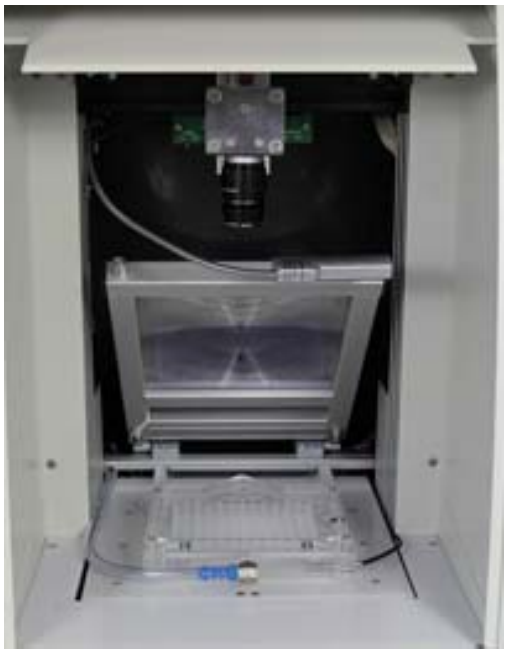
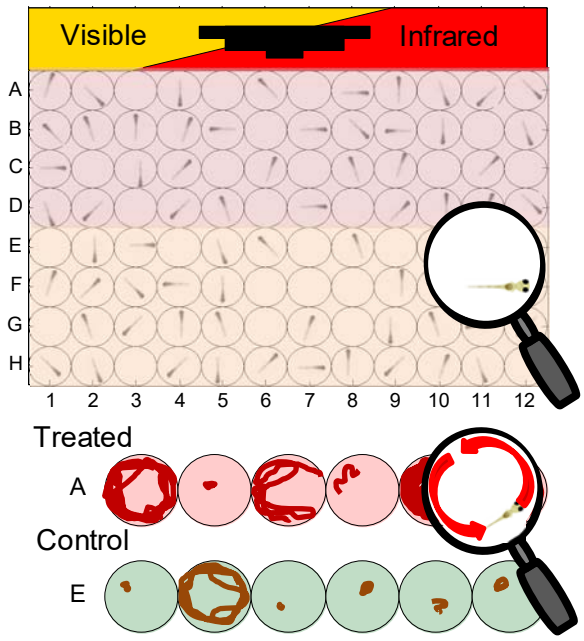
GRACE grant no 679266

D1.4 (month 18)

Report on influence of salinity and temperature on bioassay responses, and derivation of thresholds for proper application of the biounits

WP1: Oil spill detection, monitoring, fate and distribution





Prepared under contract from the European Commission
Contract n° 679266
Research and Innovation Action
Innovation and Networks Executive Agency
Horizon 2020 BG-2014-2015/BG2015-2

Project acronym: GRACE
Project full title: Integrated oil spill response actions and environmental effects
Start of the project: 01 March 2016
Duration: 42 months
Project coordinator: Finnish Environment Institute (SYKE)
Project website http://www.grace-oil-project.eu

Deliverable title: Report on influence of salinity and temperature on bioassay responses, and derivation of thresholds for proper application of the biounits
Deliverable n°: D1.4
Nature of the deliverable: Report
Dissemination level: Confidential

WP responsible: WP1
Lead beneficiary: RWTH Aachen

Due date of deliverable: 31.08.2017
Actual submission date: 31.08.2017

Deliverable status:

Version	Status	Date	Author	Approved by
1.1	draft	17.08.2017	Leonie Nüßer	WP1 members 23.08.2017
1.2	final	31.08.2017	Leonie Nüßer	Steering group 31.08.2017

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Executive Summary

In addition to the implementation of existing sensor technology a novel biosensor for the detection of oil in water is under development and verification. This biosensor utilizes specific motor responses of hatched zebrafish embryos before 120 hours post fertilization (hpf) under flow-through conditions. To adapt the biosensor to the specific parameters of the Baltic Sea, the influence of temperature and salinity on zebrafish embryo swimming behavior was determined. The data was used to derive thresholds for temperature and salinity tolerance of zebrafish embryos for behavioral monitoring.

The influence of temperatures outside the optimum niche of developing zebrafish has been investigated in several studies in the past. It has been demonstrated that early developmental stages of fish are more sensitive to temperatures outside their optimum niche than adult fish (Brett, 1970; Schirone and Gross, 1968; Rombough, 1997). It was found that for developing eggs temperatures below 23° C and above 34° C resulted in 100 % mortality of the embryos (Schirone and Gross, 1968). No experiments within GRACE were conducted to verify the findings from the literature since the reported data is consistent. The Baltic Sea as the study region within GRACE is characterized by cold climate with temperatures of 10 to 15° C maximum. Exposure of zebrafish embryos would lead to 100 % mortality. Therefore, the temperature in the assays utilizing zebrafish embryos have to be adapted to the organisms' optimum niche.

This study tested the behavioral response of hatched 96 hours post fertilization (hpf) zebrafish embryos after acute exposure to different levels of salinity (40.25 ‰; 30.25 ‰; 20.25 ‰; 10.25 ‰; 8.25 ‰; 5.25 ‰; 2.75 %; 1.5 ‰; NC 0.5 ‰). The three highest levels of salinity resulted in elevated levels of activity and ultimately the loss of normal swimming capability within 94 min. The salinities of 1.5 ‰ up to 10.25 ‰ did not affect the behavioral responses of the organisms. After 24 h of exposure to the elevated levels of salinity the embryos were tested for their capability to display normal avoidance reactions when exposed to a negative stimulus. Results show that up to the salinity of 10.25 ‰ the reaction was comparable to the control group exposed to the same stimulus. From the presented results it can be concluded that 96 hpf zebrafish embryos are tolerant to a salinity up to 10.25 ‰ and are still able to display normal avoidance reactions when exposed to a negative stimulus after 24 h. The application of zebrafish embryo swimming behavior as a biosensor on the Baltic Sea is therefore not limited by the salinity. However, since the tolerance of zebrafish embryos to temperature outside the optimum niche is rather limited, temperature needs to be controlled in the flow-through system of the biosensor to range between 26 and 28.5° C.

1. Introduction

The work within WP1 focuses on the evaluation and implementation of existing *in situ* sensor technology for the detection of oil in water into Ferrybox and smart buoy systems. The main objective is to make oil spill detection more accurate and cost-effective. At RWTH Aachen University a biosensor is developed and verified utilizing specific motor responses of hatched zebrafish embryos before 120 hours post fertilization (hpf). The concept of biological early warning systems (BEWs) is based on the continuous tracking of organisms' physiological reaction as an indicator for the presence of toxic substances (Van der Schalie et al., 2001). There are several biosensors commercially available nowadays. All of them are based on the same general principle that the utilized organisms inherit a characteristic and stable behavior. In response to a decrease in water quality caused by the presence of toxic chemicals or a change in physical chemical parameters the organisms react with a distinctive change in their behavior that can be quantified. Bae and Park (2014) reviewed BEWs utilizing aquatic organisms. They concluded that, although there exist advantages of biosensors when compared to traditional sensor technologies, quantification and interpretation of data remains to be a major challenge. For this reason, the development of the biosensor utilizing zebrafish embryos includes the designation of a robust determinative parameter that allows to distinguish between normal behavior and specific responses induced by chemical stressors.

In the first evaluation phase of the general applicability of this biosensor, hatched embryos are exposed to a set of model substances under static conditions and immediately monitored for behavioral changes. The next step includes the development and utilization of a custom made flow-through well-plate. The first prototype is currently in development and will be produced in the near future. In order to utilize the biosensor within this project, an evaluation of the response of zebrafish embryos to temperature and salinity conditions of the Baltic Sea was conducted.

The influence of temperatures outside the optimum niche of developing zebrafish has been investigated in several studies in the past. It has been demonstrated that early developmental stages of fish are more sensitive to temperatures outside their optimum niche than adult fish (Brett, 1970; Schirone and Gross, 1968; Rombough, 1997). The zebrafish is considered to be a rather eurythermal teleost species with an optimum niche between 26 and 28.5° C for laboratory cultures (Sfakianakis et al., 2011; Avdesh et al., 2012). However, it was found that for developing eggs temperatures below 23° C and above 34° C resulted in 100 % mortality of the embryos (Schirone and Gross, 1968). The authors also found that embryos raised at low concentrations within this range (e.g. 23° C) were developing slower in comparison to higher temperatures. A more detailed review of the existing data on the influence of temperature on zebrafish embryos is available in D 3.2 „Test conditions for zebrafish (month 18) Report on suitable environmental parameters for investigation of oil spills and oil spill responses using zebrafish". No experiments within GRACE were conducted to verify the findings from the literature since the reported data is consistent. The Baltic Sea as the study region within GRACE is characterized by cold climate with temperatures of 10 to 15° C maximum. Exposure

of zebrafish embryos would lead to 100 % mortality. The custom made flow-through well-plate for the biosensor utilizing zebrafish embryos will incorporate a temperature control unit that can heat up the monitored water to the organisms' optimum niche.
To our knowledge the influence of increased salinity levels on the distinctive swimming behavior of zebrafish embryos has not been assessed so far.

2. Materials and methods

2.1 Fish maintenance and egg retrieval

The breeding stock of zebrafish was maintained according to previously defined culture conditions (Braunbeck et al., 2005; Hollert et al., 2003; Nagel, 2002). Spawning trays were placed in the aquaria in the afternoon before spawning. Spawning took place the next morning after onset of light. Fertilized and normally developed eggs were selected using a binocular microscope. Eggs were then incubated in formulated water (DIN EN ISO 15088, salinity 0.5 ‰) at 26 ±1 °C until 96 hours post fertilization (hpf).

2.2 Exposure routine

Hatched and normally developed embryos were transferred individually to a 96 well-plate (Techno Plastic Products, TPPs, Zürich, Switzerland) in a volume of 100 µl formulated water at an age of 96 hpf. After a settling time of 30 min the embryos were exposed to the different exposure solutions. In order to test the organisms' response to elevated levels of salinity, water with different salinities was added into the wells of the 96 well-plates. For each treatment (40.25 ‰; 30.25 ‰; 20.25 ‰; 10.25 ‰; 8.25 ‰; 5.25 ‰; 2.75 ‰; 1.5 ‰;) 20 individuals were exposed, negative control (NC 0.5 ‰) groups were included on each plate and consisted of 16 individuals treated with formulated water only. Hence, on each plate 4 different salinities and one negative control were tested. After exposure, embryos were immediately tested for their behavioral response. After finishing the behavior assay, the embryos were incubated in the 96 well-plated for 24 h in the respective test solutions. In a next step it was tested whether the embryos exposed to the different levels of salinity were still able to react to a chemical stressor. Ethanol was chosen as model compound to induce stress behavior in zebrafish embryos. At 5 days post fertilization (dpf) the embryos were exposed to a 2 % ethanol solution and again monitored for their behavioral response. Tests were terminated before embryos reached the age of 120 hpf. After the tests were terminated, the embryos were euthanized by prolonged immersion in a benzocaine ethanol solution.

2.3 Behavioral assay

Pipetting of the exposure solutions took approximately 3 min. Immediately after pipetting of the exposure solutions into the well-plate, zebrafish embryos swimming activity was monitored using the observation system DanioVision™ (Noldus, Wageningen, The Netherlands). The software EthoVision XT (Noldus Information Technology, Leesburg, VA, USA) was used to track the locomotor activity of the embryos. In order to monitor phases of low and high swimming activity the light/dark transition test was applied (Ali et al., 2012; Schnörr et al., 2012; Legradi et al., 2015). The experimental setup in this study followed alternating 10 min light phases and 4 min dark phases. During the dark phases infrared light was used which is not perceivable by the fish embryos but allows tracking the embryos movement. Overall, the embryos were tracked for 94 min per experiment which results in 7 light phase cycles and 6 dark phase cycles. Elevated levels of embryo swimming activity are induced by the transition from light to dark. Since it is known that the swimming activity of early developmental stages of zebrafish embryos changes over the course of the day (MacPhail et al., 2009; Vignet et al., 2013) all experiments were carried out in the same time slots.

2.4 Data processing methodology

Raw data consisting of x- and y-coordinates of the individuals' position in the well over the course of the experiment duration was extracted from the EthoVision software. All further data processing steps were conducted using MATLAB, version R2013a (The MathWorks, Inc, Natick, Massachusetts, U.S.) according to methods developed in an earlier project and published in 2016 (Nüßer et al.). Behavior data is commonly evaluated based on the total distance moved by the subjects in distinct time intervals. The method applied here was developed based on the assumption that the total distance moved by one individual is not accurate to assess the individuals' specific reaction after acute exposure to a toxic substance. In Figure 1 selected data from exposure experiments with cadmium chloride are shown as the total distance moved (µm) in 1 min during a dark cycle. The trajectory of the individuals' movement was displayed by plotting the x- and y-coordinates tracked by the EthoVision software. Subject 5 and subject 56 moved approximately the same distance, 2202 µm and 2310 µm, respectively. The moving trajectory of subject 5 had a circular shape which occurs when the embryos swim in a directed way in the round well of the well-plate. The trajectory of subject 56 is point shaped, the individual did not display directed swimming activity but apparently moved around its point of origin. The data evaluation method proposed in the publication (Nüßer et al., 2016) allows to distinguish between individuals that display directed movement and individuals that, although moving a lot, do not behave in a directed manner. This parameter is suggested to be more adequate for the detection of avoidance behavior as reaction to negative stimuli like the acute exposure to toxic substances in relevant concentrations. The calculation of the parameter was carried out as follows.

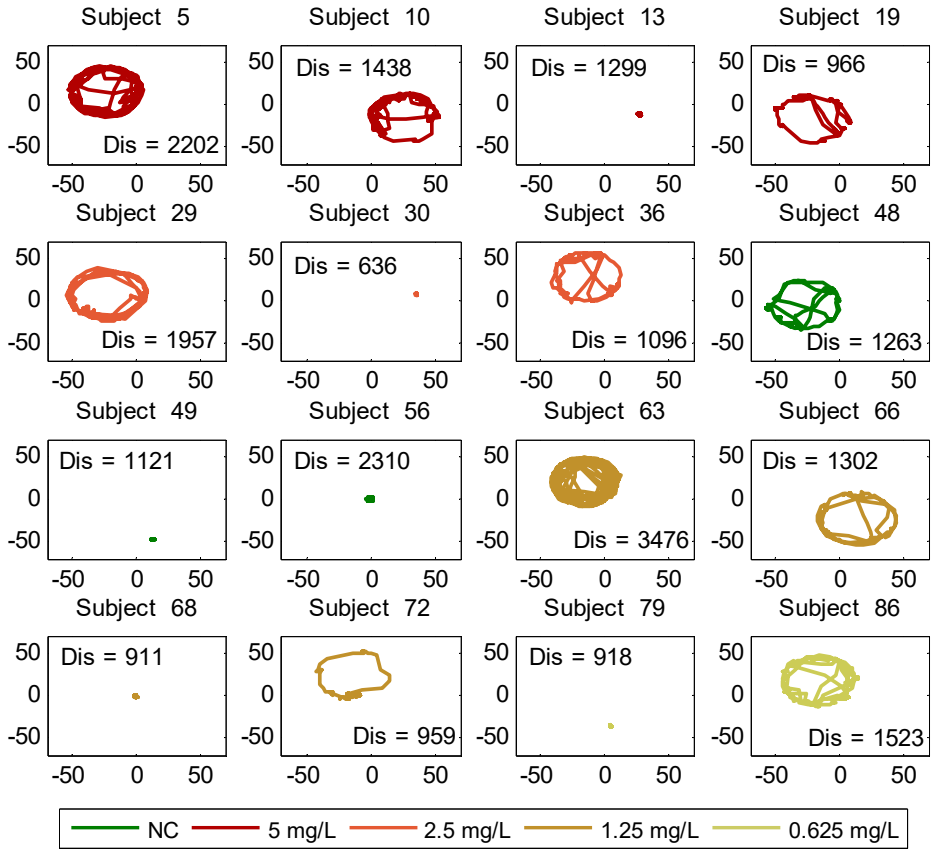

Figure 1 Selected subjects moving trajectory during the first minute of one dark cycle from a data set of experiments with cadmium chloride. Dis – total distance moved (µm) by the subject during 1 min. The trajectory of the individuals' movement is displayed by plotting the x- and y-coordinates tracked by the EthoVision software. Figure adapted from Nüßer et al. 2016.

In short, the x- and y-coordinates of the embryos swimming activity where transformed to polar coordinates, ρ (radius, µm) and θ (angle, radians) which allows to calculate the angle of the subjects' movement. In the next step maximum and minimum angles (θ max, θ min) and maximum radius (ρ max) were calculated for each subject for each time block (1 min). The subjects for whom the difference between the maximum and the minimum angle during the chosen time is larger than a certain critical angle (θ crit) and the maximum radius is larger than critical radius (ρ crit) were considered subjects with directed movements.

$$\theta_{max} - \theta_{min} > \theta_{crit}$$

$$\rho_{max} > \rho_{crit}$$

Since treatment and control groups where different sizes absolute numbers of individuals displaying directed movement could not be compared, hence, the proportion of embryos with directed movement was calculated.

3. Results and discussion

Here, the influence of the increased salinity levels of the exposure medium on the behavioral response of zebrafish embryos are presented. The raw data has been evaluated according to the methods described in chapter 2.4. The response is displayed as the proportion of embryos that displayed directed movement in relation to the total number of individuals in that treatment group. An increase in the proportion of directed movement was induced by the alternating phases of light and darkness. Onset of dark cycles are indicated by the grey lines. **Error! Reference source not found.**Figure 2 and Figure 3 show the behavioral response of the 96 hpf embryos immediately after exposure to the different levels of salinity. For all conducted replicates, the individuals in the negative control group showed normal swimming activity with a low proportion of directed movement during the cycles of light and high proportion of directed movement during cycles of darkness. All treatments with salinity greater than 5.25 ‰ induced an increased swimming activity in the individuals in the first 10 minutes. The effect was highest for the two highest levels of salinity. Individuals exposed to a salinity of 40.25 ‰ and 30.25 ‰, respectively did not respond to the darkness stimulus. The individuals' activity decreased rapidly, for the treatment group exposed to 40.25 ‰ within 20 min and for the treatment group 30.24 ‰ within 30 min to a level where ≤ 10 % of the individuals showed a directed movement.

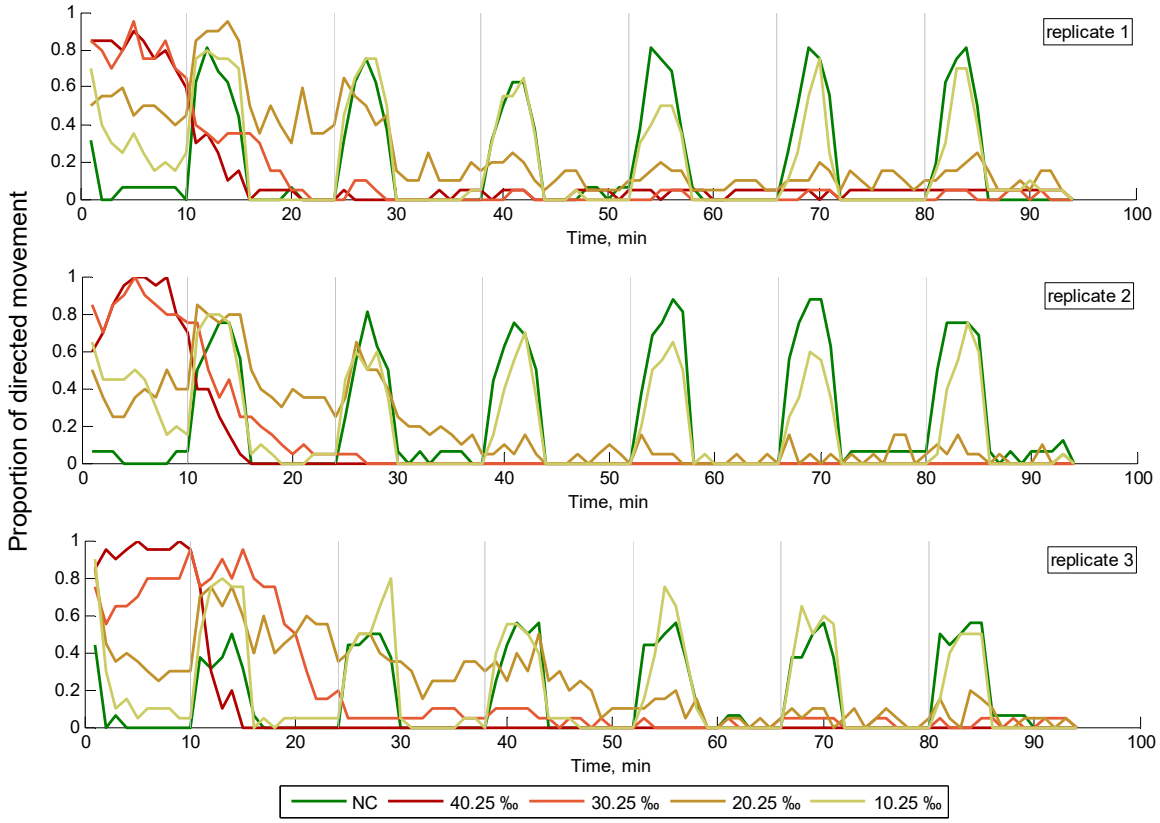

Figure 2 Proportion of directed movement of 96 hpf zebrafish embryos exposed to different levels of salinity (40.25 ‰; 30.25 ‰; 20.25 ‰; 10.25 ‰). Treatment groups n = 20; negative control n = 16

The salinity of 20.25 ‰ also induced avoidance behavior in the zebrafish embryos at the beginning of the experiment, which decreased over the course of the experiment. The decreased capability of

the embryos in the three highest treatment groups can most likely be explained by adverse effects of the salinities. The salinity of 10.25 ‰ and 8.25 ‰ led to an increase of swimming activity within the first 10 min for all replicates, 5.25 ‰ salinity induced the same effect in replicate 1. After the first 10 min all groups except the three highest treatments display comparable levels of swimming activity to the NC group.

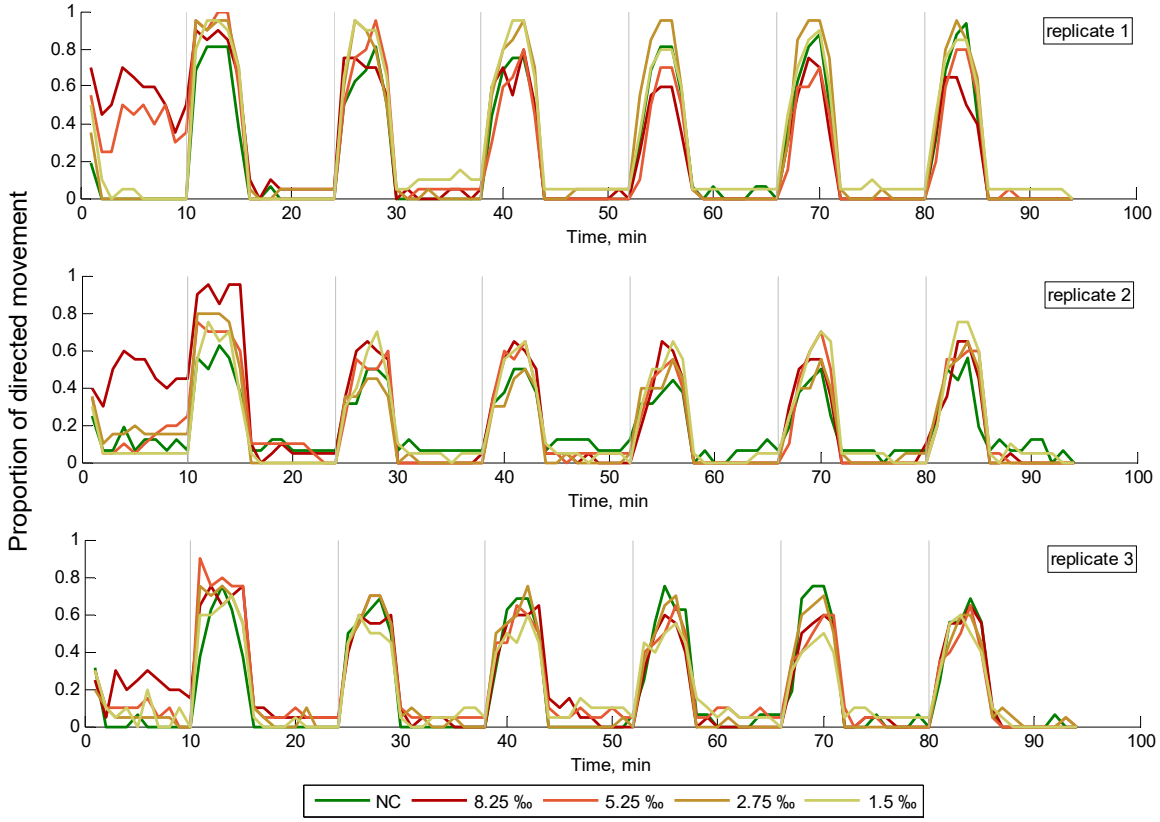

Figure 3 Proportion of directed movement of 96 hpf zebrafish embryos exposed to different levels of salinity (8.25 ‰; 5.25 ‰; 2.75 ‰; 1.5 ‰). Treatment groups n = 20; negative control n = 16

In order to test whether the exposure to the elevated levels of salinity affects the capability of the embryos to react to the exposure of a toxic substance, experiments with ethanol as a model stressor were conducted.

Figure 4 shows the results of experiments were 96 hpf embryos reared in formulated water were exposed to test solutions containing different concentrations of ethanol. The experiments were conducted to test the embryos response to a stressor under normal conditions. Exposure to 4 % ethanol led to strong avoidance behavior followed by immobility of embryos within 12 min of the experiments for all replicates. 3 % ethanol induced an increase of the directed swimming activity within the first 10 min, the avoidance behavior remained high over the course of the experiment, alternating light and dark cycles did not affect the embryos response. Exposure to 2 % ethanol also

resulted in strong avoidance behavior. For replicate 3 this effect started at the beginning of the experiment, for replicate 1 and 2 after approximately 10 min. Embryos did not respond to the transition between light and dark cycles. Treatment with 1 % ethanol led to a reduced response of the embryos in replicate 1 for the first dark cycle and for replicate 2 for all dark cycles. In replicate 3 the response of this treatment group was at negative control level.

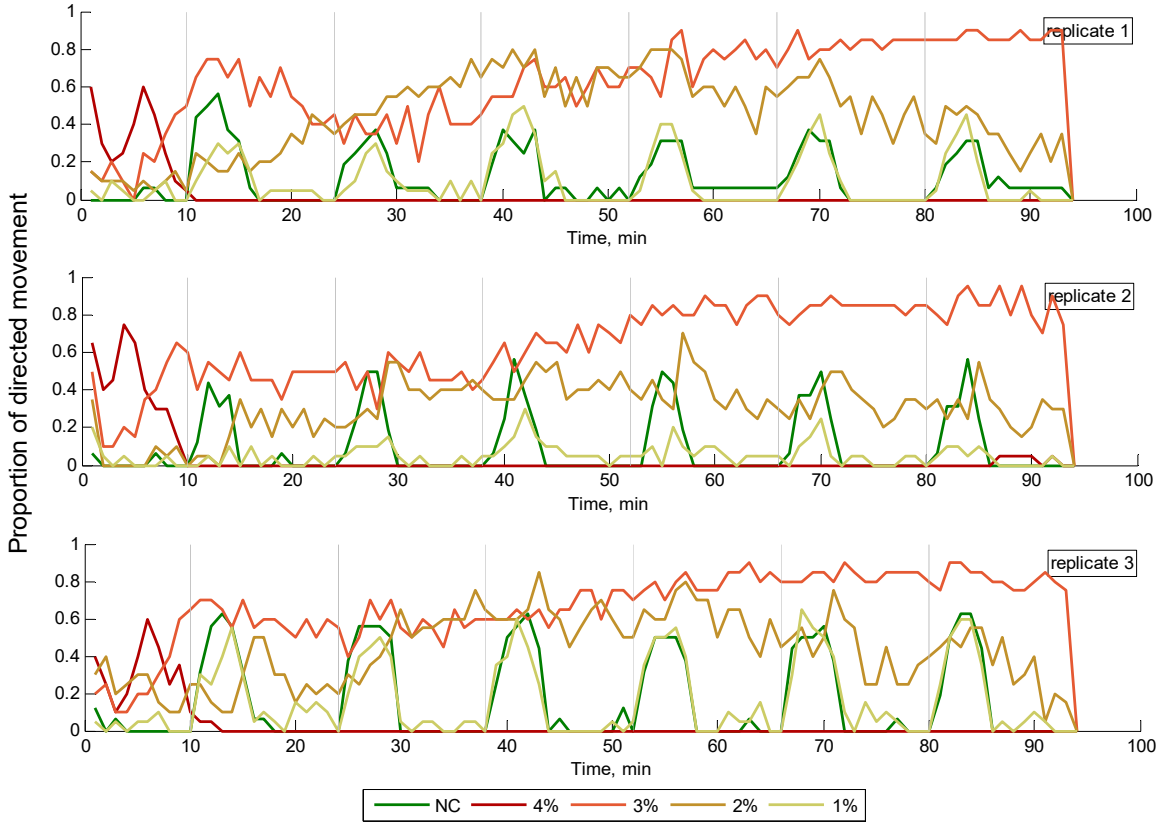

Figure 4 Figure 1 Proportion of directed movement of 96 hpf zebrafish embryos exposed to different concentrations of Ethanol (4%; 3%; 2%; 1%). Treatment groups n = 20; negative control n = 16

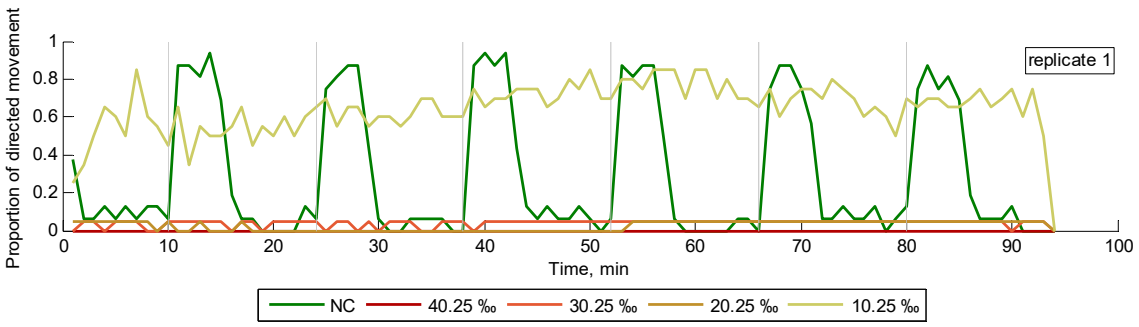

Figure 5 Proportion of directed movement of 120 hpf zebrafish embryos exposed to different levels of salinity (40.25 ‰; 30.25 ‰; 20.25 ‰; 10.25 ‰) from 96 hpf to 120 hpf and to 2 % ethanol at 120 hpf. Treatment groups n = 20; negative control n = 16

From these results the concentration of 2 % ethanol was chosen to test the embryos capability to respond to a negative stimulus after treatment with elevated levels of salinity. Hence, after exposure to the different levels of salinity for 24 h, embryos were exposed to a 2 % ethanol solution. Exposure of embryos to the three highest levels of salinity (40.25 ‰; 30.25 ‰; 20.25 ‰) resulted in 100 % mortality after 24 h, hence embryos in the wells did not show any swimming activity at 120 hpf (Fig. 5). For better comparability, the results for the embryos incubated with a salinity of 10.25 ‰ and negative control group not exposed to 2 % ethanol are presented in the same graph as the treatment groups incubated within the second well-plate (Fig. 6).

The NC group on the plate with higher salinity levels was not treated with the 2 % ethanol solution in order to demonstrate the embryos normal response to the light/dark transition test at 120 hpf. It can be noticed that the NC groups proportion of directed movement is higher and more consistent (between 80 % – 100 %) during dark phases for all replicates in comparison to results from 96 hpf embryos. The salinity treatment groups showed the same response pattern as the NC group (NC+EtOH) in response to ethanol. For all groups exposed to ethanol the general response was very comparable amongst the three replicates. The avoidance behavior was high right at the beginning of the experiment and decreased slightly within the first 10 min. With the beginning of the first dark cycle the activity increased again and reached a plateau after 40 to 50 min for all replicates. The change between light and darkness did not affect the embryos motion pattern.

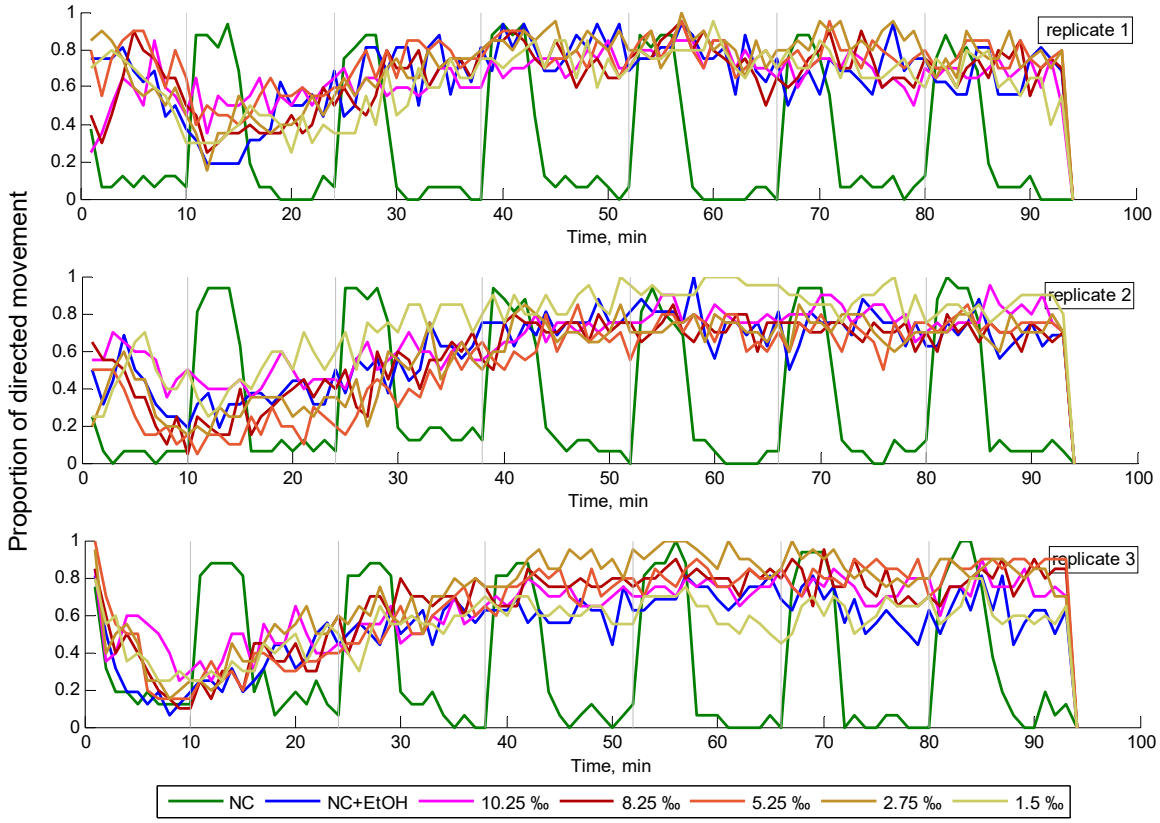

Figure 6 Proportion of directed movement of 120 hpf zebrafish embryos exposed to different levels of salinity (10.25 ‰; 8.25 ‰; 5.25 ‰; 2.75 ‰; 1.5 ‰) from 96 hpf to 120 hpf and to 2 % ethanol at 120 hpf. Treatment groups n = 20; negative control n = 16

4. Conclusion

Measurements of the surface water on the route of the FerryBox between Tallinn and Stockholm found the salinity to range between approximately 2 ‰ and 10 ‰ (http://on-line.msi.ttu.ee/GRACEferry/). This study found that hatched zebrafish embryos maintain their capability to react to exposure to a negative stimulus up to a salinity of 10.25 ‰. Based on the findings of this study it can be concluded that within this range a control of the salinity levels for the biosensor would not be necessary.

Parallel experiments at RWTH Aachen University in WP 3 investigated the tolerance of zebrafish embryos to salinity in the prolonged fish embryo toxicity test up to 120 hpf. The experiments found an $LC_{50\ 120hpf}$ of 9.4 ‰ and an $EC_{10\ 120hpf}$ of 6.6 ‰ which was in accordance to data from literature (Loerks, 2010; Sawant et al., 2001). For all further experiments within WP 3 zebrafish embryos adapted to 5 ‰ salinity were used, since this salinity did not induce adverse effects in the prolonged FET and is relevant for the brackish water conditions of the Baltic Sea. Exposure of 96 hpf embryos to elevated levels of toxicity found that the organisms were able to display normal reactions towards a negative stimulus up to 10.25 ‰. In the study by Sawant et al. (2001) it was also found that the

salinity tolerance of zebrafish embryos increases with increasing age, although for earlier developmental stages.

Since the flow-through well-plate is not available yet, no data on the behavior of zebrafish embryos under flow through conditions can be made. In future experiments the generated threshold will be tested again under flow through conditions.

From the presented results it can be concluded that 96 hpf zebrafish embryos are tolerant to a salinity up to 10.25 ‰ and are still able to display normal avoidance reactions when exposed to a negative stimulus. The application of zebrafish embryo swimming behavior as a biosensor on the Baltic Sea is therefore not limited by the salinity. However, since the tolerance of zebrafish embryos to temperature outside the optimum niche is rather limited, temperature needs to be controlled in the flow-through system of the biosensor.

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