Effects of conventionally-treated and ozonated wastewater on mortality, physiology, body length, and behavior of embryonic and larval zebrafish (*Danio rerio*)

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Abstract

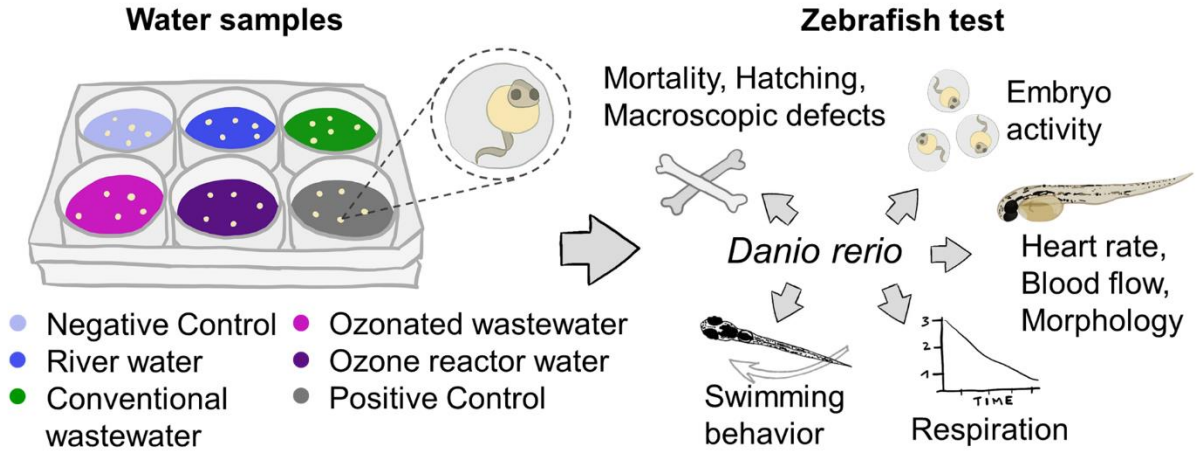
To date, micropollutants from anthropogenic sources cannot be completely removed from effluents of wastewater treatment plants and therefore enter freshwater systems, where they may impose adverse effects on aquatic organisms, for example, on fish. Advanced treatment such as ozonation aims to reduce micropollutants in wastewater effluents and, thus, to mitigate adverse effects on the environment. To investigate the impact and efficiency of ozonation, four different water types were tested: ozonated wastewater (before and after biological treatment), conventionally-treated wastewater, and water from a river (River Ruhr, Germany) upstream of the wastewater treatment plant effluent. Zebrafish (*Danio rerio*) embryos were used to study lethal and sublethal effects in a modified fish early life-stage test. Mortality occurred during exposure in the water samples from the wastewater treatment plant and the river in the first 24 hours post-fertilization, ranging from 12% (conventional wastewater) to 40% (river water). Regarding sublethal endpoints, effects compared to the negative control resulted in significantly higher heart rates (ozonated wastewater), and significantly reduced swimming activity (highly significant in ozonated wastewater and ozone reactor water, significant in only the last time interval in river water). Moreover, the respiration rates were highly increased in both ozonated wastewater samples in comparison to the negative control. Significant differences between the ozonated wastewater samples occurred in the embryonic behavior and heart rates, emphasizing the importance of subsequent biological treatment of the ozonated wastewater. Only the conventionally treated wastewater sample did not elicit negative responses in zebrafish, indicating that the discharge of conventional wastewater poses no greater risk to embryonic and larval zebrafish than water from the river Ruhr itself. The sublethal endpoints embryonic- and larval behavior, heart rates, and respiration were found to be the most sensitive endpoints in this fish early life-stage test and can add valuable information on the toxicity of environmental samples.

Keywords: wastewater treatment, fourth purification stage, ozonation, fish, sublethal effects

Highlights

- Conventionally treated wastewater showed no adverse effects on *Danio rerio*
- No detoxification effect of wastewater following ozonation was detected
- Background toxicity in the river should be considered when evaluating WWTP effluents
- Sublethal effects are the most sensitive endpoints in the fish early life-stage test

Graphical abstract



Abbreviations: WWTP: wastewater treatment plant; FET: fish early life-stage test; hpf: hours post fertilization; RW: river water; CW: conventionally treated wastewater; OW: ozonated wastewater; OR: ozone reactor water; NegC: negative control; PosC: positive control; SAC: spectral absorption coefficient; NWU: North-West University; BPM: heartbeats per minute; L: light phase; D: dark phase; Acc.: acclimatization phase

1. Introduction

Micropollutants are organic substances (e.g., pharmaceuticals, pesticides, daily-care products) usually present at low concentrations in water (in the range of pg/L to µg/L, Loos et al., 2013), but which nevertheless may have adverse effects on the biological integrity of ecosystems (Englert et al., 2013). Despite recent advances in pollution treatment, wastewater treatment plants (WWTPs) cannot fully remove anthropogenic pollutants from the wastewater and concerns have increased about possible impacts of these substances on the rivers receiving WWTP effluents. Studies have already shown that micropollutants can lead to feminization of fish (Schwarzenbach et al., 2006), alter their abundance and species structure (Mccallum et al., 2019), have adverse effects on macroinvertebrate populations (Bunzel et al., 2013; Englert et al., 2013; Stalter et al., 2013) and affect feeding rates of freshwater crustaceans (Bundschuh et al., 2011a, 2011b; Bundschuh and Schulz, 2011). This has led to a growing demand for more efficient wastewater treatment (e.g. in Germany, Switzerland, and Japan; Loeb et al., 2012) resulting in the increase of research on techniques that can enhance purification processes in wastewater treatment (Völker et al., 2019). Amongst others, these include adsorption techniques using activated carbon, membrane techniques, and advanced oxidation processes such as ozonation.

Ozonation is an oxidation technique that is used in more than 100 WWTPs globally since the first ozonation reactor was put into operation in a WWTP in Oita, Japan in 1988 (Loeb et al., 2012). Ozone (O_3) is applied in gaseous form and immediately reacts with substances present in the water. Ozonation of organic materials occurs in two different ways: either directly by reaction of ozone with structural moieties or indirectly by the formation of highly reactive hydroxyl radicals (Huber et al., 2005). Ozonation can lead to the complete mineralization of the compounds or; which is the most frequent process under economically appropriate operating conditions, to degradation into so-called transformation products (Magdeburg et al., 2014; Schmidt et al, 2011b). Some transformation products have the potential to be more toxic than their parent compounds and therefore might also have negative effects on ecosystems,

which increases concerns about emerging contaminants in water (Knoop et al., 2018; Schmidt and Brauch, 2008; Trogolo et al., 2015). However, only a small proportion of transformation products has yet been identified (Ikehata, 2006; Völker et al., 2019) and tested for possible toxicological effects (e.g. Knoop et al., 2018; Luster-Teasley et al., 2002, 2005; Rosal et al., 2009; Shang et al., 2006; Soltermann et al., 2017; Yan et al., 2014). Since the chemical structure of many transformation products is still unknown their toxicological potential cannot be determined sufficiently. Moreover, even if substances can be identified in wastewater they can often only explain parts of the effects found in corresponding bioassays (Blackwell et al., 2019; Neale et al., 2017; Völker et al., 2019). In the present study, a holistic approach was chosen to include unknown substances as well as transformation products and mixture effects and to allow the determination of the actual toxicity of the investigated water samples. Ozonated wastewater as compared to conventional wastewater treatment was tested for possible biological effects using a test setup based on the Fish Embryo Acute Toxicity Test (FET, OECD 236), with some additional analyses. Since the classical FET with the endpoints mortality and hatching rate has been identified as not sensitive enough to detect effects of micropollutants (Völker et al., 2019, Wigh et al., 2016, 2018), non-standardized endpoints such as sublethal parameters (e.g. heart rate) and swimming behaviour as endpoints of toxicity were added to the test design (see also Pohl et al., 2018; Thellmann et al., 2015, Zindler et al., 2019).

Accordingly, the aims of the present study were i) to compare the effects of conventionally treated and ozonated wastewater versus river water and a negative control with respect to mortality, physiology, body length, and behavior of embryonic/juvenile zebrafish; and ii) to evaluate the sensitivity and suitability of non-standardized endpoints of different developmental stages of juvenile zebrafish for wastewaters in addition to established endpoints described in the FET (OECD, 2013).

2. Materials and methods

2.1. Wastewater treatment plant

Water samples from different locations on the municipal WWTP Schwerte (North Rhine-Westphalia, Germany) were collected and investigated for possible toxic effects. The WWTP Schwerte receives wastewater from 50,000 population equivalents. It consists of mechanical and biological treatment steps as well as nutrient removal (nitrification and simultaneous denitrification stage for nitrogen and simultaneous chemical precipitation with $FeCl_2$ and lime for phosphorus). The biological treatment comprises two watercourses with one aeration tank for biological treatment and one clarifier each, that are operated simultaneously (Fig. 1). Parallel to the conventional wastewater treatment, the second watercourse can be operated with an ozonation step allowing for direct comparison of conventional and ozonated wastewater qualities. Ozonation is performed via dynamic recirculation (Fig. 1). Accordingly, 50 L per second of the treated wastewater from the clarifier is transferred into an ozone reactor, where the water is ozonated with approximately 5 mg O_3 per liter water injected via diffusers. The ozone is dosed dynamically according to the hydraulic inflow conditions and the relative decrease in absorbance measured before and after ozonation using the spectral absorption coefficient at a wavelength of 254 nm (SAC_{254}). Subsequently, the ozone-treated water is degassed in a second reactor and is then pumped back into the aeration tanks for further biological processing (Grünebaum, 2011; Schmidt et al., 2011a).

2.2. Water sampling

Water samples from the river Ruhr, as well as water samples from the WWTP Schwerte, were collected on 17.10.2019 in 1 L borosilicate bottles (VWR International GmbH, Darmstadt, Germany). The sampling points for the water samples from the conventional (CW) and ozonated wastewater (OW) were located directly before the wastewater discharge into the river Ruhr. The water from the ozone reactor (OR) was taken prior to the outlet into the aeration tanks, so the freshly ozonated wastewater did not have contact with microorganisms from the activated sludge treatment. The water sample for the river water assessment (RW) was taken 150 m upstream of the wastewater discharge (51°25'54.7"N 7°34'00.8"E) to exclude influence from the WWTP Schwerte.

142 The samples were transported in cooling boxes to the laboratory of University Duisburg-Essen,
143 filtered (Whatman Membrane Filters, 0.45 µm, ME 25/21, Diameter 47 mm, GE Healthcare
144 Life Sciences United Kingdom, Buckinghamshire) and subsequently frozen at -20°C prior to
145 use for approximately four months. For the toxicity tests, the samples were defrosted at 4°C
146 and an aliquot of 500 mL was transported on ice to the National Aquatic Bioassay Facility
147 laboratories at North-West University, South Africa. Following the 17-hour transport period the
148 samples were aerated for 12 hours at 27°C prior to commencement of the FET assays. It is
149 acknowledged that the handling of the samples in the described manner may influence the
150 toxicity due to altered chemical composition of the samples.

151 *2.3. Zebrafish husbandry and breeding*

152 The zebrafish, *Danio rerio*, was used as test organism since it is an established model
153 organism in environmental toxicology (Dai et al., 2013) and is a suitable organism for
154 behavioral testing (Ahmad et al., 2012; Botha et al., 2019; Kalueff et al., 2016). This species is
155 sensitive to a wide range of contaminants, (e.g. endocrine disruptors, heavy metals, and
156 organic pollutants, Dai et al., 2013) and is, therefore, an ideal candidate to indicate possible
157 adverse effects of micropollutant mixtures within differently treated wastewater qualities. All
158 procedures involving animals were approved by the AnimCare animal research ethics
159 committee (NHREC reg. number AREC-130913-015) of the North-West University, South
160 Africa. All animals were maintained and procedures performed in accordance with the code of
161 ethics in research, training, and testing of drugs in South Africa and complied with national
162 legislation (ethics approval number: NWU- 00269-16-A5). For this study, the exposures were
163 based on outlines as described in the FET test (OECD 236, 2013), with some modifications.
164 In addition to the FET endpoints survival and hatching, the sublethal endpoints embryonic
165 behavior, respiration, body length, heartbeats, blood flow, and larval swimming behavior were
166 investigated as proposed e.g. by Mandic et al. (2020); Pohl et al. (2018, 2019); Thellmann et
167 al. (2015); and Zindler et al. (2019).

Adult fish were kept at a 12/12-hour light/dark cycle at a constant incubation temperature of 27°C in a multi-linking ZebTec housing system (Tecniplast, Italy). Fish were fed 20 mg of ZM-400 granules (Protein 58.0%, Oil 14.5%, Ash 11.5%, Moisture 7.0%. vitamin A 30,000 I.U./kg, vitamin D3 2,500 I.U./kg, vitamin E 400 mg/kg, vitamin C 2,000 mg/kg, omega-3 fatty acids 30 mg/g dry weight) (ZM Systems, United Kingdom) twice per day using a Tritone Automatic Feeder.

Long-fin wild-type adult *D. rerio* were bred according to Standard Operating Procedures within the National Aquatic Bioassay Facility (Potchefstroom, South Africa). Fish were not fed the day prior to spawning and were separated into males and females the afternoon before induction of the mass spawning event. A total of 14 males and 22 females were placed into a breeding tank (ISPAWN Zebrafish, Tecniplast, Italy), female fish were placed at the bottom, male fish separated with a separation net at the top. The fish were kept in the dark until the next morning. To induce spawning the air stones and the separator were removed in complete darkness and 10 minutes later the breeding basket was lifted halfway and the lights were switched on. After another 10 min, the net was gradually lifted further until there was only one cm of water left and the fish started exhibiting mating behavior. The net was left in this position for 10 minutes until eggs could no longer be seen dropping. The adult fish were lowered halfway down twice in ten-minute steps for acclimatization and then were removed and placed back into the housing tanks.

Eggs were left in the breeding tank for approximately four hours before collection (Cassar et al., 2019; Horzmann et al., 2020; Kumar et al., 2020; Martinez et al., 2019) and then extracted using the bottom valve of the ISPAWN. Fertilized eggs in similar cell stages were identified under the stereomicroscope (Zeiss, Germany), placed in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$•$2H_20$, 0.33 mM $MgSO_4$•$7H_2O$), rinsed, and subsequently used for the fish embryo test.

2.4. Fish embryo study

The water samples (aerated overnight at 27°C to ensure sufficient oxygen levels) were tested in a static non-renewal test (Nathan and Scobell, 1993) to ensure minimal disturbance of both the chemical composition of the exposure water and the test organisms. Accordingly, there was no pH-adjustment prior to the test as well as no exchange of test solutions and no aeration of the waters during the test. Water parameters were assessed prior to the start of the test using a handheld Eutech pH 110 RS232C meter, Eutech CON 110 RS232C conductivity, and total dissolved solids meter, and Eutech DO6 DO dissolved oxygen meter, and then the samples were placed into an incubator at 27°C to ensure the waters were tempered before the insertion of the embryos. Six-well plates (Corning® Costar®, Sigma Alrich Merck, Darmstadt, Germany) were filled with 5 mL of sample per well (n=5 replicates per water sample). In addition to the four different water treatment exposure groups, a negative control (E3 medium; n=5 replicates per water sample) and positive control (4.0 mg/L 3,4-dichloroaniline; n=4 replicates per water sample) was added to the test array. The pre-sorted embryos (confirmation of fertilization and similar cell-stages) were inserted into the prepared six-well plates using five embryos per well. The six-well plates were sealed with self-adhesive, oxygen-permeable sealing film (BRAND®, Sigma Aldrich, Merck, Darmstadt, Germany) and were randomly placed in the incubator at 27°C.

All fish embryos were checked daily for endpoints defined by the OECD guideline 236 (OECD, 2013). Lethal endpoints were: (i) coagulation of fertilized embryos, (ii) lack of somite formation, (iii) non-detachment of the tail, and (iv) lack of heartbeat. Coagulated embryos were removed from the wells immediately after checking. Additionally, the fish embryos were examined daily for the following macroscopic defects: heart edema, deformations of the spine, non-development of the eyes, general developmental retardation, and lack of blood circulation or pigmentation (similar to the study by Wigh et al., 2018). The temperature in the examination room containing the stereomicroscopes and test equipment was kept at 27°C.

2.4.1 Behavioral, physiological, and morphological endpoints

For embryo activity, ten embryos per treatment group were selected randomly and recorded for 1.5 min using a stereomicroscope, a remote-controlled microscope camera, and a monitor. Embryo activity tests were performed 24 h post-fertilization (hpf). Embryo activity was assessed in bursts, i.e. spontaneous movements of the embryo. The burst activity (%) was considered the percentage of time from the total measurement duration the embryo was moving. Videos were assessed using DanioScope V1 Software (Noldus Information Technology, Wageningen, Netherlands, 2019).

Heart rate and blood flow were assessed 72 hpf using the same procedure as described above. For the heart rate assessment, usually, ten fish embryos per group were recorded under a stereomicroscope (Zeiss, Germany) using 3x magnification and a recording time of one minute. For blood flow, seven fish per group were transferred to a wetted microscope slide and recorded for 30 seconds using a 10x magnification. The body length was measured from the head to the tip of the tail for all recorded fish at 72 hpf (when larvae were relatively inactive), using the footage recorded for the heart rate assessment. Videos were assessed using DanioScope V1 Software (Noldus Information Technology, Wageningen, Netherlands, 2019).

The evaluation of the swimming behavior was performed at 120 hpf (when larvae were more active) in the DanioVision System and videos were assessed using EthoVision XT15 (Noldus Information Technology, Wageningen, Netherlands, 2019). All fish larvae of each of the treatment replicate groups, excluding obviously affected fish larvae (dead/malformed), were transferred into a common well, enabling complete randomization. Then, 12 fish per treatment group were transferred to a 12-well plate, one fish per well, and the swimming behavior was recorded during a sequence of light (L1 and L2) and dark (D1 and D2) phases after an initial 15 min acclimatization phase (Acc.) in the dark (Acc. 15 min → L1, 10 min→ D1, 10 min → L2, 10 min → D2, 5 min). The distance moved over time for each individual larvae was analyzed subsequently using GraphPad Prism (GraphPad Software, San Diego, USA, 2017).

2.4.2. Respiration assay

For the exposure of the embryos for the respiration assay, five embryos per well were transferred into six-well plates. Each treatment (six treatments in total, i.e. four water samples plus negative and positive control) consisted of three replicate wells (i.e. five fish embryos x three wells). The six-well plates were randomly placed in an incubator and exposed at 27°C.

Respiration tests were performed 24 hpf. The embryos from the different treatment groups were transferred into a 12-well respiration Microplate system (Loligo Systems, Denmark) filled with 80 µL E3 media after being washed three times with E3 media. Each respiration well contained three embryos and each of the six treatments had three replicate wells. The fish embryos were kept in E3 media during the test in order to measure only the oxygen consumption of the fish and exclude the chemical oxygen demand of the wastewaters as a disturbing factor. Additionally, the oxygen content in five blank wells filled with E3 media; but without fish, was measured as a stability control for the measurements. The respiration plate was sealed directly after the fish embryos were transferred using a compression block for microplate and the test was run until the first well reached the minimum oxygen concentration in the medium of 1.5 mg/L. Subsequently, the embryos were placed back into the exposure media. Oxygen measurements (mg/L) were acquired per well using MicroResp™ version 1 automated microplate respirometry software (Loligo Systems, Denmark, Mandic et al., 2020) and were exported to Microsoft Excel (version 2016) for further data evaluation.

2.5 Statistical analysis

The data analysis, as well as the preparation of the diagrams, was done with GraphPad Prism version 8.4.1 for Windows (GraphPad Software, San Diego, USA, 2017). Biological data obtained in the different water qualities, i.e. RW, CW, OW, OR, positive and negative control were tested for statistical significance. The data for embryo activity, heart rate, blood flow, and body length were tested for normality and subsequently analyzed for significance using a one-way-ANOVA with Tukey's multiple comparisons test (embryo activity, blood flow) or (if no normal distribution was given) Kruskal-Wallis and Dunn's multiple comparisons test (body

length, heart rate). The total distance moved was analyzed using Kruskal-Wallis and Dunn's multiple comparisons test. The significance level was $p<0.05$ for all tests. For embryo activity, heart rate, blood flow, and body length, every recorded fish was used as one replicate. For the respiration assay, three individual embryos were placed into a well, which constituted one replicate. There were three replicates per treatment. For the analysis of the larval swimming behavior (total distance moved), all five phases were analyzed separately, using each fish as one replicate. The total distance moved in the dark phases was analyzed similarly, using the values measured during the three dark phases.

3. Results

3.1. Physicochemical properties

Directly before and after exposure, pH, conductivity, and oxygen saturation were measured in the exposure media (see supplementary data, Tab. 1). The pH ranged between 6.36 (positive control) and 8.81 (CW) with a maximum deviation of 0.94 pH units (CW) between the beginning and end of the exposure. Conductivities between 719 and 833 µS/cm were found in the negative- and positive control. The values in the RW and CW were lower, ranging between 277 and 374 µS/cm. Higher values were found in the OW and OR waters, ranging between 406 and 941 µS/cm. Oxygen saturation stayed between 62.5 and 78.3% in all samples except for the positive control, where the final O_2-saturation was 30.6% at 96 hpf.

3.2. Mortality

Only coagulation was observed, while none of the other FET endpoints, i.e. lack of somite formation, non-detachment of the tail, and lack of heartbeat, were detected during the test. The experiment was considered valid since at least 80% of the negative control embryos followed normal development parameters at 96 hpf (Cassar et al., 2019). Initial mortality occurred only during the first 24 h of the test, ranging between 12% (CW) and 40% (RW), except for the positive control, which revealed a mortality of 55% 24 hpf and 75% 96 hpf. Of the investigated

299 water samples, the highest mortality occurred in the RW, followed by OW (Tables 1, 2). Lower
300 mortality rates were found in OR and CW. Hatching rates 96 hpf were 96-100% in all samples
301 except the positive control (Table 1).

302 *3.3. Embryo activity and respiration test*

303 Embryo activity was determined after 24 h exposure using burst activity as a measure of
304 spontaneous movements in the chorion, as well as oxygen consumption as a measure of
305 respiratory activity of the fish embryos. Burst activity and respiratory activity of the embryos in
306 the four water types were within the range between the negative and positive control (Fig. 2).
307 Burst activity was lowest and at the positive control level (mean: 1.8%, SD: 0.8%) in the OW
308 with a mean of 1.9% (SD:1.1%), followed by RW with 2.4% (SD: 1.8%), CW with 3.1%
309 (SD:1.2%), and OR with 4.2% (SD:2.0%) (Fig. 2A). The activity in the OR was found to be
310 similar to the mean activity in the negative control (4.0% ± 2.0%) and significantly higher than
311 the activity in the OW ($p=0.0107$).

312 The respiration test after 24 h exposure showed increased respiratory activity in all treatment
313 groups compared to the mean of the negative control (0.018 ± 0.002 O_2 mg/L/embryo/min)
314 (Fig. 2B). The highest oxygen consumption was found in the positive control (0.035 ± 0.001 O_2
315 mg/L/embryo/min), followed by the groups exposed to OW (0.033 ± 0.002 O_2
316 mg/L/embryo/min) and OR (0.029 ± 0.003 O_2 mg/L/embryo/min) followed by a mean oxygen
317 consumption of 0.024 mg/L/embryo/min (± 0.005) in the CW and 0.021 O_2 mg/L/embryo/min
318 (0.004 O_2 mg/L/embryo/min) in the RW.

319 *3.4. Body length*

320 The body length was in a narrow range and was comparable in all water samples with a mean
321 length between 2.27 mm and 2.38 mm (OR: 2.27 ± 0.22 mm, OW: 2.28 mm ± 0.22, RW: 2.29
322 ± 0.20 mm, CW: 2.38 ± 0.11 mm) (Fig. 3A). These measurements were slightly higher than in
323 the negative control (2.25 ± 0.14 mm) and the positive control (2.06 ± 0.03 mm), showing no
324 statistical differences (Table 2).

3.5. Heart rate and blood flow activity

The heartbeats per minute (BPM) were higher in all treatment groups (OW: 136.3 ± 25.3 BPM, RW: 143.4 ± 9.5 BPM, CW: 147.6 ± 9.1 BPM, OR: 156.9 ± 6.7 BPM) compared to the negative control (128.4 ± 30.7 BPM), but lower compared to the positive control (163.4 ± 17.3 BPM). A significant increase in heart rate was measured in the OR, compared to the OW group ($p=0.047$) and the negative control ($p<0.001$)(Fig. 3B).

The blood flow activity in all water samples (RW: 22.2 ± 5.1%, OW: 23.0 ± 2.4%, OR: 23.9 ± 77.1%, CW: 25.3 ± 4.0%) was lower compared to the negative control (29.6% ± 3.4%), but higher compared to the positive control (12.5% ± 7.7%), even significantly in the negative control ($p<0.001$), CW ($p=0.011$), and OR ($p=0.030$) (Fig. 3C, Tab. 2).

3.6. Larval swimming behavior

All zebrafish larvae showed a clear light/dark pattern in their swimming behavior with higher activity in the dark phases than in the light phases (Fig. 4). During the light phases, the larval activity in the different water samples showed no significant differences. However, during the three dark phases (Acc., D1, D2) fish larvae showed different activities depending on the water quality.

The total distance moved in the three dark phases decreased from Acc. to D2 parallel to the decreasing time intervals of Acc. (15 min), D1 (10 min) and D2 (5 min). In the dark phases, the mean distance moved of the larvae in all treatment groups was generally shorter than in the negative control (Fig. 4A, B) with the exception of RW in the acclimatization phase. In general, fish larvae exposed to OW and OR tended to move a shorter distance during the three dark phases, as compared to fish exposed to the negative control (Table 2). This difference to the negative control was significant in D1 (OW: $p= 0.003$; OR: $p=0.005$) and D2 (OW: $p<0.001$; OR: $p<0.001$) (Fig. 4A, B). Significant differences compared to the RW occurred in the acclimatization phase, where the fish moved in RW a significantly longer distance compared

to OW (p=0.002) and in D2, where the fish moved in RW a significantly shorter distance as compared to the negative control (p=0.035) (fig. 4B).

3.7 Sublethal macroscopic defects

Edemas in only a few embryos were detected during the FET; i.e in the groups OW (1 of 25 fish), negative control (1 of 25), and positive control (2 of 20). Spinal deformations occurred in OW (3 of 25) and OR (1 of 25), and positive control (2 of 20) (Table 2). No non-development of the eyes, general developmental retardation, or lack of blood circulation or pigmentation was detected in any of the samples (results not shown).

4. Discussion

Ozonation is a widely used method in WWTPs to eliminate micropollutants and thereby reduce possible toxic effects of the substances. Multiple studies have investigated the effects of conventionally treated and ozonated wastewater on *D. rerio* in acute or chronic test setups (Abegglen et al., 2009; da Costa et al., 2014; Pohl et al. 2018, 2019; Schmidt et al., 2011a, 2011b; Wigh et al. 2016). Toxicity of ozonated wastewater on *D. rerio* as compared to negative control has been shown for a number of endpoints, e.g. vitellogenin expression, swimming behavior of adult zebrafish (Pohl et al., 2018), genotoxicity, developmental abnormalities, endocrine-disrupting potential (Wigh et al., 2016), and mortality (da Costa et al., 2014). Usually, these studies compare the effects of ozonated versus conventional wastewater samples from a WWTP against a negative control as defined by OECD guidelines in order to describe substance-associated toxicity. However, when addressing possible adverse effects of WWTP effluents with respect to possible ecotoxicological risks for the ecosystem, the effects of chemicals already present in the receiving streams have to be considered as background ecosystem stressors. Therefore, the effects of conventionally treated and ozonated wastewater were compared with the respective effects of water from the receiving river in the present study in addition to negative and positive controls.

Mortality in the FET occurred (with exception of the positive control) exclusively within the first 24 hours. This may, however, be an underestimation of the toxic potential of the different WWTP and river samples due to the manner in which the samples were handled prior to undertaking the FET. Mortality rates were within boundaries of less than 20% (Cassar et al., 2019) except for RW (40%), OW (24%), and positive control (55% at 24 hpf, 75% at 96 hpf). At the same time, little edema (10% in positive control) and spinal deformations (12% in OW, 10% in positive control) occurred, none of them in RW (Table 2). It is possible that substances present in the river water but not in any of the wastewaters were responsible for the initial mortality in the RW since the river Ruhr is already affected by agricultural runoff and effluents of over 20 WWTPs upstream of the sampling point. Accordingly, this background toxicity has to be taken into consideration when determining the overall toxicity of differently treated WWTP effluents and their effects on the ecosystem.

To provide this ecosystem perspective, the effects of the wastewater effluents (CW, OW, OR) were compared to the RW. When analyzing the sublethal endpoints and comparing the results of the CW to the results of the RW, no significant differences were found. Thus, concerning zebrafish embryotoxicity, it is not expected that the inlet of the conventionally treated wastewater from the WWTP leads to increasing toxic effects in the receiving river. These findings coincide with a study by Abegglen et al. (2009) who (with the exception of one sample) found no toxicity in the conventional wastewater prior to and even after the ozonation step in their acute 48 h fish embryo-test with *D. rerio*. In contrast, when comparing the effects of the ozonated wastewaters with those of the RW, differences were found in the respiration assay (OW and OR) and the swimming behavior (OW), indicating that an introduction of ozonated wastewater might lead to ecosystemic effects.

In order to develop a substance-associated indication regarding the development of zebrafish, the effects of the water samples (RW, CW, OW, OR) were compared with the negative control (see Table 2). Only the fish in the CW showed no effects and thus no increased toxicity of the conventionally treated effluent. Fish in the RW showed responses in the mortality and

swimming activity endpoints, suggesting the input of toxic substances in the Ruhr catchment. In the ozonated effluents, responses were found for the endpoints mortality (OW), respiration (OW and OR), heartbeats per minute (OR), swimming activity (OW and OR), and spinal deformation (OW) (Table 2). Concerning the swimming behavior, a similar result for zebrafish exposed to ozonated wastewater compared to the negative control was reported by Pohl et al. (2018) who found significantly reduced activity of the zebrafish during the first minute after being placed in a novel vessel. It is evident that OR, as well as OW, negatively affected zebrafish behavior, indicating higher toxicity of these water qualities compared to the negative control and CW.

Ozonation can produce transformation products that are more toxic than the original substance. This has already been shown for polycyclic aromatic hydrocarbons chrysene and pyrene (Luster-Teasley et al., 2002, 2005), clofibric acid (Rosal et al., 2009), and mono-chlorophenols (Shang et al., 2006). Ozone can produce aldehydic compounds toxic to early life- stage fish (Yan et al., 2014) as well as toxic bromate (Soltermann et al., 2017), potentially toxic by-products of diclofenac (Coelho et al., 2009; Sein et al., 2008; Vogna et al., 2004) and Tamoxifen (Knoop et al., 2018). Since ozone is a volatile gas and the water samples were aerated overnight, a residual ozone concentration in the ozonated wastewater samples can be ruled out as an influence.

When comparing the effects of OW and OR, fish exposed to OR had a significantly higher embryonic burst activity and had significantly higher heart rates 72 hpf. Additionally, the mean pericardial area of fish exposed to OR was larger as compared to all other groups and at the level of the negative control. The results of these two groups point to transformation products built during the ozonation process and stress the influence of a subsequential biological treatment of the freshly ozonated wastewater. This is an important result for WWTPs lacking a subsequent biological treatment step, as demonstrated in different WWTPs (Detmold (Germany), Vienna (Austria), Rosenbergsau, and Kloten-Opfikon (Switzerland), Miehe et al., 2017). Several studies reported detoxification effects of ozonated wastewater after subsequent

biological treatment by e.g., sand filtration, fluidized or fixed bed reactors (Miehe et al., 2017), using standardized ecotoxicological test organisms like *Daphnia magna*, *Lumbriculus variegatus*, and *Oncorhynchus mykiss* (Abegglen et al., 2009; Gehrmann et al., 2018; Magdeburg et al.; 2012; Stalter et al., 2010). However, some results of studies indicated no further reduction of toxicity after subsequent biological treatment (Wigh et al., 2018; Magdeburg et al., 2012) or no toxicity of ozonated water a priori (Schlüter-Vorberg et al., 2017).

Furthermore, the comparison between OW and OR indicates that dynamic recirculation as a subsequential treatment technique has effects on the ozonated wastewater, probably due to the microorganisms present in the activated sludge of the OW as well as dilution with non-ozonated wastewater in the aeration tanks. Presumably, the OR water contained a larger amount and variety of TPs than the OW because no biological degradation was enabled in the OR water prior to the sampling. Baetz et al. (2020), also suspected transformation products in the OR water which were probably removed during the subsequent biological treatment in a study investigating water samples from the WWTP Schwerte. Moreover, approximately 50 different transformation products of the substances Metoprolol, Isoproturon, and Diclofenac were detected in ozonated wastewater samples taken in 2019 from the same sampling points on the WWTP Schwerte using non-target analysis (Wirzberger, personal communication). It was found that biological treatment decreased the amount of formed transformation products, but their results also indicated that some transformation products can also be built by biological degradation processes (Wirzberger, personal communication).

Taking into account the exposure period and with this, the developmental stage of the fish, significant differences between OW and OR were found for embryonic behavior 24 hpf and heart rates 72 hpf, whereas similar reactions of fish exposed to these two groups occurred in the swimming behavior 120 hpf. This indicates that some transformation products are unstable and are degraded during the exposure period, possibly leading to a more similar substance composition and behavioral reaction 72 hpf. Examples for unstable but toxic transformation products are 2,5-iminoquinone, 5-hyroxydiclofenac and 2,6-dichloroaniline (Coelho et al.,

2009), all transformation products from the analgesic drug diclofenac. The assumption of degradation of unstable transformation products over time is supported by the large deviations in pH (OR) and conductivity (OW and OR) measured before and after the test (see supplementary data Table S1). Thus, when investigating the effects of biological treatment after ozonation, the exposure time is an important factor, because the tested medium is influenced by chemical degradation as well as uptake and metabolism by the test organisms.

Regarding the sensitivity of the measured endpoints and their suitability for testing wastewaters, fish behavior, as well as heart rate and respiration, were more sensitive than blood flow activity and body length. Analyzing embryonic behavior can help to assess both general development and nervous system development in 24 h old zebrafish (García-Cambero et al., 2019). The locomotor activities of zebrafish are dependent on the development of the nervous system, the integrity of the brain system, and visual pathways (Ali et al., 2012; Basnet et al., 2019; Bilotta et al., 2000). The movement activity starts at 17 hpf (Colwill and Creton, 2011) and develops into a rhythmic movement of the pectoral fins and sudden changes of position, approximately every 20 seconds as they get closer to hatching (Ahmad et al., 2012). This implies an increase in embryonic activity the further the embryos develop. Taking into account that the burst activity was lower in CW, OW, and RW compared to the negative control, there might be substances in the water that led to less embryonic activity, but at the same time to earlier hatching in CW and OW as the hatching rates were at 72% in CW and OW already compared to a hatching rate of 24% in the negative control 48 hpf.

Newly hatched zebrafish larvae exhibit intermittent swimming, which develops into smoother glide swimming 96 hpf, following the development of the swim bladder (Basnet et al. 2019). Larvae 120 hpf express a pattern of increased movement in the dark and decreased movement during light phases (Basnet et al., 2019) which was also observed in the present study. The increase in locomotion during light-dark-transition can be attributed to an increased level of anxiety and stress (Basnet et al. 2019; Irons et al., 2010; MacPhail et al., 2009; Vignet et al., 2013). The considerably shorter distance moved in both ozonated wastewater groups (OW

484 and OR) compared to the negative control, points to impaired larval development, possibly
485 affecting the serotoninergic system. Stressed fish have increased serotonin levels in their
486 brains (Pohl et al., 2018; Winberg et al., 1992). Chronic activation of the brain serotonergic
487 system (Winberg and Thörnqvist, 2016) due to substances in the ozonated wastewaters,
488 therefore, might affect the stress response in exposed fish, leading to a less active swimming
489 behavior. The serotonergic system can be modulated by different psychoactive drugs like
490 serotonin-norepinephrine reuptake inhibitors (Basnet et al. 2019). A decrease in locomotor
491 activity in the dark is e.g. known for the antidepressant venlafaxine (Basnet et al., 2019;
492 Thompson et al., 2017). Hypoactivity responses in zebrafish larvae are also known for
493 ozonated carbamazepine (Pohl et al., 2019). These findings support the significantly shorter
494 distances moved in D1 and D2 by fish exposed to ozonated wastewaters compared to the
495 negative control. However, single-substance responses are not representative of the
496 environmentally relevant substance mixtures found in environmental water samples.
497 Regarding the shorter distance moved of the fish exposed to the ozonated wastewaters,
498 transformation products may be responsible for the adverse effects on swimming behavior.
499 This suggests that some stable transformation products in the OW and OR water samples are
500 more toxic than the original substances and could explain the stronger effect in the ozonated
501 wastewaters compared to the conventional wastewater (CW). In the RW, diverse effects on
502 the swimming behavior were observed in the phases Acc and D2: while the mean activity in
503 the acclimatization phase was at a similar level as in the negative control, it was significantly
504 decreased in D2 compared to the negative control. It is also visible that fish acclimated more
505 slowly in the acclimatization phase than in the negative control (Fig. 4A).

506 Thus, both embryonic and larval behavior are relatively sensitive endpoints, since they can
507 reflect measurable changes in physiological processes. This finding coincides with a study by
508 García-Cambero et al. (2019), who also measured sublethal endpoints in zebrafish embryos
509 such as morphology, heart rate, embryonic and larval behavior, exposed in differently treated
510 wastewaters. These authors found that behavioral effects were the most sensitive endpoints.

511 The endpoints heart rate, respiratory rate, and blood flow showed differences when comparing
512 the effects to the negative control. Heart rate and respiratory rate were higher in all groups
513 compared to the negative control, while blood flow was lower. These effects point to
514 background stress induced by the pollutants present in the waste- and river water, possibly
515 leading to an up-regulation of the metabolism due to detoxification processes in the exposed
516 fish. However, the only significant result was found in OR when compared to OW and negative
517 control. Heart rate responses of *D. rerio* have already been described in several studies, e.g.
518 for ozonated diclofenac and carbamazepine, platinum, trichloroethylene, and caffeine (Pohl et
519 al. 2019; Osterauer et al., 2009; Horzmann et al., 2020; Rana et al., 2010), indicating that this
520 is a parameter sensitive to a variety of substances. Although rarely observed in the present
521 study, also edemas should be assessed when investigating wastewater samples as WWTP
522 effluents can cause edema in zebrafish (Jonáš et al., 2011). The fact that a higher respiration
523 rate was measurable in the zebrafish already after 24 hpf shows that this is also a sensitive
524 endpoint that should be considered in future studies, however, more replicates to support
525 statistical analysis are recommended.

526 In the present study, the comparison of the body length did not show significant differences,
527 indicating that this endpoint is not as sensitive as swimming behavior and heart rates.
528 However, deformations of the spine occurred in the OW, causing the fish to swim in circles.
529 Macroscopic defects such as edema and spinal deformations can easily be assessed along
530 with the acute endpoints of the FET.

531 **5. Conclusion**

532 Regarding the use of ozonation in the investigated WWTP, no detoxification effect of the
533 wastewater could be found using the zebrafish test. In both types of ozonated wastewater
534 samples (OW and OR) the swimming activity of the larval zebrafish was significantly reduced
535 as compared to the negative control. Additionally, the respiration activity was highly increased
536 as compared to the negative control and the fish exposed to ozone reactor water showed
537 significantly increased heart rates. Therefore, adverse effects of ozonated wastewater on

aquatic organisms cannot be ruled out. Significant differences in embryonic behavior and heart rates were found between OW and OR, emphasizing the impact of subsequent biological treatment. None of the sublethal endpoints showed significant differences between CW as compared to the negative control as well as to RW, indicating that the conventional effluent from this treatment plant does not pose a greater threat to embryonic and larval zebrafish than the water of the river Ruhr itself, which already contains potentially toxic chemicals. However, further studies using other test organisms should be performed to verify these findings. Sensitive sublethal endpoints such as heart rates, blood flow, behavior, and respiration are useful parameters in addition to the standard FET in order to gain more insight into the physiological processes of the zebrafish.

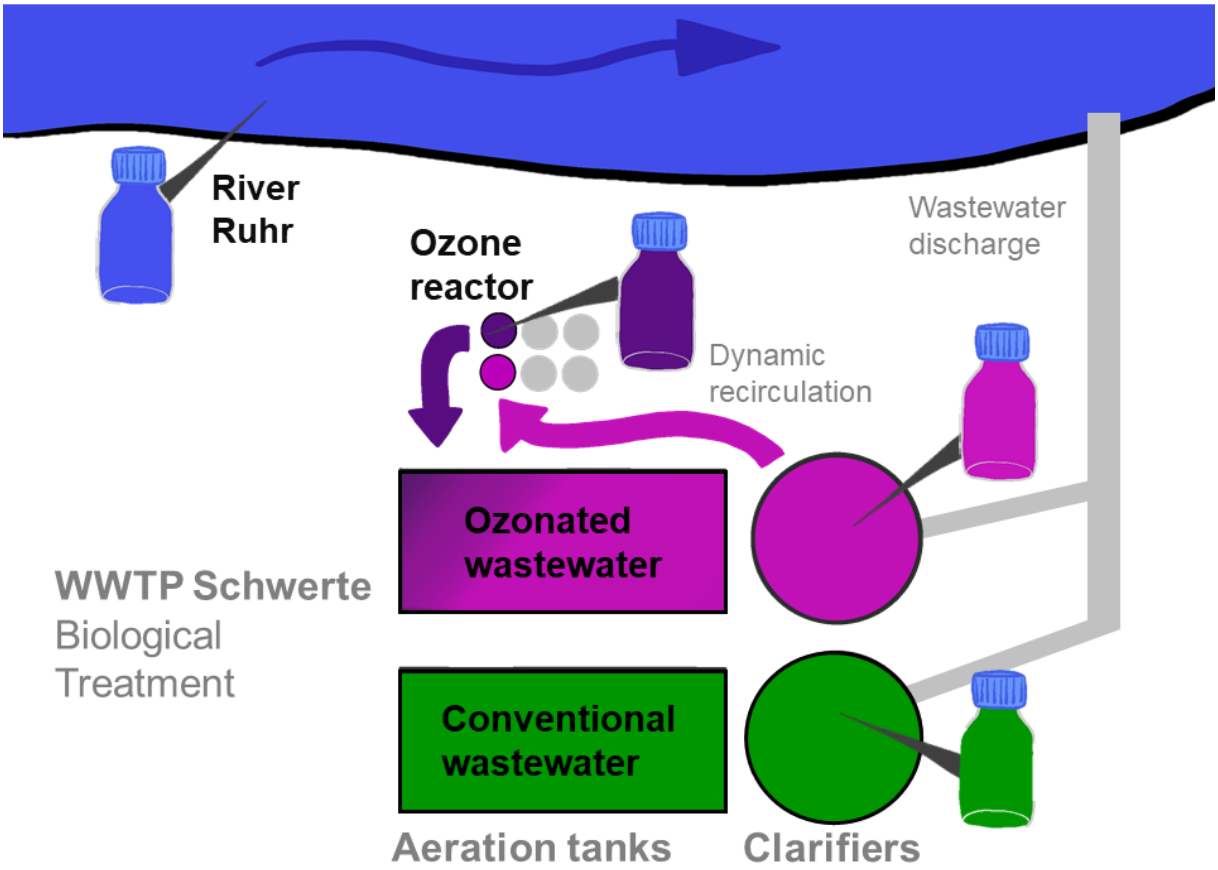


Figure 1: Conventional and ozone treatment of wastewater on the WWTP Schwerte (North Rhine-Westphalia). Ozonation is performed after biological treatment and clarifier with 50 L water per second being transferred into the ozone reactor, where the water is ozonated with approximately 5 mg/L O_3. The ozonated water can degas in a second reactor before being transferred back into the biological treatment performed in the aeration tanks (dynamic recirculation). The bottles and arrows depict the four sampling points for the water samples used in the FET.

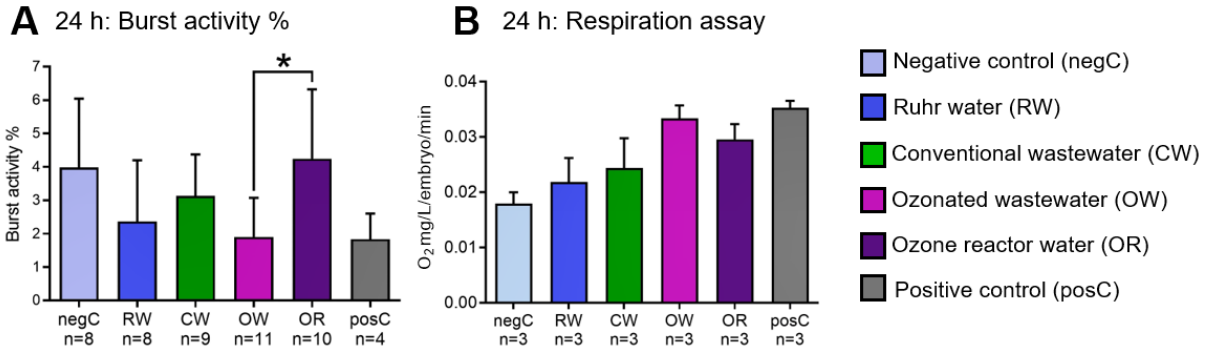


*Figure 2: Embryo activity after 24 h exposure in the different water samples: A: Burst activity (%) as a measure of the embryo movement activity and B: Oxygen consumption as a measure of respiration rate of the fish embryos. Data are presented as mean ± SD. A, *: significantly different at p<0.05 (one-way-ANOVA, followed by Tukey's multiple comparison test). B: Respiration could not be tested for statistical significance due to low n.*

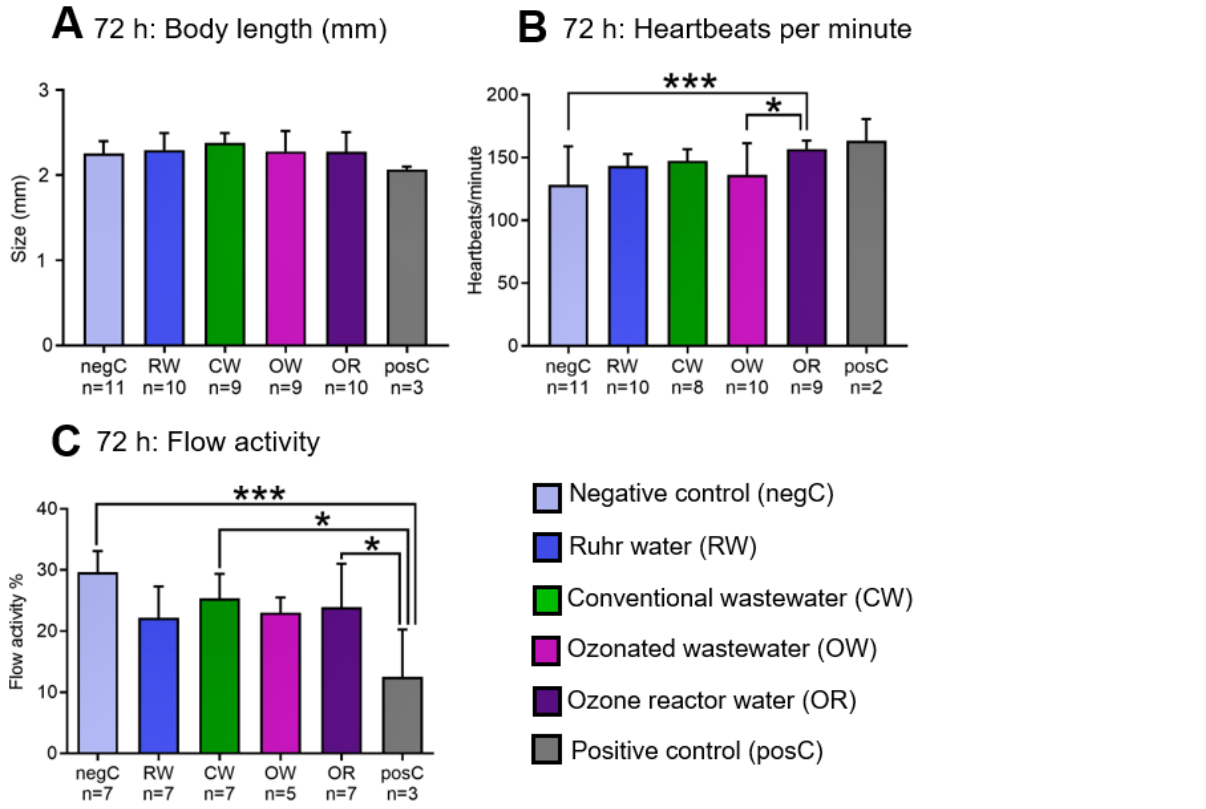


*Figure 3: Body length (A), cardiac activity, i.e. heartbeats per minute (B), and blood flow activity (C), of the zebrafish 72 hpf. Data are presented as mean ± SD. A, B, C, *: significantly different at p<0.05 (A, B: Kruskal-Wallis test, followed by Dunn's multiple comparisons test, C: one-way-ANOVA, followed by Tukey's multiple comparison test)*

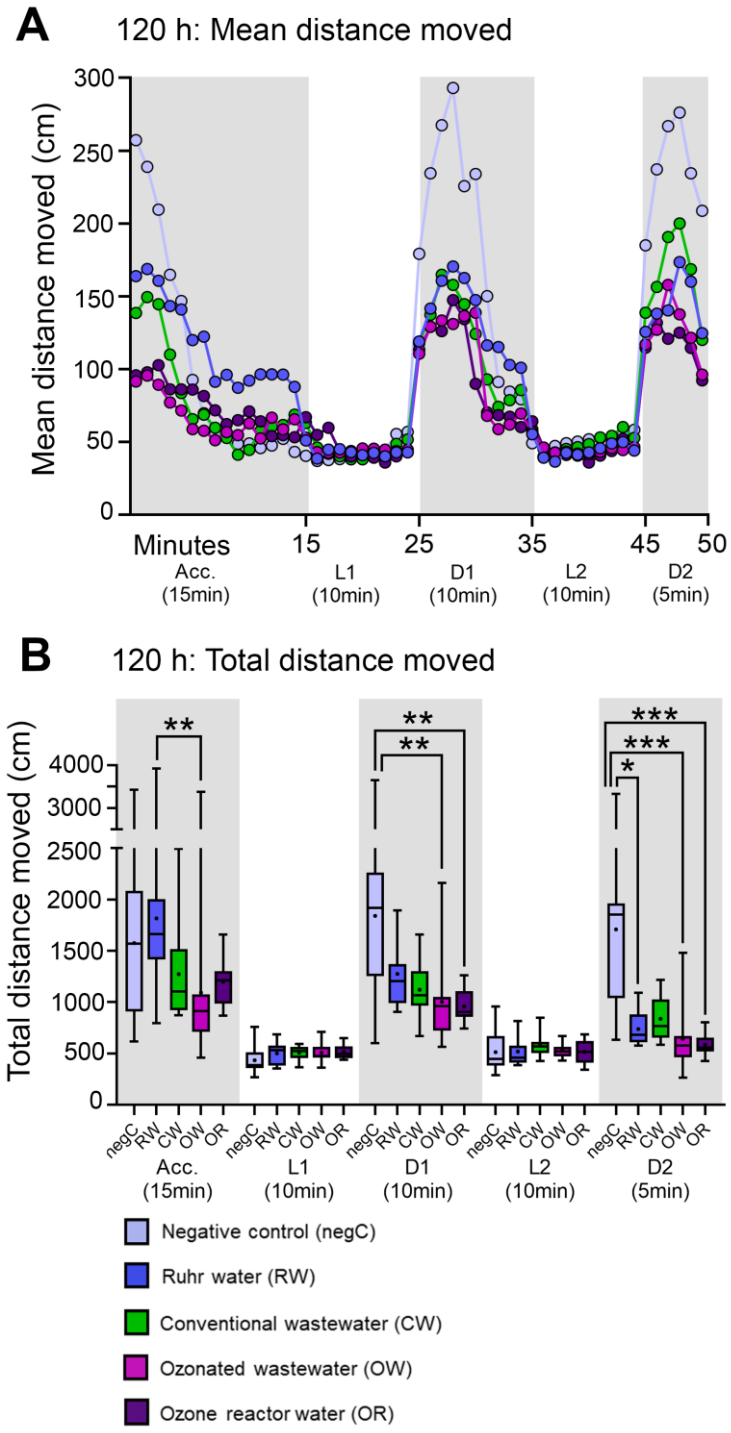


*Figure 4: Distance moved by zebrafish larvae (120 h exposure time) during the dark phases (grey background), i.e. acclimatization (Acc.), D1 and D2, as well as the light phases (white background) L1 and L2 of the behavior test. A: mean distance moved per minute. B: total distance moved during the five phases, displayed as median and whiskers, points indicate the mean of each treatment group in each phase B: * indicate significant differences at p<0.05 (Kruskal-Wallis test, followed by Dunn's multiple comparisons test). The statistical analysis was performed individually for every phase. The swimming behavior of fish larvae of the positive control could not be tested because of high mortality and low hatching rates. Due to problems with the video footage in the RW group, where only the last 5 min of D2 could be captured, the video footage of the other groups was also shortened to 5 min in D2.*

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575 *Table 1: Survival and hatching rates of D. rerio used in the FET. Abbreviations: negC: negative control, RW: Ruhr*
576 *water, CW: conventional wastewater, OW: ozonated wastewater, OR: ozone reactor water, posC: positive control.*
577 *Embryos were kept in 6-well plates with five fish embryos per well.*

Treatment	**negC**	**RW**	**CW**	**OW**	**OR**	**posC**
Number of embryos (n)	25	25	25	25	25	20
0 h						
% survival mean ± SD	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Hatching rate % mean ± SD	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
24 h						
% survival mean ± SD	84 ± 8.9	60 ± 14.1	88 ± 17.9	76 ± 8.9	80 ± 20	45 ± 25.2
Hatching rate % mean ± SD	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
48 h						
% survival mean ± SD	84 ± 8.9	60 ± 14.1	88 ± 17.9	76 ± 8.9	80 ± 20	45 ± 25.2
Hatching rate % mean ± SD	24 ± 8.9	52 ± 10.9	72 ± 33.5	72 ± 22.8	28 ± 22.8	5 ± 10
72 h						
% survival mean ± SD	84 ± 8.9	60 ± 14.1	88 ± 17.9	76 ± 8.9	80 ± 20	40 ± 28.3
Hatching rate % mean ± SD	88 ± 17.8	96 ± 8.9	100 ± 0	96 ± 8.9	100 ± 0	20 ± 28.3
96 h						
% survival mean ± SD	84 ± 8.9	60 ± 14.1	88 ± 17.9	76 ± 8.9	80 ± 20	25 ± 37.9
Hatching rate % mean ± SD	100 ± 0	100 ± 0	100 ± 0	96 ± 8.9	100 ± 0	20 ± 28.3

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*Table 2: Summary of the results of the modified FET with D. rerio. Abbreviations: RW: Ruhr water, CW: conventional wastewater, OW: ozonated wastewater, OR: ozone reactor water, posC: positive control, NA: not analyzed. Response as compared to the negative control: -: no response; +: response but not tested for significance, * , *** : significant differences as compared to the negative control ($p < 0.05$; $p < 0.001$), [a] : significant differences between two treatment groups, excluding controls. Results for non-development of eyes, general developmental retardation, lack of blood circulation, and lack of pigmentation are not shown since no effects were found.*

Sample	Endpoints									
	Mortality 96 hpf	Hatching rate % 96 hpf	Burst activity % 24 hpf	Respira-tion 24 hpf	Body length 72 hpf	Heart-beats per minute 72 hpf	Flow activity 72 hpf	Swimming activity 120 hpf	Edema 0-96 hpf	Spinal deforma-tions 0-96 hpf
RW	+	-	-	-	-	-	-	* [a]	-	-
CW	-	-	-	-	-	-	-	-	-	-
OW	+	-	- [a]	+	-	- [a]	-	*** [a]	-	+
OR	-	-	- [a]	+	-	*** [a]	-	***	-	-
posC	+	+	-	+	-	-	***	NA	+	+

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Appendix A. Supplementary data

Supplementary data to this article can be found online at XXX

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