

Hydra as a Model for Screening Ecotoxicological Effects of Plasma-Treated Water

D. Ziuzina,* C. Sarangapani, D. Boehm, & P. Bourke*

School of Food Science and Environmental Health, Environmental Sustainability and Health Institute, Dublin Institute of Technology, Dublin 1, Ireland

*Address all correspondence to: Dana Ziuzina or Paula Bourke, School of Food Science and Environmental Health, Environmental Sustainability and Health Institute, Dublin Institute of Technology, Dublin 1, Ireland; Tel.: +35314027594, E-mail: dana.ziuzina@dit.ie or paula.bourke@dit.ie

ABSTRACT: Atmospheric cold plasma (ACP) has been widely researched to generate functionalized solutions for decontamination of liquids and wastewater treatment. The focus of this work was to study the ecotoxicological effects of plasma-treated water (PTW) using a free-living brown *Hydra* as an important and prevalent component of freshwater ecosystems. We obtained PTW by subjecting 25 mL of sterile deionized water to high-voltage (80 kV) contained ACP treatment for 15 (PTW15) and 25 (PTW25) min. Toxicity measurements were based on the progressive changes in *Hydra* morphology through a scoring procedure. The effects of PTW on *Pseudomonas aeruginosa*, *Escherichia coli*, and *Bacillus atrophaeus* cells were determined using a broth microdilution method that estimated minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Results demonstrated that PTW is potentially toxic to biological systems. In *Hydra* tests, toxic effects were observed for PTW15 and PTW25 at concentrations above 50% and 25%, respectively. Similarly, PTW25 at a concentration of 25% is lethal for *P. aeruginosa* and *E. coli*, whereas an MBC of 37.5% was obtained for *B. atrophaeus*. The present investigation demonstrates that *Hydra* can be used as an additional *in vivo* tool to monitor the impact of plasma-processed solutions on the aquatic environment.

KEY WORDS: atmospheric cold plasma, plasma-treated water, toxicity, *Hydra*, antimicrobial activity

I. INTRODUCTION

Discharge of industrial effluents into the environment is a major cause of water pollution.¹ A range of industries use chemicals that, due to their complex structure and synthetic origin, are resistant to the currently available wastewater-treatment technologies.² Food-processing industries are often associated with discharge of wastewaters that can hold microbiological and chemical contaminants as well as by-products derived from various food-processing steps, such as washing, cleaning, and sanitization.^{3,4} Pharmaceutical and personal care products are continuously released into environment in large quantities.^{5,6} Pesticides and their transformation products are bioaccumulative and can often exceed ecotoxic levels for nontarget organisms in aquatic systems.⁷ The lack of efficient water-remediation technologies and wide range of processing operations performed by industries that use potentially ecotoxic substances can significantly impact the natural environment and human health. At-

atmospheric cold plasma (ACP) has been widely researched to generate functionalized solutions for biological decontamination of liquids and as part of advanced oxidative remediation processes to improve wastewater treatment. ACP is composed of several excited atomic, molecular, ionic, and radical species coexisting with numerous reactive species including electrons, positive and negative ions, free radicals, gas atoms, molecules in the ground or excited state, ultraviolet (UV) light, shockwaves, and pyrolysis. All contribute to chemical and physical effects that can subsequently attack pollutant molecules and decompose them into more environmentally friendly products.^{3,8,9} ACP has been successfully applied to remove dyes,¹⁰ pesticides,^{11,12} aflatoxins,¹³ antibiotics,^{14,15} and pathogenic bacteria.^{4,16,17} To date, studies have focused on the decontamination and degradation capacity of ACP against environmental pollutants in aqueous phase; however, very limited studies have been performed to evaluate toxicity of plasma-processed effluents on terrestrial or aquatic environmental indicators. Therefore, the main focus of this work is to evaluate ecotoxicological effects of plasma-treated water (PTW) as a simple model, using the free-living brown *Hydra*, as an important, environmentally relevant, and prevalent component of freshwater ecosystems. The associated toxic effects of PTW on microbial cells are also evaluated.

II. METHODOLOGY

A. Generation of PTW

We generated PTW using an atmospheric air high-voltage dielectric barrier discharge in-package ACP system that is described in Sarangapani et al.¹⁰ and is fully characterized in Moiseev et al.¹⁸ For each experiment, 25 mL of water was subjected to direct ACP (i.e., samples were placed between aluminum electrodes within plasma discharge) generated at 80 kV with a treatment duration of 15 or 25 min to produce PTW15 and PTW25, respectively. Treatment was carried out at ambient temperature (~ 20°C). Samples were stored for 24 h post-treatment at room temperature. The pH of each PTW was monitored using a glass electrode pH meter (Orion Optics UK; Staffordshire, UK) before and after plasma treatment. Nitrate concentrations and hydrogen peroxide concentration in PTW were determined according to the procedure of Boehm et al.¹⁹ and Lu et al.²⁰

B. *Hydra* Maintenance and Toxicity Assay

A free-living brown *Hydra* was used to study the toxicity of water treated with ACP. *Hydra* was maintained in *Hydra* medium (HM) containing 110 mg/L N-Tris (hydroxymethyl) methyl L-2-aminoethanesulfonic acid (TES) and 147 mg/L CaCl₂·2H₂O (pH 7) and was kept at room temperature (~ 20°C) in glass beakers that were autoclaved before use. We used deionized water in the preparation of all solutions required throughout the

study. *Hydra* was fed daily with freshly hatched *Artemia* nauplii. After feeding, HM was replaced with fresh medium to avoid culture contamination.^{21–23}

Hydra was exposed to untreated deionized water (control), PTW15, or PTW25. Concentrations of PTW were set to 25%, 50%, 75%, and 100% using HM. Experiments were conducted using six-well plates. Each well, containing three nonbudding 1-d-starved *Hydra*, was filled with 2 mL of either PTW samples or the control. Samples were spiked with TES and CaCl₂ to match HM concentrations of these compounds, and the pH was adjusted to 7.4 ± 0.2 with 1 M NaOH. Before each experiment, samples were passed through 0.2- μ m membrane filters. Observations of *Hydra* were conducted using an Olympus SZ51 stereoscope (Olympus Corporation of the Americas; Center Valley, PA). Results were recorded at 1 and 24 h after the exposure and daily when required. Toxicity measurements of progressive changes in *Hydra* morphology were taken based on a scoring procedure using a scale from 10 to 0 devised by Wilby in 1988²⁴ and reproduced later by Quinn et al.²² With this method, a score of 0 corresponds to the highest degree of toxicity. Experiments were performed in triplicate and repeated twice ($n = 6$).

C. Bacterial Strains, Culture Conditions, and Antimicrobial Activity of PTW

Three single-species test microorganisms, namely, *Pseudomonas aeruginosa* of American Type Culture Collection (ATCC) 27853, *Escherichia coli* of ATCC 25922, and *Bacillus atrophaeus* var. *niger* of ATCC 9372, obtained from stock cultures of the School of Food, Science, and Environmental Health of the Dublin Institute of Technology, were used to determine antimicrobial activity of PTW. Each bacterium was inoculated in tryptic soy broth (TSB; Biokar Diagnostics; Ireland) and incubated overnight at 37°C. The density of each overnight culture was adjusted to McFarland 0.5. The minimum inhibitory concentration (MIC) of PTW25 or untreated water for *P. aeruginosa*, *E. coli*, and *B. atrophaeus* was determined using a broth microdilution method.²⁵ Briefly, PTW was twofold diluted in TSB containing bacterial cells (final cell concentration, $5 \log_{10}$ colony-forming units/mL), with resulting concentration of PTW ranging from 50% to 0.01%. Controls included PTW or untreated water and TSB without bacterial cells (blank) and TSB with bacterial cells (positive control). Plates were incubated for 24 h at 37°C. The MIC of either treated or untreated water for three microorganisms was determined as the lowest concentration showing no turbidity when compared to corresponding blank controls. Plasma-treatment experiments were replicated three times, and two biological replicates were included in each plate. The lowest PTW concentration that inhibited growth of the microorganism was detected by the lack of visual turbidity (matching the corresponding blank control) and was designated MIC.²⁶ To determine minimum bactericidal concentration (MBC), 10- μ L aliquots of MIC and at least two dilutions of higher concentration from each well were plated on tryptic soy agar (TSA Biokar Diagnostics; Ireland) in duplicate, and plates were incubated for 24–48 h at 37°C.

MBC was determined to be the lowest concentration of PTW at which no bacterial growth was observed.

III. RESULTS AND DISCUSSION

Considering potential impacts of industry wastewater effluents on the environment, numerous studies have focused on optimization of plasma technology to achieve efficient biological and chemical pollutant control. Because of the demonstrated performance of plasma processing in pollutant removal, it becomes clear that apart from the biological and chemical degradation capacity of ACP, other important aspects of sustainability including ecotoxicological effects of plasma-processed effluents, and the by-products require holistic assessment. This work investigated ecotoxicological effects of PTW, as a simple matrix, using *Hydra* as a model invertebrate that represents an important member of the aquatic food web in many freshwater ecosystems. *Hydra* species requires no license requirements or animal ethics approval, it is associated with low costs, and it is relatively easy to culture and maintain in laboratory environments.²⁷ *Hydra* has been used to monitor freshwater toxicity and wastewater quality^{28–30} and evaluate toxicity of pharmaceuticals³¹ and nanomaterials.^{32,33} Moreover, it was demonstrated that sensitivity of *Hydra* to different water sources associated with agricultural and mining sectors was strongly correlated to sensitivity of the *Daphnia* standard assay.³⁴

In this work, the response of *Hydra* to PTW was measured at the physiological level by evaluating morphological features of the *Hydra* body and tentacles and scoring from 10 to 0. Results demonstrated that both PTW15 and PTW25 at 100% were very toxic to *Hydra* (Fig. 1). Exposure to PTW15 for 1 h resulted in body elongation (Fig. 1[b]) that was accompanied by slow body reactions, whereas the *Hydra* body was totally contracted after exposure to PTW25 (Fig. 1[c]), receiving a score of 5 according to the classification scheme (Table 1). Further retaining *Hydra* in PTW15 and PTW25 at 100% for 24 h led to complete disintegration of polyps (Fig. 1[e] and [f], respectively), resulting in a score of 0 for both solutions (Table 1). No alterations in *Hydra* morphology were observed in control samples after 1 h and 24 h (Fig. 1[a] and [d], respectively). PTW15 at a concentration of 75% was still lethal to *Hydra* (Fig. 2[a]). Moreover, *Hydra* exposed to PTW15 at 75% could not recover after prolonged retention in HM. Morphological features of test animals were altered to a reduced degree after exposure to lower concentrations of PTW15 at 50% and PTW15 at 25% receiving scores of 6 and 7, respectively (Fig. 2[b] and [c]). These PTW15 concentrations were sublethal, because all *Hydra* attained normal morphological appearance and feeding behavior after incubation for 4–5 d in standard culture conditions. In contrast, all concentrations of PTW25 resulted in a score of ≤ 5 (Fig. 2[d]–[f]), and *Hydra* death occurred more frequently during subsequent incubation in HM after exposure to PTW25 at 25%.

The difference in the response of *Hydra* to test solutions could be related to the chemical composition of PTW15 and PTW25, with higher concentrations of hydrogen peroxide and nitrate generated after 25 min than after 15 min of treatment (Table

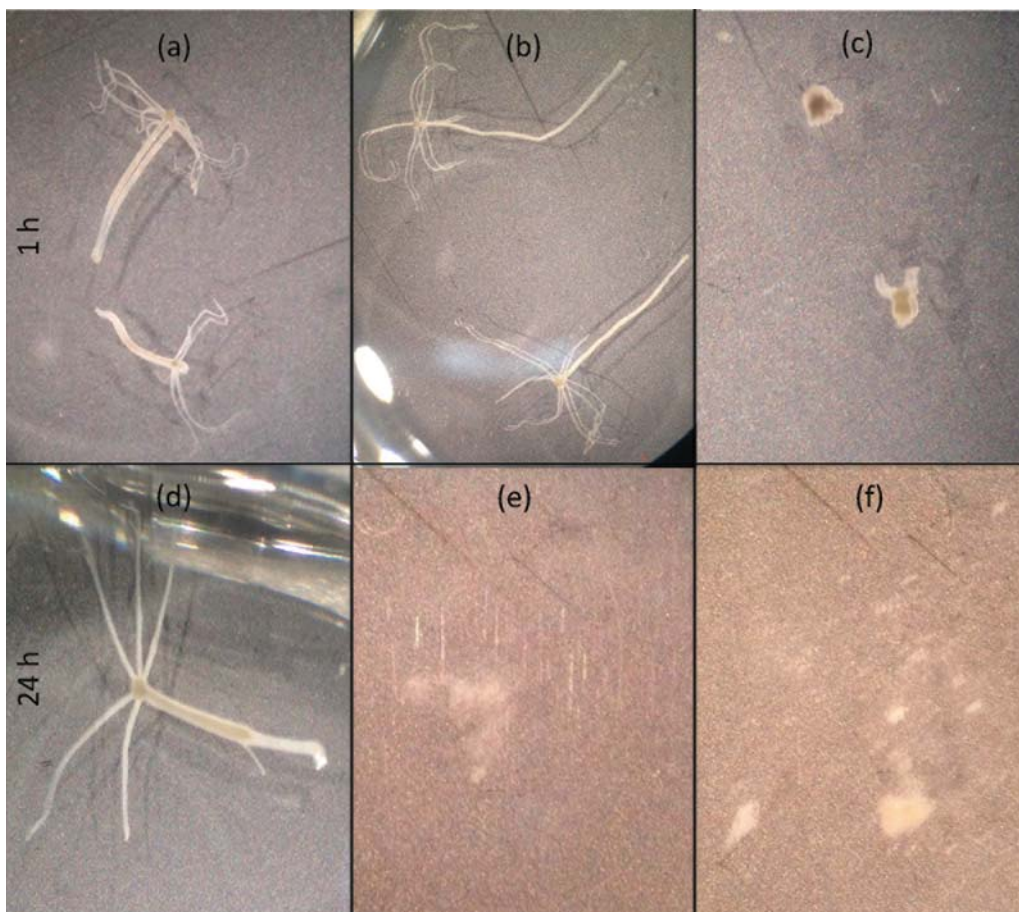


FIG. 1: Images of *Hydra* reflecting progressive changes in body morphology due to exposure of PTW15 and PTW25 at 100% recorded after 1 h (a–c) and 24 h (d–f): (a, d) Control; (b, e) PTW15; (c, f) PTW25

2). The toxicity of plasma-treated or -activated solutions on cell culture systems have been widely reported, and hydrogen peroxide appears to have a crucial role in causing cytotoxic effects.³⁵ Haertel et al.³⁶ demonstrated that mainly hydrogen peroxide generated during extended plasma treatments (5 min) was implicated in mammalian cell death. Boehm et al.¹⁹ showed that plasma-treated buffer solution inhibited cell growth in a treatment-time-dependent manner, producing a linear correlation to the solutions' peroxide concentration, which correlates with current results. Furthermore, the pH of water was reduced from ~ 5.5 to ~ 2.79 and ~ 2.63 after 15 and 25 min of treatment, respectively; therefore, to exclude toxic effects of an acidic environment, the pH of PTW was adjusted to ~ 7.4 before each experiment. Preliminary research demonstrated that when *Hydra* was exposed to PTW generated during shorter plasma durations (1–5

TABLE 1: Changes in *Hydra* morphology due to exposure to PTW15 and PTW25 according to *Hydra* toxicity classification scheme

	PTW Concentration (%)	Characteristics	Score
PTW15	100	Disintegrated	0
	75	Totally contracted; no visible tentacles	4
	50	Tentacles and body shortened	6
	25	Shortened tentacles; body slightly contracted	7
PTW25	100	Disintegrated	0
	75	Dead but intact (ball-shaped body)	1
	50		1
	25	Totally contracted; tentacles visible	5
Control	—	Extended tentacles and body; reactive	10

Score 0 corresponds to highest degree of toxicity. Results were recorded after 24 h from the beginning of the experiment.

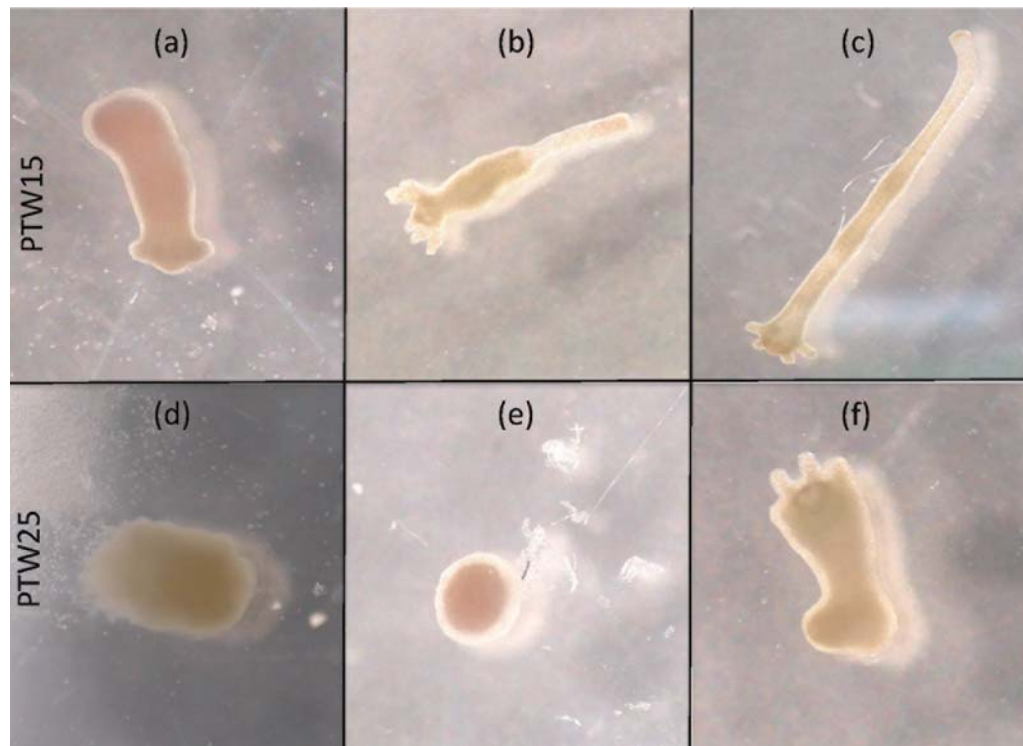


FIG. 2: Images of *Hydra* reflecting progressive changes in body morphology due to exposure of PTW15 (a–c) and PTW25 (d–f) at concentrations of (a, d) 75%, (b, e) 50%, and (c, f) 25%, recorded after 24 h

TABLE 2: Concentration of hydrogen peroxide and nitrate (mM) of PTW

		Method	Concentration
PTW15	Hydrogen peroxide	KI	0.54
		TiOSO ₄	0.51
	Nitrate	Merck nitrate assay	0.84
PTW25	Hydrogen peroxide	KI	0.83
		TiOSO ₄	1.10
	Nitrate	Merck nitrate assay	1.30

PTW, Plasma-treated water

min and PTW1–PTW5, respectively) with unadjusted pH, the changes in animal body morphology could be observed within 3 h. After this period, if PTW1 and PTW2 were sublethal to *Hydra*, exposure to PTW3–PTW5 (with more acidic pH) resulted in *Hydra* death (data not shown). This observation could be useful in terms of selecting plasma treatment parameters, because higher voltage levels and longer treatment durations will likely be associated with low pH and substantial concentrations of hydrogen peroxide and nitrite/nitrate in plasma-treated solutions, which can potentially affect the ecotoxicity profile for aquatic life.

One of the standard methods for determining toxicity of liquids or single substances is to assess growth inhibition of the bacterium *Pseudomonas putida* (International Organization for Standardization 10712).³⁷ *P. putida* is commonly used to research toxicity of silver nanoparticles,^{38,39} metals, and pesticides,^{40,41} although the use of other microorganisms, such as *E. coli*, *S. aureus*, and *Bacillus*, has also been reported.^{42,43} In this work, the toxic effect of water subjected to plasma treatment for 25 min was also examined using three bacterial strains, namely, *P. aeruginosa*, *E. coli*, and *B. atrophaeus*. Using a broth microdilution assay, MIC and MBC values were established (Table 3). As shown in Fig. 3(a) and (b), PTW25 at concentrations of $\geq 25\%$ were lethal to both *P. aeruginosa* and *E. coli* (MBC values for both microorganisms corresponded to 25% of PTW25), which correlated with *Hydra* response. A slightly lower sensitivity to PTW25 was observed for *B. atrophaeus*, the growth of which was eliminated at concentrations above 25% (Fig. 3[c]). Antimicrobial efficacy of plasma-activated water has been widely reported showing lethal effects against a wide range of microorganisms.^{44–46} Although this property of plasma-treated solutions may offer a novel and effective decontamination approach for a range of industrial sectors, care must be taken regarding potential unintended adverse effects. Studies by Patange et al.⁴ demonstrated that although ACP treatment could effectively inactivate key indicator microorganisms in model dairy and meat wastewater, prolonged contact with plasma-processed effluents (that retained longer-lived secondary reactive oxygen or nitrogen species) of up to 48 h was toxic to *Daphnia magna*. The tests involved in the current work are not widely standardized; however, the demonstrated PTW toxicity in the *Hydra* test and antimicrobial assay indicates that ecotoxicological impact must be

TABLE 3: MIC and MBC values (%) of PTW25

	MIC	MBC
<i>P. aeruginosa</i>	25 ± 0	25 ± 0
<i>E. coli</i>	12.5 ± 0	25 ± 0
<i>B. atrophaeus</i>	18.5 ± 7.5	37.5 ± 14.4

MBC, minimal bactericidal concentration; MIC, minimal inhibitory concentration; PTW, plasma-treated water treated for 25 min.

considered when plasma is used for remedial treatment or functionalization of liquids. This research also supports the possibility of integrating *Hydra* as a relevant bioindicator, because its sensitivity to PTW was closely correlated with bacterial growth inhibition, and such an assay could therefore be used as an additional biomonitoring tool in a test battery applied for ecotoxicity screening of plasma-processed solutions. In addition, with the *Hydra* assay, sample toxic effects can be measured at different levels, including acute/chronic toxicity, regeneration and reproduction ability, feeding behavior and attachment to a substrate, and teratogenic and genotoxic effects.²² However, to ensure that the *Hydra* assay realistically reflects toxicity of PTW or plasma-treated industrial effluents, validation studies involving other aquatic invertebrate models and elucidation of PTW ecotoxic profiles are still required. Thus, further studies are warranted to ascertain how to avoid negative impacts of plasma-processed solutions on nontarget aquatic organisms, eliminate environmental pollution, sequentially benefit human health, and progress safe applications of this technology in water-remediation processes.

In summary, this study investigates ecotoxicological effects of PTW using *Hydra* as a model organism. Based on toxicity tests, we found that water exposed to either 15 or 25 min of treatment is highly toxic to *Hydra*. We observed complete disintegration of culture after contact with PTW of 100% for 24 h. We also found that water exposed to a longer treatment duration of 25 min exhibited higher toxicity in comparison to effects induced by water treated with shorter (15 min) treatment. These observations were possible when *Hydra* was exposed to lower concentrations of PTW15 and PWT25 (25% and 50%). Sensitivity of *Hydra* to PTW was correlated with a bacterial growth inhibition assay indicating potential ecotoxicological impacts of plasma-processed solutions. This research highlights the importance of implementing ecotoxicity risk assessments in relevant investigations of novel plasma technologies. We also demonstrate that *Hydra* could be used as an additional *in vivo* tool to monitor the impact of plasma-processed solutions on the aquatic environment.

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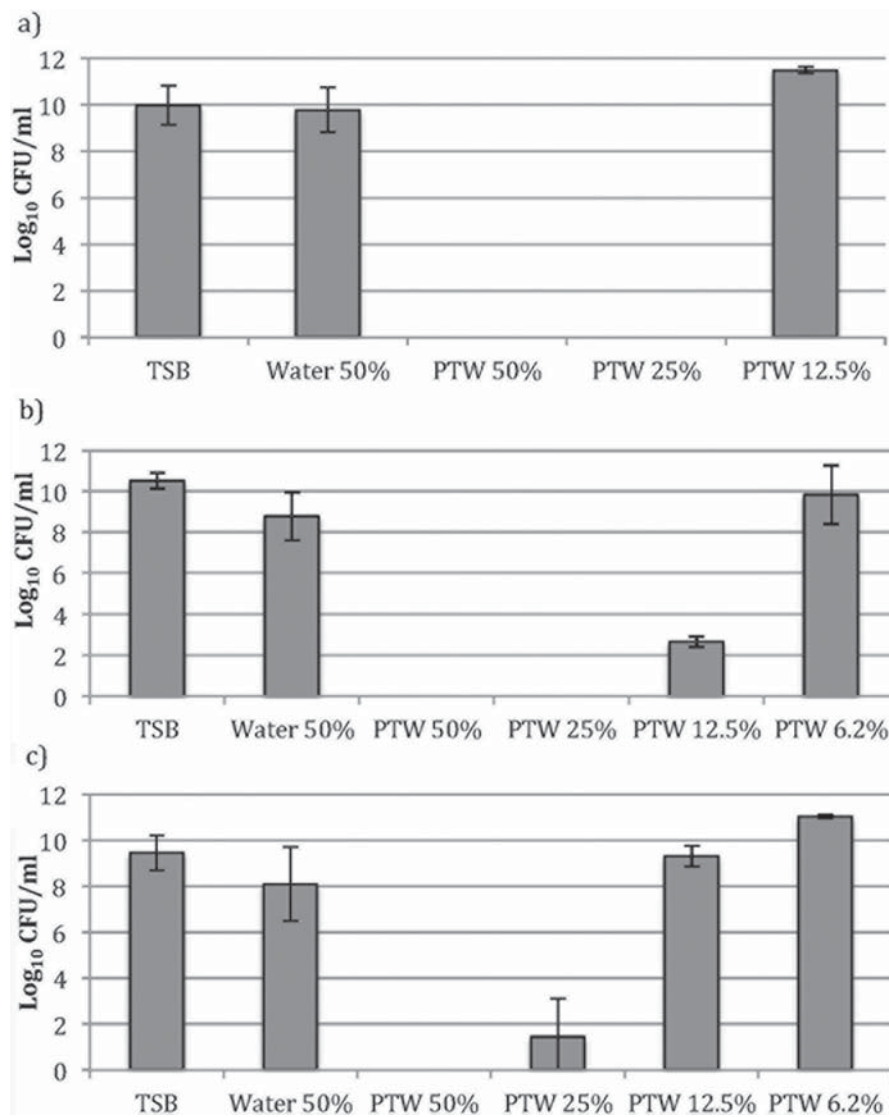


FIG. 3: Effect of PTW25 at different concentrations assessed using the broth microdilution method on (a) *P. aeruginosa*, (b) *E. coli*, and (c) *B. atrophaeus*. (Vertical bars, standard deviation.)

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