Developmental toxicity of nonylphenol in zebrafish (Danio rerio) embryos

Tamer El-Sayed Ali1* & Juliette Legler2

1Oceanography Department, Faculty of Science, Alexandria University, Alexandria, Egypt.
2Institute for Environmental Studies, VU University, 1081 HV Amsterdam, The Netherlands

*E.mail: tameraly@yahoo.com

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Present study reports the response of zebrafish embryos exposed in vivo to graded concentrations of Nonylphenol (NP), ranging from high lethal levels to lower, more environmentally relevant levels. Embryos treated with NP showing developmental abnormalities and morphological alterations in a dose-dependent pattern. Results suggest that early exposure to NP can cause direct and delayed mortalities or even non-lethal malformations at doses more than 0.3 µM, while EC50 was shown to be 1 µM after 6 days of exposure.

Keywords: Nonylphenol, Zebrafish, Toxicity, Non-estrogenic action, Developmental abnormalities.

Introduction

Nonylphenol (NP) is a widely used industrial organic compound that enters the environment as a product of microbial degradation of nonylphenol polyethoxylates (NPEs). Majority of research on NP has concentrated on the estrogenic effects of NPs. The endocrine disrupting chemicals on several fish species, where plasma vitellogenine (VTG) gene expression has been used as a biomarker for fish exposure to oestrogens. Kinetics of hepatic VTG mRNA expression, plasma VTG accumulation and VTG clearance have been determined after exposure to NP in zebrafish (Danio rerio), rainbow trout (Oncorhynchus mykiss), Atlantic salmon (Salmo salar), Japanese medaka (Oryzias latipes), Fathead minnows (Pimephales promelas), common carp (Cyprinus carpio). However, to our knowledge, reporting the non-estrogenic action of such compounds is poorly studied. NP can cause developmental toxicity in aquatic organisms and it was demonstrated in killifish (Fundulus heteroclitus) and zebrafish (Danio rerio) causing both lethal and sublethal developmental abnormalities after 96 h, 48 h of exposure, respectively.

This study aimed to determine concentration-dependent effects of graded series of NP on the development of zebrafish embryos according to gross morphology. It also examine the extent to which the magnitude of the effects is dependent on the concentration of NP with which the embryos are treated.

Materials and Methods

Chemicals

Seven stock solutions (0.1, 0.3, 1, 3, 10, 30 and 100 mM) of NP (a mixtures 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

Zebrafish were raised and kept under standard laboratory conditions at about 28°C and a photoperiod of 14:10 h. light:dark. Fish were fed with dry fish feed, Tetra-Pro Flakes (Tetra GmbH, Germany) in the morning and hatched brine shrimp (Artemia cysts from INVE, Grantsville, UT, USA) in the afternoon. Fish were acclimated in glass aquaria containing copper free water. 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.

Zebrafish embryo test

Selected eggs (1 hour post fertilization, hpf) were placed in 24-well cell culture sterilized plates (one
embryo/well). Embryos were exposed to these concentrations of NP at the 4:8 – cell stage (1:1.25 hour post fertilization, hpf). Ten embryos/concentration were used and incubated at 28°C. Embryos/larvae were screened daily - till 6 days - and scored for survival, alterations in morphology, developmental abnormalities and endpoints of toxicity\(^ {14} \). Toxic/lethal end points (coagulation, missing heart beat, 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. For the later stages, (6 days), larvae from each replicate (n = 20 per concentration) were immobilized in 2% methylcellulose. The experiment was repeated twice.

Developed embryos/larvae were examined and photographed daily by a stereo microscope. Paintshop 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 6-days 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 GraphPad Prism 5.01.

**Results**

To gain more insight in the embryotoxic effects of NP, zebrafish embryos were exposed from 1 hour post fertilization (hpf) for the first 6 days of development to follow up the developmental alterations caused by graded levels of NP (Figs.1-5).

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**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).
For the groups treated with 0.01, 0.03 and 0.1 µM, NP has no effect during all the experimental period. Otherwise, NP leads to lethal and non-lethal malformations in embryos varied according to the concentration and duration of exposure. Respecting to 1 and 3 µM, the NP started its toxic non-lethal action 24 hpf with simple oedema which increased stepwise leading to severe head, yolk sac and heart oedemas after 6 days of exposure at the first concentration level and death at the second one. Concentration 10 µM was toxic within the first three hours of exposure, all embryos stopped their development in the epiboly stage, while at 0.1 µM, 20% of the exposed embryos died (Fig. 6). The developmental effects of NP were dose dependent with an EC_{50} value of 0.8 µM for all endpoints (Fig. 7).

24 hpf

The concentrations of 0.01, 0.03, 0.1 and 0.3 µM have not caused morphological alterations in embryos compared with those of the control group during the first 24 h of development, showing well developed healthy embryos with somites, yolk sac, tail, head, eyes, prominently sculptured brain and few 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 yolk sac oedema and extended unstraight notochord. Embryos exposed to 3 µM group seems more affected with the time, showing delayed growth “un hatched” with oedema all around the body “eye, pericardial and yolk sac oedema” (a, arrows, ×4), or hatched with a curved notochord (b, ×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).

48 hpf
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, eyes and pigment extends the whole length of the body, similar to the control group embryos. The 1 µM treated-group showing bigger oedema and a slightly unstraight notochord. Embryos exposed to 3 µM group seems more affected with the time, showing
line oedema and blood clotting around yolk sac accompanied with growth retardation (small head and eyes) and malformed tail (curved, short, no tail fin). However, Blood circulates through a closed set of channels and clear heart beats were measured and ranged between 119-120 beats/min., as all other groups (Fig. 2).

72 hpf

Hatched larvae 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 group except 20% of those of the 1 μM treated group. Focusing on the 3 μM treated group, malformed-coiled tail, necrosis in brain and other body tissues, blood clotting in yolk sac and reduction in heart beats number (80 beats/min.) were also detected (Fig. 3).

96 hpf

Further regular development was shown in 0.01, 0.03, 0.1 and 0.3 μM treated groups. Also those of 1 and 3 μM treated embryos have presented 100% and 50% hatching, respectively. However the hatched larvae of the later group were severely curved.

120 hpf

For the groups treated with 0.01, 0.03 and 0.1 μM, hatched larvae have completed most of their morphogenesis, started to grow rapidly and swim about actively, showing inflated swim bladders and protruded mouths. The ventral yolk sac extending in both directions and nearly empty. For 0.3 μM treated group, although the larvae were healthy and behaved as the control group from the developmental point of view, a slight scoliosis (curvature in notochord and tail fin) was shown. Embryos exposed to 1 μM showing curved notochord, less active heart beating and delayed development of caudal fin. Embryos exposed to 3 μM presenting only 60% survival, 50%
of these living are so curved, with thinner caudal fin than the control group, so big yolk sac, not so far in the development with small head and jaws are mal formed. The rest of living embryos are unhatched with head, heart and yolk sac oedema (Fig. 4).

6 days post fertilization
For the groups treated with 0.01, 0.03 and 0.1 µM, active swimming larvae have been demonstrated with an absorbed yolk sac and a more ventrally dropped gut tube. The larva also moves its jaws, opercular flaps, pectoral fin and eyes. Partial recovery of the tail and notochord curvature was shown in embryos treated with 0.3 µM and the development was parallel to those of the control and previously mentioned treated groups. Severe oedema was shown around different body organs with a slight curved notochord was shown in the larvae of the 1 µM treated group. For those treated with 3 µM, all larvae were dead with no further growth than presented at 120 h pf stage (Fig. 5).

Discussion
Chronic and acute toxicities of NP on aquatic organisms have been recently reviewed by Staple.\(^{15}\) The degree of toxicity of NP varies according to the dose and exposure period. Additionally, the nature of effects in the zebrafish embryo test differs according to the embryonic/larval phase. According to\(^{11}\), acute toxicity tests with zebrafish embryo can only be a preliminary step for the assessment of the environmental risk of NPs. In the present study, NP caused abnormal development at nominal concentration of 1 µM at the beginning of the test, reached to severe oedemas after 6 days of exposure, whereas higher concentrations led to full development arrest and mortality. The action of lethality varied according to the concentration, meaning that, for the highest nominal concentration of 10 µM, the experiment was terminated at 24 h examination, whereas for 3 µM the lethality was shown on the sixth day with a delayed-hatched larvae, explaining the acute immediate toxicity of the first concentration and the non-lethal action (endpoints are inhibition of the embryonal development and oedemas) of the second one during the first 120 hpf. This study demonstrates that the developmental effect of NP is dose dependent with a LC\(_{50}\) value of 1 µM. Very recently,\(^{11}\) demonstrated that NP caused lethal as well as non-lethal malformation during zebrafish embryo development, presenting EC\(_{50}\) for lethal endpoints of 6.7 mg/L, after 48 hours of exposure\(^{16}\) demonstrated that exposure of zebrafish juveniles of 17dpf to 0.01-1 µM NP, strongly enhanced the expression of CYP19A2 gene in dose-dependent manner.

Conclusion
The recorded abnormalities, lethal and non-lethal malformations occurred at different concentrations levels may be due to the ability of NP to be metabolized in the fish causing numerous direct and indirect effects. It ranges from changes in gene expression\(^{17,18}\) through induction of estrogen responsive genes\(^{19}\) and protein\(^{19}\) and effects on brain muscarinic receptor\(^{20}\) to increased apoptosis\(^{21}\), expression of acute phase protein\(^{22}\) and changes in phase II detoxication\(^{23}\). This study confirms the action of NP as a toxic compound causing internal and morphological malformation and mortality in zebrafish at dose rates approximately equal to the LC\(_{50}\) (1 µM) at 6 days post fertilized larvae level. The range of responses of NP shows that exceeding a thresholds concentration of 0.3 µM would put the embryos in risk.

References
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