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RESEARCH ARTICLE

Assessment of eco-toxic effects of commonly used water disinfectant on zebrafish (*Danio rerio*) swimming behaviour and recovery responses: an early-warning biomarker approach

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Abstract

Eco-toxicity profiles for commonly used disinfectants were lacking. Available traditional toxicity techniques have some limitations (assessments and ethical issues). Behaviour toxicology is a promising research area towards early warning and non-invasive approaches. We studied the potential eco-toxic effects of sodium hypochlorite (NaOCl) on the swimming behaviour of zebrafish. Zebrafish were exposed to different concentrations (Treatment I, Treatment II, Treatment III, and Treatment IV) of NaOCl for 360 h. Recovery study (144 h) was conducted for NaOCl treatment groups. The swimming behaviour of zebrafish was quantified efficiently using an online monitoring system (OMS). OMS dataset was processed for determination of behavioural differences by MATLAB and SPSS. Compared to the control group, the swimming strength of zebrafish under NaOCl treatments declined significantly ($p < 0.001$). Avoidance behaviour has occurred on zebrafish under NaOCl exposure periods. Furthermore, NaOCl toxicity also adjusted circadian rhythms on zebrafish. Zebrafish swimming strength was significantly ($p < 0.001$) improved under-recovery periods. Moreover, normal diurnal patterns have occurred. NaOCl could cause behavioural abnormalities in non-target organisms. Continuous exposure to common disinfectants could cause external and internal stress on non-target organisms, resulting in behavioural changes and circadian rhythm adjustments. Continuous changes in behavioural and circadian rhythms might reduce organisms' fitness and adaptation capacity. This study highlights (1) the importance of computer-based toxicity assessments, and (2) swimming behaviour is an early warning biomarker for eco-toxicity studies.

Keywords Disinfectant · Environmental pollution · Aquatic concern · Stress indicator · Real-time assessment

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Zongming Ren and Yaxin Yu contributed equally to this paper.

Highlights

- Sodium hypochlorite (NaOCl) is potentially a behavioural disruptor.
- Swimming behaviour could be reliable non-invasive biomarkers for eco-toxicity studies.
- A concentration-dependent toxicity was observed on zebrafish under NaOCl treatments.
- Avoidance behaviour resulted on zebrafish under prolonged exposure to NaOCl.
- Recovery responses are an auxiliary study for eco-toxicity studies.

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Introduction

Water demand and pollution lead to the treatment and reuse of water resources (Senthil Rathi et al. 2021). Failure of water treatment systems in inactivating pathogens has resulted in outbreaks of many waterborne diseases and illnesses. Disinfectants are the best option for eradicating microbes in water treatment systems and other fields (aquaculture, agriculture, domestic purposes, and health care centres) (Holm et al. 2019; Yun et al. 2020; Choi et al. 2021; Huang et al. 2021). Different kinds of disinfectants were commercially available (totally 131 household products) for use (Goh et al. 2021). Chlorine is predominantly disinfectant because of its significant antimicrobial activity and least expensive advantages (Dastagiri Reddy and Elias 2021). Sodium hypochlorite (NaOCl) is a mostly used chlorine-based disinfectant; since the seventeenth century, it has been capable of destroying pathogens (including persistent pathogens) more efficiently (Ujimine et al. 2017). Thus, most household bleach contains NaOCl (up to 9%) as the active ingredient (CDC-https://cfpub.epa.gov/giwiz/disinfectants/index.cfm). NaOCl-based products are recognised as essential germicides (US-EPA 1967). They are used in different (domestic, industrial, aquaculture, agriculture, veterinary practices, scientific, and biomedical) applications (dwell time: 30 s, pathogens: bacteria and viruses) (Emmanuel et al. 2004; Tudela et al. 2019; Duerschner et al. 2020). The EPA recently recommended NaOCl (EPA itemised: 72 products, formulation type: dilutable, wipe, ready to use, Viking, electrostatic spray; surface type: hard nonporous, food contact post rinse required) as the active ingredient to inactive SARS-CoV-2 (US-EPA 2021). The WHO also recommended NaOCl as biocides at 0.1% and 0.5% for general environmental and blood spill disinfections, respectively, for SARS-CoV (WHO 2020). Overuse of disinfectants (e.g., NaOCl) resulted in the mortality of 17 different free-living species in Chongqing, China (Nabi et al. 2020). Environmental factors govern the decomposition process of NaOCl, decompose slower with air, and the process is accelerated under direct light conditions. NaOCl has been categorised under Class-I toxic substance by US-EPA due to its developmental effects on biota (as mentioned in Elia et al. 2006). The concern is that approximately 80% of wastewater (including aquaculture wastewater) is rereleased to environmental compartments without proper treatment (Xie et al. 2019; Deere et al. 2020). Global use of disinfectants is also escalating (up to 0.78 billion during 2020) after the pandemic (Subpiramaniyam 2021). Generally, a high concentration (1000 ppm) of NaOCl is required to neutralise pathogens in the contaminated zones (bathroom and toilet) (Rim 2021). Hence, it is essential to measure disinfectant performance and the potential impacts on non-target organisms to ensure their safety and sustainability (Macedo et al. 2020; WHO 2020).

Increasing the biological effects of waterborne contaminants has led the scientific community to develop biomarkers using biological models (Makaras et al. 2018; Falcao et al. 2019). Biological monitoring methods provide information on waterborne contaminants' potential impacts on ecological risk assessment (Makaras et al. 2020) . Zebrafish is the most recommended model for various (behavioural, neuro-, and ecotoxicology) aquatic biological monitoring studies (Hong and Zha 2019; Loring et al. 2020; de Oliveria et al. 2021). Available traditional biological endpoints have some limitations: toxicity tests conducted at high concentrations; no early-warning biomarkers are focused (Magalhaes et al. 2007; Hong and Zha 2019). Behavioural activity is the primary response of organisms to reveal the initiation of changes in physiological and ecological processes. Thus, an organism's behavioural activity can be recognised as valid biomarkers to assess the water quality of an aquatic ecosystem and the toxicity of waterborne contaminants (Xia et al. 2016). Additionally, behaviour biomarkers do not require animal sacrifices even at long-term toxicity monitoring. The swimming behaviour is a routine process of fish that governs other essential behavioural activities such as searching for feed, protecting from predators, reproduction, *Etc*. The swimming behaviour of a fish might affect under stress conditions. Thus, we can observe different stages (safe, acclimation, adjustment, or toxic) on the swimming response of fish (Ren et al. 2018). These behavioural changes could result in hyper- or hypo-activity. Generally, agitated (hyperactive) behaviour could occur under stress conditions when fish habitat is in the open environment. Suppose fish habitat is in an unescapable milieu. To elude themselves from the external stimuli, they could minimise the gill movement (increase distance between gill epithelium and the external environment) and water flow. This action could result in hypoactivity. This situation is an indication of the avoidance behaviour of fish. Noteworthy, avoidance behaviour could reveal fish's sensing capability to stressors. The significance of these endpoints could increase the ecological significance of risk assessment procedures. Behaviour anomalies associated with toxicant treatment can recover once fish are introduced to a toxicant free environment; thus, recovery responses are recognised as an assisting parameter in behavioural toxicology (Ren et al. 2021).

The continuous contamination of water systems has led to the development of reliable real-time monitoring approaches. Real-time ecological or biological monitoring is possible only with the computer-based apparatus. Thus, researchers developed different types of computer-based real-time monitoring sensors and systems in recent years. Noteworthy, online monitoring systems are inexpensive, user-friendly, require no human interference, and harmless to test specimens (Pasternak et al. 2017; Anas et al. 2020). Real-time monitoring technologies based on fish swimming behaviour are improved much in recent years, which signifies the significance of behavioural toxicology (Zhang et al. 2015). Long-term eco-toxicity assessment is possible using online behaviour monitoring systems. The generated behaviour data can be analysed using computer software, making online behaviour assessments more manageable and efficient monitoring systems (Xia et al. 2016).

This study's hypothesis is to use fish swimming behaviour as an early warning non-invasive biomarker to assess the impacts of waterborne contaminants. We also highlight the importance of online monitoring systems in continuous eco-toxicity assessments in this study. To evaluate the eco-toxicity of commonly used disinfectants on non-target organisms, we exposed zebrafish to different concentrations of NaOCl for 360 h. We analysed swimming behaviour using an online monitoring system (OMS). The recovery study (after NaOCl treatment period, zebrafish were introduced to normal water) conducted for 144 h.

Materials and methods

Zebrafish care

Healthy *Danio rerio* (AB wild-type, 5–6 months old, length 3.4 ± 0.4 cm, weight 0.4 ± 0.1 g) used in this study were obtained from our fish breeding laboratory (location: Institute of Environment and Ecology, Shandong Normal University, Jinan, China). Zebrafish were maintained in a recirculating aquaculture system (water was aerated by air bubblers and purified with activated carbon and filter cotton, continuously) at constant photoperiods (light - 12 h: dark - 12 h). Rearing conditions were set optimum with the following water-quality parameters, temperature (27 ± 0.5 °C), water hardness (250 ± 20 mg/L), and pH (7.8 ± 0.2). Zebrafish were fed twice a day with commercially available fish feed. We removed uneaten feed and fish faecal matters manually at regular (at least 1 h later to feeding) intervals. We used both sexes of zebrafish for tests. We avoided abdomen elongated zebrafish for tests.

Procurement of NaOCl and preparation of test concentrations

NaOCl was purchased from NAIS Group, Co., Ltd., China. NaOCl contaminated water was prepared by mixing NaOCl (appropriate amount, based on the requirement) in water (collected from the rearing system). Control: 0 μL of NaOCl, Treatment I: 0.0025% v/v, Treatment II: 0.005% v/v, Treatment III: 0.0075% v/v, and Treatment IV: 0.01% v/v. NaOCl contaminated water renewed (5 L) at every 96 h of the study periods.

Online behavioural toxicity assessment

We used an OMS to assess the zebrafish's swimming strength (Fig. 1). The main components of OMS are a fish chamber with metal sensors, a water-flow system (water tank, peristaltic pump), and a normal computer (central processing unit-CPU, monitor, and software). Fish chamber (material: high-grade plastic, size: 5 cm in diameter and 7 cm in length, shape: cylindrical) has two opening edges (top and bottom) and two pairs of metal electrodes (sensors) on its inner surface. Metal sensors are connected to the CPU through copper-coated PVC cables. The water-flow system connects the fish chamber with rubber tubes. Water from the tank pumped at 30 mL/min flows to the fish chamber through the peristaltic pump. The bottom valve of the fish chamber receives water from the tank, and the top valve ousts the water back to the same tank to complete a water cycle. The metal sensors are the automatic samplers of the OMS. The CPU and software process the samples through A/D transformers (analogue to digital convertor) and display them on the computer monitor. System calibrations were performed with and without zebrafish in the fish chamber at constant water flow. We managed the sensing capacity of the sensors to harvest behaviour strength of fish between 0 and 1 to determine no movement and full movement, respectively. The signal acquisition and transmission of the OMS are shown in Fig. 1. The sensor senses the fish movement by touch evoked electrical impulse. The sensors generate electrical signals. One pair of sensors sends a high frequency of the electrical signal of altering current, and the electrical signal was received by another pair of sensors in the fish chamber. The A/D transformers digitalised this electrical signal. The software in the computer analyses these digitalised signals. The result is displayed as a line graph on the computer monitor.

Main group: zebrafish (three numbers) were randomly collected from the rearing system and housed in the OMS fish chamber (single chamber). Zebrafish were starved for 24 h before the commencement of the behavioural tests. Nevertheless, they were fed once a day with regular feed during the tests. An appropriate amount of NaOCl (Treatment I: 0.0025% v/v) was mixed in the tank connected to the fish chamber. Replicates: Simultaneously, three replicates were also maintained. Similar setups were upheld for the control (without adding NaOCl), Treatment II (0.005% v/v), Treatment III (0.0075% v/v), and Treatment IV (0.01% v/v) groups. We used water from the rearing system to perform all tests. We also maintained the water-quality parameters and photoperiods similar to the rearing system.

We continuously monitored the behavioural toxicity of NaOCl for 360 h with normal photoperiods (light-12 h : dark-12 h). We conducted the recovery capability of zebrafish to NaOCl toxicity for 144 h with regular photoperiods (light-12 h : dark-12 h).

Data analysis

We used MATLAB Environments (MATLAB 2009, @ 1984–2009, The Math-Works, Inc.) to calculate the Mean value from the OMS dataset. Standard deviation (SD) was calculated using an MS-Excel worksheet. We used Statistical Product and Service Solutions (SPSS 16.0) to execute a general linear model (univariant analysis and Duncan's multiple range tests-DMRT). We used MATLAB to process the real-time data and obtain figures for swimming strength, autocorrelation, and self-organising map (SOM). The Mean

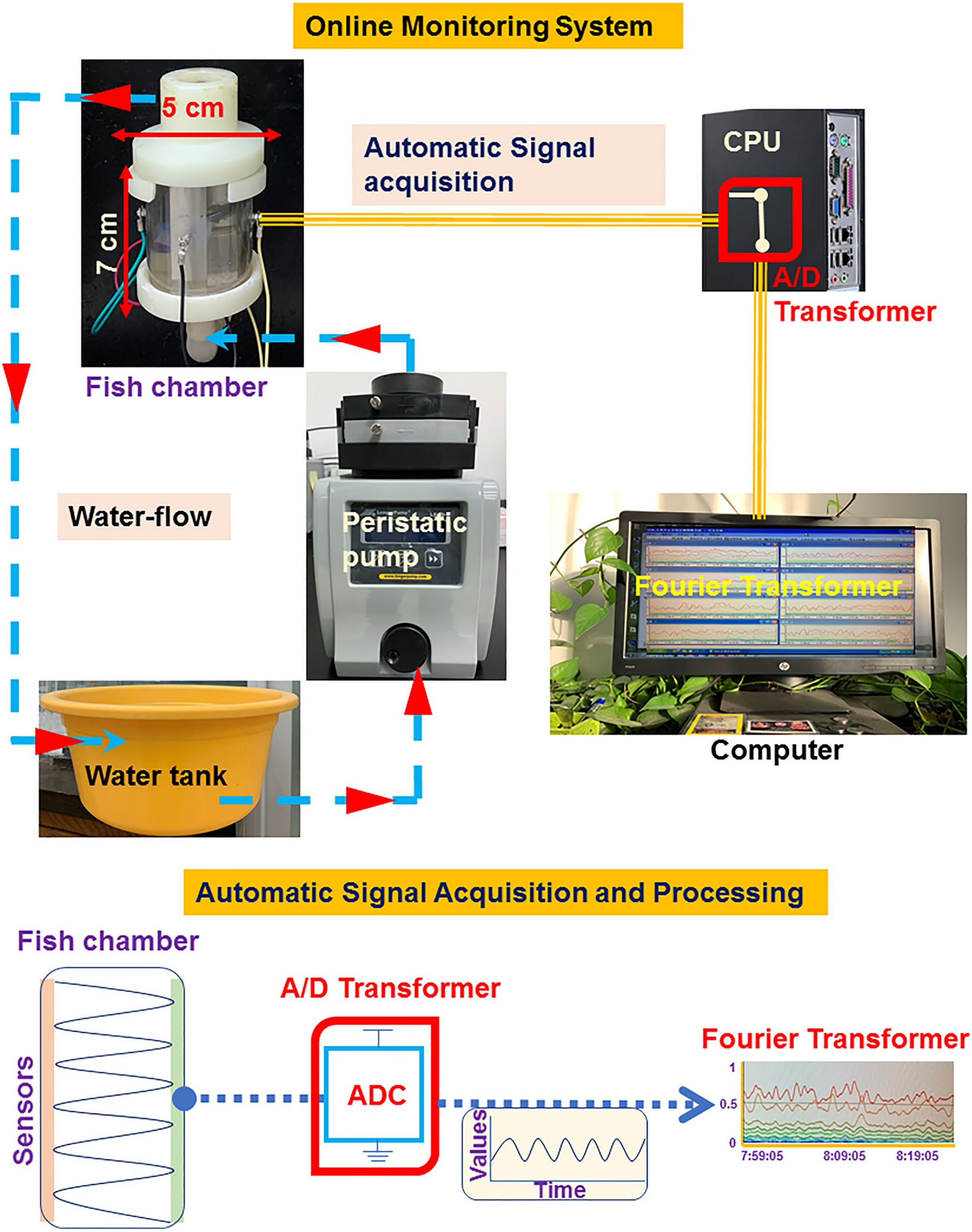


Fig. 1 Water-flow and automatic signal acquisition processes of online monitoring system (OMS)

of every hour was used for all analyses. We used different file format for SOM (text-tab delimited.txt), autocorrelation-MS Office-Excel worksheet-.xlsx) (pls. see Suppl Material.docx).

Circadian differences were calculated by using the formula in MS-Excel worksheet:

$$\text{Time delay D (AVN} - \text{AVD)} = \frac{(\text{AVD} - \text{AVN})}{\text{AVN}} \times 100\%$$

where AVN and AVD are the average value of night and day time, respectively.

Results

We showed real-time swimming behaviour (360 h) of the control and NaOCl treatment (Treatment I, II, III, and IV) groups in Fig. 2. The behaviour strength of the control group was found higher during the daytime and lowered during the night. A series of auxiliary actions have also resulted in the control group during the daytime. The behaviour strength of the NaOCl treatment groups was affected when compared to the control group. We visualised a series of abnormal, readjustment, and undifferentiated actions on the NaOCl treatment groups. However, we visualised a few auxiliary actions in the NaOCl treatment groups. The behaviour strength of the NaOCl Treatment I group is predominantly similar to the control group. Behaviour strength was partially identical

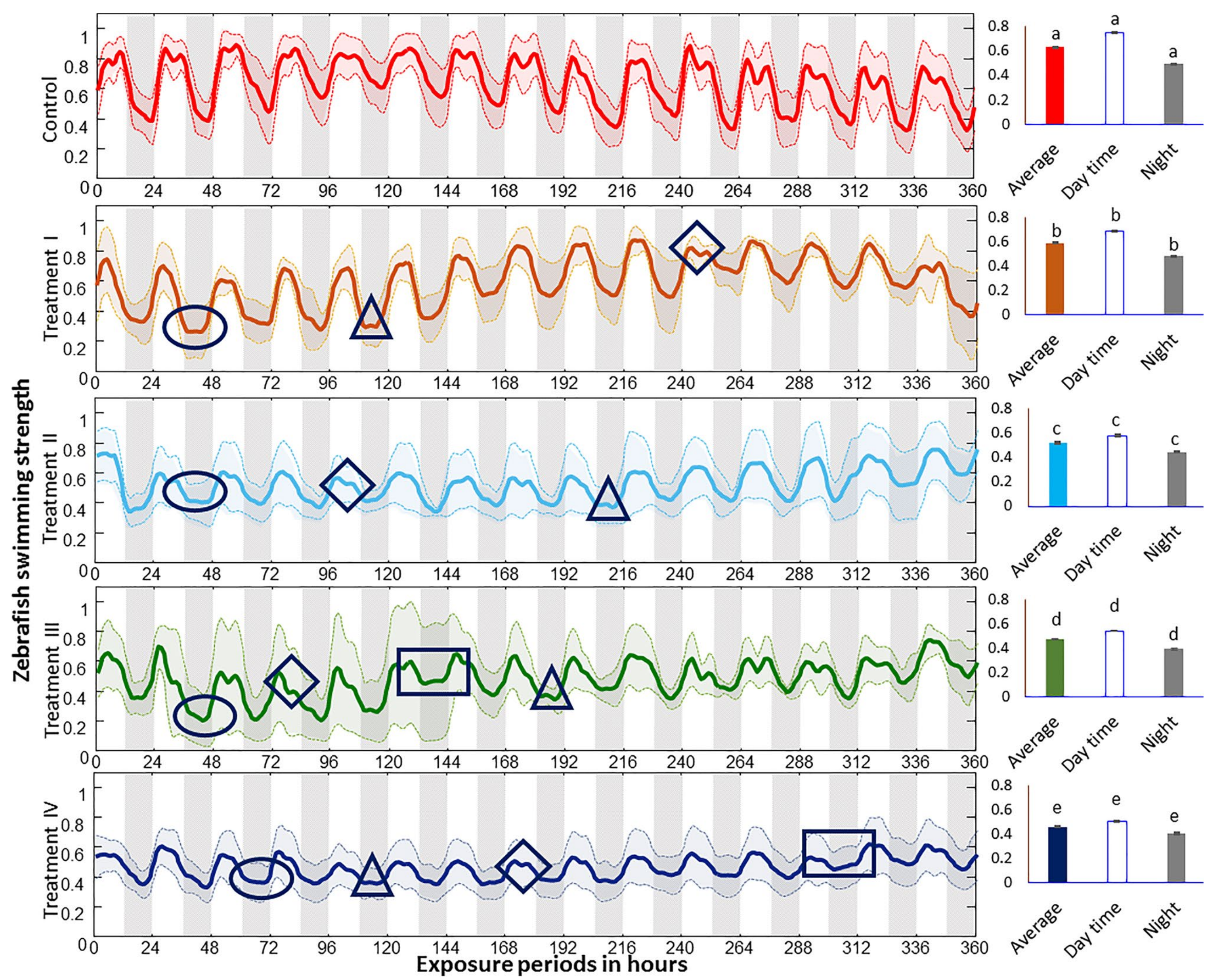
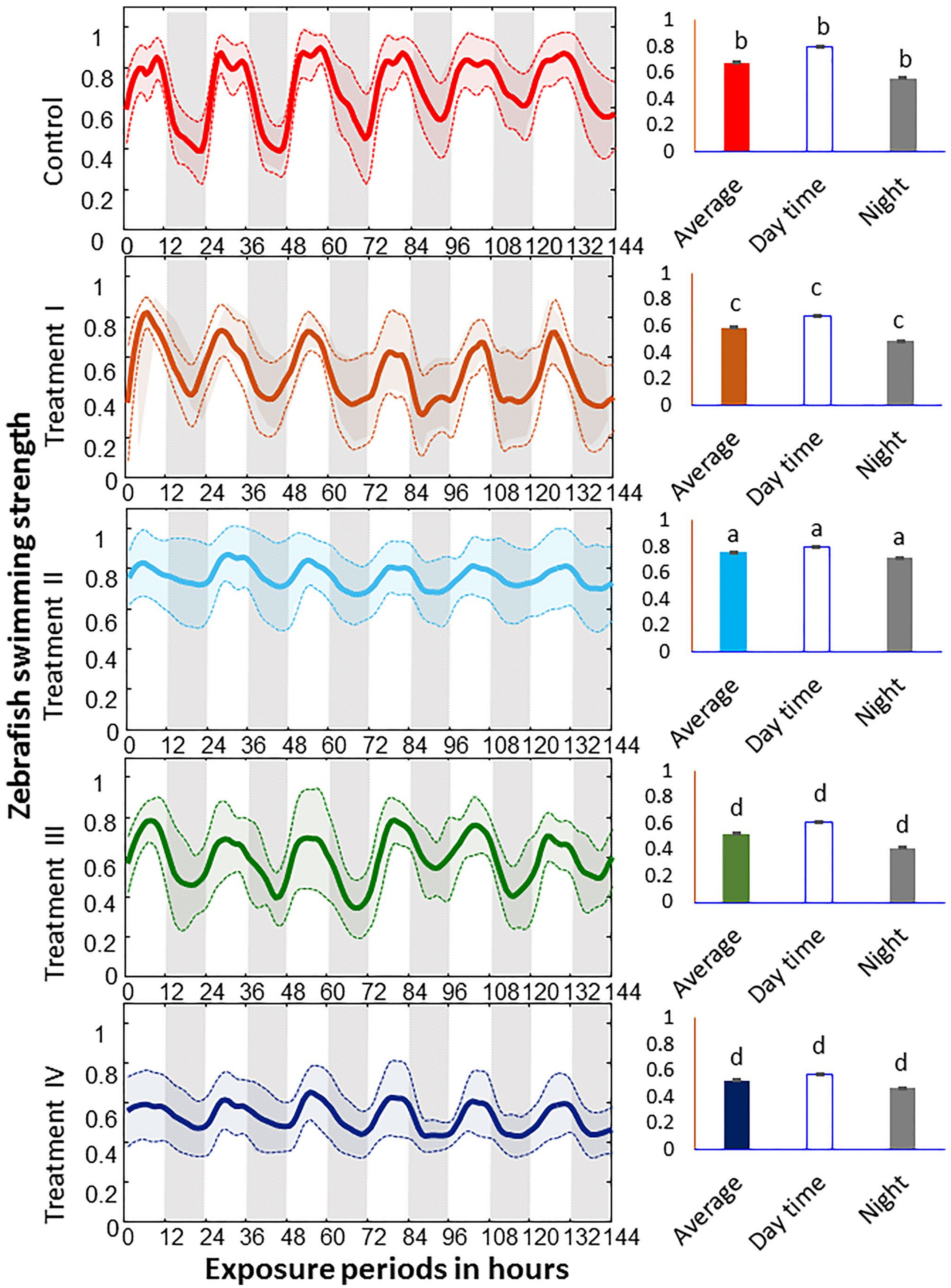


Fig. 2 Real-time swimming behaviour of zebrafish for 360 h of study periods. Solid lines and shaded dotted lines signify real-time swimming strength and S.D., respectively. Night of the study periods represented in shadow bars on the behaviour strength. Bar graphs beside behaviour strength of each group illustrate the differences in the behaviour strength at different photoperiods. Lowercase alphabets above the bars reveal statistical differences (DMRT, $p < 0.001$) among other groups. Symbols on the behaviour strength: oval, diamond, square box, and triangle to represent abnormal, auxiliary, undifferentiated, and readjustment actions


Zebrafish swimming strength
Control
Treatment I
Treatment II
Treatment III
Treatment IV
0 0.2 0.4 0.6 0.8 1
0 12 24 36 48 60 72 84 96 108 120 132 144
Exposure periods in hours
Average
Day time
Night
b b b
c c c
a a a
d d d
d d d

◂**Fig. 3** Recovery responses of zebrafish (144 h). Solid lines and shaded dotted lines signify real-time swimming strength and S.D., respectively. Night of the study periods represented in shadow bars on the behaviour strength. Bars beside behaviour strength of each group represent the differences in the behaviour strength at different photoperiods. Lowercase alphabets above the bars reveal statistical differences (DMRT, $p<0.001$) among other groups

to the control group in both NaOCl Treatment II and III groups. A series of undifferentiated actions have occurred in both Treatment III and IV groups. Among NaOCl treatment groups, behaviour strength affected the Treatment IV group immensely. Differences in behaviour strength of each group at different photoperiods are illustrated in bars. Overall, swimming behaviour declined significantly ($p<0.001$) in NaOCl treatment groups compared to the control group. Bars reveal that zebrafish swimming activity was higher during the daytime when compared to the night.

We illustrated recovery responses of zebrafish for 144 h study periods in Fig. 3. Zebrafish swimming strength improved under-recovery study periods in all NaOCl treatment groups. However, NaOCl Treatment groups (except NaOCl Treatment I) were not similar to the control group. Not many alterations have occurred under control and NaOCl Treatment I exposure compared to 360 h of study periods. When compared among NaOCl treatment groups, zebrafish swimming strength greatly improved under Treatment II. Observed recovery responses were statistically significant ($p<0.001$).

As shown in Table 1, differences in swimming strength were statistically significant ($p<0.001$) among the factors such as groups, days, photoperiods, groups and days, groups and photoperiods, days and photoperiods, and groups and days and photoperiods. Adjusted "*r*" squared for 360 h and 144 h (recovery study) study periods were 7.72% and 7.96%, respectively, which reveal a strong association between the factors.

The light period is 8:00 a.m. to 7:59 p.m. every day, and the dark period is 8:00 p.m. every day to 7:59 a.m. on the next day. Values represent Mean ± S.D for each group.

We tabulated zebrafish swimming differences at the different time-delayed trials in Table 2. Statistical analysis reveals that zebrafish swimming strength was higher during daytime (AVD) in 360 and 144 h exposure periods. The control group AVD maximum percentage change resulted for NaOCl Treatment IV group, at 360 h (32.7%) and 144 h (28%) study periods. A minimum percentage change occurred for NaOCl Treatment I (360 h: 8.7%) and Treatment II (144 h: 0.2%). A maximum AVN percentage change resulted for NaOCl Treatment III (360 h: 20.1%) and Treatment II (144 h: 28.3%) groups. A minimum AVN percentage change occurred for NaOCl Treatment I for 360 (2.8%) and 144 (12.8%) h of study periods. Among the NaOCl treatments, maximum percentage differences between AVN and AVD resulted in the Treatment I group (43.3%) for 360 h of study periods and the Treatment III group (47.8%) for 144 h of study periods. A minimum percentage change occurred for the Treatment IV group (24.9%) and the Treatment II group (11.7%) for 360 and 144 h study periods. Observed behavioural differences for AVD (daytime) and AVN (night) were statistically significant at $p<0.05$.The light period is 8:00 a.m. to 7:59 p.m. every day, and the dark period is 8:00 p.m. every day to 7:59 a.m. on the next day. Values represent Mean ± S.D for each group. [a] Indicates $p < 0.05$ of different treatments compared to the control group.

SOM profiles for the control and different NaOCl treatment groups are illustrated in Fig. 4. Daytime and night of the study periods were differentiated by ordination map, in which the upper zone is night and the lower zone is daytime. Six clusters are classified in this study. We represented each cluster in different colours in the dendrogram. The Ward linkage method calculates the closeness between each cluster. Clusters 2 and 6 represent the daytime of the study periods. And, Clusters 3 and 4 signify the night of the study periods. Figure 4 illustrates SOM profiles for the control, NaOCl Treatment I, II, III, and IV groups. Zebrafish (under 0% NaOCl) swimming activity was higher during the daytime and decreased at night. Similar responses have resulted in NaOCl treatment groups. However, SOM profiles for NaOCl treatment groups did not completely match the control group SOM.

SOM profiles for zebrafish under-recovery study periods are exemplified in Fig. 5. The upper and lower zones of the ordination map signify night and daytime, respectively. We visualised six clusters in the dendrogram. The closeness between clusters 1 and 4 indicates day, and clusters 2 and 3 imply the night of the study periods. We visualised differences and partial matching of swimming strength in this study. SOM profiles for NaOCl treatment groups were predominantly matching with the control group.

We exemplified autocorrelation analysis to determine periodicity changes of zebrafish swimming strength for 360 h and 144 h study periods in Fig. 6. Clear swinging peaks (diurnal pattern) represent that the circadian rhythms of the control group were not affected throughout the study periods. The circadian rhythms were affected under NaOCl treatments (360 h study periods) (Fig. 6a). Swinging peaks for NaOCl treatment groups were not similar to the control group. Nocturnal behaviour resulted in NaOCl treatment periods. We noticed a gradual biphasic trend at minus series (nocturnal) for NaOCl Treatment I group. In other NaOCl treatment groups, peaks at minus series fluctuate recurrently. The control and NaOCl treatment groups under-recovery study (144 h) periods showed clear swinging peaks throughout the study periods, indicating that the circadian rhythm was not affected under normal (recovery) conditions (Fig. 6b).

Table 1 Tests between subjects' effects for NaOCl treatment and recovery groups

Factors	NaOCl treatment		Recovery	
	F value		*F* value	
Groups	359.5	Significance $p < 0.001$ Adjusted ***r*** squared 7.72%	352.0	Significance $p < 0.001$ Adjusted ***r*** squared 7.96%
Days	28.4		1.0	
Photoperiods	2.5		1.1[#]	
Groups and Days	24.6		9.3	
Groups and Photoperiods	67.6		34.9	
Days and Photoperiods	3.8		3.2[*]	
Groups and Days and Photoperiods	1.8		2.6	

[#]Not significant

[*]Significant at $p < 0.05$

Discussion

The environmental spreading of the virus that causes severe acute respiratory syndrome coronavirus (SARS-CoV-2) on humans can be prevented by disinfectants. Thus, regulatory agencies and public health organisations made recommendations to disinfect regularly handling objects and boarding places with household bleach, soaps, alcohol wipes, and handwash (Rivera et al. 2020; Eldeirawi et al. 2021). Among different kinds of disinfectants (alcohols, aldehydes, bases, biguanides, chlorine, glycols, iodophors, metal ions, organic acids, phenolic compounds, surfactants, thiazoles), the use of chlorine-based disinfectants (> 0.5 mg/L residual free chlorine) increased globally during the SARS-CoV-2 pandemic (Christen et al. 2017; Dhama et al. 2021). Furthermore, the recommended levels for chlorine-based disinfectants on medical applications were 500 mg/L (without obvious contamination) and 1000 mg/L (with obvious contamination) (Wang et al. 2020). Thus, the occurrence of disinfectants in the water ecosystem is well predictable (Amorim et al. 2017). A maximum concentration of 2 mg/L of NaOCl was used in WWTP at Junglang (Park et al. 2016). The concentration of NaOCl in the aquatic environment might occur at several hundreds of microlitres. No observed effect concentration and lowest observed effect concentration for chlorine-based disinfectants are 0.002 and 0.004% v/v, respectively (as mentioned in Subpriamaniyam 2021). We chose 0.0025, 0.005, 0.0075, and 0.01% v/v concentration to study the potential ecotoxicity of NaOCl. Disinfectants can persist in the water system (Ton et al. 2012). The occurrence of NaOCl in the aquatic environment might pose health effects on biota (Deere et al. 2020). Hence, different aquatic biological models, such as fish, mussels, planktons, and amphibians, were considered for water quality monitoring. Among these bioindicators, fish are recognised as toxicity models among aquatic organisms because fish are the higher trophic organism, susceptible to environmental changes, biochemical functioning is similar to humans, easy to maintain/handle, and inexpensive. Among fishes, zebrafish are widely used to assess waterborne disinfectants' potential impacts (as mentioned in Ton et al. 2012). In this study, we have chosen zebrafish as an animal model to

Table 2 Comparison of zebrafish swimming strength under NaOCl and recovery Mean values in different photoperiods

Exposure periods	Groups	Parameters				
		AVD	*D* (compared to control) %	AVN	*D* (compared to control) %	*D* (AVN—AVD) %
360 h (8 a.m. of 1st day to 7:59 am of 16st day)	Control	0.75 ± 0.06		0.49 ± 0.07		52.4
	Treatment I	0.68 ± 0.10[a]	8.7	0.48 ± 0.12[a]	2.8	43.3
	Treatment II	0.58 ± 0.05[a]	22.6	0.45 ± 0.06[a]	9.3	30.1
	Treatment III	0.54 ± 0.07[a]	27.7	0.39 ± 0.09[a]	20.1	37.9
	Treatment IV	0.50 ± 0.04[a]	32.7	0.40 ± 0.04[a]	17.8	24.9
144 h (8 a.m. of 1st day to 7:59 am of 7st day)	Control	0.80 ± 0.02		0.56 ± 0.08		43.6
	Recovery I	0.67 ± 0.03[a]	15.8	0.48 ± 0.06[a]	12.8	38.8
	Recovery II	0.80 ± 0.02[a]	0.2	0.71 ± 0.02[a]	28.3	11.7
	Recovery III	0.61 ± 0.05[a]	23.4	0.41 ± 0.05[a]	25.6	47.8
	Recovery IV	0.57 ± 0.01[a]	28.2	0.47 ± 0.02[a]	16.1	23.0

The light period is 8:00 a.m. to 7:59 p.m. every day, and the dark period is 8:00 p.m. every day to 7:59 a.m. on the next day. Values represent Mean ± S.D for each group. Indicates p<0.05 of different treatments compared to the control group assess the toxic effects of NaOCl on non-target organisms. Exposure to waterborne contaminants affects fish swimming behaviour even at lower concentrations (μg/L) (Brodin et al. 2013, 2017). Concentration-dependent behaviour anomalies resulted in zebrafish under NaOCl treatments. Magalhaes et al. (2007) reported similar behaviour anomalies on zebrafish under NaOCl treatments, where the swimming activity decreased with increasing NaOCl concentration. In our previous studies, we also noticed a correlation between declined swimming activity of zebrafish with chemical concentrations (Zhao et al. 2020; Poopal et al. 2021; Ren et al. 2021). Declined swimming strength under (360 h) NaOCl treatments reveals the avoidance behaviour of zebrafish. Avoidance or escaping sign is a common response of fish to reveal stress in its environment. Continuous exposure to environmental stressors could affect the normal behavioural activity of fish; hyperactivity or hypoactivity is a sign of avoiding stress (Hong and Zha 2019). The fractal dimension of zebrafish swimming velocity increased under NaOCl-based aqueous solutions (0 to 0.005% v/v) treatments (Nimkerdphol and Nakagawa 2008). The authors also noticed that water quality parameters increased zebrafish swimming trajectories under NaOCl treatments.

We visualised a series of abnormal, auxiliary, undifferentiated, and readjustment actions under NaOCl treatments. Normally, fish swimming activity requires higher energy consumption. Under continuous environmental stress conditions, fish might cease swimming to conserve energy to cope with the stress, which could decline the swimming strength; thus, we can visualise abnormal behaviour strength. The term abnormal (Latin *abnormis*) means deviating from the normal. A series of abnormal actions were shown under NaOCl exposure periods, indicating that zebrafish sensed NaOCl toxicity and might activate its coping mechanisms against the toxicity. Acclimatisation is a typical survival strategy for fish under tolerable stress conditions. During acclimatisation, fish might undergo a series of adjustments. The occurrence of adjustments on fish purely depends on the strength of the stressors. The term auxiliary actions mean the occurrence of additional actions (adjustments). The occurrence of auxiliary actions during the daytime signifies acclimatised state of zebrafish to their current environment. Adjustment is the state of being adjusted to the current environmental change. Readjustment is adapting oneself again to the current environmental change. The occurrence of readjustment actions at night under NaOCl treatments indicates internal stress (the activation of detoxification and ejecting mechanisms). Under normal or tolerable stress conditions, the behavioural strength could be higher in the daytime and lower at night; this results in *zig-zag* patterns. When the

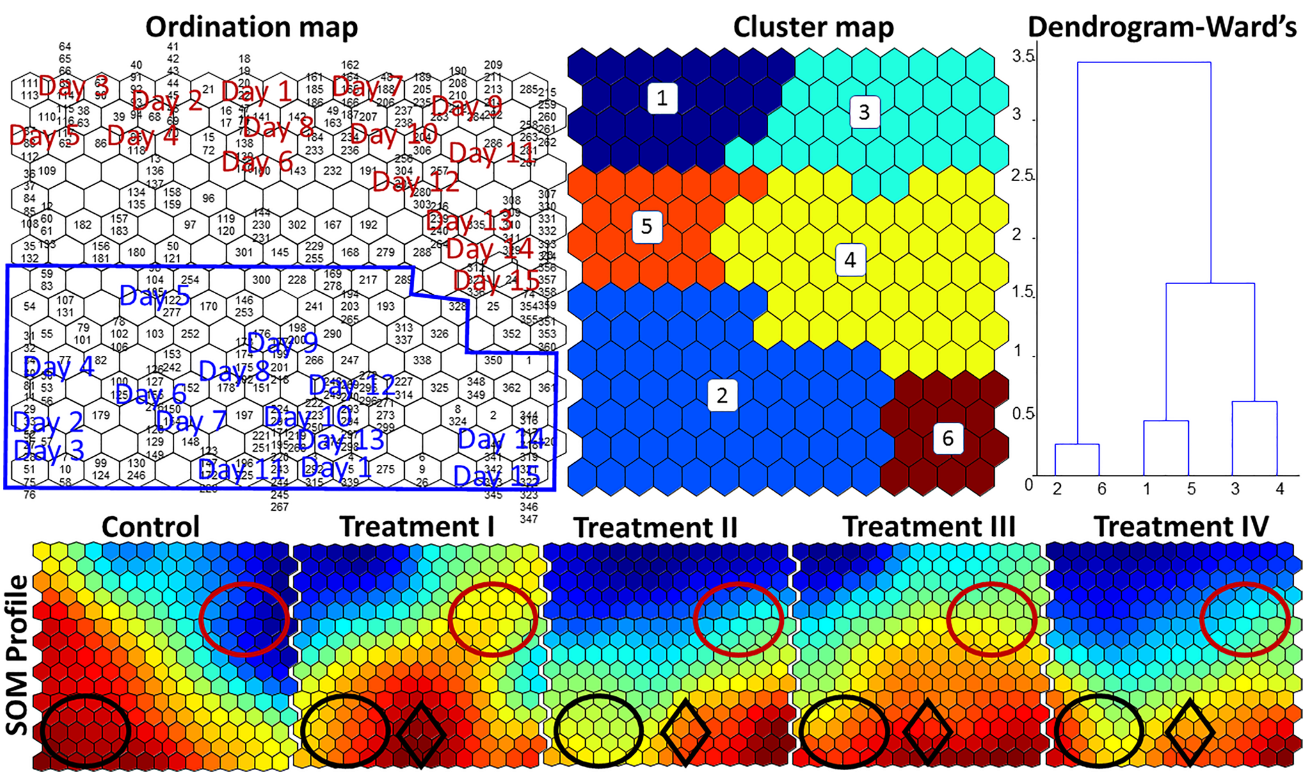


Fig. 4 SOM analysis for zebrafish swimming strength under 360 h study period. Ordination map-blue colour text and red colour text represents daytime and night of the study. SOM profiles—dark blue to dark red colour indicates lower to higher swimming strength. The symbol ring (red—night, black—daytime) and the diamond represent differences and partial matching in swimming strength

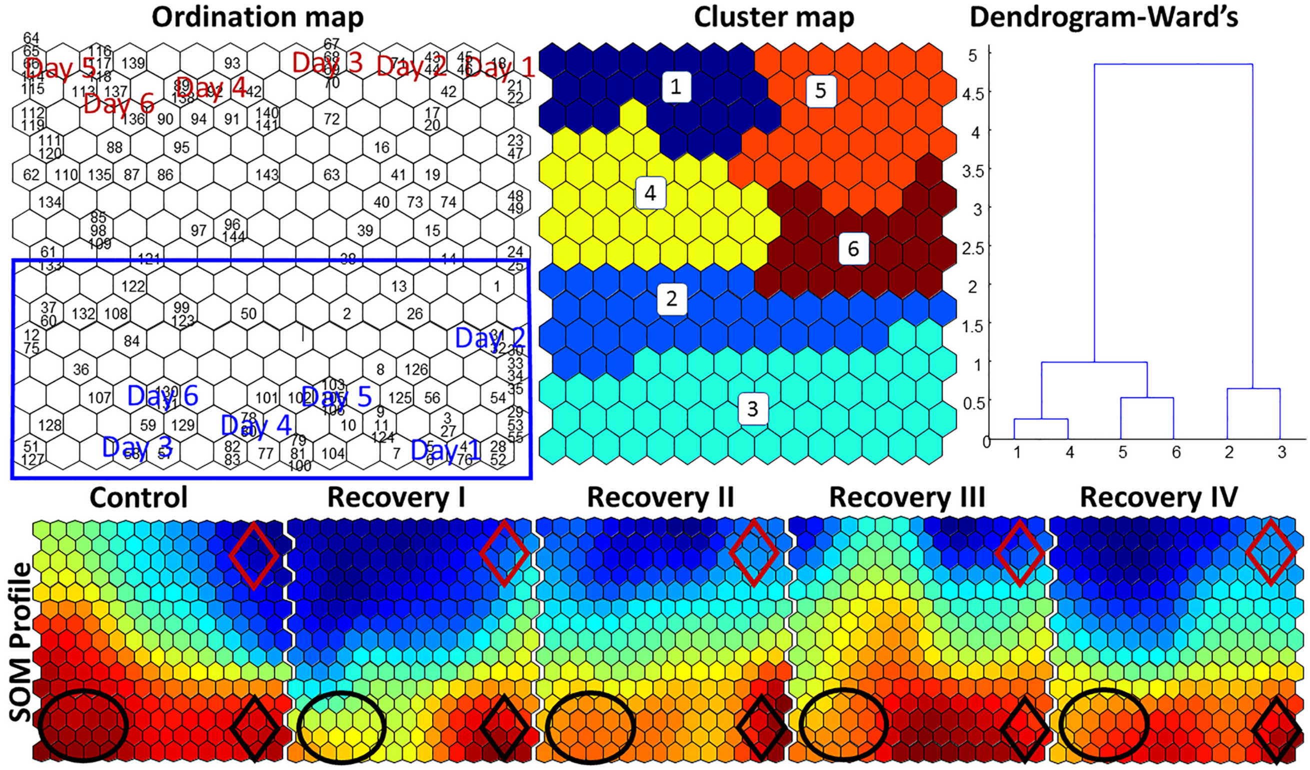


Fig. 5 SOM analysis for zebrafish swimming strength under 144 h (recovery) study period. Ordination map—blue colour text and red colour text represent daytime and night of the study. SOM profiles—dark blue to dark red colour indicates lower to higher swimming strength. The symbol ring and the diamond (red—night, black—daytime) represent differences and partial matching in swimming strength

stress overwhelms the tolerance capacity of fish, the behaviour strength could be unspecifiable, not able to differentiate the action. We witnessed a series of undifferentiated behaviour strengths under higher concentrations of NaOCl (Treatment III and IV). The undifferentiated area on real-time data reveals that zebrafish's coping capacity was overwhelmed by NaOCl stress at higher concentrations.

Swimming performance is essential for survival for fish; chemical exposure could affect ion homeostasis, resulting in behavioural changes in fish (as mentioned in Goulding et al. 2013). NaOCl forms hydrogen cations and hypochlorite ions in the aquatic ecosystem, which could damage the membrane potential of cells and result in disruption of biochemical (including enzymes) and ion homeostasis mechanisms (Lopez-Galindo et al. 2010a,b). NaOCl entry through gill tissues might affect the tissue morphology, resulting in disruption of routine metabolism (ion-regulation mechanism, respiration, and excretion) of zebrafish; thus, alteration in swimming behaviour might occur. Exposure to commonly used disinfectants can generate reactive oxygen species in tissues and affect the antioxidant defence system of fish. Additionally, disinfectants can affect the biochemical and hormonal functioning of fish (Elia et al. 2006; Kim and Ji 2019). Swimming activity depends on the energy metabolism (biochemical activities) of an organism. The decline of swimming strength might result from internal stress (imbalanced energy metabolism of zebrafish) caused by NaOCl. Painter et al. (2009) reported that waterborne chemicals could impair nervous cell signalling by binding or blocking synaptic receptors that could affect nerve impulse communication transmission; as a result, behaviour anomalies (declined behavioural performance) could occur on organisms. NaOCl treatment inhibited acetylcholine activity on *Mytilus galloprovincialis* under long-term exposure (Lopez-Galindo et al. 2010a). Thus, a decline in swimming activity indicates the neuro-toxic effect of NaOCl on zebrafish. However, internal stress (histological, biochemical, and hormonal changes) could recover considerably under normal conditions (without environmental stressors). It is shown through the recovery response of zebrafish. Zebrafish swimming strength increased after reintroducing to normal conditions (water without NaOCl). A concentration-dependent improvement occurred in zebrafish swimming strength under-recovery periods. A similar result was observed on zebrafish under chemical treatments (Poopal et al. 2021).

Online biological behaviour monitoring systems are considered an important tool in early warning pollution assessments (Magalhaes et al. 2007). Noticeably, the observed behavioural anomalies have occurred at lower concentrations

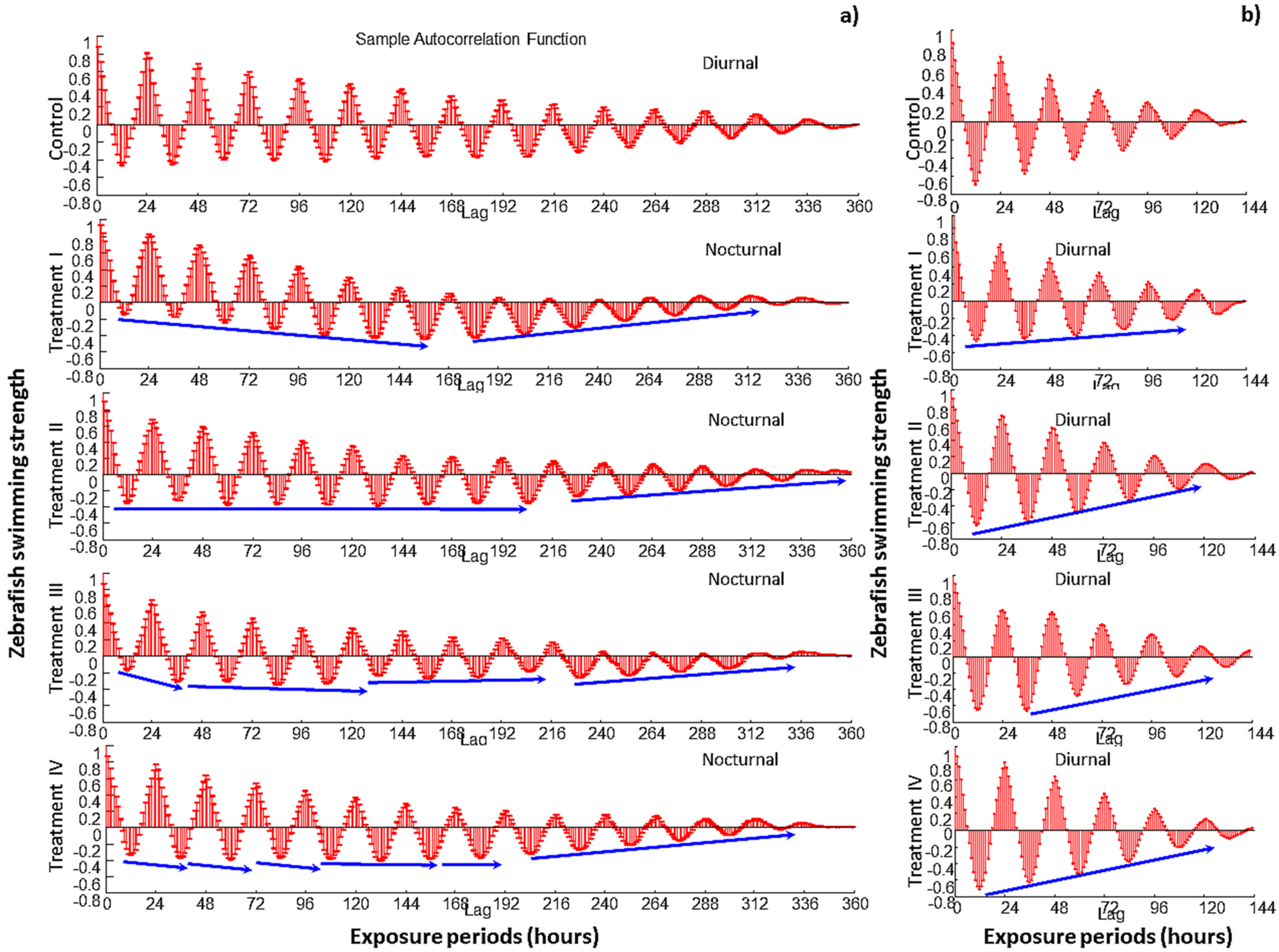


Fig. 6 Autocorrelation analysis. Periodicity changes occurred on zebrafish swimming strength at **a**) 360 h and **b**) 144 h study periods. Arrows specify continuity of behaviour strength at minus series

(even at 0.002% v/v) of NaOCl treatments when compared to the concentration (0.1 mg/L) reported by Lopez-Galindo et al. (2010b) to cause histological, enzymological, and antioxidants changes on *Solea senegalensis*. A considerable amount of data was generated in this study using OMS, and the complexity in the data was solved by SOM. Changes in the behaviour activity of zebrafish were visualised on SOM. SOM differentiates the photoperiods of the study. We further analysed the data to determine changes in a circadian rhythm by autocorrelation analysis using MATLAB. Circadian rhythms are a fundamental behavioural trait and an essential factor governing organisms' physiology and behavioural activities (Melvin 2017; Zheng et al. 2021). Circadian rhythms have an essential role in sleep cycles, hormone secretion, blood pressure, and other routine life processes. Zebrafish is a good model organism for circadian rhythms studies (Doria et al. 2018; Yang et al. 2019). The circadian rhythms are endogenously driven, controlled by genes. Waterborne chemicals can affect circadian genes (Liang et al. 2019). Shi et al. (2019) reported that transcriptional alteration of circadian rhythm genes on zebrafish could occur even at nanogram concentration of chemicals. Waterborne chemicals induce the circadian rhythms of fish through neuroendocrine pathways (Melvin 2017). Zhao et al. (2018) noticed a correlation between locomotory behaviour changes and disruption in clock genes of fish. Biocide exposure enhanced the circadian gene (*clock 1a*) expression, which resulted in behavioural changes in zebrafish (Yang et al. 2019). Changes in circadian rhythms reflect the adaptation of an organism. Circadian rhythms of NaOCl treatment groups were affected under 360 h study periods. This indicates that zebrafish recognised stressors and initiated their adaptation mechanisms. Circadian rhythms regulate immune processes in an organism (Ren et al. 2018), which can be adjusted due to chemical-mediated changes in internal homeostasis towards adaptation (Yang et al. 2018). Cellular free radicals and the circadian clock are interconnected; thus, alterations in antioxidants' defence mechanisms result in fish circadian disruption (Zheng et al. 2021). NaOCl has the potential to cross-link fibrinogen and affect blood plasma antioxidants defence mechanisms (immunostimulatory properties) (Manucat-Tan et al. 2021). Bao et al. (2019) reported that waterborne contaminants (perfluorooctane sulfonate) affect networking genes (hypothalamus-pituitary–gonadal-liver axis) of female zebrafish. Biotransformation and detoxification processes mainly occur in the liver tissue; exposure to NaOCl might accelerate the formation of free radicals in the liver tissue of zebrafish. These free radicals might distribute through blood and cause an oxidative imbalance in zebrafish. Thus, circadian rhythm adjustments might result from internal stress (the antioxidant homeostasis).

In summary, under closed conditions, swimming behaviour could be an ideal indicator for ecotoxicological studies. The real-time swimming behaviour of fish can be quantified efficiently using OMS. We can analyse the OMS dataset for statistical differences using MATLAB and SPSS software. Zebrafish are highly sensitive to NaOCl toxicity, even at lower concentrations. The NaOCl is potentially a toxic substance at studied concentrations. A concentration-dependent toxic effect has resulted in this study. However, the NaOCl-mediated toxic effects are reversible (based on concentration) even at higher concentrations.

Conclusion

We exemplified the toxicity of NaOCl by monitoring (online) the swimming behaviour of zebrafish. The swimming strength of zebrafish was quantified efficiently using OMS. NaOCl induced behaviour anomalies on zebrafish even at lower concentrations (0.0025% v/v). We observed avoidance behaviour under 360 h of NaOCl exposure periods. Escaping or avoidance behaviour is not only stimulus–response but also reflects fitness of fish. Under prolonged toxicant exposure periods, avoidance behaviour might be overwhelmed by internal stress (morphological and biochemical changes), which could affect the fitness of fish. In that aspect, continuous contamination of NaOCl is a severe threat to aquatic biota. Circadian rhythm of zebrafish was affected under 360 h NaOCl treatment periods; this is also a risk to organisms because adjustments in endogenous timing mechanism can affect organisms' adaptive capacity. The recovery responses (improvement in zebrafish swimming strength) reveal that the resulting behaviour anomalies under 360 h of study periods are based on stressor effects. This study highlighted the importance of behaviour toxicology towards water quality assessments. This study also signifies that swimming behaviour is a non-invasive biomarker in early warning signal approaches for toxicity studies. More biological behaviour studies are warranted to assess eco-toxic effects of emerging and unintentional or accidental pollutants. Future scope: Mathematical modelling based on the OMS dataset will be a promising method for eco-toxicity prediction assessments.

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Data availability Online monitoring dataset-supplementary file (data original.xlsx). Guidance for SOM and autocorrelation analysis (Suppl Material.docx).

Declarations

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