable model for detecting early metabolic disturbances induced by tetracycline, while chronic exposure reveals adaptation thresholds that cannot be captured by short-term assays. Such integrative approaches are essential for improving ecological risk assessment of antibiotics in aquatic environments.

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