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Effects of a Branched *p*-Nonylphenol Isomer (4(3'-,6'-dimethyl-3'-heptyl)-phenol) on Embryogenesis in *Lymnae stagnalis L.*

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Abstract. The tertiary branched alkyl-chain isomers of *p*-nonylphenol (NP) are perceived to have more estrogenic potency than its constituent secondary and primary straight alkyl-chain isomers. Investigations with single tertiary branched isomers of NP can therefore contribute toward the elucidation of the mechanisms of toxicity and estrogenicity of NP. A single tertiary branched alkyl-chain isomer (4(3'-,6'-dimethyl-3'-heptyl)-phenol) was used in studies to determine its effects on embryonic growth and mortality in *Lymnaea stagnalis L.* Egg masses were exposed to the test compound for 20 days in a static waterborne-exposure regime with an average NP concentration of 105 μg/L and water temperature range of 18–20°C. Observations were made under a microscope and pictures were taken with a digital camera to determine the various developmental stages of growth, the duration of growth in each stage, embryo hatchability, and embryo mortality. The isomer was found to cause significant delay in all stages of growth and more significantly in the Morula and Veliger stages. An increase in embryo mortality, from the third day until the end of the experiment, was observed in exposed egg masses compared to controls. The hatching success of embryos was also significantly reduced by exposure, with 81% hatchability in exposed egg masses compared to 93% in the controls, after 18 days of continuous exposure. The encapsulating jelly strand that completely covers the rows of egg masses may have prevented the isomer residues from effectively penetrating into the embryos as shown by the observed low bioconcentration factors of the isomer in egg masses during exposure, resulting in unexpectedly lower observed estrogenic effects. However, this factor was not investigated. *In vivo* biotransformation of some of the residues of the isomer into catechol metabolites by the embryos during exposure could also result in the reduction of its estrogenic potential. To understand more fully the extent of toxicity and estrogenicity of this isomer, *in vitro* estrogenic assays are recommended. It would also be necessary to investigate its estrogenic effects on embryo development after *in vivo* maternal exposure.

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Introduction

The endocrine effects of synthetic estrogenic compounds on aquatic organisms have mainly been observed in fish sampled from effluents coming out of sewage treatment plants and from rivers and estuaries linked to these effluents (Jobling and Sumpter 1993; White *et al*. 1994; Harries *et al*. 1997; Gagne and Blaise 1998; Helaleh *et al*. 2001; Koerner *et al*. 2001; Spengler *et al*. 2001; Thomas *et al*. 2001; Van Aerle *et al*. 2001). Some of the observed estrogenic effects include elevated blood plasma levels of the yolk precursor protein (vitellogenin), sexual disruption expressed in form of intersex, decreased fecundity and hatchability of embryos, inhibition of testicular growth in male fish, and reduction in gonadal sizes in male and female fish (Gray and Metcalf 1996; Jobling *et al*. 1996). The ecological implications of these effects on freshwater fish and other aquatic organisms can therefore be devastating. Nonylphenol (NP) is one of the most studied synthetic estrogenic compounds since the first report of its ability to displace 17β-estradiol from the estrogen receptor in the 1970s (Mueller and Kim 1978; Soto *et al*. 1991; White *et al*. 1994; Jobling *et al*. 1996; Czech *et al*. 2001; Madigou *et al*. 2001; Thomas *et al*. 2001). It is a persistent degradation product of nonylphenolpolyethoxylate (NPE), a surfactant often used in the manufacture of detergents, paints, pesticides, and plastics and forms one of the potent estrogenic compounds in sewage and waste water (Jobling and Sumpter 1993; Harries *et al*. 2001; Spengler *et al*. 2001). It was because of the detection of these estrogenic effects of NP that NPE use in the manufacture of household detergents has been discontinued in countries such as Germany and United Kingdom (Jobling and Sumpter 1993).

The mechanisms of NP estrogenicity have been investigated to some extent, and it is now known that it competes with the natural estrogen, 17β-estradiol (E2), in binding to the estrogen receptor (White *et al*. 1994; Brzozowski *et al*. 1997; Madigou *et al*. 2001). Both *in vivo* and *in vitro* studies show that its

potency as an estrogen receptor is weaker compared to that of 17β-estradiol, with significant effects being reported at concentrations of between 10^3 and 10^4 orders of magnitude higher compared to the effective concentration of the natural estrogen (White *et al*. 1994; Czech *et al*. 2001). According to literature reports, the binding potency of NP to the estrogen receptor only ranges between 9×10^{-6} and 2×10^{-2} relative to 17β-estradiol (Jobling and Sumpter 1993; Arcaud-Hoy and Benson 1997). However, it is assumed that the potential estrogenic effects of NP can be equally considerable as a consequence of its wider distribution in freshwaters at much higher concentrations, its persistence in aquatic sediment and macrophytes as well as its ability to bioaccumulate in tissues and various organs of aquatic organisms (Naylor *et al*. 1992; Kvestak and Ahel 1994; Harries *et al*. 1997; Spengler *et al*. 2001; Lalah *et al*. 2003a, 2003b). NP is also reported to be three times more estrogenic than DDT and despite its relatively lower environmental persistence and bioaccumulation potential, it may manifest more endocrine disruption in aquatic organisms than DDT (Jobling and Sumpter 1993; Lalah *et al*. 2003a). Some of the reported tissue bioaccumulation factors of NP in fish and aquatic invertebrates under laboratory and field conditions range between 13 and 3,400, depending on species, method of exposure, and exposure concentrations (Ekelund *et al*. 1990; Lalah *et al*. 2003b). Recently, Madigou *et al*. (2001) have reported on stimulation of vitellogenin and induction of trout estrogen receptor gene in rainbow trout as well as sexual reversion of male trout embryos by NP and 17β-estradiol at 10^{-6} M and 10^{-8} M concentrations, respectively. Czech *et al*. (2001) have reported lower fecundity and significant histopathological changes in the lungs and foot of *L. stagnalis* after semistatic waterborne 12−day continuous exposure to NP at concentrations of 100 μg/L and 10 μg/L, respectively. These reports show that NP has the potential to alter hormonal pathways that regulate reproductive and growth processes in fish and other aquatic organisms. The developmental or reproductive toxicity of NP appears to occur at various stages, during embryo development, at juvenile and at adult stages (Gray and Metcalf 1996; Arukwe and Goksoyr 1997; Lalanzer 1999; Pedersen *et al*. 1999; Czech *et al*. 2001; Yokota *et al*. 2001).

The complex chemical structure of NP makes it difficult to understand the mechanisms of its toxicity and estrogenicity. Studies have shown that commercial technical NP and, consequently, the residual NP formed as a degradation product of NPE during waste treatment, is composed of at least 22 different para-isomers (Bhatt *et al*. 1992; Routledge and Sumpter 1997; Wheeler *et al*. 1997). Results of *in vitro* experiments indicate that in order to get optimal estrogenic activity, an alkylphenol such as NP should be composed of at least a single tertiary branched six- to eight-carbon alkyl chain attached to the para-position of the phenol group (Routledge and Sumpter 1997). The structural requirement for activity of alkylphenols, with respect to estrogen-receptor binding ability, is also reported to be determined by both the size and the hydrophobic character of the alkyl group (Mueller and Kim 1978; Jobling and Sumpter 1993; Brzozowski *et al*. 1997; Madigou *et al*. 2001). The nature of the alkyl group and, as a direct consequence, its solubility and lipophilicity, also influence NP bioavailability and metabolism (Meldahl *et al*. 1996; Staples *et al*. 1998; Arukwe *et al*. 2000; Ferreira-Leach and Hill 2001; Lalah *et al*. 2003b). The potency of NP mixture is therefore expected to be highly dependent on the individual isomer structure and content. The alkyl chain of an NP isomer can vary in size, degree of branching, and in the attachment position on the phenol. It is now perceived that, in general terms, the branched-chain isomers have more estrogen-like toxicity than the straight-chain isomers because of their relative structural resemblance to natural estrogens, in addition to being relatively more resistant to biodegradation in the aquatic environment (Pedersen *et al*. 1999; Hu and Aizawa 2003). Our present study focused on a single tertiary isomer of NP, 4(3',6'-dimethyl-3'-heptyl)-phenol, which we synthesized in the laboratory (Lalah *et al*. 2001a, 2001b; Klaus Günther 2000; Institute of Applied Physical Chemistry, Juelich, Germany, personal communication) and used to investigate NP effects on mortality, hatchability, and delay in growth of developing embryos of the hermaphrodite pond snail, *L. stagnalis*. This is the first time this isomer was synthesized and used in such endocrine studies. Based on the presumption that branched isomers have more estrogenic potency, this single branched isomer would be expected to have higher estrogenic potential and therefore to cause higher effects on embryo growth, mortality, and hatchability in *L. stagnalis.* compared to the technical NP mixture at similar concentrations. *L. stagnalis.* was chosen as a test organism because of its abundance and usefulness in biochemical research as has been discussed in detail in the literature (Canton and Slooff 1977; Luchtel *et al*. 1997; Lanzer 1999; Atienzar *et al*. 2002). In addition to being widely distributed in freshwaters, these common pond snails grow rapidly and have very high fecundity. The existence of Cytochrome P450 (Cyt P450) enzyme system in *L. stagnalis.* and use of this organism in ecotoxicological studies to understand endocrine disruption have also been previously reported (Canton and Slooff 1997; Lanzer 1999; Czech *et al*. 2001). A number of studies using technical NP to investigate estrogenic effects on developing embryos of *L. stagnalis.* which could provide comparison with our study have also been reported (Lanzer 1999; Czech *et al*. 2001).

Materials and Methods

Uniformly ring radiolabeled ^{14}C-nonylphenol isomer, 4(3'-, 6',-dimethyl-3'-heptyl) phenol (>95% pure by high-performance liquid chromatography) with a specific activity of 16.205 megabecquerels/mg and a normal standard of the same NP isomer (99% pure by nuclear magnetic resonance) were synthesized in our laboratory as has been reported (Lalah *et al*. 2001a, 2001b). The radiolabeled ^{14}C-NP isomer (1% of the normal cold material) was mixed with the standard NP isomer before dosing into the aquarium. This isomer is known to constitute 10% of technical p-NP, which is a mixture of more than 20 differently branched- and linear-chain isomers.

Radioanalysis to determine the concentration of the test chemical was performed on a Packard Tri-Carb Scintillation Analyzer Model 2100 TR. The solid egg mass samples were combusted to $^{14}CO_2$ in a Packard Sample Oxidizer Model 307. The egg masses were exposed to the test chemical in tap water in duplicate small glass aquaria (29 cm × 29 cm × 45 cm) and another aquarium glass tank of similar size was set up for control (Fig. 1). The chemical concentration in the water was monitored daily and kept constant with a stirrer at an average of 105 μg/L (range: 93–116 μg/L) by a high-performance liquid chromatography generator column method as described elsewhere (Lalah *et al*. 2003b).

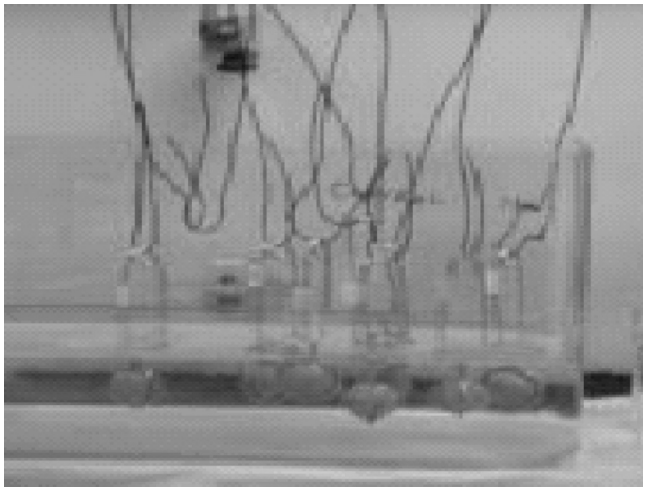

Fig. 1. The photograph of one of the aquarium glass tanks, showing the immersed hanging automatic-closing sieves containing the egg masses

This concentration was chosen because previously reported studies with technical NP used 100–110 μg/L concentration range, and this would provide comparison with ours (Lanzer 1999; Madigou *et al*. 2001; Yokota *et al*. 2001). The embryos were observed under a microscope and pictures were taken with a digital camera.

Fifty adult hermophrodite pond snails, *L. stagnalis*, were purchased from Münchner Zoo Fachmarkt in Munich, Germany, and maintained in a large laboratory glass aquarium with 80 L of water, 3–4-cm sediment depth (sediment size: 1–2 mm, 3–4 mm and 5–8 mm, mixed in equal masses) in a controlled room at 19–20°C and 72% relative humidity with 12 hours light and 12 hours darkness cycle for 6 months, feeding with Sera Premium® Spirulina 20% dead algae tablets, and changing 20% of the water every week. The pH of the water was 8 and dissolved oxygen content was 6.7 mg/L (74.5% saturation) and additional mild pump aeration was provided. During the spawning period, egg masses were collected and kept in glass beakers until hatching. The hatchlings were transferred to a smaller glass aquarium containing tap water only and kept under the same temperature and light/darkness conditions in the control room, feeding them regularly with Spirulina, and changing the water every 2 days, until maturity (approximately 3–4 months). For each aquarium tank, freshly laid egg masses (20 in total, selecting same-size samples) of *L. stagnalis* from this matured first generation were carefully removed with a spatula, placed gently in automatic-closing tea sieves (stainless steel, Fackelmann®) obtained from Münchner Zoo Fachmarkt, Munich, Germany (Fig. 1) and then transferred to the aquarium for continuous exposure to the test compound at an average concentration of 105 μg/L until the snails hatched. The NP concentrations in the water were checked regularly and kept constant by continuous circulated dosing from the high-performance liquid chromatography column. The number of fertilized eggs per capsule was counted six times weekly and, simultaneously, the embryos were examined for deformations, their developmental stages, and for any mortality under a microscope ($8_{\times}$–$16_{\times}$ magnification) (from Hewlett Packard, Munich, Germany) and by taking pictures (Fig. 2, Table 1) using a digital camera. The control tank with the same number of egg masses and subjected to the same experimental conditions was not treated. The water temperature during the test period ranged from 18°C to 21°C. The water in both control and treated aquaria was filtered once every 2 days to prevent any accumulation of calcium carbonate as a result of evaporation, as had been experienced by Lanzer (1999).

The entire embryonic development and life cycle of *L. stagnalis* until a young snail hatches takes place inside single eggs and can be divided into four stages (Table 1). The eggs are encapsulated within a protective transparent jelly strand, with the eggs arranged longitudinally. In our experiment, spawning (after pairing) took place in the aquarium where three to four egg masses were laid on the glass walls. Scalpels were used to collect the egg masses. The egg masses (approximately 2- to 4-cm long) contained three to four rows of several eggs covered in a jelly strand matrix. Because the jelly was clear, the eggs and the embryos were clearly visible and could easily be observed and counted under the microscope. Normally, each egg contains one embryo, but can sometimes contain more than one. Up to eight embryos in some eggs of *L. stagnalis* were identified in one reported previous study (Lanzer 1999). In our study, we identified two and three embryos, respectively, in some eggs. The duration of embryo development depends on the temperature and the amount of dissolved oxygen and can take place over a period of 10–20 days, as reported by three authors who have studied embryonic development and growth of *L. stagnalis* and have been able to distinguish the four different stages of growth (Canton and Slooff 1977; Muenzinger 1987; Lanzer 1999).

In another experiment to determine the accumulation of ^{14}C-nonylphenol isomer in developing embryos of *L. stagnalis*, the egg masses, at different embryonic developmental stages, were exposed to the test chemical in 200 mL of tap water in 250-mL glass beakers. The water concentration was monitored every day and an average exposure concentration was calculated (Table 2). The egg masses were sampled after 1, 2, 3, and 4 days of exposure, respectively, to determine the amount of ^{14}C residues accumulated for each developmental stage. The sampled egg masses were rinsed several times with distilled water before analysis and were analyzed by complete combustion to $^{14}CO_2$ in an oxidizer, with the released $^{14}CO_2$ residues being quantified in the liquid scintillation counter. The disintegrations-per-minute values were converted to nanogram equivalents, and the accumulated residues were expressed as nanograms per gram wet weight of egg mass (Table 2).

Statistical Analysis

Bar chart ratios were drawn and medians of mortality in egg masses were calculated to show differences in embryo mortality in egg masses counted in both NP-treated tanks and in control (Fig. 3a,b and Fig. 4). The percentage (%) number of identified embryos in each growth stage, based on initial total number of exposed embryos, and the length of embryonic stages, calculated based on 75% of determined embryos in each stage, were plotted to show differences in extent of growth in Morula, Trochophora, Veliger, and Hippo stages of growth in NP-treated egg masses and in controls (Figs. 5 and 6). Significant differences in embryo mortality and life cycle development (indicated by % number and extent of growth in each stage) between NP-treated egg masses and controls were determined using analysis of variance (ANOVA).

Results and Discussion

Stages of Growth and Development of Embryos in L. stagnalis

The different stages of growth and development in *L. stagnalis* and some of the characteristic features are summarized in Table 1. We were able to identify all of these four stages of growth and use them to investigate the effects of exposing the egg masses to the NP isomer. The observed four stages of embryonic development in our experiment are illustrated in Figure 2.

Fig. 2. The four embryonic stages of growth (samples from control tank in *Lymnaea stagnalis L.* as observed under the microscope (×16)

Table 1. Embryonic growth, stages of development and characteristic features in *Lymnaea stagnalis L.*

Embryonic stage	Duration	Characteristic features
1. Morula stage	60–72 hours (2.5–3 days)	Embryo is yellow, very slow motions
2. Trochophora	Starts after 3 days Lasts between 48 and 60 hours (2–2.5 days).	Clear to whitish, ''grainy'' embryo, uninterrupted rotation
3. Veliger stage	Starts after 5 days Lasts 48 hours (2 days)	Eyes can be seen, embryo loses its round form, soft body and shell can be distinguished
4. Hippo stage	Starts after 7 days Lasts between 48 and 72 hours (2–3 days)	Complete metamorphosis to young snail, embryo fully developed, eyes and heartbeat can be seen, shell and foot clearly separated

Note Based on information extracted from Canton and Slooff (1977), Bluzart and Seuge (1981), Muenzinger (1987), Lanzer (1999)

Table 2. The bioconcentration of the branched NP isomer in exposed egg masses (ng/g wet weight) at different stages of embryonic growth

Stage of growth	Morula	Trochophora	Veliger	Hippo
NP (μg/L)	105.3 ± 31.46	120.1 ± 68.56	99.03 ± 40.33	124.6 ± 12.59
1 day	69.03 ± 49.62	116.36 ± 12.22	152.02 ± 50.10	243.11 ± 94.11
2 days	118.41 ± 34.55	199.20 ± 15.94	181.59 ± 46.34	294.75 ± 117.46
3 days	266.32 ± 128.51	nd	nd	244.34 ± 54.66
4 days	nd	120.95 ± 63.26	132.67 ± 32.24	nd

Note $n = 3$ eggs; exposure temperature: 20 ± 1°C *nd* not determined

Effects of the Branched p-NP Isomer on Mortality of Embryos

The average number of embryos per egg mass was 74 in both control and in the NP-exposed samples. The total number of egg masses in each tank was 20. To determine mortality, the numbers of dead embryos were counted and compared with the total numbers of embryos in all the egg masses counted, as shown in Figure 4a and b. Embryo mortality was significantly higher in the egg masses exposed to NP isomer compared to controls ($p > 0.05$, Student's *t*-test). The median of the total number of dead embryos was also significantly higher in the NP-exposed egg masses (Fig. 3). In the egg masses exposed to NP, mortality on average of two deaths per day (based on total number of dead embryos per egg mass) was also higher than in the controls (one death per day). The maximum number of observed dead embryos in NP-exposed egg masses was above those in the controls. In the NP-exposed aquarium, embryo death started after the third day and remained higher than in control until the end of the experiment.

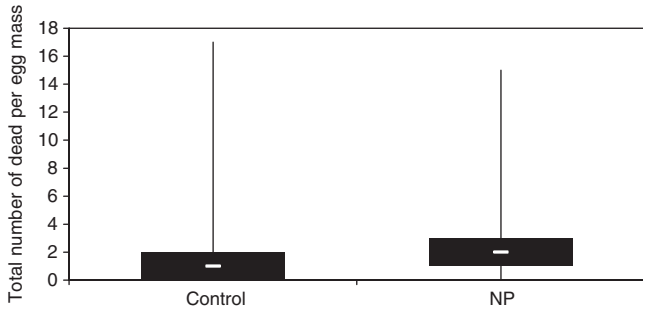

Fig. 3. The median of mortality in the egg masses of *Lymnaea stagnalis* L. under NP-exposure and in the control. (Note: The vertical bars show the total number of egg masses observed (based on same y-axis scale))

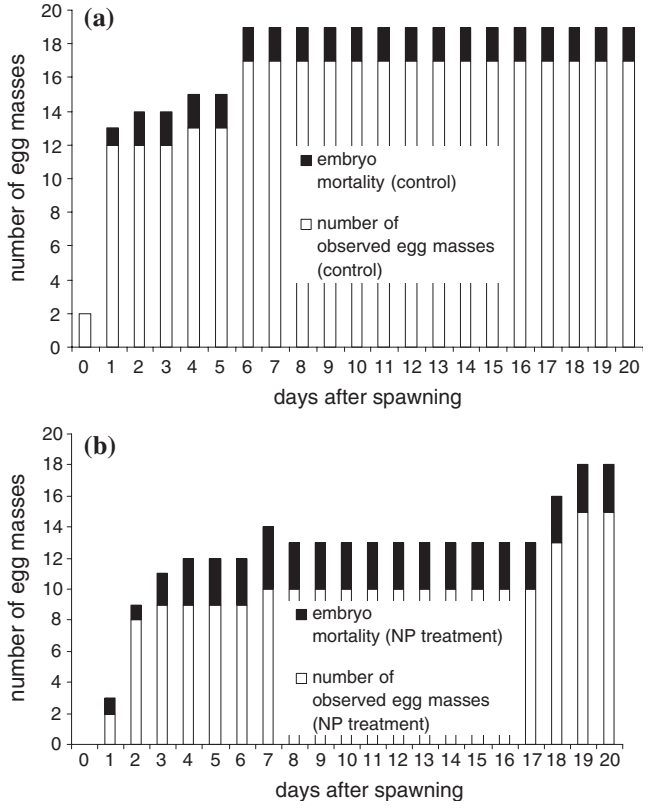

Fig. 4. **a** Bar chart of ratios showing embryo mortality in *Lymnaea stagnalis* L. in counted egg masses (control tank). **b** Bar chart ratios showing embryo mortality in *L. stagnalis* in counted egg masses (NP isomer-exposed tank)

Effects of the Branched NP Isomer on Embryo Growth and Life Cycle Development in L. stagnalis

Life cycle development (extent of growth) was evaluated based on observations made on the 3rd, 6th, 9th, 12th, 15th and 20th day from the start of exposure (Fig. 5). On the third day, 61.8% of the embryos in the control tank were in Morula stage and 36% were in the Trochophora stage, whereas in the NP-exposed egg masses, 76.5% of the embryos were in Morula and 21.7% in Trochophora, showing that over the 3-day growth period, development was significantly delayed in NP-exposed embryos. On the sixth day, 3.2% and 93.8% of the embryos in the control were in Trochophora and Veliger stages compared to 28.8% and 68.5% in NP-exposed embryos, respectively. Again, significant delay was manifested in NP-exposed embryos as they stayed longer in the Trochophora stage ($p > 0.05$, Student's *t*-test). On the ninth day, 2.2% (of the 93.8%) embryos were still in the Veliger stage in the control tank, whereas in the NP-exposed egg masses, 17.6% (of the 68.5%) of the embryos were still in the Veliger stage. On the ninth day, 94.8% of all embryos in the control were in Hippo stage compared to 79.1% in the NP-exposed embryos. Observations made on the 12th day showed that 42% of all embryos in the NP-exposed egg masses were in the Hippo compared to 76.6% in the control. In the control (on the 12th day), 54.9% of the embryos were hatched compared to 19.8% in the NP-exposed egg masses. This is an important and significant estrogenic effect. Three days later (15th day), 9.3% of the remaining Hippos in NP-exposed egg masses were hatched. The percentage hatching rose to 87.7% and to 76.3% in the control and in the NP-exposed embryos, respectively, after 15 days. On the last day of the experiment (20th day), 0.3% and 2.8% of the embryos were still in the Hippo in the control and in the NP-exposed egg masses, respectively. Therefore, although the results show that the effects on the delay were distinct, the effects on percentage hatchability after 20 days were less noticeable.

The results presented in Figure 6 indicate that, in total, the embryos in the NP-exposed egg masses experienced delay in development compared to the untreated controls. In 75% of the embryos in the controls, the Morula stage lasted for less than 4 days while the Morula stage in 75% of the NP-exposed embryos lasted for less than 5 days. The duration of Trochophora stage was less than 2 days for both the controls as well as the NP-exposed egg masses. Seventy-five percent of the embryos in the controls were in the Veliger stage for 2 days, whereas for 75% of the NP-exposed embryos the Veliger stage lasted 3 days. The duration of the Hippo stage was between 4 and 6 days for the NP-exposed embryos and between 5 and 6 days for the untreated controls (Fig. 6). As shown in Figure 6, the delay was more significant in the Morula and Veliger stages of growth.

The results show that the branched NP isomer residues were taken up by the exposed egg masses in all of the four stages (Table 2). However, the bioconcentration factors (BCF) were low despite the fact that the eggs are known to be rich in vitellins and lipids and therefore presumably very lipophilic. The low BCF values indicate that the egg masses may have been protected against the penetration of the chemical by the encapsulating jelly around them. This factor needs to be investigated for clarity. The BCF values were Morula: 0.66 (1 day), 1.13 (2 days), 2.54 (3 days); Trochophora: 1.66 (2 days), 1.01 (4 days); Veliger: 1.54 (1 day), 1.83 (2 days); and Hippo: 2.37 (2 days). Most residues were accumulated by the Morulas and the Hippos after 2 days and 3 days, respectively, of continuous exposure. The accumulation increased with duration of exposure in Morula.

Our results show that the NP isomer (4(3'-,6'-dimethyl-3'-heptyl)-phenol) affected the duration of growth of the embryos and caused embryo mortality compared to controls. Other workers working with embryo exposure in *Lymnaea* have

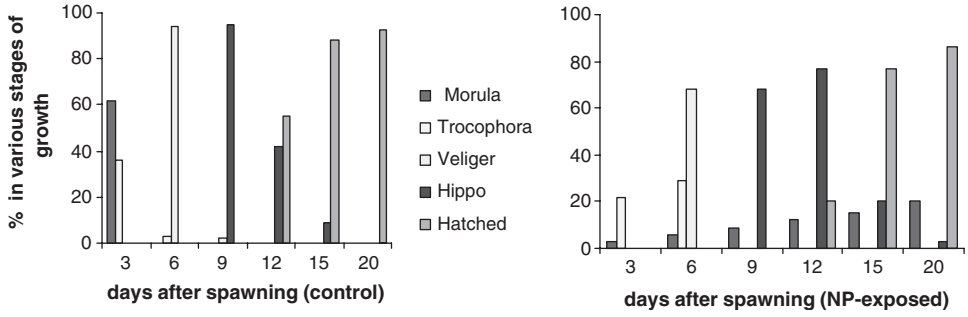

Fig. 5. The percentage number of embryos (based on initial total number of embryos) identified in various stages of growth in *Lymnaea stagnalis L.* in control and in NP-exposed egg masses

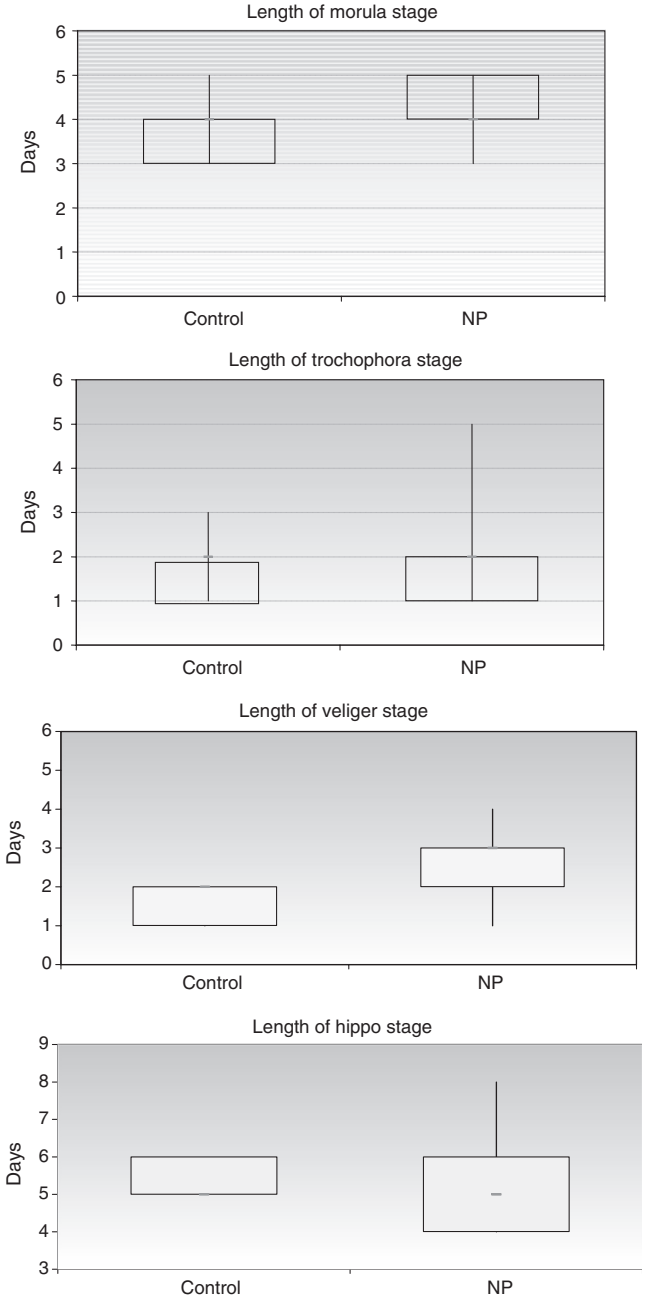

Fig. 6. Length of different embryonic stages (based on 75% of embryos) in the NP-isomer-exposed egg masses and the controls

shown similar effects. Lanzer (1999), working with an exposure concentration of 110 μg/L of technical NP over an exposure period of 20 days, observed similar effects on developing embryos. She found a no-effect concentration of 120 μg/L and a lowest observable-effect concentration of 160 μg/L for the embryos. Our results seemed to differ to an extent from those of Lanzer (1999), especially based on observations made on the 18th day. On the 18th day of exposure, Lanzer (1999) recorded almost similar hatching successes of 89% and 86% in the control and in the NP-exposed egg masses, respectively. We recorded a hatching success of 93.3% and 81.8% in the control and in the NP-exposed egg masses, respectively, on the 18th day, which indicated a more significant effect by the branched isomer compared to the technical NP mixture.

The BCF values show that the branched ^{14}C-NP isomer was taken up by the egg masses in all the exposed stages. However, the egg masses appeared to have been protected from the chemical by the jelly strand because the relatively low BCF value obtained does not correspond with the high lipophilic nature of the egg masses (Table 2). Most residues were accumulated by the Hippos after 2 days of continuous exposure. In previous studies, we found a rapid internal *in vivo* distribution of this isomer in various organs in *Lymnaea*, with highest bioaccumulation factor of 290 in the digestive gland/albumen gland after 3 days of continuous exposure of live snails to an average 105 μg/L concentration of the isomer in water (Lalah *et al*. 2003b). The live snails were able to accumulate the ^{14}C-isomer residues from water and through food intake. In the same experiment, we also found that the isomer could be transferred to other organs as well, including the gonads, which had a bioaccumulation factor of 49 after 6 days. Because the gonads are connected to the albumen gland, it was possible to get some of the residues transferred from this gland to the gonads. The accumulation in the gonads is considered significant with respect to estrogenic toxicity in this organism because the accumulated residues can then be responsible for transfer of estrogenic effects to next-generation offspring after maternal exposure. In the same study (Lalah *et al*. 2003b), we found that the isomer was metabolized to a catechol, which could also reduce its estrogenic potential, especially in presence of high concentrations of Cyt P450s, which are responsible for the Phase 1 biotransformation of such compounds in aquatic organisms. A high concentration of the isomer in the egg masses (and in embryos through penetration and binding) could cause endocrine disruption, but if the concentration of the Cyt P450s was significantly high in the embryos, then the resultant biotransformation of this

isomer to a catechol could reduce its estrogenic potential. At the concentration used in this experiment, NP mainly appeared to have had a significant effect on the length of the total embryogenesis in *L. stagnalis*. Our results also suggest that the encapsulating jelly surrounding the eggs could be responsible for reduction of mortality and life-cycle development effects of NP. However, this factor needs to be investigated. This isomer could therefore be expected to cause more estrogenic and toxic effects in *in vitro* assays as well as by *in vivo* exposure to maternal organisms. This needs to be investigated.

Other Previously Reported NP Estrogenic Effects

Similar studies, involving embryo exposure to investigate the effects of technical NP has been previously reported (Gray and Metcalf 1996; Pedersen *et al*. 1999; Czech *et al*. 2001; Madigou *et al*. 2001; Meldahl *et al*. 1996; Yokota *et al*. 2001). Gray and Metcalfe (1996) studied the effects of NP on Japanese medaka, *Oryzias latipes*, by exposing their eggs (n = 10–20), separated individually, at 25°C and examining the developing embryos under a dissecting microscope to determine the various stages of growth, hatching success, and mortality from day 0, after spawning, to day 7 (swim up). They reported embryo toxicity, LC50 of 460 μg/L, lower mean lengths, and lower mean weights of hatchlings, respectively. They also found that 50% (when exposed to 50 μg/L) and 86% (when exposed to 100 μg/L) of male fish developed testis-ova, an intersex condition characterized by both testicular and ovarian tissue in the gonads of male and juvenile fish, and a change in male:female sex ratio, showing that NP exposure to embryos can interfere with growth and sexual development in Japanese medaka. Yokata *et al*. (2001) studied the chronic effects of technical NP on reproductive status of Japanese medaka over two generations, after continuous, flow-through exposure to embryos within 24-hour postfertilization. They found reduced embryo survival, induction of testis-ova in adult male offspring, and female-skewed sex ratio at an exposure concentration ranging from 51.5 μg/L to 183 μg/L. In addition, Yokata *et al*. (2001) found that embryo exposure at concentrations ranging from 4.2 μg/L to 17.7 μg/L affected fecundity and fertility, based on reduction in fertility of exposed organisms to 76% compared to controls, in both first- (F0) and second- (F1) generation fish, showing that the estrogenic effects can be transferred *in vivo* to the next generation. Madigou *et al*. (2001) exposed rainbow trout embryos to NP (100 μg/L, for 1 hour per day for 10 days) and studied the effects of NP on activation of 17β-estradiol hormone (E2)-dependent gene and sexual differentiation on male embryos and found that apart from induction of intersex, NP exposure increased trout estrogen-dependent mRNA in embryo liver.

Endocrine effects of NP, in a continuous exposure concentration of 100 μg/L, on reproductive capacities (fecundity and fertility) have also been studied in *L. stagnalis* by exposing adults (Czech *et al*. 2001). Czech *et al*. (2001) reported reduction effects on egg production and hatching rate after 6–12 weeks of exposure. They also reported transfer of the endocrine effect, from maternal exposure to the next generation, by analysis of symptoms in F1 generation snails. Relating to specific isomer structural-effect differences, some recently reported NP isomer effects include studies by Pedersen *et al*. (1999), who compared *in vivo* estrogenic activities of two branched-chain isoforms of alkylphenols (*tert*-octylphenol and technical NP) and two linear-chain isoforms (*n*-octylphenol and *n*-nonylphenol) in juvenile rainbow trout. By both intraperitoneal injection (50 mg/kg) and waterborne continuous exposure (150 μg/L) for 9 days, they reported significantly higher induction of plasma vitellogenesis by the two branched-chain alkylphenols compared to the two linear-chain isomers. In their study, Pedersen *et al*. (1999) also found that *tert*-octylphenol (which has a similar tertiary structure to our isomer) was eight times as potent as the technical NP. Similar higher estrogenic potency of *tert*-octylphenol (relative to technical NP) was also observed by Jobling *et al*. (1996) as demonstrated by stimulation of vitellogenin synthesis and inhibition of testicular growth in rainbow trout.

Although structural-property relationship indicates more estrogenic potency for the tertiary branched NP isomers such as 4(3'-,6'-dimethyl-3'-heptyl)-phenol) (Hu and Aizawa 2003; Schutz and Sinks 2002), *in vivo* metabolism and biotransformation of such isomers to catechols can make them less active (Meldahl *et al*. 1996; Ferreira-Leach and Hill 2001; Lalah *et al*. 2003b). In previous studies in our laboratory, *in vivo* metabolism of this isomer to a catechol (4(3'-6'-dimethyl-3'-heptyl)-catechol)) was found to occur in the digestive gland and in the gonads of *L. stagnalis* after continuous exposure at a concentration of 105 μgL in water and food for 8 days (Lalah *et al*. 2003b). Similar metabolic transformation of *tert*-octylphenol to catechol (*tert*-octylcatechol) in juvenile rainbow trout was demonstrated by Ferreira-Leach and Hill (2001), at exposure concentration of 4 μg/L ^{14}C-octylphenol (flow-through) for 10 days. The tertiary alkyl group is a good electron-donating group and it makes the phenolic ring more susceptible to electrophilic attack. Hydroxylation of the tertiary isomer of NP would therefore be expected to occur on the phenolic ring instead of on the alkyl chain. The metabolic pathway for catechol formation occurs by hydroxylation of the phenolic group and by glucuronidation of the phenol and the catechol groups mediated by the cytochrome (Cyt) P450 phase I enzymes (Arukwe *et al*. 2000). On the other hand, *in vivo* hydroxylation of linear-alkyl chain and secondary-alkyl chain NP isomers has been found to occur on the alkyl chain (by ω- and β-oxidation at various positions on the alkyl chain) instead of on the phenolic group, albeit their perceived less estrogenic potential, showing differences in the mechanisms of metabolism and toxicity between the straight-chain and branched-chain isomers (Meldahl *et al*. 1996; Arukwe *et al*. 2000). The existence of Cyt P450 in *Lymnaea* is known, with the highest concentration in the digestive gland and a specific Cyt P450 CYP10 isozyme isolated from this gland has been reported (Czech *et al*. 2001). It is also known that the endocrine glands (dorsal bodies (DB)) located in the central nervous system of *Lymnaea* are involved in regulation of vitellogenesis and development of sex organs as well as expression of Cyt P450 genes (Geraarts and Joose 1975; Luchtel *et al*. 1997). These metabolic mechanisms of NP isomers in *L. stagnalis.* can influence their estrogenic potential. Other metabolic mechanisms that can influence NP estrogenic effects include binding, by alkylation mediated by Cyt P450, to other cellular proteins such as GSH and DNA (Gallagher *et al*. 2001). Toxic effects of NP at the DNA level, including DNA damage and genomic mutations, have also been reported in developing

Barnacle larvae after NP exposure as endocrine functions of NP and these changes at DNA level may be precursors of some of the reported effects at higher biological organization (Gallagher *et al*. 2001).

Conclusion

This study shows the susceptibility of embryogenesis of the hermaphrodite pulmonate *L. stagnalis* to the branched NP isomer, 4(3',6'dimethyl-3'heptyl)-phenol, in a screening test procedure. The results show that the NP isomer caused a significant increase in embryo mortality and delay in embryonic development, particularly in Morula and Veliger stages of growth, shown by length of duration taken to pass from a corresponding stage of growth to the next, when compared with controls. The NP isomer also caused significant delay in hatchability of the embryos of *L. stagnalis* after continuous exposure to an average concentration of 105 μg/L, with 81% hatchability obtained in NP-isomer exposed embryos after 18 days' continuous exposure compared to 93% in the controls.

The low BCF values of the NP isomer found in egg masses in all of the four stages of growth suggest that our NP isomer may not have been able to penetrate the lengthwise encapsulating jelly strand that surrounds the eggs, thereby preventing the embryos from adequate exposure to the test compound, leading to unexpectedly lower estrogenic effects observed in this study. However, this hypothesis was not investigated in this study. Therefore, there is a need to investigate further the estrogenic potential of this NP isomer by *in vitro* assays (*e.g.*, by yeast assays) and *in vivo* estrogenic effects on embryo development after *in vivo* maternal exposure.

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