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The value of Zebrafish (*Danio rerio*) as an integrative model in ecotoxicology

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Abstract

Nonylphenol (NP) is a widely used industrial chemical that exists heavily in the aquatic environment and it is considered a severe contaminant in the ecosystem. Evidence of the severe effects of NPs on different aquatic organisms exists in a number of axes which may be estrogenic and non-estrogenic. In this study, we report the effect of NP on zebrafish (*Danio rerio*) embryos. One hour post fertilized (hpf) eggs were exposed to graded levels of NP (0.01, 0.03, 0.1, 0.3, 1, 3 and 10 µM), ranging from lower more environmentally relevant levels to high lethal levels. The embryos/larvae were checked at 24, 48 and 72 hpf to study the development and survival rates. Morphological abnormalities in different organs were photographed and examined. Embryos were highly affected by NP in a dose-dependent pattern. Through a comparative approach of the treated embryos with the control group, it could be revealed that 0.01, 0.03, 0.1 and 0.3 µM have no side effect on the embryos, meanwhile 1 and 3 µM caused severe oedema and less development, moreover 10 µM was lethal from the beginning. The results of the current study show that more attention should be given to assess the risk of these compounds in the aquatic environment.

Keywords: zebrafish, nonylphenol, toxicity, teratogenecity, developmental abnormalities

1. Introduction

Fish embryos represent a good model for environmental risk assessment of chemicals since they offer the possibility to perform small-scale, high-throughput analyses [1]. Zebra fish (*Danio rerio*) is an emerging toxicity screening model for both medicine and ecology [2]. It is a suitable experimental organism in toxicological studies due to its short spawning intervals, easily manipulation and adaptation to the laboratory conditions, rapid development and generation time, transparent eggs and can be maintained in a relatively small space [3, 4, 5, 6]. facilitating visual identification of morphogenetic movements and organogenesis with a standard dissecting microscope. Embryos of zebra fish have often been used in toxicity studies of environmentally relevant substances [7, 1, 8, 9, 10]. Beyond their application for determining the acute toxicity, fish embryos are also successful models for studies aimed to understanding of toxic mechanisms and the indication of possible adverse and long-term impacts [1]. Several endpoints can be detected in parallel, thereby enhancing the toxicity spectrum covered by the assay [8].

Nonylphenol (NP) is a widely used industrial compound that enters the environment as a microbial degradation product of nonylphenol polyethoxylates (NPEs). Evidence of the severe effects of NPs on different aquatic organisms exists in a number of axes including endocrine and non-endocrine. The majority of research has focused on the estrogenic effects of NPs as endocrine disrupting chemicals on several fish species, where plasma vitellogenine (VTG) gene expression has been used as a biomarker for fish exposure to estrogens [11], [12], [13]. However, studies concerning the non-estrogenic action of such compounds are poorly studied. NP can cause developmental toxicity in aquatic organisms and it was demonstrated in zebrafish causing both lethal and sublethal developmental abnormalities after 96 h and 48 h of exposure, respectively [14, 8].

The present study aimed to evaluate the role of zebrafish embryos in ecotoxicological studies. This could be realized by determining the concentration-dependent effects of graded series of NP on the development of zebrafish embryos

2. Materials and Methods

Compounds

Seven stock solutions (0.1, 0.3, 1, 3, 10, 30 and 100 mM) of NP (a mixture of isomers, CAS Number: 84852-15-3, Sigma-Aldrich, Netherlands) were dissolved in dimethyl sulfoxide (DMSO, 0.01%) immediately prior to use and then directly diluted 10000 times in Dutch standard water (nominal concentrations: 0.01, 0.03, 0.1, 0.3, 1, 3 and 10 µm). Solvent (DMSO, 0.01%) and negative controls were incorporated in the experiment.

Fish Maintenance

Zebra fish were raised and kept under standard laboratory conditions at about 28°C and a photoperiod of 14:10 h. light: dark [15]. Fish were fed with dry fish feed, Tetra-Pro Flakes (Tetra GmbH, Germany) in the morning and hatched brine shrimp (Artemia cysts from INVE, Grantsvillle, UT, USA) in the afternoon. The fish were acclimated in glass aquaria containing copper free water. Typically, the eggs were spawned synchronously at dawn of the next morning. One hour later, eggs quality has been checked under the microscope (Leica MZ 75), being sure to select the healthy, fertilized eggs for the experiment. Fish breeding and embryo manipulation were conducted according to Westerfield *et al.* (1997) [16].

Embryo Test

Fertilized eggs (1 hour post fertilization, hpf) were selected and placed in 24-well cell culture sterilized plates (one embryo/well). Embryos were exposed to the previously mentioned concentrations of AP at the 4:8 – cell stage (1:1.25

hour post fertilization, hpf). 24 embryos/concentration were used and incubated at 28°C. Embryos/larvae were screened daily - till 72 hpf - and scored for survival, alterations in morphology, developmental abnormalities and endpoints of toxicity [3]. Toxic/lethal end points (coagulation, missing heartbeat, missing somites, missing tail detachment, missing spontaneous movement) and non-lethal malformations (pericardial or yolk sac oedema, bent notochord, fin malformation, no pigmentation, incomplete head and eye development) were reported separately.

Morphological observation of embryos

Developed embryos/larvae were examined and photographed daily by a stereo microscope. Paint shop Pro. 8 image analysis software was utilized to control a Roper digital camera on the microscope. Images were depicted at all treatment levels to complete the picture of the morphological abnormalities in different organs.

Calculation of LC_{50} and EC_{50}

The LC_{50} and EC_{50} were calculated at 120 h post fertilization from concentration-% lethality and concentration-% effect curves, respectively for all end points separately as well as for the sum of lethal affected embryos. Logistic curves with binomially distributed errors were used to describe the relationships. From these, LC_{50} and EC_{50} values and their 95% confidence intervals were calculated using Graph Pad Prism 5.01.

3. Results

Embryo-toxicity assay in the developing zebra fish was optimized. The dose-related effects of NP were determined following 72 h exposure starting at 1 hour post fertilization (hpf). The results showed a follow up of the developmental alterations caused by graded levels of NP (Figures 1-3).

For the groups treated with 0.01, 0.03 and 0.1 μM NP, no effect was observed during all the period of the experiment. Meanwhile, higher concentrations of NP leads to lethal and non-lethal malformations in embryos varied according to the concentration and duration of the exposure. Respecting to 1 and 3 μM, the NP started its toxic non-lethal action 24 hpf with a minute oedema which increased stepwise leading to severe head, yolk sac and heart oedemas after 72 hours of exposure at the first concentration level and death at the second one. Concentration 10 μM was toxic within the first few hours of exposure, all embryos stopped their development in the first (epiboly) stage while at 0.1 μM, 22% of the exposed embryos died (Figure4). The developmental effects of NP were dose dependent with an EC_{50} value of 0.8 μM for all endpoints (Figure 5).

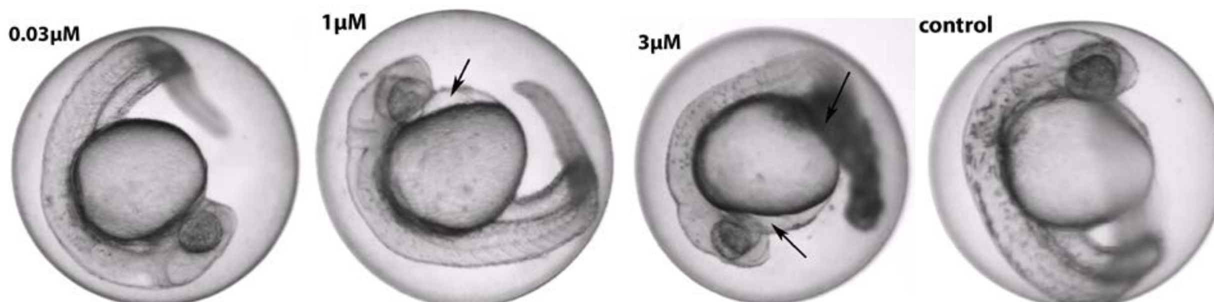

Fig 1: Morphological changes in zebrafish embryos exposed to different concentrations of NP and were photographed live in lateral orientation through a stereomicroscope at 24 h post fertilization (hpf). Embryos exposed to concentrations of 0.01, 0.03, 0.1, 0.3 μM, showing well developed embryo with yolk sac, tail, head, eyes and pigmentation similar to the control group embryos. Embryos exposed to 1 μM showing yolk sac oedema (arrow). Embryos exposed to 3 μM, showing extended mal-formed yolk sac accompanied with oedema (×4).

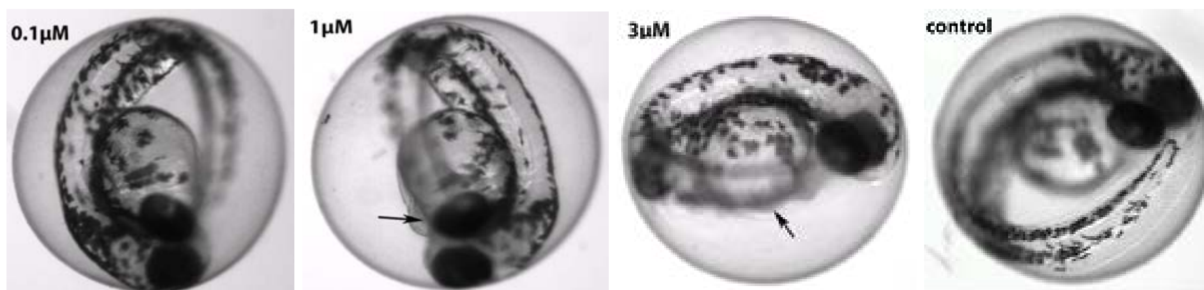

Fig 2: Morphological changes in zebrafish embryos exposed to different concentrations of NP and were photographed live through a stereomicroscope at 48 h post fertilization (hpf). Embryos exposed to concentrations of 0.01, 0.03, 0.1, 0.3 μM, showing embryos with well-developed notochord with muscles, otolith, caudal fin, head, eyes and pigmentation similar to the control group embryos. 1μM group, showing bigger oedema and a slightly unstraight notochord. 3 μM group, (a) line oedema around yolk sac (arrow), growth retardation (small head and eyes), (b) mal formed tail (curved, short, no tail fin), blood clotting around yolk sac (arrow, visualized as tissue discoloration), simple scoliosis was shown (×4).

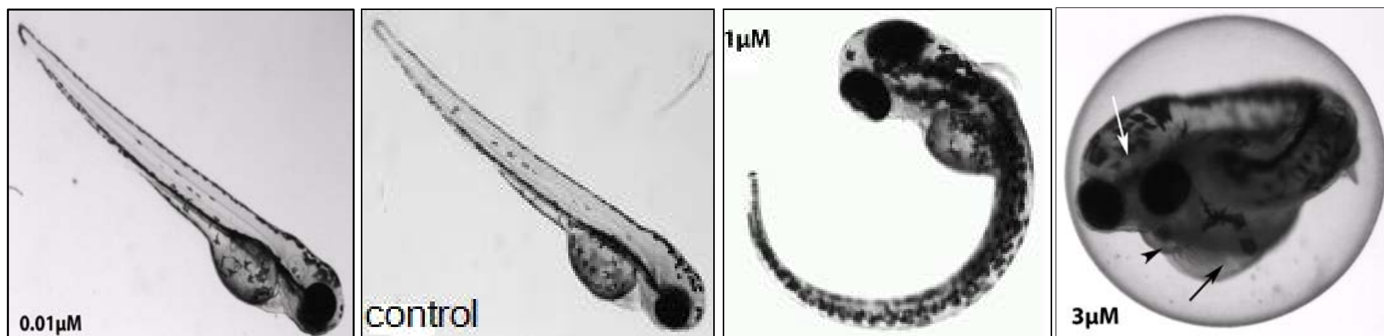

Fig 3: Morphological changes in zebrafish embryos exposed to different concentrations of NP and were photographed live through a stereomicroscope at 72 h post fertilization (hpf). Embryos exposed to concentrations of 0.01, 0.03, 0.1, 0.3 µM, showing well developed hatched larvae similar to the control group larvae (×4). 1µM treated group, showing delayed growth "un hatched" with oedema or hatched with a curved notochord (×2). 3 µM group, necrosis in body tissue and brain (white arrow, detected by condensed spots of pigments), oedema with blood clotting in yolk (arrow) and heart (head arrow), coiled tail, but the heart still beating. (×4).

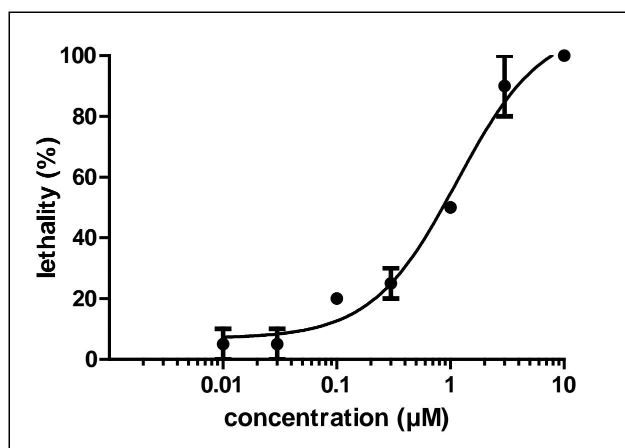

Fig 4: Dose-effect curve of lethal malformation (% relative to control) of zebrafish embryos caused by different concentrations of NP.

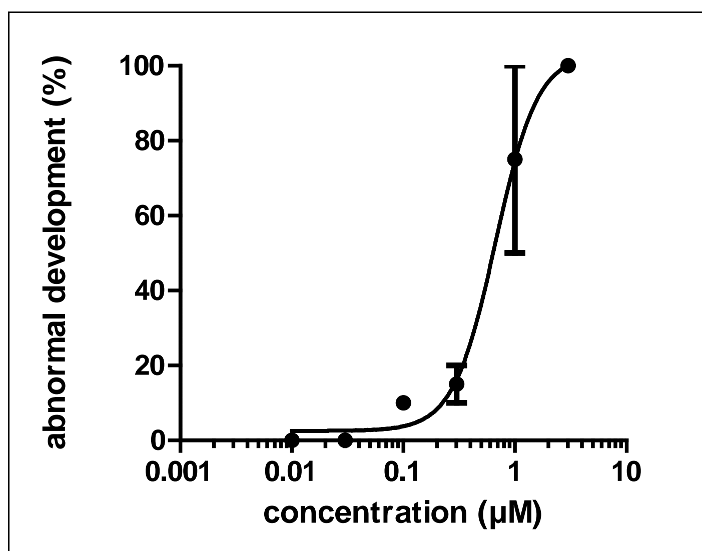

Fig 5: Abnormal development (% relative to control) of zebrafish embryos caused by different concentrations of NP at 72 hour post fertilization.

3.1. 24 hours post fertilization

Figure 1 represents embryos subjected to the concentrations of 0.03, 0.1 and 0.3 µM were not altered morphologically, compared with those of the DMSO control group during the first 24 h of development, showing well developed healthy embryos with somites, yolk sac, tail, eyes, head, prominently sculptured brain and a number of pigment cells are present along the axis dorsal to the yolk extension and on the dorsal part of the yolk ball, similar to the control ones. While, embryos exposed to 1 and 3 µM showed coagulation resulted from development stop in epiboly stage.

3.2. 48 hours post fertilization

Embryos exposed to concentrations of 0.01, 0.03, 0.1 and 0.3

μM showing embryos with well-developed notochord, otolith, caudal fin, head, clear eyes and pigmentation extends to the whole length of the body, similar to the control group embryos. The 1 μM treated-group showed bigger oedema and a slightly unstraight notochord. Embryos exposed to 3 μM group seems more affected with the time, showing severe oedema and blood clotting around yolk sac accompanied with growth retardation noticed from small head and eyes and mal formed tail (curved, short, no tail fin). However, blood was circulated through a closed set of channels and clear heart beats were measured and ranged between 119-120 beats/ min., as all other groups (Figure 2).

3.3. 72 hours post fertilization

Larvae were hatched with quite elongated pectoral fin buds and vigorous heart beats were observed in the control group and those treated with 0.01, 0.03, 0.1 and 0.3 μM of NP. Also, it was shown that the yolk sac started to be shrunk making the pericardial cavity more conspicuous. For the embryos treated with 1 and 3 μM, severe oedemas all around the body accompanied by growth retardation and curved notochord were shown. The embryos of these treatments still looked like those that were 48 h old and approximately no hatching was recorded in these groups except 20% of those of the 1 μM treated group. Respecting to the 3 μM treated group, malformed-coiled tail, necrosis in brain and other tissues of the body, blood clotting in yolk sac and reduction in the number of heart beats (80 beats/min.) were also detected (Figure 3).

4. Discussion

The toxic effect of NP has been reviewed and studied in different aquatic organisms and adult fish species [17, 6, 13]. Limited information concerning the effect of such class of chemicals on fish embryos [18]. The degree of toxicity of NP varies according to the dose and exposure period. Additionally, the nature of its effects on fish embryos differs according to the embryonic or larval stage. To gain more insight in the embryotoxic effects of NP, zebrafish embryos were exposed from one hour post fertilization (hpf) for the 72 hpf of development to follow up the developmental alterations caused by graded levels of NP. According to Kammann *et al*. (2009) [8]. Acute toxicity test with zebrafish embryo can only be a first step for the assessment of the environmental risk of such compounds. In the current study, NP caused developmental abnormality at a dose of 1 μM at the beginning of the test, reached to severe oedema after 72 hours of exposure, whereas higher concentrations led to full development arrest and mortality. The degree of lethality varied according to the concentration, meaning that, for the highest nominal concentration of 10 μM, the experiment was terminated at 24 h of examination, whereas for 3 μM the beginning of lethality was shown at 72 h with a delayed-hatched larvae. This explained the immediate toxicity of the first concentration and the non-lethal action- endpoints are inhibition of the development and oedemas- of the second one during the first 72 hpf. This study indicated that the developmental effect of NP is dose dependent with a LC_{50} value of 1 μM.

The recorded abnormalities, lethal and non-lethal malformations occurred at different concentrations may be due to the ability of NP to be metabolized in the fish causing numerous direct and indirect effects ranging from changes in gene expression [19, 20]. Through induction of estrogen responsive genes [21]. And protein [22]. Kazeto *et al*. (2004) [23]. Demonstrated that exposure of zebrafish juveniles to 0.01-1 μM NP, strongly enhanced the expression of CYP19A2 gene in dose-dependent manner.

This work explores the potential of zebrafish and its sensitivity to be a successful model capable of monitoring the effect of NP even if at micromolar levels. Also, the present study confirms the action of NP as a toxic compound causing internal and morphological malformation as well as mortality in zebrafish at dose rates approximately equal to the LC_{50} (1 μM) at 72 hour post fertilized larvae level. Such results would be valuable for better estimating the risk of such class of chemicals and for following up their mode of actions.

5. References

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