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## Comparative study of acetaminophen and 4-methoxyphenol toxicity

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**Abstract:** Acetaminophen (APAP, paracetamol) is one of the most widely used human drugs, and simultaneously, one of the most frequently detected pharmaceutical micropollutants in environmental matrices. The major metabolites of acetaminophen are generally considered to be glucuronide and sulfate conjugates, p-aminophenol, hydroquinone, and NAPQI, while 4-MP may occur as one of the transformation products under specific environmental degradation pathways. Determining and comparing the toxicity of these compounds is crucial for environmental and public health. In this study, the toxicity of APAP and 4-MP was assessed using two model microorganisms, *Escherichia coli* ATCC-25922 and *Candida albicans* ATCC-1023, based on growth inhibition. The susceptibility of microorganisms belonging to the human microbiome (*E. coli*, *E. hormaechei*, *S. epidermidis*, *L. rhamnosus*, and *C. albicans*) to both compounds was determined by calculating the minimum inhibitory concentrations (MICs). Genotoxicity was estimated using the genotoxin-sensitive *recA* promoter in *E. coli* RFM443 *recA::luxCDABE*. Reactive oxygen species (ROS) production was measured in *E. coli* exposed to both compounds at concentrations of 0.1, 1, 10, 100, and 1000 mg·L<sup>-1</sup> as an oxidative stress indicator. Comparative analysis revealed similar sensitivity of *E. coli* and *C. albicans* to both compounds at 100 mg·L<sup>-1</sup>, though at 10 mg·L<sup>-1</sup>, 4-MP exerted a stronger inhibitory effect on both strains than APAP. MIC assays showed that *E. coli* and *E. hormaechei* were more sensitive to 4-MP than to APAP, while no significant differences were observed for the remaining strains. APAP demonstrated a stronger genotoxic effect than 4-MP in *E. coli* cells. ROS data indicate that oxidative stress is the primary molecular mechanism underlying the toxicity and genotoxicity of both compounds. Future studies should investigate the toxicity of APAP and 4-MP mixtures under environmentally relevant exposure conditions.

## Introduction

The contamination of water resources with pharmaceuticals and their metabolites is a significant global problem. Pharmacologically active compounds, including antibiotics and hormones, have been detected in all aquatic and water-related compartments (Lunghi et al. 2025), causing negative effects on living organisms and having long-term consequences for human health through the development of antimicrobial resistance (AMR) (Ossowicz-Rupniewska et al., 2023). In response

to these threats, the European Union has established a comprehensive legal framework for the protection of water resources, most notably through the Water Framework Directive 2000/60/EC and Council Directive 98/83/EC on the quality of water intended for human consumption, implemented in Poland through the Water Law Act (Dz. U. poz. 2294, 2017, Cybulski et al. 2021, Szymański et al., 2026).

The environmental concern is acetaminophen, a fully synthetic aromatic amide of the aniline derivative class, with

a para-substituted benzene ring bearing a hydroxyl and an acetamido group. APAP is one of the most widely consumed over-the-counter analgesic and antipyretic agents worldwide (Chacón et al. 2022), marketed under several trade names, of which acetaminophen, paracetamol, and N-acetyl-p-aminophenol (APAP) are the most widely recognized. Its molecular formula is  $C_8H_9NO_2$ , and its molecular weight is  $151.16 \text{ g}\cdot\text{mol}^{-1}$ . The compound is a white, odorless, crystalline solid with moderate water solubility (1:70 at room temperature; 1:20 at  $100^\circ\text{C}$ ), a  $pK_a$  of 9.5–9.7, and an octanol-water partition coefficient of 3.2. These physicochemical properties facilitate its mobility and persistence in aquatic environments (IARC, 1990; Mészáros et al. 2022).

Conventional wastewater treatment technologies, including the activated sludge process, frequently fail to achieve complete removal of APAP, resulting in its continuous discharge into surface waters and soils. Concentrations in surface waters reach up to  $10 \text{ }\mu\text{g}\cdot\text{L}^{-1}$ , in groundwater used for drinking water supply up to  $1.89 \text{ }\mu\text{g}\cdot\text{L}^{-1}$  (Li et al. 2014; Pandey et al. 2024), in contaminated soils below  $100 \text{ }\mu\text{g}\cdot\text{kg}^{-1}$ , and in biosolids up to  $1400 \text{ }\mu\text{g}\cdot\text{kg}^{-1}$  (Chacón et al. 2022; Tomczyk and Urbaniak 2025), with the highest recorded concentration reaching approximately  $107 \text{ }\mu\text{g}\cdot\text{L}^{-1}$ . Evidence of paracetamol bioaccumulation in both flora and fauna, together with its detection at substantial concentrations in multiple environmental matrices, underscores the risks this compound poses to ecological balance and human health (Pandey et al. 2025). Numerous studies have demonstrated that APAP induces reproductive disruption, DNA damage, and oxidative stress in aquatic organisms, and has been associated with nephrotoxicity, myocardial infarction, hepatic damage, and impaired fetal development in humans and animals (Li et al. 2014; Pandey et al. 2024). Paracetamol use during pregnancy may increase the risk of attention-deficit/hyperactivity disorder (ADHD) and autism spectrum disorder (ASD; Bühner et al. 2021). Furthermore, regular paracetamol use has been associated with a 28% higher risk of developing liver cancer (Tian et al. 2024).

Experimental studies have shown that the major metabolites of APAP include glucuronide and sulfate conjugates, p-aminophenol, hydroquinone, and NAPQI. In turn, a study by Li et al. (2014), which utilized both  $^{14}\text{C}$  isotope labeling and LC-MS/MS techniques, and other studies confirmed the presence of 4-MP as one of the transformation products formed through specific environmental degradation pathways (Li et al. 2014; Žur et al. 2018; Chacón et al. 2022; Yisau et al. 2024; Pandey et al. 2024, 2025; Fig. 1). The possible biodegradation pathway of APAP, with 4-MP as a one of the metabolites formed, is presented in Fig. 1 (adapted from Žur et al. 2018 and Pandey et al. 2025).

4-MP is widely used across multiple industrial sectors, including the pharmaceutical, agricultural, and cosmetic industries, and has been detected in aquatic and terrestrial environments at concentrations ranging from  $\text{ng}\cdot\text{L}^{-1}$  to  $\mu\text{g}\cdot\text{L}^{-1}$ . Studies have shown that 4-MP inhibits the synthesis of DNA, RNA, and proteins, causing skin irritation, disruption of reproductive function, and exhibiting mutagenic, genotoxic, and carcinogenic properties in both humans and animals (Asakawa et al. 1994; Miyachi et al. 2005; Rahman et al. 2017; Saeed et al. 2024). Despite the widespread environmental occurrence of paracetamol, comparative toxicological data on its effects on soil microorganisms and the human microbiome remain scarce. In this experimental work, we tested the following research hypothesis: 4-MP, the transformation product formed as a result of acetaminophen degradation in soil, exhibits toxic, genotoxic, and oxidative stress-inducing activity. The present study comparatively assessed the toxicity, genotoxicity, and oxidative stress-generating potential of APAP and 4-MP using strains of *E. coli* ATCC-25922 and *C. albicans* ATCC-10231. Based on minimum inhibitory concentration (MIC) values, the susceptibility of microorganisms constituting the human microbiome was further compared among *E. coli*, *E. hormaechei*, *S. epidermidis*, *L. rhamnosus*, and *C. albicans*. The results contribute to understanding the impact of paracetamol and its

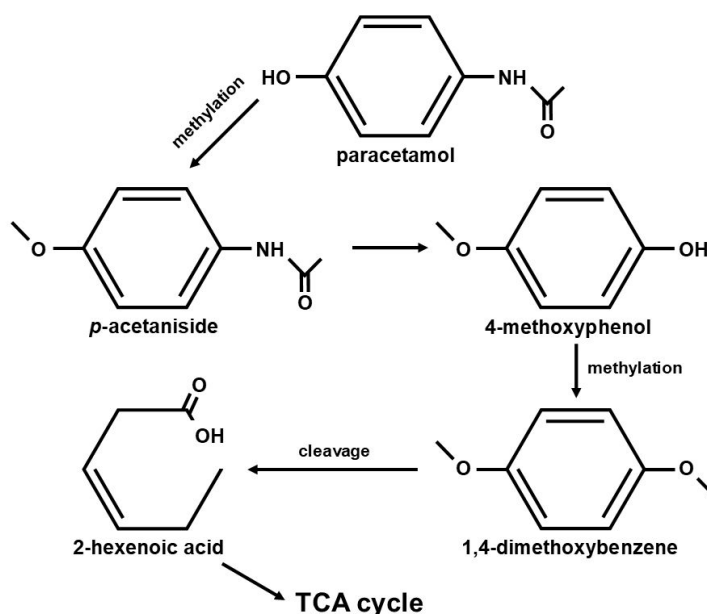


**Fig. 1.** Possible biodegradation pathway of APAP with 4-MP as a one of the metabolites formed (adapted from Žur et al. 2018 and Pandey et al. 2025).

transformation product on environmental microorganisms and biodegradation efficiency.

## Materials and Methods

### Chemical Compounds

The concentrations of 0.01, 0.1, 1, 10, 100, and 1000 mg·L<sup>-1</sup> were selected based on literature data reporting environmentally detected levels of paracetamol and its biodegradation metabolites (Li et al. 2014; Pandey et al. 2024). The inclusion of higher concentrations was intended to determine whether the toxicological mechanism of both compounds is concentration-dependent. Acetaminophen and 4-methoxyphenol were purchased from Sigma-Aldrich (UK) and dissolved in dimethyl sulfoxide (DMSO) prior to use. In the samples studied, the final DMSO concentration was 10%. Solvent controls were included in the experimental systems used in the studies.

### Microorganisms

The study was conducted during the academic year 2024/2025 using microorganisms purchased from the American Type Culture Collection (ATCC, USA). The toxicity of APAP and 4-MP was assessed based on the antimicrobial activity of both compounds against two model microorganisms: the bacterium *E. coli* ATCC-25922 and the fungus *C. albicans* ATCC-10231. Oxidative stress, based on reactive oxygen species (ROS) generation, was additionally measured in *E. coli* ATCC-25922. The genotoxicity of both compounds was determined using the *E. coli* RFM443 *recA:luxCDABE* biosensor strain. For the determination of MIC values, a broader panel of microorganisms representing the natural human microbiome was employed, comprising *E. coli* ATCC-25922, *Enterobacter hormaechei* ATCC-700323, *Staphylococcus epidermidis* ATCC-12228, *Lactocaseibacillus rhamnosus* ATCC-53103, and *C. albicans* ATCC-10231.

### Rationale for the Selection of Microorganism Strains

The study was conducted using microorganisms belonging to the human microbiome to investigate the effects of the drug and its transformation product, 4-MP, on the natural human microbiota. When ingested as a drug, APAP is metabolized in the human body, enters the bloodstream, and reaches the tissues and organs, affecting not only the gut microbiome but also the microbiome of the entire body. Furthermore, when immunity is compromised, there is excessive proliferation of microbiome microorganisms and the emergence of opportunistic infections, which are also caused by strains of *E. coli*, *C. albicans*, and *S. epidermidis* - the subjects of our study.

### Justification for the Selection of Biological Assays Used in the Experiments

The toxicity (inhibition of microbial culture growth and determination of MIC values), genotoxicity, and oxidative stress (based on ROS synthesis values) tests conducted in this study were aimed at determining the toxic and genotoxic potential of both tested chemical compounds (APAP and 4-MP). The determination of ROS synthesis levels served as the basis for confirming oxidative stress as the primary mechanism of toxicity and genotoxicity of the chemical compounds studied in this work.

### Measurement of Antimicrobial Activity

Antimicrobial activity was established based on the growth inhibition of both microorganisms, cultivated at 37°C in Luria-Bertani (LB) medium containing tryptone (10 g·L<sup>-1</sup>), yeast extract (5 g·L<sup>-1</sup>), and NaCl (10 g·L<sup>-1</sup>) (pH 7.0). On the following day, the cultures were refreshed by dilution with fresh LB medium and incubated in a shaking water bath (130 rpm) at 37°C until reaching the logarithmic growth phase (OD<sub>600</sub> = 0.2). Subsequently, in 96-well plates, 100 µl of bacterial or fungal cultures were mixed with 100 µl of appropriate concentrations of APAP and 4-MP. Control samples contained no chemicals. The plates were then incubated at 37°C for 24 and 48 h. Nalidixic acid (NA), a quinolone antibiotic that acts by inhibiting bacterial DNA gyrase and disrupting DNA replication, was used as a genotoxic standard. The growth of bacterial and fungal cultures following treatment was assessed spectrophotometrically at 600 nm using a GloMax® microplate reader (Promega, MA, USA), and the results were expressed as a percentage of growth inhibition or stimulation relative to the control. All experiments were performed in at least three independent series, with six replicates per sample in each series.

### Determination of Minimum Inhibitory Concentration (MIC)

MIC values for APAP and 4-MP were determined using *E. coli* ATCC-25922, *E. hormaechei* ATCC-700323, *Staphylococcus epidermidis* ATCC-12228, *Lactocaseibacillus rhamnosus* ATCC-53103, and *C. albicans* ATCC-10231. Microorganisms were cultivated overnight in Müller-Hinton medium (MHM) at 37°C. The following day, cultures were refreshed with fresh MHM and incubated at 37°C until reaching the logarithmic growth phase (OD<sub>600</sub> = 0.2). Subsequently, a drop of each culture was placed on Müller-Hinton agar (MHA) containing appropriate concentrations of the drugs, ranging from 10 mg·mL<sup>-1</sup> to 0.01 µg·mL<sup>-1</sup>. Control samples contained no chemicals. Plates were incubated at 37°C for 24 and 48 h, after which the presence or absence of microbial growth was recorded.

### Genotoxicity Assay

To determine the genotoxic potency of both compounds, the *E. coli* RFM443 *recA:luxCDABE* biosensor strain, carrying a plasmid construct with a genotoxin-sensitive *recA* promoter fused to the *luxCDABE* reporter gene, was used. The strain was cultivated overnight in LB medium at 37°C. The following day, the culture was diluted with fresh LB medium and incubated at 37°C with shaking (130 rpm) until reaching the logarithmic growth phase (OD<sub>600</sub> = 0.2). Subsequently, 100 µl of *E. coli* RFM443 *recA:luxCDABE* culture was mixed with 100 µl of appropriate concentrations of APAP and 4-MP in white 96-well plates and incubated for 2 h at 37°C. Luminescence was measured at time 0 (immediately upon addition of the tested compounds) and after 2 h of incubation using a GloMax® microplate reader (Promega, MA, USA). The luminescence signal was normalized to the OD<sub>600</sub> value according to the formula:  $L_c = L / OD_{600}$ , where  $L_c$  is the corrected luminescence and  $L$  is the raw luminescence value. Results are expressed as a percentage of *recA* promoter activity relative to the control at time 0 (100%). All experiments were

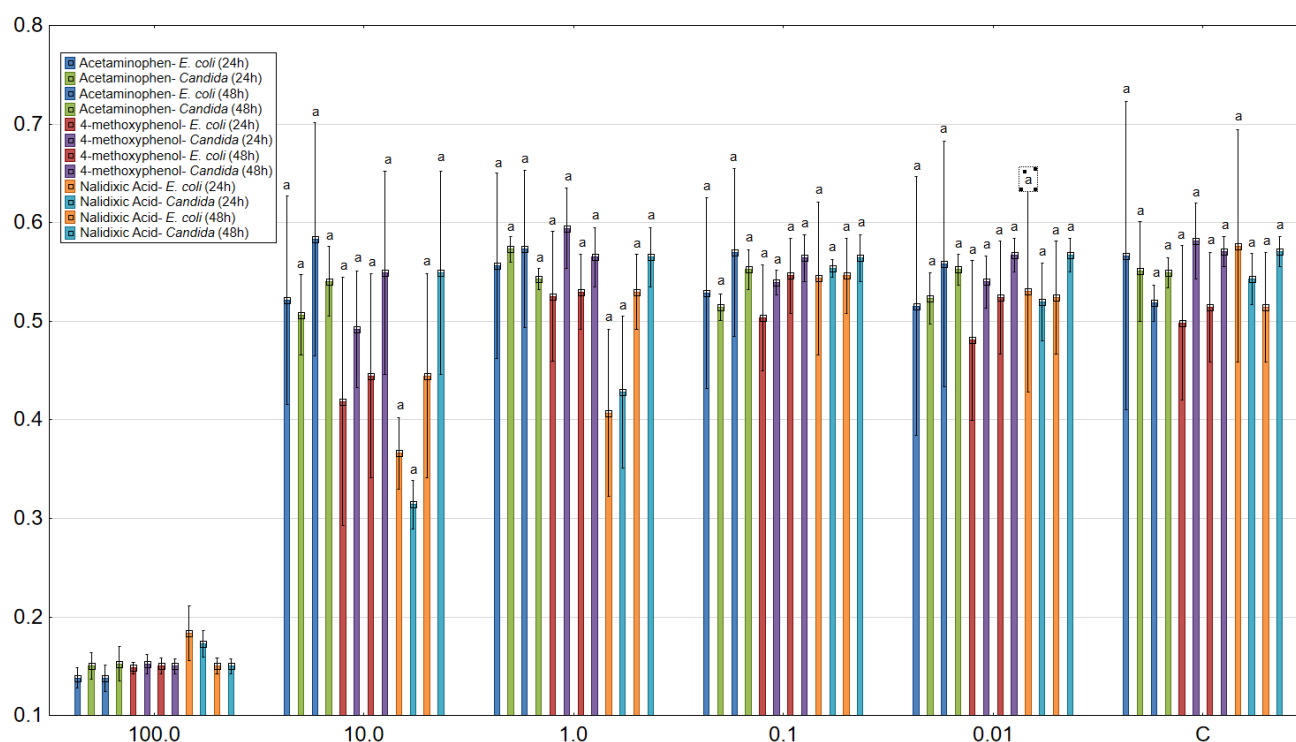


**Fig. 2.** Percentage inhibition of *E. coli* and *C. albicans* culture growth after 24 h and 48 h exposure to acetaminophen, 4-methoxyphenol, and nalidixic acid at concentrations of 0.01, 0.1, 1, 10, and 100 mg·L<sup>-1</sup> compared to the control (100%).

Note: a – statistically significant differences at  $p \leq 0.05$ . Data are presented as mean  $\pm$  SD; error bars indicate the SD of three independent replicates ( $n = 3$ ).

performed in three independent series, with six replicates per sample in each series.

### Determination of Intracellular ROS Levels

To confirm oxidative stress as a mechanism underlying the toxicity and genotoxicity of the tested compounds, intracellular ROS levels were determined in *E. coli* ATCC-25922 cells using 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich, UK). Both compounds were tested at concentrations of 0.01, 0.1, 1, 10, and 100 mg·L<sup>-1</sup>. *E. coli* was cultivated overnight in LB medium at 37°C, then refreshed with fresh LB medium and incubated at 37°C with shaking (130 rpm) until reaching the logarithmic growth phase ( $OD_{600} = 0.2$ ). Subsequently, 200  $\mu$ l of a mixture of *E. coli* culture and appropriate concentrations of APAP and 4-MP were added to black 96-well plates. Control samples contained no chemicals; NA was used as a reference standard. DCFH-DA was added to a final concentration of 5  $\mu$ M, and the plates were incubated at 37°C for 3 h. Thereafter, DCF fluorescence intensity was measured using a GloMax® microplate reader (Promega, MA, USA) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Results were expressed as a percentage of ROS increase relative to the untreated control. All experiments were performed in three independent series, with six replicates per sample in each series.

### Statistical Analysis

Prior to statistical analyses, the assumptions of normality and homogeneity of variance were verified. Data normality was assessed using the Shapiro–Wilk test, while homogeneity

of variances was evaluated using Levene's test. In cases where the assumption of homogeneity of variance was not met, the Brown–Forsythe test was applied. For growth inhibition experiments, one-way ANOVA was applied, followed by Tukey's honestly significant difference (HSD) post hoc test. For *recA* promoter induction in *E. coli* RFM443 *recA::luxCDABE* and ROS synthesis, Student's t-test for independent samples was applied. All statistical tests were two-tailed and evaluated at a significance level of  $\alpha \leq 0.05$ . Statistical calculations were performed using Statistica software, version 13.3.

## Results

### Antimicrobial Activity of APAP and 4-MP

At 100 mg·L<sup>-1</sup>, both *E. coli* and *C. albicans* showed growth inhibition exceeding 70% after 24 and 48 h of incubation (Fig. 2). At APAP concentrations of 0.01–10 mg·L<sup>-1</sup>, slight growth inhibition was detected after 24 h, reaching a maximum of 8% in *C. albicans* at 10 mg·L<sup>-1</sup>. In *E. coli*, APAP at concentrations of 0.01–10 mg·L<sup>-1</sup> stimulated growth after 48 h, reaching over 12% at 10 mg·L<sup>-1</sup>. At 10 mg·L<sup>-1</sup>, 4-MP inhibited the growth of both strains by over 16% after 24 and 48 h, with stronger inhibition observed in *E. coli* after 24 h compared to APAP.

### Minimum Inhibitory Concentration (MIC) Values for APAP and 4-MP

The MIC analysis revealed comparable antimicrobial activity of acetaminophen and 4-methoxyphenol against the tested bacterial strains and *C. albicans*. An exception was observed for *L. rhamnosus*, against which acetaminophen demonstrated greater growth inhibition (MIC = 5 mg·mL<sup>-1</sup>)

**Table 1:** MIC values (mg•mL<sup>-1</sup>) of APAP, 4-MP, and kanamycin (control) against microorganisms after 24 and 48 h of incubation.

| Chemicals       | <i>E. coli</i> (Gram–) | <i>E. hormaechei</i> (Gram–) | <i>S. epidermidis</i> (Gram+) | <i>L. rhamnosus</i> (Gram–) | <i>C. albicans</i> |
|-----------------|------------------------|------------------------------|-------------------------------|-----------------------------|--------------------|
| Acetaminophen   | 5                      | 5                            | 10                            | 5                           | 10                 |
| 4-methoxyphenol | 5                      | 5                            | 10                            | 10                          | 10                 |
| Kanamycin       | 0.1                    | <0.1                         | 0.1                           | <0.1                        | 0.2                |

than 4-MP (MIC = 10 mg•mL<sup>-1</sup>). Comparison of the MIC values with those of the positive control, kanamycin, indicated that the antimicrobial efficacy of the classic antibiotic exceeded that of acetaminophen and 4-MP by more than an order of magnitude (Table 1).

**Genotoxic effect of APAP and 4-MP**

Genotoxic activity after 2 h of exposure indicated that APAP elevated *E. coli* RFM443 *recA::luxCDABE* expression more than 4-MP, with differences of approximately 20% at 1 and 10 mg•L<sup>-1</sup> and over 10% at 100 mg•L<sup>-1</sup> (Fig. 3). NA at 1 mg•L<sup>-1</sup> elicited the highest level of induction among the tested compounds, exceeding 150% relative to the control.

**Intracellular ROS Levels**

At concentrations of 0.1, 1, 10, and 100 mg•L<sup>-1</sup>, APAP increased ROS levels in *E. coli* cells by 13%, more than 10%, 14%, and more than 18%, respectively, compared to the corresponding ROS levels induced by 4-MP. The strongest

ROS induction was observed in cultures treated with NA at 1, 10, and 100 mg•L<sup>-1</sup>, reaching over 120%, over 150%, and approximately 200% of the control, respectively (Fig. 4).

**Discussion**

Paracetamol (APAP, acetaminophen) is one of the most frequently detected pharmaceutical contaminants in water and soil environments, where it undergoes biodegradation, resulting in the formation of its metabolites, including 4-methoxyphenol (4-MP). Both APAP and its metabolite have adverse effects on living organisms and bioaccumulate in their cells (bacteria, fungi, and unicellular algae) and tissues (plants and animals), thereby disrupting the proper functioning of ecosystems. This study compares the biological activity of paracetamol and its transformation product, 4-MP. Drug metabolites often exhibit higher mobility, distinct toxicities, and greater persistence than their parent compounds; therefore, studying their activity is very important for determining environmental safety.

In this experiment, a comparative analysis of the antimicrobial activity of APAP, 4-MP and nalidixic acid (NA,

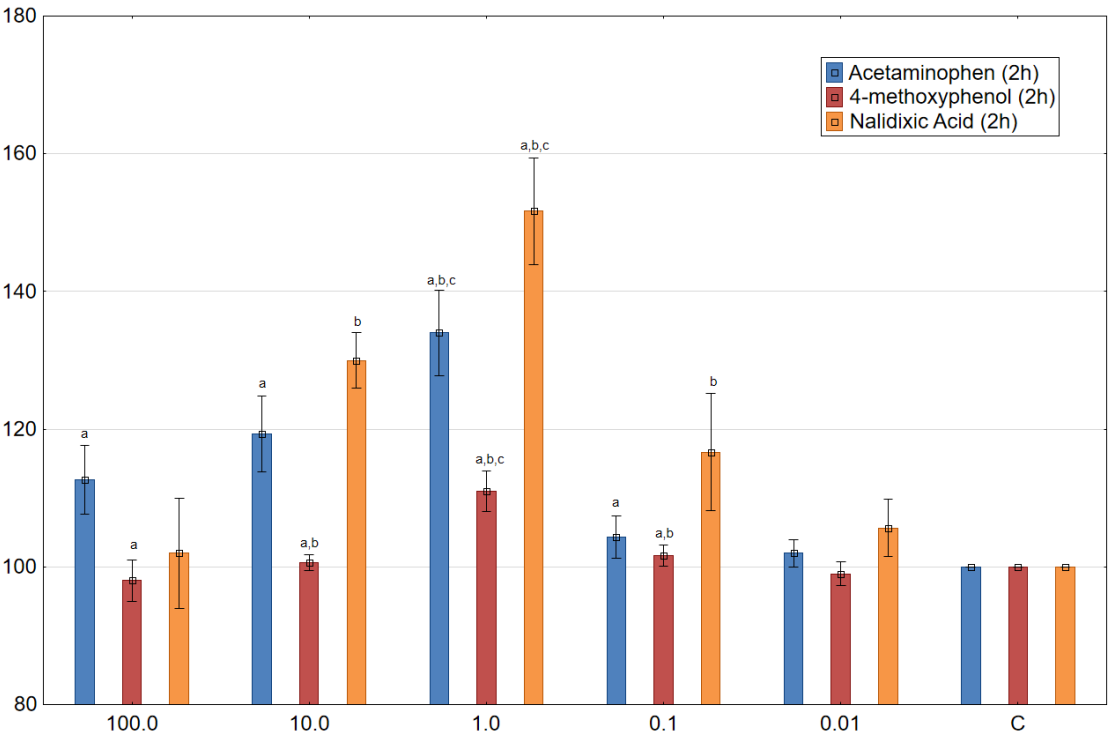


**Fig. 3.** Activity of *E. coli* RFM443 *recA::luxCDABE* after 2 h exposure to acetaminophen, 4-methoxyphenol, and nalidixic acid at concentrations of 0.01–100 mg•L<sup>-1</sup>, relative to the control (100%). Statistically significant differences ( $p \leq 0.05$ ): (a) acetaminophen vs. 4-methoxyphenol; (b) 4-methoxyphenol vs. nalidixic acid; (c) nalidixic acid vs. acetaminophen. Data are presented as mean  $\pm$  SD (n = 3); error bars indicate SD

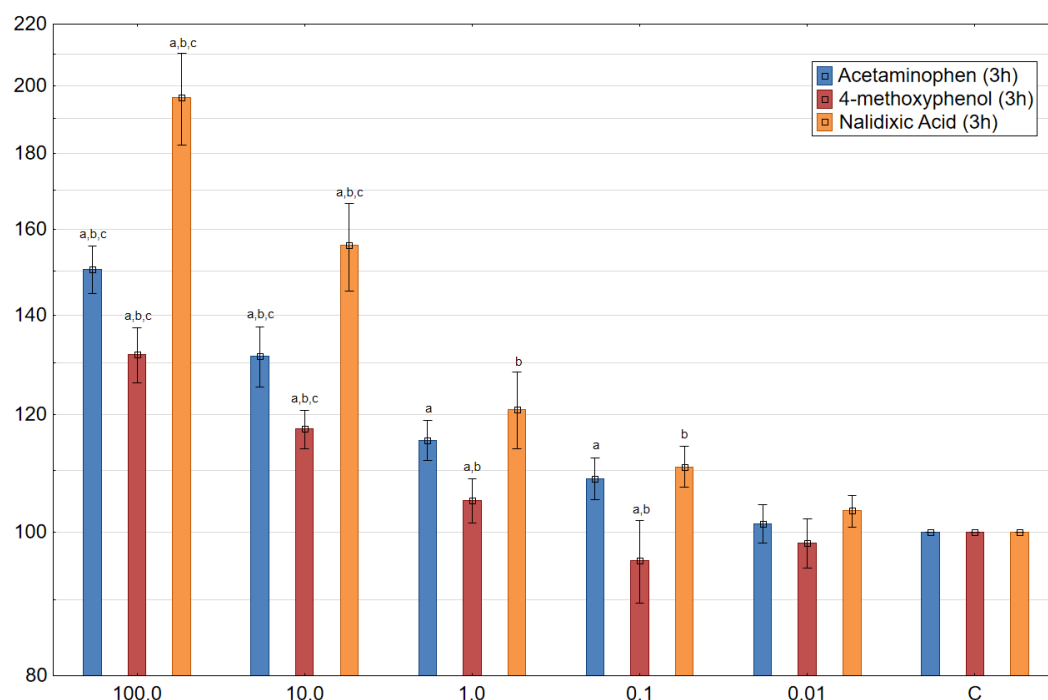


**Fig. 4.** ROS levels (%) in *E. coli* after 3 h exposure to acetaminophen (APAP), 4-methoxyphenol (4-MP), and nalidixic acid (NA) at concentrations of 0.01–100 mg·L<sup>-1</sup>, relative to the untreated control (100%). Statistically significant differences ( $p \leq 0.05$ ): (a) APAP vs. 4-MP; (b) 4-MP vs. NA; (c) NA vs. APAP. Data are presented as mean  $\pm$  SD ( $n = 3$ ); error bars indicate SD.

used as a positive control) revealed the sensitivity of both *E. coli* and *C. albicans* strains to paracetamol and 4-MP, with the highest toxic effect observed at a concentration of 100 mg L<sup>-1</sup> after 24 h and 48 h. 4-MP applied at a lower concentration of 10 mg L<sup>-1</sup> exerted a stronger inhibitory effect on both *E. coli* and *C. albicans* strains compared to APAP, indicating broader and more potent biological activity of the transformation product relative to APAP. For all three compounds, i.e., APAP, 4-MP, and nalidixic acid (NA, used as a positive control), a concentration-dependent inhibition of microbial growth was observed, whereby increasing concentrations of each compound consistently and predictably suppressed microbial viability, regardless of exposure duration. Furthermore, varying sensitivities to acetaminophen, APAP, and NA were observed in the two microorganisms studied, *E. coli* and *C. albicans*. This is likely due to differences in the chemical structures and activities of the compounds tested, as well as structural differences between the cells: *E. coli* is a bacterium, whereas *C. albicans* is a eukaryotic fungal cell. MIC tests were performed to determine the sensitivity of a larger group of microorganisms (*E. coli*, *E. hormaechei*, *S. epidermidis*, *L. rhamnosus*, and *C. albicans*) to the chemical compounds under investigation. The results showed that the sensitivity of the tested microorganisms to APAP and 4-MP is species-dependent and dose-dependent. We demonstrated that as the concentration of APAP and 4-MP increases, a bactericidal effect occurs in a greater number of the tested microorganisms. These results are consistent with those reported by previous authors (Torequell et al. 2025), who demonstrated that paracetamol and its derivatives exhibit antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi, and drug-resistant strains. Moreover,

antiviral and antiprotozoal activity has been reported for this compound. Toxicological profiling of acetaminophen has revealed that this drug has the potential to damage organs, particularly the liver and lungs, via a mechanism involving oxidative stress.

Using the genotoxicity-sensitive *recA* promoter assay in *E. coli*, we demonstrated that paracetamol, 4-MP, and NA, tested individually, exhibited genotoxic effects. Among the chemical compounds tested (except at a concentration 100 mg L<sup>-1</sup>), the strongest genotoxic response was detected for NA, a known genotoxicity standard. Comparative analysis of the genotoxic potential of APAP and 4-MP revealed a stronger genotoxic effect for the parent drug relative to 4-MP. Within the range of APAP, 4-MP, and NA concentrations tested, a positive correlation was observed between the measured ROS concentration and the level of genotoxicity of the compounds in *E. coli*. ROS generation and oxidative stress represent the primary molecular mechanism underlying the toxicity and genotoxicity of test compounds, as confirmed by earlier studies (Žur et al. 2018; Kirkland et al. 2021; Jaeschke et al. 2021; Torequell et al. 2025). ROS and oxidative stress lead to DNA damage and genotoxic effects. The strongest genotoxic effect and induction of ROS synthesis were detected for APAP. These findings demonstrate a robust and consistent concentration-dependent induction of oxidative stress, indicating that ROS overproduction constitutes a central and reproducible mechanism of cellular damage for both the parent drug and its metabolite across the tested concentration range (0.01–100 mg L<sup>-1</sup>).

Analysis of the relationship between test compound concentration and *recA::luxCDABE* promoter activity in *E. coli* (genotoxicity assay) provided additional insight into the

nature of these genotoxic responses. The result for APAP was negligible, suggesting the absence of a concentration-dependent genotoxic response, which may reflect the cytotoxicity-masking effect at higher concentrations, as proposed by Kirkland et al. (2021). A statistically non-significant positive result was observed for 4-MP indicating a lack of a relationship between concentration and DNA damage induction. In contrast, NA also displayed statistically non-significant results, which may reflect the complex and non-dynamic nature of genotoxic responses at higher concentrations, potentially attributable to cytotoxic interference. Taken together, these data suggest that while oxidative stress induction follows a concentration-response pattern for all compounds, genotoxic effects, as measured by *recA* promoter activation, exhibit more complex, compound-specific relationships with concentration.

In the period between 2019 and 2025, an increasing number of studies have addressed the possible carcinogenic effects of paracetamol. In 2019, the California Office of Environmental Health Hazard Assessment (OEHHA) initiated an investigation into the potential carcinogenic risk associated with paracetamol use. In 2021, an independent research group led by Jaeschke et al. published findings demonstrating that, at recommended therapeutic doses, only small amounts of the paracetamol metabolite N-acetyl-p-benzoquinone imine (NAPQI) are formed in the human body and that such quantities are unlikely to exert harmful effects under normal conditions. It is only in cases of acetaminophen overdose that substantially higher levels of NAPQI are produced and glutathione is depleted, potentially resulting in mitochondrial dysfunction and DNA damage, although exclusively at doses that induce cell death (Jaeschke et al. 2021; Torequell et al. 2025). Kirkland et al. (2021), however, argue that the cytotoxic effects of this drug manifest prior to the induction of permanent genetic damage, suggesting that the genotoxic and carcinogenic risk under therapeutic conditions remains limited. More conclusive evidence of toxicity and genotoxicity has emerged for the metabolite 4-MP. Based on the current state of knowledge, methoxyphenols are recognized as genotoxins and mutagens capable of inhibiting the synthesis of nucleic acids and proteins (Saeed et al. 2024). The results obtained in the present study, confirming the genotoxic nature of 4-MP, are consistent with previously published findings.

## Conclusions

1. The results of the conducted studies demonstrated that both compounds – the parent drug, paracetamol, and its transformation product, 4-MP, exhibit toxic and genotoxic properties in *Escherichia coli* bioassays.
2. The results obtained support the hypothesis that 4-MP, as a transformation product of APAP, exhibits toxic and genotoxic activity and generates oxidative stress in *E. coli* cells similarly to the parent drug.
3. The diverse chemical structures of the tested compounds - APAP, 4-MP, and the standard (NA) - affected their activity against the tested *E. coli* strains. The analysis of ROS level detected oxidative stress, which is likely one of the main mechanisms underlying the toxicity and genotoxicity of APAP and 4-MP. The relationship between ROS synthesis, as an indicator of oxidative stress, and the genotoxic effect is most clearly observed at a concentration of 10 mg

$L^{-1}$  for the tested chemical compounds APAP, 4-MP, and NA.

4. There is an environmental risk that paracetamol and 4-MP, because of their toxic and genotoxic activities, may disrupt the quantitative and qualitative composition of soil and activated sludge microbiota, leading to impairment of their biological functions.
5. Another possible situation in the environment is that drugs and their transformation products present in environmental matrices may interact in various ways with other micropollutants (pesticides, pharmaceutical residues), forming complexes of unknown and unpredictable biological activity, which may negatively affect the proper functioning of ecosystems and pose a threat to living organisms.

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## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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## Porównanie toksyczności acetaminofenu i 4-metoksyfenolu

**Streszczenie:** Acetaminofen (APAP, paracetamol) jest jednym z najczęściej stosowanych leków przeznaczonych dla ludzi, a jednocześnie jest jednym z najczęściej wykrywanych mikrozanieczyszczeń farmaceutycznych w matrycach środowiskowych. 4-MP jest jednym z produktów transformacji acetaminofenu powstającym w określonych ścieżkach degradacji środowiskowej. Porównanie toksyczności tych związków ma kluczowe znaczenie dla ochrony środowiska i zdrowia publicznego. W niniejszym badaniu toksyczność APAP i 4-MP oceniono przy użyciu dwóch modelowych mikroorganizmów, *E. coli* ATCC-25922 i *C. albicans* ATCC-1023, w oparciu o hamowanie wzrostu hodowli. Wrażliwość mikroorganizmów należących do mikrobiomu człowieka (*E. coli*, *E. hormaechei*, *S. epidermidis*, *L. rhamnosus* i *C. albicans*) na oba związki określono poprzez obliczenie minimalnych stężeń hamujących (MIC). Genotoksyczność oszacowano przy użyciu wrażliwego na genotoksyny promotora *recA* w *E. coli* RFM443 *recA::luxCDABE*. Wytwarzanie reaktywnych form tlenu (ROS) zmierzono w *E. coli* poddanej działaniu obu związków w stężeniach 0,1, 1, 10, 100 i 1000 mg·L<sup>-1</sup>, jako wskaźnika stresu oksydacyjnego. Analiza porównawcza wykazała podobną wrażliwość *E. coli* i *C. albicans* na oba związki w stężeniu 100 mg·L<sup>-1</sup>, chociaż w stężeniu 10 mg·L<sup>-1</sup> 4-MP wywierał silniejsze działanie hamujące na oba szczepy niż APAP. W testach MIC wykryto, że szczepy *E. coli* i *E. hormaechei* były bardziej wrażliwe na 4-MP niż na APAP, natomiast w przypadku pozostałych szczepów nie zaobserwowano istotnych różnic. APAP wykazał silniejsze działanie genotoksyczne niż 4-MP w komórkach *E. coli*. Dane dotyczące ROS wskazują, że stres oksydacyjny jest głównym mechanizmem molekularnym leżącym u podstaw toksyczności i genotoksyczności obu związków. Biorąc pod uwagę uzyskane wyniki przyszłe badania powinny dotyczyć toksyczności mieszanin APAP i 4-MP w warunkach narażenia odpowiadających warunkom środowiskowym.