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Molecular evidence for suppression of swimming behavior and reproduction in the estuarine rotifer *Brachionus koreanus* in response to COVID-19 disinfectants

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ABSTRACT

The increased use of disinfectants due to the spread of the novel coronavirus infection (e.g. COVID-19) has caused burden in the environment but knowledge on its ecotoxicological impact on the estuary environment is limited. Here we report *in vivo* and molecular endpoints that we used to assess the effects of chloroxylenol (PCMX) and benzalkonium chloride (BAC), which are ingredients in liquid handwash, dish soap products, and sanitizers used by consumers and healthcare workers on the estuarine rotifer *Brachionus koreanus*. PCMX and BAC significantly affected the life table parameters of *B. koreanus*. These chemicals modulated the activities of antioxidant enzymes such as superoxide dismutase and catalase and increased reactive oxygen species even at low concentrations. Also, PCMX and BAC caused alterations in the swimming speed and rotation rate of *B. koreanus*. Furthermore, an RNA-seq-based ingenuity pathway analysis showed that PCMX affected several signaling pathways, allowing us to predict that a low concentration of PCMX will have deleterious effects on *B. koreanus*. The neurotoxic and mitochondrial dysfunction event scenario induced by PCMX reflects the underlying molecular mechanisms by which PCMX produces outcomes deleterious to aquatic organisms.

1. Introduction

When a novel coronavirus (SARS-CoV-2) caused the COVID-19 outbreak, disinfection of surfaces was recommended as one of the most effective ways to prevent infection (Ijad et al., 2021; Nowak et al., 2021). However, the chemicals recommended for hard-surface disinfectants and hand sanitizers for COVID-19 are classified as biocide and could have adverse effects, especially when high demand for them causes wide spread release (Merchel Piovesan Pereira and Tagkopoulos, 2019; Dev Kumar et al., 2020; Tan et al., 2021). In fact, the increase in disinfectant usage caused by the COVID-19 pandemic has created a large effluent of these chemicals into aquatic environments, and inappropriate use leads to severe environmental problems caused by the chemicals

themselves or their byproducts (Yadav et al., 2020).

Chloroxylenol (para-chloro-meta-xylenol, PCMX), commonly known as Dettol, is a chemical currently recommended to inactivate viruses, including COVID-19. It is also used to remove bacteria, algae, and fungi (Dev Kumar et al., 2020) and commonly used in households, hospitals, and other institutions to sanitize bathrooms, laundry equipment, bedding, and pet living quarters (EPA, 1994; Dev Kumar et al., 2020). In particular, it has long been used because of its low toxicity to target vertebrates such as humans, fish, and birds (EPA, 1994). Benzalkonium chloride (BAC) is another widely used chemical with broad-spectrum antimicrobial characteristics against bacteria, fungi, and viruses (Elersek et al., 2018; Merchél Piovesan Pereira and Tagkopoulos, 2019). Aquatic ecosystems are a major target of increased disinfectant impacts

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caused by COVID-19, and non-target organisms are likely to be affected (Zhang et al., 2020). In fact, these disinfectants were measured in sediment (Li and Brownawell, 2010; Khan et al., 2017) and found to persist in soil and estuarine environments. However, no studies have been conducted on the adverse effects that these disinfectants have on aquatic organisms. Furthermore, information is lacking about the genotoxicity and carcinogenicity of these chemicals, even though they show minimal systemic toxicity in vertebrates (EPA, 1994).

To date, the global expression of defensive genes by aquatic organisms in response to disinfectants has been reported in only a few species. For example, in the rainbow trout *Oncorhynchus mykiss*, toxic results were found in response to three general disinfectants (triclosan, PCMX, and methylisothiazolinone) (Capkin et al., 2017), demonstrating that these disinfectants cause DNA damage to the transcriptional regulation of superoxide dismutase (SOD), glutathione peroxidase, glutathione S-transferase, heat shock protein, catalase (CAT), and heat shock 70 cognate genes. Furthermore, in the Japanese medaka *Oryzias latipes*, significant upregulation of the vitellogenin gene was shown in juvenile fish following exposure to BAC (Kim et al., 2020). However, transcriptional profiling studies in response to disinfectants (to evaluate ecophysiological effects and uncover toxic mechanisms) have not been reported, although molecular studies have been deemed a promising approach to molecular ecotoxicological studies (Anderson et al., 1994; Cannon et al., 2012).

Brachionus spp. are rotifers, fundamental microzooplankton in the ecological structure of aquatic ecosystems that make energy available for other trophic levels (Dahms et al., 2011). They link the food web from primary producers to large consumers and also participate in the microbial loop by consuming bacteria (Turner and Tester, 1992; Yúfera, 2002). These organisms are thus suitable species for conducting life history research in a short time frame (Snell and Janssen, 1995; Dahms et al., 2011). In ecotoxicological studies, the advantages of using these organisms to assess adverse effects have been fully addressed for metals, pharmaceuticals, and persistent organic pollutants due to their short life cycle (~1 day) and ease of maintenance in the laboratory (Dahms et al., 2011; Lee et al., 2020; Li et al., 2020).

In this paper, several *in vivo* endpoints of the estuarine rotifer *Brachionus koreanus* were measured to evaluate the ecotoxicological and ecophysiological effects of two disinfectants, PCMX and BAC. We focused on the defensive role of antioxidant enzymes in response to PCMX and BAC. In addition, to evaluate the relationship between molecular mechanisms and COVID19 disinfectant-induced adverse effects on behavior and reproduction, an ingenuity pathway analysis (IPA), a powerful method using web-based software for data analysis in genomics, was conducted to examine the signaling pathways under PCMX exposure.

2. Materials and methods

2.1. Test organism

A population of the estuarine monogonont rotifer *B. koreanus* has been stably maintained at the Department of Biological Sciences in Sungkyunkwan University in South Korea for several years. This species was originally collected at Uljin (36°58'43.01"N, 129°24'28.40"E) and kindly provided by Prof. Heum Gi Park, Gangneung-Wonju National University, South Korea. The species was identified using both morphological information and the *COI* and *ITS1* genes (Hwang et al., 2013).

2.2. Chemicals

All chemicals, including 4-chloro-3,5-dimethylphenol (PCMX), alkylbenzyltrimethyl ammonium chloride (BAC), and dimethyl sulfoxide (DMSO), were purchased from Sigma-Aldrich (St. Louis, MO, USA) at HPLC grade (>99%). The analytical kits for the SOD (EC 1.15.1.1) and

CAT (E.C.1.11.1.6) assays were purchased from Abcam (ab65354, Cambridge, UK) and Sigma-Aldrich (CAS 9001-05-2), respectively. All reagents for molecular studies and chemical exposure were of ultrapure grade for molecular biology and analytical grade.

2.3. *In vivo* toxicity test: lethal concentrations and life table parameters

B. koreanus were exposed to PCMX and BAC to measure the median lethal concentrations (LC₅₀-24 h) with waterborne exposure. Stock solutions of PCMX and BAC (conc. 100 mg PCMX mL⁻¹ and 20 mg BAC mL⁻¹) were prepared using DMSO and distilled water, respectively, because of their molecular weight and water solubility (Table S2). The final concentration of DMSO was 0.001% in each PCMX exposure group, the lowest concentration that showed no adverse effects in the preliminary study. For the test organisms, 2 h post-hatching individuals (n = 10) were used in biological triplicates, and mortality was measured after 24 h of exposure following the reported procedures for rotifers (Snell and Persoone, 1989).

The effects of PCMX (0, 1, 2, 5, and 10 mg L⁻¹) and BAC (0, 0.03, 0.06, 0.15, and 0.3 mg L⁻¹) on the population growth, reproduction, and lifespan of *B. koreanus* were measured as shown in Lee et al. (2018) and Kang et al. (2021). Briefly, population growth of *B. koreanus* following exposure to PCMX and BAC was measured by counting the number of individuals produced by ten individuals for 7 days. Reproduction was measured by counting the number of neonates produced by one adult for 6 days. Lifespan indicates the average number of days from hatching to death. The individuals used for the lifespan study were selected from stable individuals hatched simultaneously. The details for measuring the life table parameters are presented in Text S1.

2.4. Concentrations of disinfectants in water and *Brachionus koreanus*

The dissolved concentrations of PCMX and BAC were analyzed to validate the exposure concentrations. The chemical analyses of PCMX and BAC were conducted using liquid chromatography (LC) and LC-mass spectrometry (MS)/MS, respectively and the detailed conditions are described in Text S2.

The PCMX and BAC that accumulated in *B. koreanus* were also analyzed. For this step, a large-volume exposure test was conducted using approximately 50,000 *B. koreanus* individuals. In brief, 600 mL of test solution was prepared for exposure, and the *B. koreanus* were damp-dried at 24 h post-exposure. For analysis, biological samples were sonicated in 1 mL of methanol (40 °C, 30 min). Then, the samples were filtered with an appropriate filter and injected into the LC system (Text S2). All tests were conducted in triplicate.

2.5. Measurement of reactive oxygen species and antioxidant enzymatic activity

The intracellular reactive oxygen species (ROS) levels were measured using 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) as described in Jeong et al. (2017). The experimental groups were sampled 24 h after exposure to PCMX and BAC at the NOEC level. Additionally, *N*-acetyl-L-cysteine (NAC), a ROS scavenger, was administered to confirm scavenging of the ROS generated in response to PCMX and BAC.

Approximately 5000 individuals were used to analyze antioxidant enzymatic activity, and ten individuals were used for microscopic observation. Fluorescent images of the organisms were observed using a fluorescence microscope (Olympus IX71, Olympus Corporation, Tokyo, Japan). The enzymatic activities of SOD and CAT were measured using cellular activity assay kits according to the manufacturers' protocols. The enzymatic activities were normalized with proteins as explained in the manufacturers' protocols. Protein quantitation followed the Bradford assay as described in Park et al. (2020).

2.6. Swimming behavior

Swimming behavior (swimming speed and rotation rate) were measured with a minor modification of the procedures of Garaventa et al. (2010). The swimming pattern was recorded under a stereomicroscope (M205A, Leica Microsystem Ltd., Wetzlar, Germany), and alterations in swimming speed and rotation rate were compared with the control. Briefly, the movement was recorded using LAS software ver. 4.3 (<https://www.leica-microsystems.com>; M205A, Leica Microsystems Ltd.) after placing rotifers onto slide glass and covering them with a cover glass; movies were acquired in AVI file format (.avi). Then, the recorded videos were processed using ImageJ software (<https://imagej.nih.gov/ij/>). A binary 8-bit image converted from the colored video was used to determine the boundary between the rotifers and the background based on a user-defined threshold value. To measure the movement of 2D tracks of the rotifer *B. koreanus* in response to NOEC levels of PCMX and BAC, the particle tracking plugin MTrack2 was used as described in Lee et al. (2020). In brief, MTrack2 (<https://imagej.nih.gov/ij/plugins/multitracker.html>) identifies objects based on the defined particle size and determines which objects in successive frames are closest together. The traveled distance was calculated using the x and y coordinates of successive frames and then divided by the total play time of the video to calculate movement ($\mu\text{m/s}$).

2.7. RNA-seq and bioinformatic analyses

To examine molecular signaling pathway changes in *B. koreanus* in response to PCMX, we chose the NOEC level and a lower concentration that produced significant adverse effects on the lifespan parameters of *B. koreanus*. First, RNA-seq was conducted using samples treated with PCMX at concentrations of 1, 5, and 10 mg L^{-1} , which was lower than the NOEC-24 h. For this analysis, RNA was extracted in triplicate from PCMX-exposed *B. koreanus* and the control and converted to complementary DNA (Text S3). Then, sequencing libraries were constructed. Sequencing was conducted using an Illumina Novaseq 6000 at DNA LINK (Seoul, South Korea). The paired-end raw data were manufactured, normalized, and trimmed by Trimomatic v0.38. To compare gene expression, the RNA-seq data were analyzed by the new tuxedo protocol (Pertea et al., 2016). The cleaned RNA-seq data were mapped onto the reference genome by HISAT v2.2.1. From the mapped files, fragments were counted on the gene-level with annotated genome files using Stringtie v2.1.4, and estimated expressions were calculated and normalized as Fragments Per Kilobase Million and Transcripts Per Kilobase Million (TPM) values. To check the normalized TPM value calculation and variation of samples within replicates and within groups, a principal component analysis (PCA) was used and visualized as a plot. Using the counted data, the differential analysis proceeded with the edgeR method using the TCC package in R (Sun et al., 2013). Significant fold change differences (absolute $\log[\text{fold change}] > 1$, $\text{FDR} < 0.05$) between the control group and the PCMX treatment groups (1, 5, and 10 mg L^{-1}) were counted as differentially expressed genes (DEGs), and those results were visualized as volcano plots with $\log\text{FC}$ and $\log\text{FDR}$ axes.

2.8. Ingenuity pathway analysis

To acquire detailed information about the functions and pathways changed by the DEGs, we conducted gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses. We used functional annotation information about the *B. koreanus* genome (Park et al., 2020) and did a BLAST search in the Swiss-Prot database with high correctness ($e\text{-value} < 1e^{-10}$). The enriched GO term and KEGG pathway information was analyzed using the DAVID v6.8 functional annotation analysis tool (Huang et al., 2009). Moreover, from the enriched biological processes identified using GO terms and KEGG pathways, we filtered significant pathways at Fisher's $P < 0.05$.

The IPA web-based software (Ingenuity Systems, Redwood City, CA, USA) was used to visualize the biological functions of genes and molecules and find the canonical pathways and disease and function responses of *B. koreanus* to PCMX. Using the fold change value of the DEGs from each experiment, the enriched canonical pathways and networks between genes were analyzed using an IPA core analysis of the Ingenuity database. To observe the biological states of networks across all experiments, we used an IPA comparison analysis to compare canonical pathways and functions. In all processes, the networks and canonical pathways were explored as significant at $P < 0.05$.

2.9. Statistical analysis

Data are expressed as the mean value (\pm S.D). All statistical analyses were performed using SPSS ver. 17.0 (SPSS Inc., Chicago, IL, USA). First, the homogeneity of variance and normal distribution of all data were analyzed by Levene's test. Significant differences between exposure groups were analyzed by one-way analysis of variance (ANOVA) with a *post hoc* analysis following Tukey's method ($P < 0.05$).

3. Results

3.1. In vivo effects of disinfectants in the rotifer *Brachionus koreanus*

The LC_{50} -24 h of PCMX and BAC in *B. koreanus* was approximately 24.264 mg L^{-1} and 0.483 mg L^{-1} , respectively (Fig. 1A and B). A reverse S-curves analysis of each test on the survival rate produced 15 mg L^{-1} of PCMX and 0.3 mg L^{-1} of BAC as the NOEC level. Those concentrations were used directly in this study as marginal levels of exposure for measuring biological responses in *B. koreanus*.

In the exposure test, the initial concentrations of dissolved PCMX and BAC were $8.7 \pm 0.2 \text{ mg L}^{-1}$ and $0.39 \pm 0.05 \text{ mg L}^{-1}$, respectively, which differed slightly from the expected concentrations (10 mg L^{-1} of PCMX and 0.3 mg L^{-1} of BAC), but were within the range of concentration used for exposure when considering the recovery rate. The concentrations of these two disinfectants did not show any significant changes in the experimental condition (Fig. 1C and Fig. S1), although the half-life of PCMX in the environment was reported in several studies (NCBI, 2021). The bioaccumulated concentrations of these two disinfectants increased significantly ($P < 0.05$) during 24 h of exposure, whereas no levels were detected in the controls. In particular, PCMX, a disinfectant that was observed to be less toxic at the lethal concentration, had a lower concentration than BAC in *B. koreanus* (Fig. 1C and Fig. S1).

In the control, the population of *B. koreanus* increased by up to approximately 300 individuals during 7 days of incubation, and that population growth was significantly retarded ($P < 0.05$) in response to all concentrations of PCMX, even though all the concentrations of PCMX were less than the NOEC. In the groups treated with 2 and 5 mg L^{-1} of PCMX, the final number of rotifers was less than 50. Interestingly, BAC did not cause any conspicuous retardation in population growth at any exposure, although statistical differences were observed at the highest concentrations of BAC (Fig. 2A, Table S3). In the fecundity experiments, PCMX produced a significant decrease ($P < 0.05$) in reproduction ability. The number of newly hatched neonates decreased in a dose-dependent manner (Fig. 2B), resulting in zero offspring at 10 mg L^{-1} PCMX. However, the number of hatched individuals did not change significantly (ANOVA, Tukey's *post hoc*, $P > 0.05$) in response to BAC exposure.

The lifespan, a representative parameter to examine failure beyond compensation in stress conditions, also decreased significantly ($P < 0.05$) in response to PCMX, by up to 33% compared with the control. However, the lifespan did not change significantly ($P > 0.05$) upon BAC exposure (Fig. 2C).

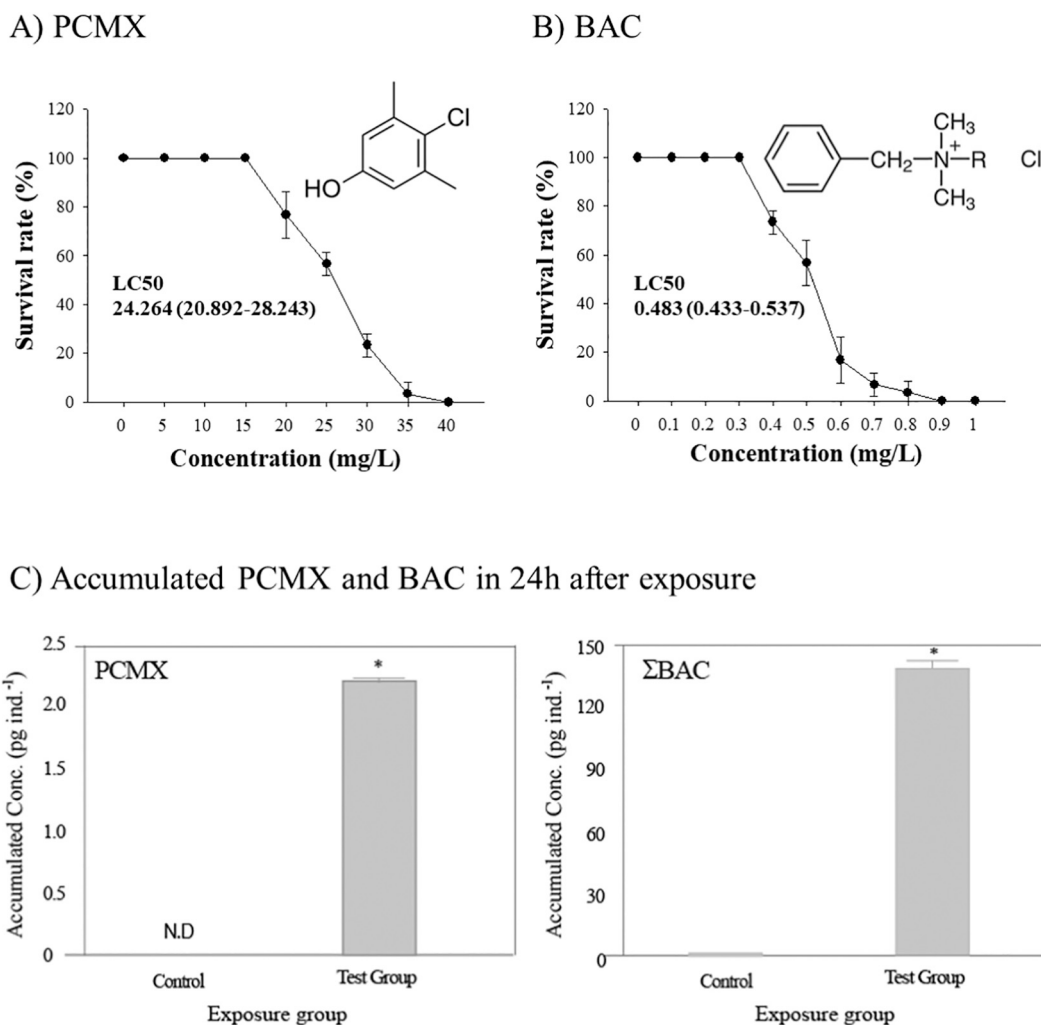


Fig. 1. Acute toxic effects of (A) chloroxylenol (PCMX) and (B) benzalkonium chloride (BAC) on the survival rate (%) of *Brachionus koreanus*. (C) The concentrations of PCMX and BAC in *B. koreanus* after exposure to PCMX and BAC for 24 h.

3.2. Generation of reactive oxygen species

H₂DCFDA staining showed that only PCMX without NAC produced fluorescence under the green stimulus (Fig. 3A). The ROS levels in *B. koreanus* were increased by approximately 10% in response to PCMX, whereas lower ROS levels compared with the control were measured following BAC exposure (Fig. 3B). Experiments with NAC, a ROS-scavenging chemical, support our finding that PCMX caused ROS generation in *B. koreanus* (Fig. 3B).

3.3. Antioxidant enzymatic activities

The increase in the antioxidant enzymatic activities of SOD and CAT were in accordance with the results observed in the ROS experiments (Fig. 4A and B), showing a series of these enzymes as antioxidant mechanisms to oxidative stress (Fig. 4C).

3.4. Swimming behavior

Both swimming speed and movement patterns were altered in response to PCMX and BAC exposure (Fig. 5). Swimming speed was measured as 840.4 $\mu\text{m/s}$ in normal conditions and decreased to 422.4 and 672.0 $\mu\text{m/s}$ upon exposure to PCMX (10 mg L^{-1}) and BAC (0.3 mg L^{-1}), respectively. The alterations in swimming speed are expressed as percentages relative to the control and were reduced by

both disinfectants (Fig. 5A). However, BAC reduced the swimming speed by only 15% compared with the control (Fig. 5B), whereas PCMX exposure caused a difference of about 40%, which explains the abnormal swimming in the figure showing the movement path. The changes in sinuosity observed in the swimming track were also significant ($P < 0.05$), and the rotation rate was significantly enhanced (ANOVA, Tukey's *post hoc*, $P < 0.05$) in response to PCMX (Fig. 5C). These results are well matched with the swimming path measured by MTrack2, which showed abnormality in the rotifers' swimming around in place (Fig. 5D).

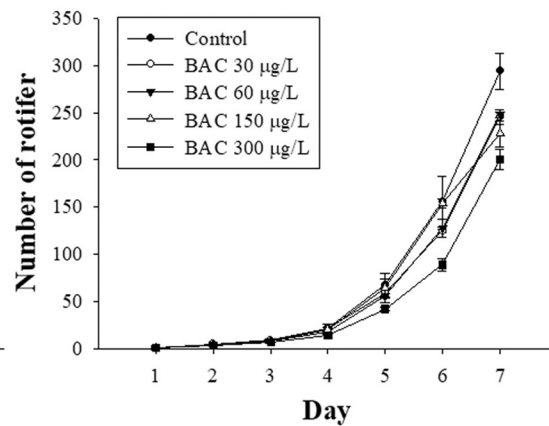
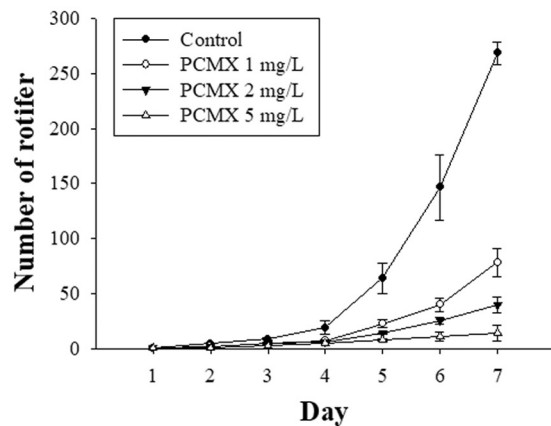
3.5. Differential gene expression and gene ontology and Kyoto Encyclopedia of Genes and Genomes analyses

In transcriptomic analysis, we identified 12,250 expressed genes, of which 399, 1329, and 1069 were differentially expressed upon exposure to three different concentrations of PCMX (1 mg L^{-1} , 5 mg L^{-1} , and 10 mg L^{-1} , respectively) (Table S4). The PCA showed clear differences across PCMX concentrations, suggesting that PCMX induced global gene expression differences between treatment groups in a concentration-dependent manner (Fig. 6A). Apparent differences in upregulated and downregulated genes were also exhibited upon exposure to 1 mg L^{-1} PCMX (upregulated: 92, downregulated: 148), 5 mg L^{-1} PCMX (upregulated: 145, downregulated: 379), and 10 mg L^{-1} PCMX (upregulated: 145, downregulated: 379) ($P < 0.05$) (Fig. 6B, Fig. S2, and Table S5). It is particularly noteworthy that the highest concentration of PCMX

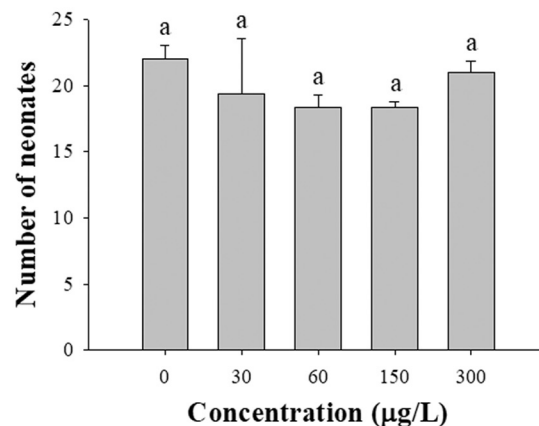
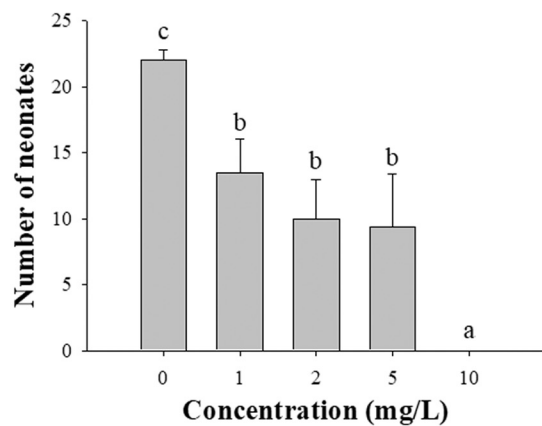
Chloroxylenol (PCMX)

Benzalkonium chloride (BAC)

A) Population



B) Reproduction



C) Mean lifespan

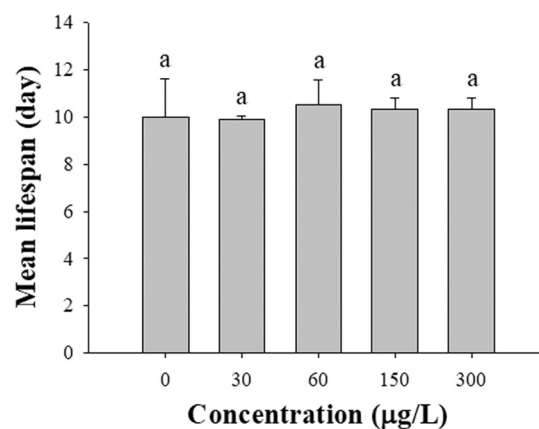
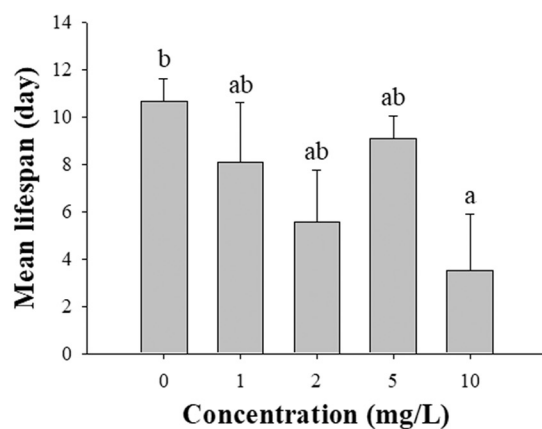
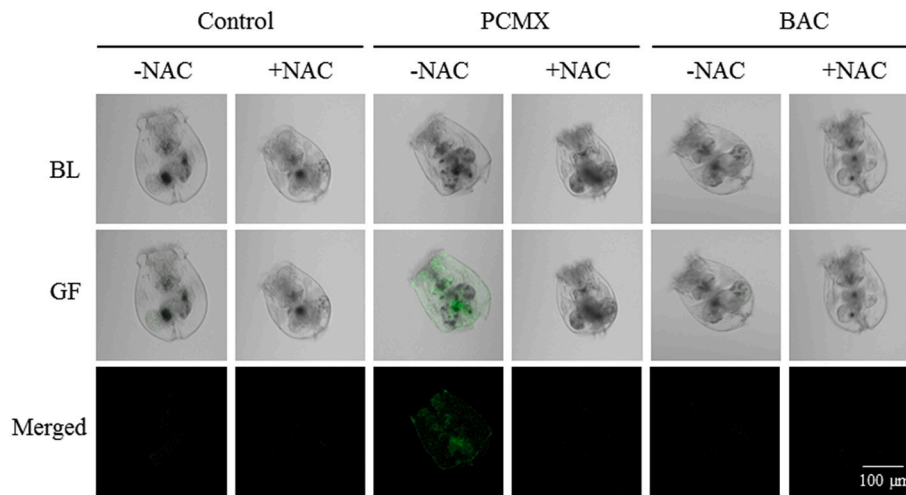


Fig. 2. Effects of exposing *Brachionus koreanus* to different concentrations of chloroxylenol (PCMX) and benzalkonium chloride (BAC): (A) Population growth, (B) Reproduction, and (C) Mean life span. *B. koreanus* was exposed to PCMX (left) and BAC (right) for 7 days. Differences between exposed groups were analyzed for significance using Tukey's *post hoc* analysis. Different letters above columns indicate significant differences ($P < 0.05$).

(10 mg L⁻¹) caused a significant increase ($P < 0.05$) in the number of downregulated DEGs, compared with the control group. The DEGs shared among the three PCMX treatment groups accounted for only 14.9% and 12.6% of the total upregulated and downregulated DEGs, respectively (Fig. 6C and Table S4).

The DEGs among the three different PCMX concentrations were further examined using GO term and KEGG (Fig. 6C) analyses. Clearly,

5 mg L⁻¹ and 10 mg L⁻¹ PCMX had significant effects ($P < 0.05$) on metabolic process-related biological processes such as oxidation-reduction, hydrogen sulfide metabolism, lipid catabolism, phospholipid biosynthesis, glucose metabolism, and long-chain fatty-acyl-CoA biosynthesis. Similar characteristics were observed in the KEGG path enrichment analysis. At all three PCMX concentrations, DEGs were significantly enriched ($P < 0.05$) in the metabolic pathway. Notably,

A) H₂DCFDA staining

B) Reactive oxygen species

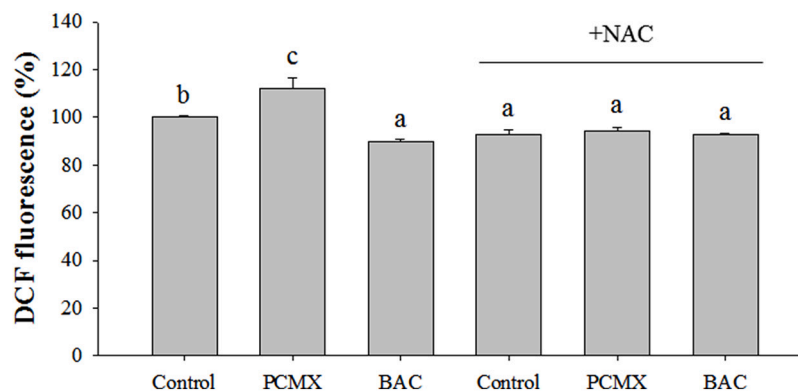


Fig. 3. Effects of exposing *Brachionus koreanus* to chloroxylenol (10 mg L⁻¹) and benzalkonium chloride (0.3 mg L⁻¹): (A) Reactive oxygen species (ROS), (B) Superoxide dismutase activity, (C) Catalase activity. Each group was treated with 0.5 mM *N*-acetyl-L-cysteine (NAC). ROS levels are represented as relative fluorescence (%) of the control without NAC. Different letters above columns indicate significant differences ($P < 0.05$) as analyzed by ANOVA (Tukey's *post hoc* test).

compared with the lower concentrations (1 mg L⁻¹ and 5 mg L⁻¹ PCMX), the DEGs in the lysosome pathway of *B. koreanus* exposed to the highest concentration of PCMX were significantly enriched ($P < 0.05$). In combination with autophagy, lysosomes play a crucial role in resistance to exogenous substances by recognizing foreign materials and attempting to degrade them (Medina et al., 2011). The excessive oxidative stress induced by a high PCMX concentration can negatively affect lysosomal membrane stability (Pascua-Maestro et al., 2017). As a result, several DEGs were related to the expression of the lysosome pathway.

3.6. Ingenuity pathway analysis

To further explore the functions and interactions among DEGs unique to the PCMX-exposed rotifers, we performed an IPA at all three PCMX concentrations (1, 5, and 10 mg L⁻¹). When we compared those three concentrations of PCMX, we found that 10 mg L⁻¹ PCMX inactivated more canonical pathways than the two lower concentrations (Fig. 7A). The canonical pathway analysis revealed that calcium signaling, estrogen receptor signaling, notch signaling, and NRF-2-mediated oxidative stress response were significantly enriched ($P < 0.05$) upon exposure to 10 mg L⁻¹ PCMX. The disease and function analysis predicted disorders of energy metabolism, movement disorders, and organismal death (Fig. 7B). Notably, the top enriched functional

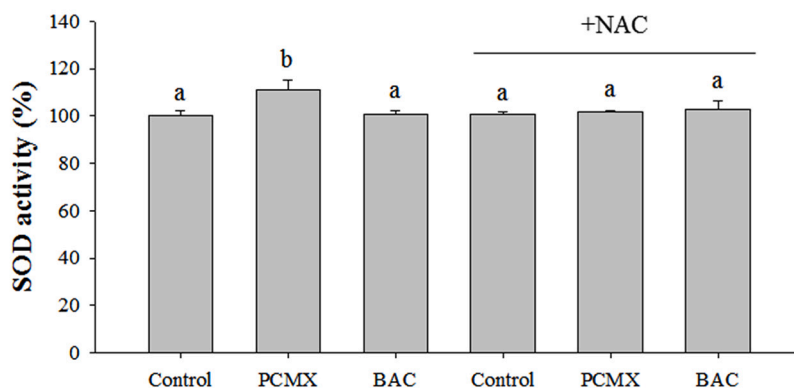
network in the 10 mg L⁻¹ PCMX-treated groups consisted of cellular assembly organization, lipid metabolism, and reproductive system disease (score above 30) (Fig. S3). The other intriguing enriched network involved embryonic development, nervous system development and function, and neurological disease (score 37) (Fig. 8A). *HSPA5* and *SOX2* were the main hub genes connected to several genes in this network. IPA also provided a biological picture of the calcium signal pathway associated with behavioral impairments in the 10 mg L⁻¹ PCMX-treated groups (Fig. 8B). DEGs (*CREB3L1*, *MYH7*, *SLC2A1*, and *SPR*) associated with movement disorder in the neurological disease category in the 10 mg L⁻¹ PCMX-treated groups are presented in a heat map (Fig. 8C and Table S6).

4. Discussion

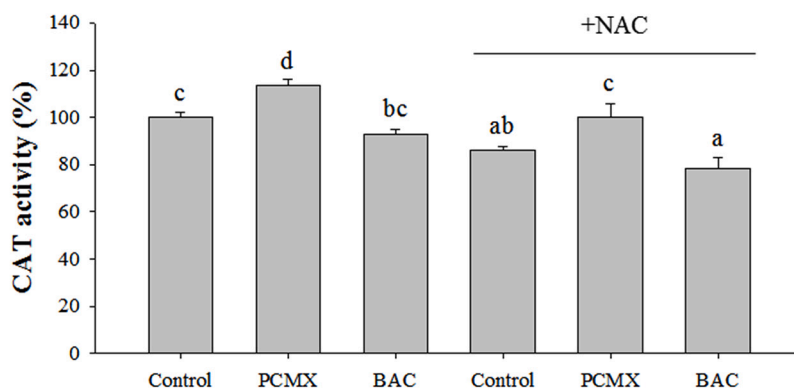
4.1. Increased use of disinfectants and toxic effects

In previous studies, the environmental relevant levels of disinfectants have wide ranges according to the environment from ng L⁻¹ up to several hundreds of μg L⁻¹ (Table 1). In recent years, the use of disinfectants has increased dramatically due to frequent epidemic outbreaks. In fact, the use of those antimicrobials, which are permitted by United States Food and Drug Administration, has increased significantly since

A) Superoxide dismutase



B) Catalase



C) Schematic diagram of SOD and CAT-mediated antioxidant mechanism

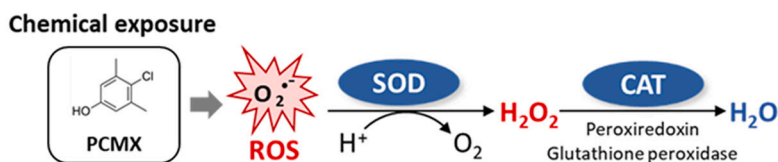


Fig. 4. Image of *Brachionus koreanus* under blue light (BL), green fluorescence (GF), and merging after H2DCFDA staining in response to chloroxylenol and benzalkonium chloride without 0.5 mM *N*-acetyl-L-cysteine (NAC) and with NAC as a negative control. The scale bar indicates 100 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the outbreak of the COVID-19 pandemic. Of them, PCMX in particular is widely used in antibacterial hand sanitizers and household disinfectants in several countries (DeLeo et al., 2020; Kimura et al., 2014; Tan et al., 2021), whereas BAC is widely used for medical purposes. Near wastewater sites, including hospitals, laundries, dairies, swimming pools, and paper production facilities, the concentrations of these disinfectants were near or higher than the lethal concentrations measured in our study. Furthermore, as the degradation rates of these chemicals in municipal sewage treatment plants is less than 100% (Yu et al., 2006; Choi and Oh, 2019; Ebele et al., 2017; Tan et al., 2021), the fate of these disinfectants raises environmental issues in aquatic ecosystems (DeLeo et al., 2020). Therefore, it is timely to examine the adverse effects of PCMX and BAC in aquatic environments.

In our study, the mortality data in *B. koreanus* indicate that these two disinfectants cause toxic effects. The different lethal concentration and NOEC levels (more than one order of magnitude) in response to the two chemicals indicate that PCMX is less toxic to *B. koreanus* than BAC (BAC $LC_{50} = 0.483 \text{ mg L}^{-1}$ and PCMX $LC_{50} = 24.264 \text{ mg L}^{-1}$ at 24 h). This difference is similar to previously reported data that screened several

species, including both land and aquatic organisms (Table S7). BAC is more toxic than PCMX, with acute lethality occurring at environmentally relevant concentrations or less (Sreevidya et al., 2018). BAC at lower concentrations induced similar adverse effects on the survival, reproduction, lifespan, and germline toxicity of two model organisms (worm *Caenorhabditis elegans* and zebrafish *Danio rerio*) compared with PCMX and benzethonium chloride (Sreevidya et al., 2018). Those differences are well reflected in the maximal permissible concentrations of these chemicals in ready-to-use PCPs. Typically, BAC is an acceptable preservative in cosmetics with a maximum allowable concentration of 0.1% in ready-for-use formulations (EU Cosing, ANNEX V), whereas PCMX can be in the range of 0.3% to 3.75% and 0.5% according to EU Cosing ANNEX V and WHO, respectively (EU, 2021; WHO, 2009). Those results reflect the potentially different modes of action of these two chemicals. However, the rotifer *B. koreanus* had different susceptibility to PCMX than the other tested organisms, particularly more resistance than the freshwater cladoceran species *D. magna* (Table S8). Although toxicity results are likely chemical- and species-specific, *D. magna* were more resistant to lindane, endosulfan, pentachlorophenol, 3,4-

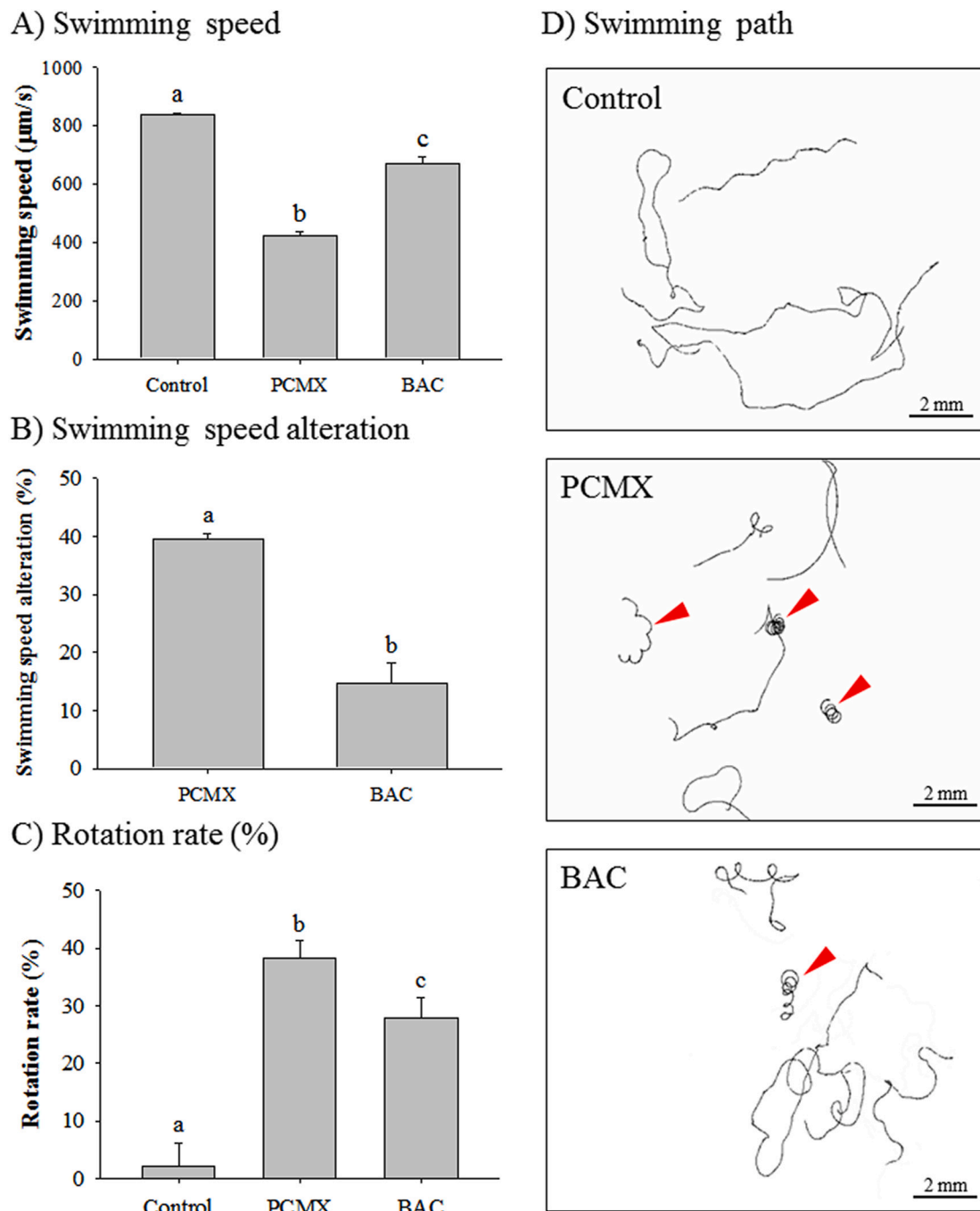
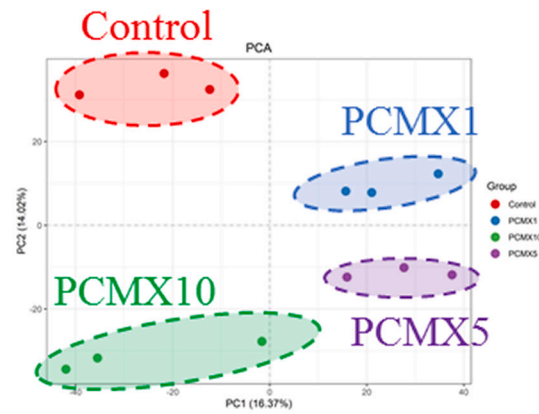


Fig. 5. Alterations in the swimming behaviors of *Brachionus koreanus* in response to chloroxylenol (PCMX) (10 mg L^{-1}) and benzalkonium chloride (BAC) (0.3 mg L^{-1}): (A) Swimming speed, (B) Swimming speed alteration, (C) Rotation (%), and (D) Swimming path for the control, PCMX-, and BAC-exposed groups.

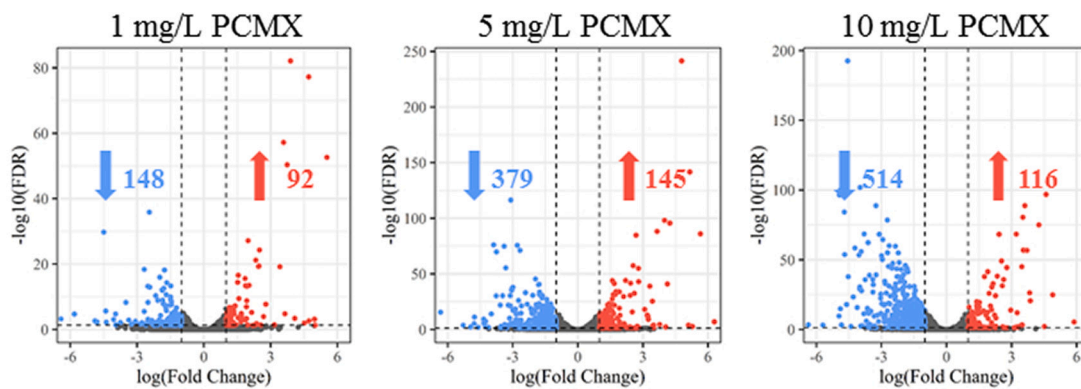
dichloroaniline, and copper sulphate than the freshwater rotifer *Brachionus calyciflorus* (Ferrando et al., 1992). Contrary to the lethal concentrations, the results observed upon long-term exposure (>4 days) were significant. Thus, the growth, reproduction, and lifespan were prominently reduced in response to PCMX in a dose-dependent manner, whereas the NOEC levels of BAC did not cause any significant alterations to *B. koreanus*. Particularly, the population-growth rate was significantly reduced at 5 mg L^{-1} PCMX, which is one-third of the NOEC level (NOEC, 15 mg L^{-1} PCMX), whereas no mortality was observed at even the highest concentration of BAC (NOEC, 0.3 mg L^{-1} BAC). In terms of accumulated PCMX, an average individual exposure of 2.3 pg of PCMX was calculated. Actually, in a study conducted in vertebrates (rat and dogs), rapid excretion of PCMX was found, with almost complete

elimination within 24 h (Havler and Rance, 1977; EPA, 1994). However, the lipophilic and longer chain BACs used in this study (C12, C14, and C16) showed metabolic stability, with high accumulated concentrations. Nonetheless, the inhibitory effect on growth in response to BAC did not differ significantly from that of the control group after 7 days of exposure. The two disinfectants tested here are classified differently: BAC is a quaternary ammonium compound, and PCMX is a phenol antiseptic compound. Therefore, the mode of toxic action might be different, although the general responses in organisms can be similar due to integrated stresses. For example, a previous study of PCMX and other disinfectants, including BAC, showed that these chemicals caused hatching delays (hatching inhibition), embryo mortality, morphological malformations, and neurotoxicity in zebrafish (Sreevidya et al., 2018).

A) PCA



B) Volcano Plot



C) GO term and KEGG pathway enrichment analysis of DEGs

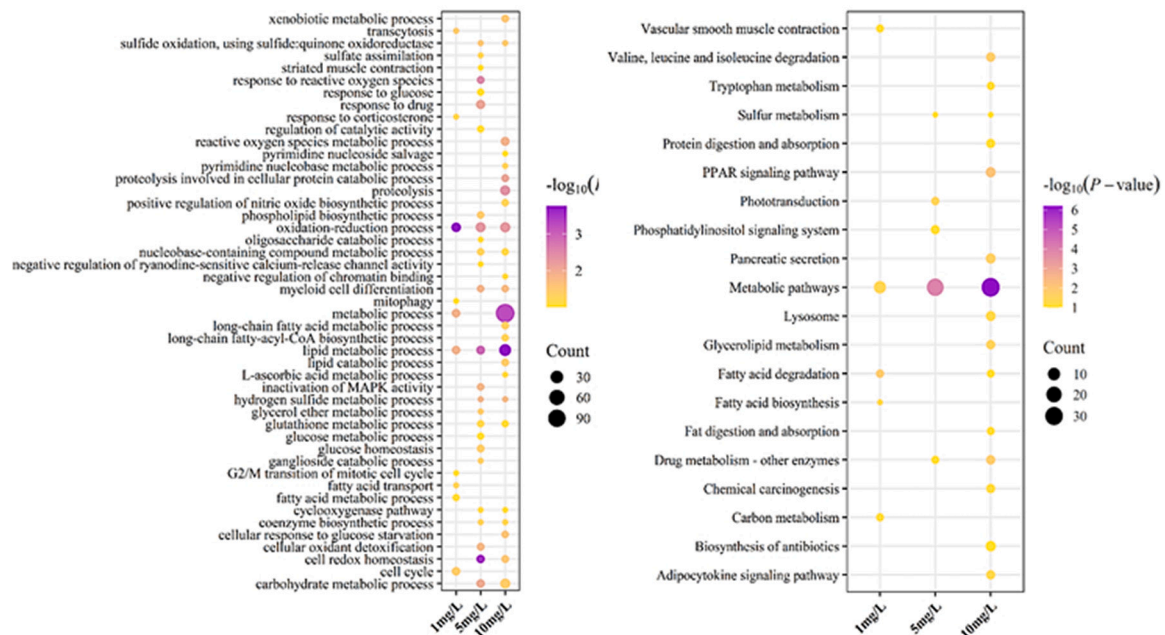
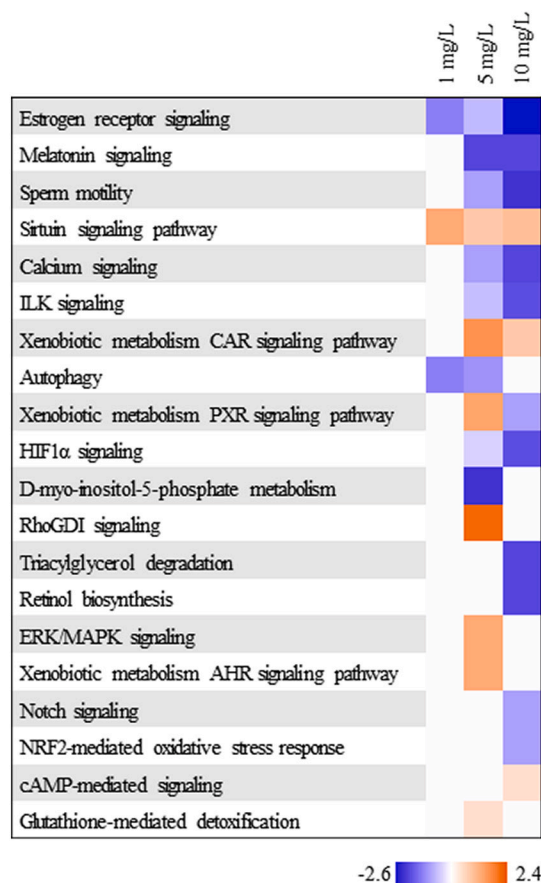


Fig. 6. Validation of RNA-sequencing data. (A) Principal component analysis for each concentration-based set of differentially expressed genes (DEGs). (B) Volcano plot comparing the levels of gene expression between the control and chloroxyleneol (PCMX)-exposed groups. Red and blue dots represent up- and downregulated DEGs, respectively, with $FDR \leq 0.05$ (>2.0 -fold change) compared with controls. Gray dots represent nonsignificant genes out of the filter criteria. (C) Gene ontology (GO) term and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses of DEGs. The x-axis represents the exposed PCMX concentration, and the y-axis indicates GO (left) and KEGG (right) terms with significantly enriched DEGs in *Brachionus koreanus* exposed to PCMX ($P < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

A) Canonical pathways



B) Diseases and bio-functions

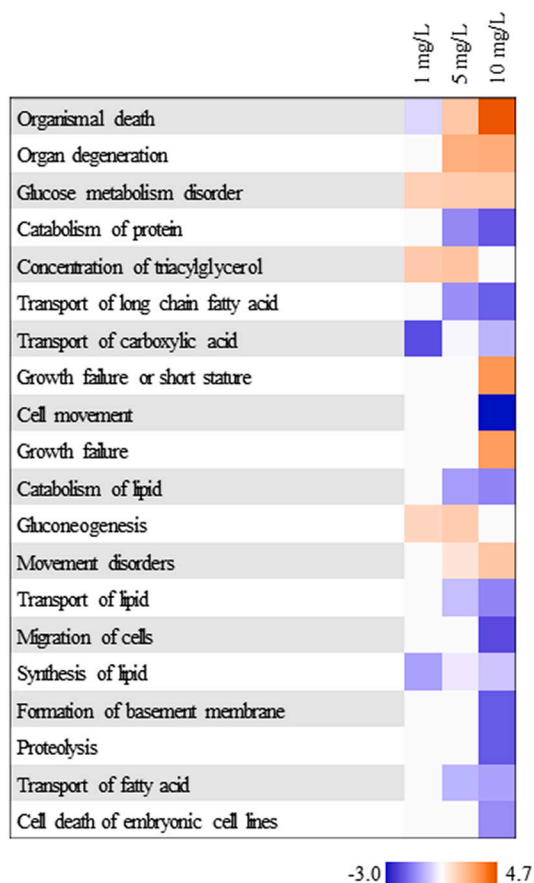


Fig. 7. Ingenuity pathway analysis (IPA) heat map of (A) canonical pathways and (B) diseases and bio-functions altered by chloroxylenol (1, 5, and 10 mg L⁻¹) treatment. Orange and blue indicate that the zscore is positive and negative, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The toxic results measured in the zebrafish *D. rerio* showed that, among five different disinfectants, only PCMX induced a 'body curvature' phenotype, suggesting that it has a unique mode of toxic action in zebrafish.

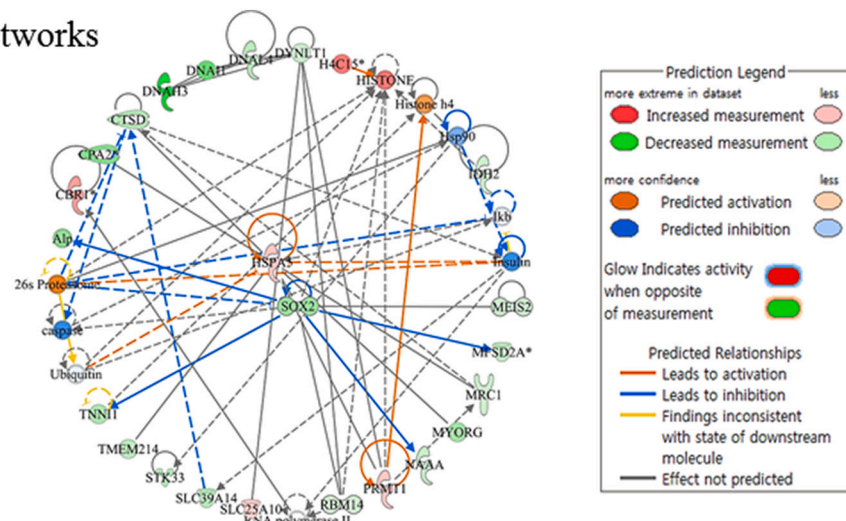
4.2. Disinfectants: an oxidative stress inducer

The increased ROS patterns measured in *B. koreanus* in response to these two disinfectants also differed. PCMX is widely used because it has low toxicity to humans and is biodegradable (Nowak et al., 2021). However, in aquatic environments, PCMX is classified as a moderately toxic chemical based on its low LC values (7.7 mg L⁻¹ for *D. magna* LC₅₀-96 h) for invertebrates, unlike in vertebrates such as birds and fish (Table S7) (EPA, 1994). Various *in vivo* and *in vitro* studies of its toxicity have demonstrated a lack of toxic effects in several organisms, including humans (Yost et al., 2016). Non-target invertebrates were not expected to be exposed to PCMX due to its indoor usage patterns. However, our results show that PCMX induced oxidative stress in the estuarine rotifer *B. koreanus*. The general reactions to PCMX in bacteria show that hydroxyl (-OH) groups of the chloroxylenol (Fig. 1A) bind directly to and disrupt bacterial cell membranes. In addition, the protein and nucleic acid contents of targeted bacterial cells also become coagulated and cease to function, leading to rapid cell death (NCBI, 2021). Similarly, after four days of exposure to PCMX, the number and fertility (reproduction) of *B. koreanus* were noticeably affected in a dose-dependent manner. Furthermore, the antioxidant enzymatic activity of SOD and CAT in *B. koreanus* increased upon exposure to 10 mg L⁻¹ of PCMX for

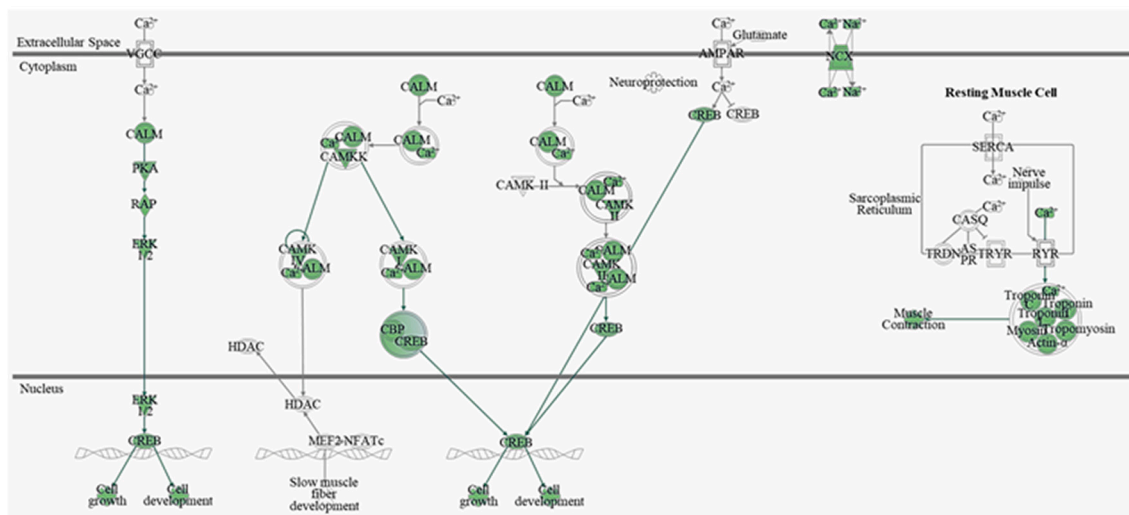
24 h. The increase in antioxidant enzyme levels in the PCMX-exposed groups supports the finding of physiological alterations with significant repercussions on various life cycle parameters (e.g., population growth and fecundity) in chronically exposed populations. However, in the BAC-exposed groups, ROS and consequent antioxidant enzymatic responses were not induced, implying that BAC exposure within the NOEC range did not induce ROS or ROS-related physiological changes in *B. koreanus*, as supported by the H₂DCFDA fluorescence observed in the presence of NAC, a ROS scavenger.

Oxidative stress, its antioxidant responses, and adverse outcomes such as delayed growth, productivity, and lifespan are a common series of responses to environmental stressors in aquatic invertebrates (Buttemer et al., 2010; Lister et al., 2016; Jeong et al., 2017). They can also be interpreted as changes in energy allocation: the stress generated by PCMX causes the reallocation of the cellular energy budget from reproduction and growth to the expression of defensive components (e.g., antioxidative stress-related genes and enzymes), leading to delayed growth, productivity, and lifespan. For example, in the copepods *Tigriopus japonicus* and *Paracyclopsina nana*, crude oil extraction and microplastics caused oxidative stress, which reduced fertility by reducing energy allocation for reproduction (Han et al., 2014; Jeong et al., 2017). In our findings, the lifespan of *B. koreanus* was significantly reduced in response to PCMX, indicating that cellular defense mechanisms activated by ROS are closely related to changes in life parameters *in vivo* through a modulated energy budget. In *B. koreanus*, the antimicrobial agents triclosan and triclocarban produced similar effects on the endpoints of lifespan and oxidative stress-mediated expression of the

A) Enriched networks



B) Calcium signaling pathway and relevant genes



C) Heatmap of DEGs

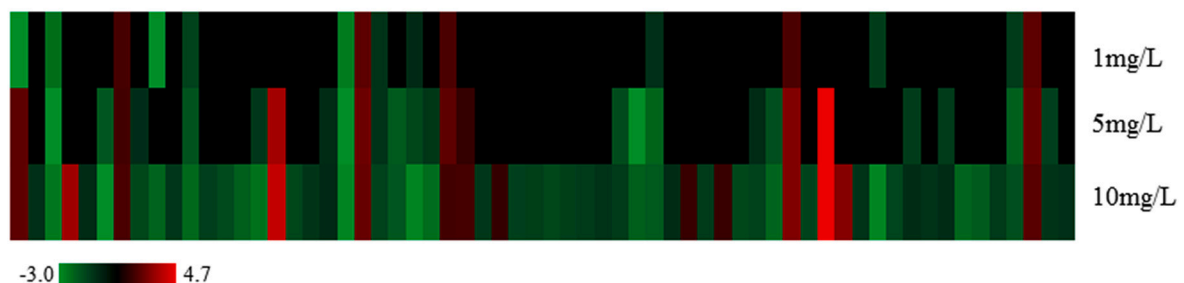


Fig. 8. (A) Enriched networks related to embryonic development, nervous system development and function, and nervous system diseases and (B) Calcium signaling pathway and relevant genes altered after chloroxylenol (PCMX) (10 mg L^{-1}) exposure. Green indicates decreased expression. (C) Heatmap of differentially expressed genes associated with motor impairment in the category of neurological disorders in the 10 mg L^{-1} PCMX treatment group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

defensome (Han et al., 2016). Conversely, in the freshwater rotifer *Asplanchna brightwellii*, the antioxidant agent thiazolidine-4-carboxylic acid extended the lifespan by 7.8% (Bozovic and Enesco, 1986), demonstrating that ROS is both directly and indirectly associated with longevity. Of the variables measured in *B. koreanus*, the reproduction rate (the number of neonates produced by one adult during 6 days) is

highly likely to be related to the population increase (total number of rotifers after 7 days of incubation) because *B. koreanus* have an absolute parthenogenetic reproductive system that shows no resting eggs. Moreover, a shortened lifespan can predict the possible consequences of long-term exposure to PCMX in *B. koreanus*. Taken together, the reduced fecundity and shortened lifespan observed in PCMX-exposed rotifers

Table 1

Environmentally relevant concentrations and lab-based experiment levels of PCMX and BAC studied in previous studies.

	Study objectives	Media (samples)	Study sites	Measured (or studied) range	Reference
PCMX	Monitoring	Surface (river) water	South Wales, UK	0.03–0.36 $\mu\text{g L}^{-1}$	Kasprzyk-Hordern et al., 2008
	Monitoring	Surface (river) water	Jakarta, Indonesia	0.06–1.2 $\mu\text{g L}^{-1}$	Dsikowitzky et al., 2016
	Evaluating the efficiency of wastewater plant technology	Wastewater and adjacent surface waters	River Ely, South Wales, UK	WWTP Coslech Influent: 7811–65,381 Effluent: <84–888 Upstream: <30–99 Downstream: <30–358	Kasprzyk-Hordern et al., 2009
			River Taff, South Wales, UK	WWTP (Cilfynydd) Influent: 4068–33,974 Effluent: 441–3010 Upstream: <30–254 Downstream: <30–225 >500 $\mu\text{g L}^{-1}$	
	Screening	WWTP industrial impacted Drinking waters and urban stream waters	Guangzhou, China	1.62–9.57 $\mu\text{g L}^{-1}$	Thomas et al., 1999 Tan et al., 2021
	Monitoring and develop screening techniques To evaluate removal efficiency Removal efficiency	Sea waters from offshore sites Lab-based work (Biodegradation)	Eighteen marine sites, Kuwait –	0.06–43,070 $\mu\text{g L}^{-1}$ 5 mg L^{-1} 5–50 mg L	Smith et al., 2015 Choi and Oh, 2019 Nowak et al., 2021
BAC	Monitoring & Ecological risk assessment	Surface (stream) water WWTP	Hwaseong, Korea Inf./Eff.	0.0115–57.4 (C12 + C14) $\mu\text{g L}^{-1}$ 61.5/0.493 (C12 + C14) $\mu\text{g L}^{-1}$	Kim et al., 2020
	Monitoring	Estuarine sediments	Long Island Sound, NY Bowery Bay, NY Estuarine Sediment	30 $\mu\text{g Kg}^{-1}$ 5325 $\mu\text{g Kg}^{-1}$	Li and Brownawell, 2010
		River sediments	Forge River, Moriches Bay, NY	55 $\mu\text{g Kg}^{-1}$	
	Screening	River sediments	21 sites, Austria	1.02 $\mu\text{g g}^{-1}$	Martínez-Carballo et al., 2007
	Degradation by bacteria	Lab-based work (Biodegradation)	–	30–200 mg L^{-1}	Patrauchan and Oriol, 2003

could contribute to reduced population growth.

4.3. Behavioral impact and molecular evidences

Swimming is important for aquatic organisms (Charoy and Janssen, 1999; Obertegger et al., 2018). For rotifers, swimming abnormalities can affect several aspects of fitness in terms of encountering food and predators (Charoy and Janssen, 1999; Chen et al., 2014; Obertegger et al., 2018). In this study, the locomotory behavior of *B. koreanus* was affected after relatively short exposures to PCMX and BAC. Particularly, the abnormal circular motion measured in response to PCMX and BAC indicates that these two disinfectants disrupt the balance in rotifer swimming patterns. PCMX had stronger effects on rotifer swimming behavior than BAC. In PCMX-exposed rotifers, the ROS level increased significantly in a concentration-dependent manner, which is closely related to intracellular metabolic disorders (Liang et al., 2021). As mentioned in previous studies, metabolic capacity is closely associated with swimming performance (Yan et al., 2013). Rotifers use ciliary locomotion, which requires them to spend energy. A theoretical and calculational study conducted by Epp and Lewis (1984) reported that rotifers use about 62% of their energy for ciliary movement. The demand for metabolic energy in response to PCMX might exceed the energy supply available from a rotifer's accumulated energy resources, which could make the animal unable to sustain its routine metabolism (Sokolova et al., 2012). The detoxification and metabolic pathways of xenobiotics (e.g., toxicants) also require energy and could interfere with the energy-making process in mitochondria. Thus, stress and the reallocation of energy budgets in response to PCMX might also affect swimming behavior.

Although PCMX is less toxic than BAC, it reduced reproduction and lifespan and led to abnormal swimming behavior in *B. koreanus*. Furthermore, fundamental changes in the transcriptional landscape of

the rotifers were observed with all PCMX treatments, as demonstrated by the numerous DEGs identified in this study. Nevertheless, the specific functional response was concentration-dependent: the 10 mg L^{-1} PCMX treatment produced the largest treatment-specific response. Several macromolecule metabolic process-related genes, including *TCEA2* and *RVSE2*, were overexpressed in the group that received the highest PCMX concentration. The upregulation of those metabolic process pathways is likely associated with energy homeostasis for energy metabolic regulation in PCMX-exposed rotifers. The available energy that organisms use for growth and reproduction can be affected by external factors such as toxicants (De Coen and Janssen, 2003) and microbial agents (Maynou et al., 2021). Those previous studies are consistent with our findings, indicating that PCMX causes changes in the metabolic processes of rotifers. That process can change both the amount and ratio of energy stored in macromolecules (carbohydrates, lipids, and proteins) (Sancho et al., 2009). Lipids in particular constitute an important source of energy that is typically allocated to offspring (Goulden and Place, 1993). In cladocerans, for example, the offspring consume approximately 50% of the lipid reserves allocated in the brood pouch of parthenogenetic females, and the remaining reserves are maintained as a major energy source during the postembryonic-period (Goulden and Place, 1993). Therefore, our result suggests that PCMX adversely affects metabolic processes in rotifers, leading to *in vivo* toxicity.

In our RNA-seq analyses, oxidative stress-induced DNA damage was predicted, with consequent adverse effects on individuals. In addition, a series of toxic effects was observed when we used various approaches to uncover adverse outcomes such as acute and chronic toxicity. To further confirm the PCMX-induced adverse effects on cellular and molecular responses, we performed IPA. The results indicate that PCMX disrupts swimming behavior by affecting the calcium signaling pathway and nervous system (Fig. 9). The contraction of muscles associated with movement depends on an increase in the concentration of cytoplasmic

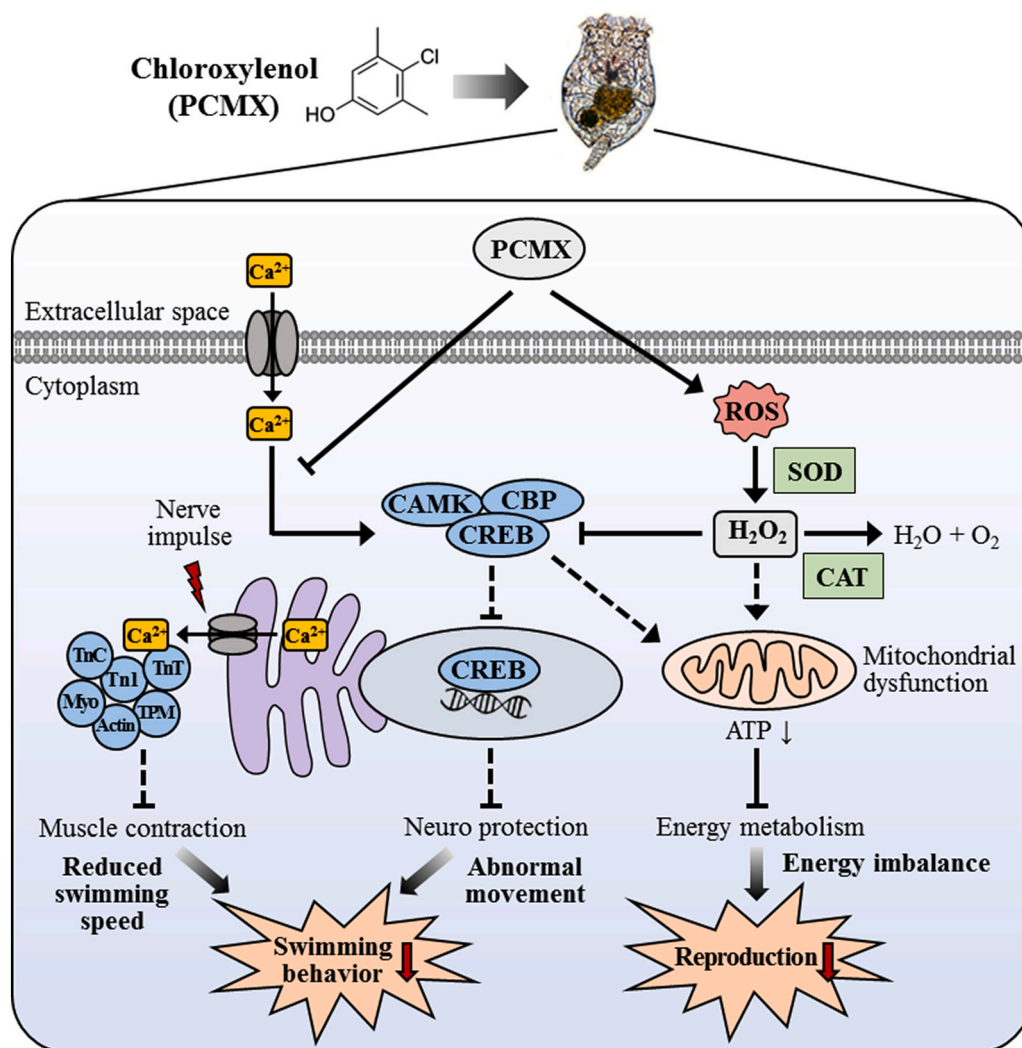


Fig. 9. Schematic summary of the suggested chloroxylenol-mediated molecular mechanism for suppressed swimming behavior and reproduction in *Brachionus koreanus*.

calcium. First, calcium is stored in the endoplasmic reticulum through the activity of the sarcoendoplasmic reticulum Ca^{2+} ATPase (Brennan et al., 2005). The cascade of neurotransmitter released from nerve terminals induces action potentials, and that process produces postsynaptic calcium signals for muscle contraction through mechanical binding of ryanodine receptors (Altringham and Ellerby, 1999). The increasing cytosolic calcium promotes the contraction of cardiac and skeletal muscle, along with troponin and myosin complexes (Schiaffino and Reggiani, 1996). Those reactions are also important mediators of swimming behavior in marine organisms. For example, in the fathead minnow *Pimephales promelas*, alterations in swimming behavior can involve changes in calcium signaling that can be measured by disruption of ryanodine and dihydropyridine receptors, which are directly related to muscle function in response to the antimicrobial chemical triclosan (TCS) (Fritsch et al., 2013). TCS, which is in wide use, causes sporadic wriggling movement in the larvae of rainbow trout (*Oncorhynchus mykiss*) (Capkin et al., 2017). Ajao et al. (2015) suggested that the interference in calcium-dependent K^+ leakage and excitation-contraction of skeletal muscles induced by TCS are the main causes of abnormal swimming behavior in rainbow trout. Our canonical pathway results indicate that calcium signaling and muscle-related genes (actin, myosin, and troponins) were downregulated, indicating that PCMX hindered muscle contraction and calcium signaling processes, resulting in decreased rotifer movement.

Furthermore, changes in intracellular calcium concentration can lead to reductions in the function and viability of neurons (Jones and Smith, 2016). As expected, the IPA predicted a significant effect on movement disorders in the neurological category in rotifers exposed to 10 mg L^{-1} PCMX. Moreover, embryonic and nervous system development and neurological disease were identified as the main canonical pathways associated with PCMX exposure. Indeed, our swimming behavior analyses indicate that PCMX not only reduced swimming speed but also caused abnormal swimming patterns in rotifers. These results indicate that neurotoxic environmental pollutants can affect the behavior of marine organisms. In zebrafish, the neurotoxicants copper chloride and chlorpyrifos produced freeze responses (an anti-predator behavior) and a decrease in swimming speed, respectively (Tilton et al., 2011). Moreover, the freshwater rotifer *B. calyciflorus*, which possesses a well-developed neuromuscular system despite its small size, exhibited uncoordinated movements upon exposure to neurotoxic cyclodiene-type toxicants (Charoy and Janssen, 1999). Therefore, PCMX-induced abnormal swimming behavior might be entirely due to changes in muscle function and neurotoxicity mediated by the calcium signaling pathway (Fig. 9). Taken together, the adverse effects of PCMX on rotifers at the behavioral, physiological, and molecular levels reveal that this compound might pose a much more serious threat to aquatic animals than currently perceived.

5. Conclusions

PCMX and BAC are widely used as topical antiseptic products in over-the-counter medications and hard-surface disinfectants. However, our data indicate that these compounds can have serious repercussions in aquatic environments. In previous studies, the modes of toxic action of PCMX and BAC were reported in terms of their genotoxic, cytotoxic, and neurotoxic effects in other species. However, although ROS plays a central role in cell signaling for oxidative stress and regulating the main pathways of apoptosis mediated by mitochondria, previous studies did not directly demonstrate ROS generation in response to PCMX. In this paper, we are first to report that PCMX induces ROS generation in *B. koreanus* and produces various adverse effects in *in vivo* life parameters, producing deleterious effects on the molecular responses required for swimming behavior and reproduction. Furthermore, this study can cover the relevant concentrations of disinfectants, while many works on this chemical, focusing on its degradation or elimination by bacteria, and its biological toxicity, have been conducted at much higher levels of concentration. The expected mode of action described by IPA after ROS generation supports all the results observed at the *in vivo* and *in vitro* levels, including behavioral and life table parameters and alterations in enzymatic activity. It is also consistent with the chemical structural properties of PCMX, which contains hydroxyl groups that affect cell membranes. In conclusion, *in vivo* life parameter observations, antioxidant responses, and RNA-seq-based IPA all shed light on how PCMX induces deleterious effects at various levels that produce the observed *in vivo* and *in vitro* endpoints. These findings can be used to examine the underlying molecular mechanisms of rotifers using to cope with PCMX-induced oxidative stress and its resulting effects.

CRedit authorship contribution statement

J.-S.L. and K.-H.S. designed and supervised the project. E.-J.W., E.B., Y.H.L., H.J., Y.L., M.-S.K., H.-W.J., J.-K.M., and M.W. collected and generated the data. E.-J.W., E.B., Y.H.L., J.-S.L., and K.-H.S. wrote the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.marpolbul.2022.113396>.

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