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Performing acute phytotoxicity of widely used drugs on germination and root elongation of *Lactuca sativa* L.

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ABSTRACT

The aim of this study was to investigate the phytotoxic effects of six most common pharmaceuticals exposed at four concentrations in bioassays. The pharmaceuticals presented are classified as five antibiotics and one anticonvulsant drug. Toxicity tests were carried out using diluted model wastewater to evaluate the toxic potential using *Lactuca sativa L.* as a bioindicator. The highest inhibition effect was observed in the range of 0.1 to 1000 mg L^{-1}. *Lactuca sativa* seeds were used for this study and germination percentage, sprout as well as root length were evaluated. All the formulations used in this bioassay had inhibitory effects on root growth, radicle and hypocotyl elongation, and germination of *Lactuca sativa* seeds. The results showed that in the tests with *Lactuca sativa* seeds, sulfamethoxazole (SMZ) 8.6% and trimethoprim (TMP) 3.2% at 0.1 mg L^{-1} dilution showed the least inhibition, while tetracycline (TC) at concentrations of 500 and 1000 mg L^{-1} was fatal to lettuce seeds. Overall, this study emphasizes the importance of proper disposal of water containing antibiotics as a strong exposure factor for biological organisms.

Keywords: antibiotic pollution, antibiotic resistance, bioassay, *Lactuca sativa L.,* phytotoxicity, wastewater.

INTRODUCTION

Today, there is a global problem of the spread of antimicrobial drugs (Yang et al., 2021) and their derivatives, which are used in all sectors of human activity: medicine, pharmaceuticals, livestock (Pan and Chu 2016), agriculture, food production and more. In addition to this, further treatment and disposal of these xenobiotic components into the environment (Vasquez et al., 2014; Régo et al., 2018; Gangar and Patra, 2023). Pharmaceutical compounds enter the terrestrial environment through the discharge of treated wastewater (Zhang and Li, 2011) and sludge into agricultural soils. Antibiotics are considered pseudo-persistent due to continuous discharge and poor biodegradability (Ben et al., 2019). Therefore, those compounds accumulate in water bodies and plants; for example, (Geng et al., 2022) assessed antibiotic residue accumulation in edible plants and detected antibiotics accumulation in plant roots caused by residual concentrations in soils. Moreover, (Wilkinson et al., 2022) showed that sulfamethoxazole (SMX) and trimethoprim (TMP) are frequently detected in aquatic environments worldwide, with TMP found on every continent except Antarctica and SMX having one of the highest concentrations found in rivers worldwide.

This situation leads to a more crucial consequence – an increase in the number of antibiotic-resistant bacteria (Cantas et al., 2013; Carvalho and Santos, 2016; Bombaywala et al., 2021; Sun et al., 2022), as well as antibiotic-resistant infections and diseases that are not sensitive to antibiotics (Hernando et al., 2006; Ventola, 2015; Gangar and Patra, 2023).

This work was aimed at studying the phytotoxic potential of six (clarithromycin, sulfamethoxazole, trimethoprim, tetracycline, sulfadiazine and carbamazepine) widely used drugs by bioassay. These six pharmaceuticals were selected because of high consumption and the presence in wastewater (Kortesmäki et al., 2020). The results of these studies will enable to develop and implement biodegradable and combined methods for the inactivation of antibiotics from wastewater,

as presented in work (Vasenko et al., 2020). This knowledge will be used for a new approach to biodegradation method by immobilized biocenoses on inert surfaces (Tsytlishvili et al., 2018) based on previous research for inactivation antibiotics from wastewater (Baaloudj et al., 2021).

For this study, *Lactuca sativa L.* seeds were used and germination percentage, sprout as well as root length were evaluated. All the pharmaceuticals used in this bioassay had inhibitory effects on root growth, radicle and hypocotyl elongation and germination of *Lactuca sativa* seeds (Dutka, 1995; Valerio et al., 2007; Chan-Keb et al., 2018). A sensitive and accessible ecotoxicological method widely used for assessing the phytotoxicity of water, model wastewater, soil and industrial effluents (Garcia et al., 2009). There are known high sensitivity bioindication methods (bioassay) using a set of animal and plant biotests, which consists of test organisms of three trophic levels (Forget et al., 2000). The following test objects were used: *Allium cepa*, *Lactuca sativa*, test with the nematode *Panagrellus rediviru* and test with *Hydra attenuata* and *Daphnia magna*, as representatives of invertebrate animals. As well as enormous number of studies focused on marine organisms (phytoplankton, marine bacteria, algae, *Danio rerio* or fish). According to (Chan-Keb et al., 2022), the advantage of using lettuce seeds (*Lactuca sativa*) in tests for toxicity in wastewater with antibiotics (Pino et al., 2016) due to its rapid growth and the fact that it requires little energy for germination, as well as being inexpensive vegetables, simple in cultivation, availability throughout the years and ability to use them as in laboratory and field trials.

This study is interesting because it provides information about the behavior and subsequent fate of antibiotic-containing wastewater treatment in WWTPs using biological stage, since activated sludge uses mainly as mechanisms of removal: biodegradation and adsorption (Li and Zhang, 2010).

MATERIALS AND METHODS

Test organisms and chemicals

Lettuce seeds (*Lactuca sativa* NL-364334274) were obtained from EU System St. (product of Holland) and were kept in the dark at 4 °C shielded from large modifications of temperature and moisture. Acute toxicity tests were performed on certified bioreagents: clarithromycin (CLAR) (purity ≥ 95% HPLC), sulfamethoxazole (SMZ) (purity ≥ 99%), trimethoprim (TMP) (purity ≥ 98% HPLC), tetracycline (TC) (purity 98.0–102.0%), sulfadiazine (SDZ) (purity 99.0–101.0%), and carbamazepine (CBZ) (purity ≥ 98% HPLC) which were purchased from Sigma-Aldrich, Steinheim, Germany. Fresh stock solutions of pharmaceuticals were prepared in deionized water (resistivity > 5 MΩ·cm (typically 10–15 MΩ·cm) obtained from an Elix® Essential 15 Water Purification System (Millipore; Molsheim; France). The model solutions were stored in the dark at 4 °C to avoid photodegradation and have been used within 24 h after preparing.

Dilution preparation

The model samples with drugs were diluted four-fold with distilled and deionized water to 1000 mg L^{-1}, 500 mg L^{-1}, 100 mg L^{-1}, and 0.1 mg L^{-1}.

Experimental design

The experimental procedure for acute ecotoxicity tests with *Lettuce* seed was set up according to Dutka (1989, 1995) "Seed Germination and Root Elongation (*Lactuca sativa*)", described by Gabriela Castillo (2004) based on section 6.3.2 *Lactuca sativa*, Sobrero & Ronco (2004) Priac & Badot (2017) and from Charles et al., (2011) with some adaptations. Control and toxicity test. Assay protocol (Trautmann et al., 2001):

1. First, the lettuce seeds were submerged in a 10% bleach solution for 20 minutes, then rinsed five times with deionized or distilled water. This killed fungal spores that can interfere with seed germination.
2. Secondly, paper filter disks (Whatman No. 3, Ø = 90 mm) were placed in polystyrene Petri dishes (Ø = 100 mm) previously marked with the corresponding dilution start and end dates of the bioassay.
3. To each Petri dish, 2 ml of the appropriate test solution were added. In the control dishes, deionized water was used as a test solution.
4. Twenty seeds were placed on the filter paper with a pair of tweezers with enough space to allow root growing. The plates were covered by foil and placed in plastic bags to prevent the loss of humidity. The seeds were incubated in the dark at constant temperature (preferably

21.0 ± 2.0 °C) for 5 days (120 hours). Each condition was tested in triplicate.

5. At the end of this time (120 h), the number of germinated seeds in was counted, and the root as well as sprout length of each were carefully measured to the nearest mm with electronic stainless steel calliper (accuracy 0.02 mm).

Characteristics of pharmaceutical test products

The experiment was conducted at the Lab of Water and Environmental Engineering, Aalto University in Espoo, Finland. *Lactuca sativa* seeding and analysis of samples were carried out in April-May 2024.

The bioassay was performed using *Lactuca sativa* seeds and six pharmaceutical products (clarithromycin (CLAR), sulfamethoxazole (SMZ), trimethoprim (TMP), tetracycline (TC), sulfadiazine (SDZ) and carbamazepine (CBZ)) Sigma Aldrich CHEMIE GmbH, the physico-chemical properties of which are shown in Table 1.

For this bioassay, 200 ml of 1000 mg L^{-1} sample solution of each of the six preparations were prepared with distilled water (Fig. 1).

Despite their simplicity and wide use of acute phytotoxicity assay, they are not standardized and may include adaptive parameter variability as shown in Table 2.

Bioassay (Ratsch and Johndro,1984; Dutka, 1989; Bolonhesi and Lopes, 2018):

1) After 120 hours (5 days) germination of seeds was counted (GR%), where GSS is the number of germinated seeds in the sample and GSC the number of germinated seeds in the control. The percentage of germination for each dilution was calculated according to Equation 1.

Table 1. Physicochemical properties of the six pharmaceutical drugs

Chemical group	Compound	Drug class	Formula molecular	Molar mass [g·mol^{-1}]	CAS registration number	ATC code
Macrolides	Clarithromycin (CLAR)	Antibiotic	$C_{38}H_{69}NO_{13}$	747.95	81103-11-9	J01FA09
Sulphonamide antibiotic	Sulfamethoxazole (SMZ)	Antibiotic	$C_{10}H_{11}N_3O_3S$	253.28	723-46-6	J01EE01
Aminopyrimidine antibiotic	Trimethoprim (TMP)	Antibiotic	$C_{14}H_{18}N_4O_3$	290.323	738-70-5	J01EA01
Subclass of polyketides	Tetracycline (TC)	Antibiotic	$C_{22}H_{24}N_2O_8$	444.440	60-54-8	A01AB13
Sulfonamide antibiotic	Sulfadiazine (SDZ)	Antibiotic	$C_{10}H_{10}N_4O_2S$	250.28	68-35-9	J01EC02
Dibenzoazepine and a member of urease	Carbamazepine (CBZ)	Anticonvulsant medication	$C_{15}H_{12}N_2O$	236.274	298-46-4	N03AF01

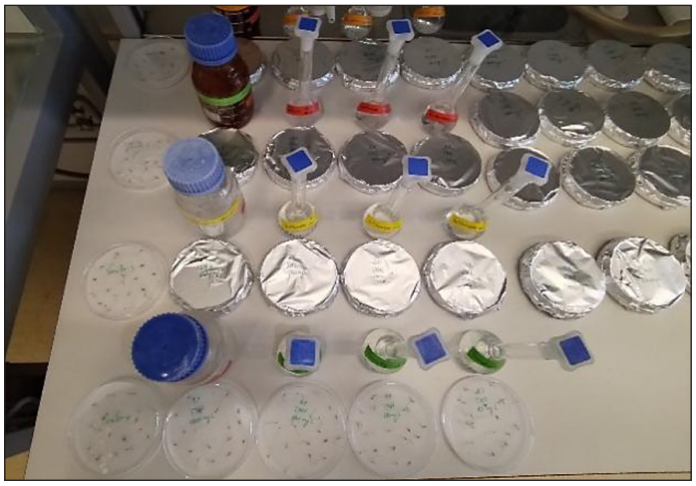


Figure 1. Preparation of sample drug solutions from each of the 4 concentrations to determine acute toxicity, experiments were carried out with four concentrations of the drug, i.e. 0.1, 100, 500 and 1000 mg L^{-1}, which were obtained from serial dilutions in triplicate, and distilled water was used for control growth in a total amount of 78 experimental units (Fig. 2).

Table 2. Parameters of seed germination for bioassays experiment

Parameters	Examples
Bioassay test object	*Lactuca sativa L.*
Support for cultivation	Filter paper Whatman® No. 3
Seed pre-treatment	10% bleaching solution hypochlorite
Temperature (in °C)	21 ± 2
Dish	Polyethylene Petri dish 90 × 14 mm
Number of seeds	20
Amount of sample	2 ml
Duration	120 h
Control water	Distilled and deionized
Condition of germination	In a dark, covered by foil, prevent remoisturise

$$GR\% = GSS / GSC \times 100 \quad (1)$$

where: $GR\%$ – the relative germination percentage; GSS – number of germination seeds in samples; GSC – number of germination seeds in control.

2) Percentages of roots growth inhibition (% RGI) were calculated by the medium values for each sample, using Equation 2.

$$RGI\% = RGC - RGS/RGC \times 100 \quad (2)$$

where: RGC – root growth average in control; RGS – root growth average in samples.

3) Percentages of hypocotyl (sprout) growth inhibition (HGI %) were calculated by the medium values for each sample, using Equation 3.

$$HGI\% = HGC - HGS/HGC \times 100 \quad (3)$$

where: HGC – hypocotyl growth average in control; HGS – hypocotyl growth average in samples.

4) Germination index (GI) (Ranal et al., 2009; Rede et al., 2019) were used to assess the response variability between Lettuce seeds treatment. Calculations of these indices were performed using the Equations 4 where RLS is the root length of the sample, RLC the root length of the control.

$$GI = RLS \times GSS/RLC \times GSC \quad (4)$$

The germination index tells us about the percentage of germination and the rate of germination. GI seems to be the most comprehensive measurement parameter, combining both the percentage of germination and the root growth and elongation after 5 days (120 h) between the samples and the control seeds of the treatment in triplicate. Then, a higher GI value indicates a higher percentage and rate of germination.

RESULTS AND DISCUSSION

Table 3 presents the germination rate of *Lactuca sativa* for the six drugs diluted at four concentrations.

According to the data obtained from bioassay in Table 3 shown the data of the percentage of germination of lettuce seeds for five antibiotics and one anticonvulsant in four dilutions of *Lactuca sativa* seeds germinated in the control water presented up to 100% germination. An acute phytotoxic effect was observed with an increase in the concentration of the drug exposure, especially

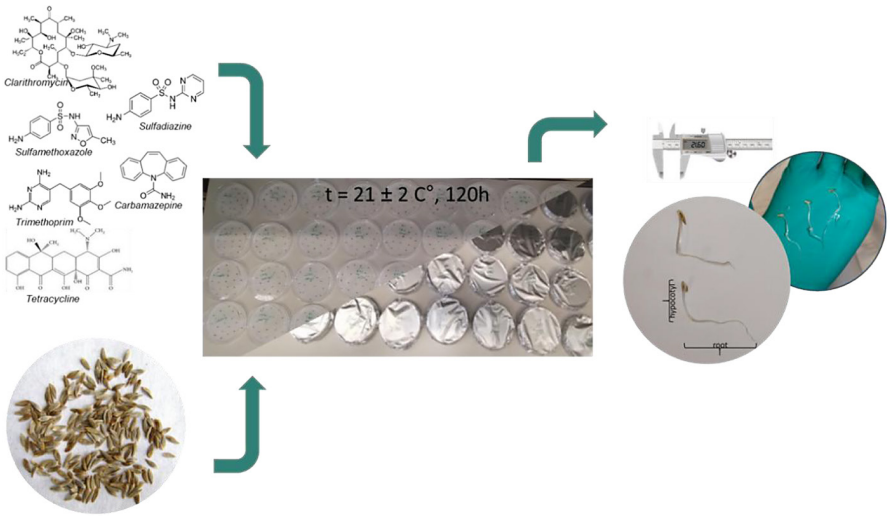


Figure 2. Experimental schematic diagram of effects on *Lactuca sativa* seeds in different treatments of six drugs

Table 3. Germination rate (GR%) for *Lactuca sativa* using six drugs (n = 3 replicates).

Parameter	Samples	Tested concentration (mg L^{-1})			
		1000	500	100	0.1
Germination rate GR (%)	Clarithromycin (CLAR)	72.3±6.8	82±5	85±1.7	96±3.3
	Sulfamethoxazole (SMZ)	82±5	83.3±5	97±1.7	100
	Trimethoprim (TMP)	80±5	93±1.7	95±2.4	100
	Tetracycline (TC)	0	0	70±5	75±6.2
	Sulfadiazine (SDZ)	92±3.3	93±1.7	95±2.4	95±2.4
	Carbamazepine (CBZ)	77±2.4	85±6.2	87±5	90±5

with a tetracycline treatment concentration of 1000 and 500 mg L^{-1}. A noticeable effect was manifested in the form of reddening of seeds and their deformation under the influence of Tetracycline inhibition as shown in Figure 3.

The exposure of *L. sativa* seeds for clarithromycin, sulfamethoxazole, trimethoprim, sulfadiazine and carbamazepin in a range of concentrations between 0.1 and 1000 mg L^{-1} did not cause statistically significant effects on percentage of seed germination, roots, and sprouts elongation comparatively to the control. Moreover, the lowest germination to concentrations 1000 mg L^{-1} at 72.3% was found for clarithromycin.

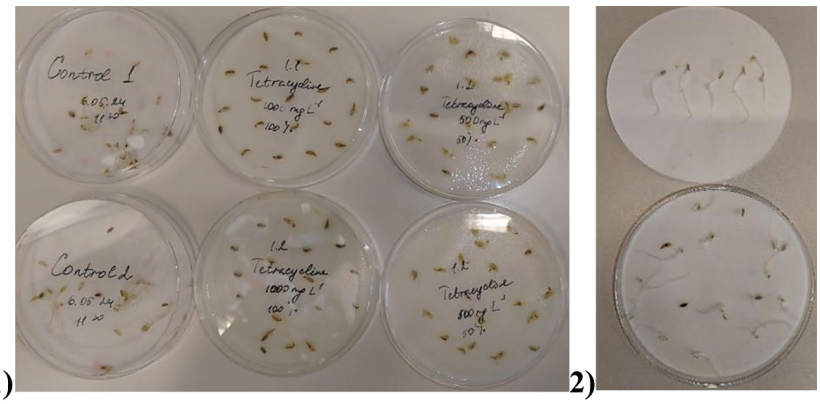
This was confirmed by the results described in Table 3 and Figure 3. Nonetheless, percentages of root growth inhibition (RGI %) has inversely proportional connection with percentage of germination. It means that inhibition growth of roots increases with concentration. Especially, it is visually reflected for trimethoprim from 3.2 to 86% and sulfamethoxazole from 8.6 to 60% (Fig. 4a). Significant differences were observed among all drugs for concentrations 500 and 1000 mg L^{-1}.

For hypocotyl (sprout) growth inhibition (HGI%), a gradual increase of 52 to 81%, on average, can be observed when the exposure concentration increases (Fig. 4b), but for tetracycline this parameter changes significantly at high chronic concentrations.

However, given all concentrations of the six drugs, there was a reduction in root elongation with respect to control, as treatment per 1000 mg L^{-1} was represented by the greatest effect (Fig. 4b). It was also noted that in the tetracycline treatments they were totally inhibited at those concentrations.

Foremost, in Figure 4b it can be seen that significant inhibition of hypocotyl length was exerted by tetracycline at concentrations of 500 and 1000 mg L^{-1}, however, at concentrations from 0.1 to 1000 mg L^{-1}, no significant differences were observed between five drugs clarithromycin, sulfamethoxazole, trimethoprim, sulfadiazine and carbamazepine.

According to the calculation of the germination index shown in Figure 5 it can be concluded that all preparations promote strong inhibitory action on germination of seeds, especially for tetracycline, clarithromycin and sulfamethoxazole in any concentration. The profound effect on fatality for lettuce seeds is performed by tetracycline in concentrations 500 and 1000 mg L^{-1}.



1) 2)

Figure 3. Petri dishes contain *Lactuca sativa* seeds after 120 hours exposure tetracycline (1) with dilutions 1000 and 500 mg L^{-1} and control (2)

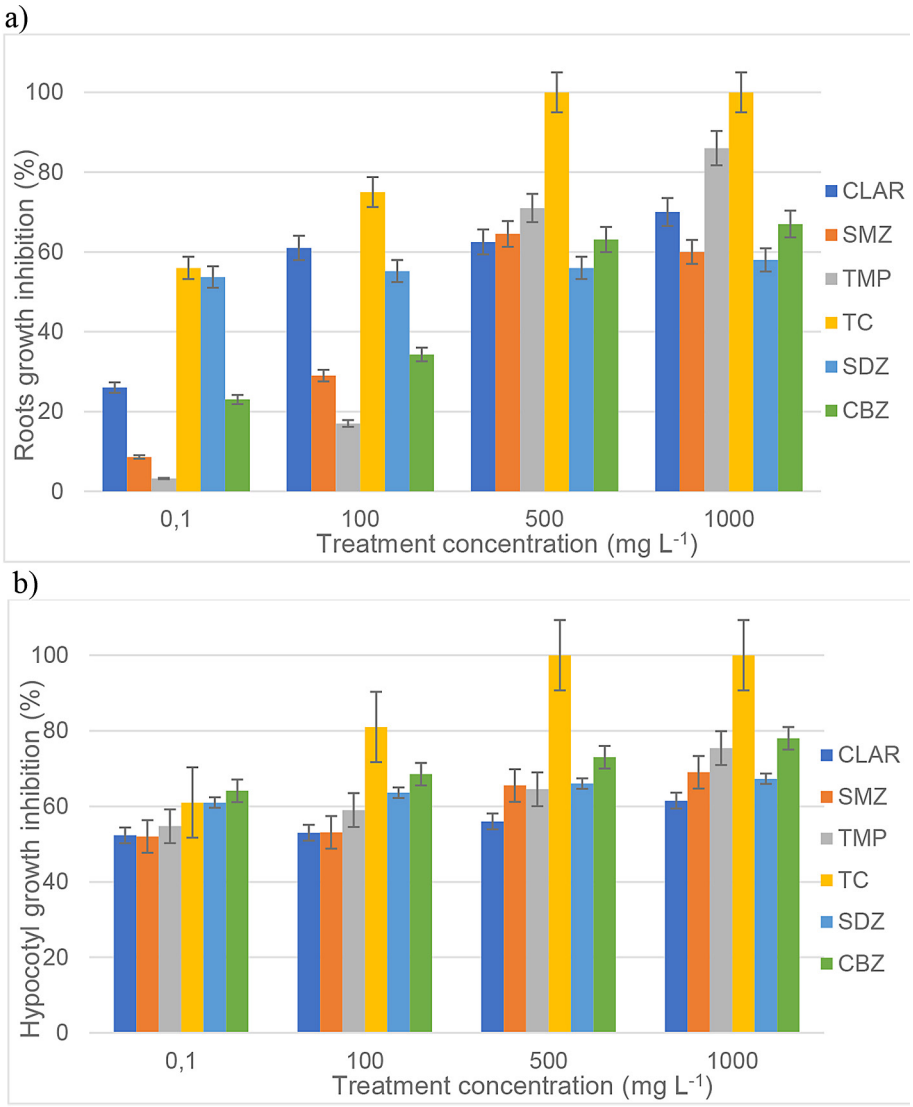


Figure 4. Percentages of root growth inhibition (RGI %) of *Lactuca sativa* fore four concentrations exposed to 6 drugs (a), * the error bars represent the standard deviation; percentage of hypocotyl (sprout) growth inhibition (HGI %) of *Lactuca sativa* between the concentration exposed to 6 drugs (b), * the error bars represent the standard deviation

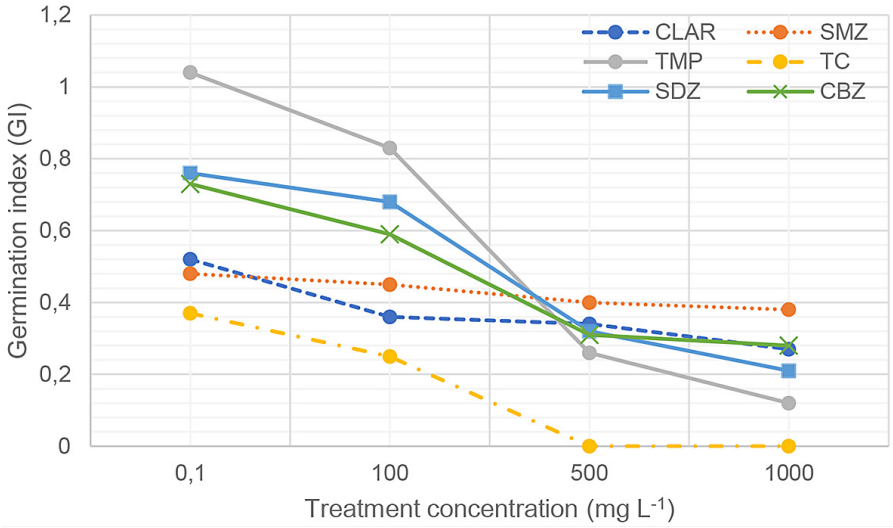


Figure 5. The effect on *Lactuca sativa* seeds is presented as a germination index between concentrations of 6 drugs

CONCLUSIONS

This experimental bioassay of acute toxicity with seeds of *Lactuca sativa* was indicative and sensitive for six frequently used drugs. This study showed that the germination and elongation tests under different conditions and with different growing parameters were dependent on the concentration and treatment terms, affecting the percentage of Lettuce germination and total length.

Tetracycline showed the greatest toxic effect: germination percentage, sprout and root length were the lowest. This suggests that the development of a methodology for inactivating antibiotics from wastewater by biological and advanced oxidation processes (AOP) requires knowledge of phytotoxicity.

The authors would like to emphasize that the presence of antibiotics in water has a strong impact on biological indicator organisms. These problems of antibiotic resistance and ecotoxicological impact are becoming more common and are found worldwide. For further study, the authors decided to use other bioindication organisms, parameters and natural samples of effluent/influent water from WWTP's containing antibiotics.

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