Effects of 4-\textit{n}-nonylphenol on the Prosobranch Snail 
\textit{Marisa cornuarietis}

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\textbf{Abstract.} The awareness of endocrine disrupting chemicals (EDC’s) has increased throughout the last decade due to great amounts released into the environment by the industry. In this study we have investigated the effect of the abundant EDC, 4-\textit{n}-nonylphenol, on the gastropod \textit{Marisa cornuarietis}, as very little attention has been dedicated to the invertebrates responses to EDC’s. \textit{M. cornuarietis} was exposed to a nominal concentration of 4-\textit{n}-NP of 100\(\mu\)g/L for a period of 34 days. The experiment was performed as a static renewal test ensuring that the snails were continuously exposed to 4-\textit{n}-NP. During the experiment eggs were collected and counted three times a week to investigate 4-\textit{n}-NP’s effect on the fecundity. Water samples from exposed aquaria were taken several times a week to measure the exact concentration of 4-\textit{n}-NP as it was expected that the concentration would decrease over time. After three weeks clutches of eggs from exposed and unexposed snails were set to hatch for the purpose of investigating the hatchability success of the two types of eggs. Furthermore the concentration of 4-\textit{n}-NP in the exposed snails was measured as well as their content of lipid. Our results showed no significantly difference in fecundity between the exposed and unexposed snails. A significantly higher hatchability success of the eggs from unexposed compared to exposed snails after eight days of hatching was detected. A relatively low content of 4-\textit{n}-NP, approximately 10ng/g, were found in eight exposed snails after 34 days of exposure to 4-\textit{n}-NP which conflicts with the high content of lipid, approximately 30\%, found in the same eight snails. Uncertainties in the extraction procedure made concentration results from extraction of water samples and snails unreliable.

\textit{Keywords:} \textit{Marisa cornuarietis}, 4-\textit{n}-nonylphenol, endocrine disruptors, GC-MS, prosobranchs

\textbf{Introduction}

Endocrine disrupting chemicals (EDCs) are released into the environment in great amounts by industry [Newmann & Unger, 2000] and the awareness of these substances have increased through the 1990s [Kojima, 2003]. But most of the attention has been dedicated to the study of vertebrate response to the EDCs even though invertebrates make up 95\% of the animal kingdom and provide key species for ecosystem functioning. This explains the limited number of examples of
endocrine disruption (ED) in invertebrates [Oehlmann & Schulte-Oehlmann, 2003]. The EDCs are shown to have the potential to perturb sensitive hormonal pathways that regulate reproductive capacities (fecundity and fertility) in exposed organisms [Czech et al., 2001]. The well documented case study about the antifouling biocide tributyltin (TBT) and its masculinising effects in about 150 species of prosobranch molluscs, shows that inhibition of aromatase activity ending up in a final sterilisation of affected females can have drastic effects up to the population and community levels [Oehlmann et al., 2000], and there is no reason to suppose that such far-reaching changes are unique for TBT according to Oehlmann & Schulte-Oehlmann, [2003].

The purpose of this paper is therefore to investigate the known EDC 4-n-nonylphenol’s effect on the gastropod Marisa cornuarietis.

Alkylphenols such as octyl- and nonylphenol are known EDCs that and mimic estrogens and therefore regulate the activity of estrogen-responsive genes by binding to estrogen receptors. Males will develop female traits or female endocrine-related features might change, when exposed to these substances [Newmann & Unger, 2000]. Alkylphenols are the toxic degradation products of alkylphenol ethoxylates (APEO) that represent the third largest group of surfactants in use worldwide. Nonylphenol polyethoxylates (NPEO) account for >80% of the APEOs [Hansen et al., 1999]. Nonylphenols (NP) are used for the synthesis of nonionic surfactants NP-polyethoxylates (NP-PEs) and are used in smaller amounts as stabilizers and antioxidants in the manufacture of plastics such as polystyrene and polyvinyl chloride, several of which are used as food contact plastic [Zalko et al., 2003]. The estimated annual consumption of APEO was in 1995 in Denmark 1,936 t, the majority used as cleaning materials [Dansk Planteværn, 1998], and 18,500 t in the UK in 1992, [Oehlmann et al., 2000]. The consumption of APEOs on a global scale exceeds 300,000 tons annually [Bennett & Metcalfe, 1997].

NP consists of a lipophilic part and a hydrophilic part, where the benzene structure and the alkyl chain makes up the lipophilic part and the hydroxy molecule accounts for the hydrophilic part [Dansk Planteværn, 1998]. Surfactants are most commonly used in aqueous solutions, and these compounds are therefore discharged directly into the environment via industrial and municipal wastewater treatment systems [Bennett & Metcalfe, 1997] and the distribution of NP in the environment is very much determined by the partitioning of the compound between the water and the sediment phase expressed as the partitioning coefficient log K \(_{ow}\). When log K \(_{ow}\)> 3 for a compound, the compound is considered as bioaccumulative thus log K \(_{ow}\) is an expression of its lipophilic character [Newmann & Unger, 2000]. NP has a log K \(_{ow}\) on 4.48 and therefore tends to accumulate in biota and in sediment where organic content determines the amount that adsorbs [Ying et al., 2003; Porter & Hayden, 2002]. NP also binds to biosolids and solids dissolved in the water phase [Porter & Hayden, 2002]. Concentrations of NP found in river estuary sediment in the southern part of the North Sea were 300μg/kg and concentrations in the range of 5-375μg/kg have been reported from sediment from the Danish sea [Mellergaard et al., 2002]. Concentrations of NP in sewage effluent have been reported internationally to lie between 25ng/l to 330μg/l and for surface water between 5ng/l and 180μg/l. A single river contamination reported from Spain had a concentration of 600μg/l however concentrations in surface water does not typically exceed concentrations of 1 μg/l [Miljøstyrelsen, 2002]. A survey of molluscs in Italy found NP at concentrations from 67 to 696ng/g [Porter & Hayden, 2002]. NP is moderately volatile – while its vapour pressure is relatively low, its Henry’s law constant is in the moderately volatile range
which is defined as between $10^{-5}$ - $10^{-7}$ atm*m$^3$/mole. The Henry’s law constant for NP ranges from $1.55*10^{-5}$ to $4*10^{-5}$ atm*m$^3$/mole. NP therefore tends to move great distances in the atmosphere by air currents [Porter & Hayden, 2002].

NP exhibits a high content of isomeric compounds determined by their para or ortho orientation, where 4-n-nonylphenol (4-n-NP) is a straight-chained isomer [Yukiko et al., 1999] An experiment performed by Gabriel et al. showed that branched 4-NP serves as growth substrate and thereby stimulates growth of the Gram-negative bacteria Sphingomonas xenophaga, whereas 4-n-NP which is straight –chained as indicated by the $n$ did not stimulate the growth of bacteria [Gabriel et al., 2005].

4-n-NP is readily biodegradable in freshwater and is half-life in aquifer material (sediment and groundwater) under aerobic conditions is calculated to be 7 days by Ying et al., 2003.

The animal used in this experiment is the prosobranch M. cornuarietis (Mesogastropoda; Architaenioglossa; Ampullariidae) [Purchon 1977, Grantham et al. 1995]. Through several experiments the snail has shown to be a promising organism on which to test endocrine disruptors as referred to in Oehlmann et al. from 2000. M. cornuarietis has its natural habitats in South- and Central America, where it can be found in freshwater areas [Grantham et al. 1993]. The snail can reach a diameter of 40 to 60 mm in diameter and feeds on both freshwater plants [Grantham et al. 1993] and eggs from other prosobranchs [Cowie, 2001]. The animal is dioecious - i.e. it has sexual reproduction. Eggs are laid in gelatinous clutches and are typically attached to a fixed point in the snail’s environment. The gelatinous material has several purposes. Besides keeping the eggs together and protected it serves as food material for the growing embryos. Thus it is possible for the juveniles to skip the veliger stage and instead emerge at the crawling stage [Purchon 1977].

The estrogenic potential of 4-n-NP and 4-NP has been tested on several invertebrate organisms. An experiment involving the freshwater mud snail Potamopyrgus antipodarum has shown an increase in fertility and juveniles lacking shells [Duft et al., 2003]. 4-n-NP has also been tested on other organisms besides gastropods. An experiment on the crustacean Daphnia magna has revealed that exposing maternal daphnids to 4-NP produced several developmental abnormalities in offspring [LeBlanc et al., 2000]. Another example exposing the barnacle Elminius modestus to 4-n-NP showed that exposure caused disruption of the timing of larval development [Billinghurst et al. 2001]. Furthermore a LOEC value for acute toxicity of 4-n-NP down to 6.7μg/L for shrimps have been reported [Hansen et al., 1999].

Some chemicals with k$\text{ow}$ values similar to that of NP are known to be bioaccumulated in the environment as is demonstrated by their very high BCF. For example, the BCF of DDT is 34,000, 1,400 for anthracene, 3,200 for fluoranthene and 6,100 for pyren [Porter & Hayden, 2002].

In our project we have investigated the effect of 4-n-NP on the gastropod M. cornuarietis. On the basis of the introduction we hypothesize that 4-n-NP will act as an agonist on M. cornuarietis and 1) will have an effect on fecundity, 2) will have an effect on fertility/hachtability and 3) will bioaccumulate in M. cornuarietis.

Materials and methods

The basis of our project was the article “Effects of endocrine disruptors on prosobranch snails (Mollusca: Gastropoda) in the laboratory. Part I: bisphenol A and octylphenol as xeno-estrogens” by Oehlmann et al., [2000]. The purpose of their article was to investigate bisphenol A’s (BPA) and
octylphenols (OP) effect on reproduction of the freshwater snail *M. cornuarietis* and the marine snail *Nucella lapillus*. In our experiment we chose to use the known EDC 4-n-NP because NPEO are the most widely used surfacants in the industry and are present in the environment. In the article by Oehlmann et al., they did not check the development of the concentration of OP during their experiment and therefore we decided that it would be interesting to see if the 4-n-NP was removed from the water phase as a result of biotransformation, adsorption to solids within the aquarium or evaporation and thus we took water samples on selected days. Aware of the risk that the compound will be removed from the water phase we also added 4-n-NP on selected days.

### Experimental setup

For each 50 liter glass aquarium 40 liters of MilliQ water was added. Furthermore 16 mL of 11,11 g/l KNCO₃, 14,98 g/l KNO₃ and 96,13 g/l MgSO₄, 64 mL of 79,89 g/l NaHCO₃ and 17,84 mL of Ca²⁺ was added to the water in each aquarium to create artificial freshwater. Each aquarium was separated in four sections by attached glass walls. We used ten Eheim Ecco power pumps with a biological filter without coal because that would remove the 4-n-NP from the water. An air tube was attached to each of the four sections in the ten different aquaria. To regulate the water temperature we placed a temperature regulator (Jäger, Aquarium Regler-Heizer, 230 V~ 50 watt, Typ TSRH 50) in each of the aquaria. The temperature was kept at 25°C and the light:dark cycle was adjusted to 12:12 h. To our fertility /hatchability test we used two 40 liter aquaria one for the controls and one for the eggs coming from the exposed females. In the hatchability trial no 4-n-NP was added.

We used the isomer 4-n-NP (Riedel-de Haën, CAS no.104-40-5) as the test compound. The general physicochemical properties of NP are shown in the table below:

<table>
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<th>Property</th>
<th>Value</th>
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<tbody>
<tr>
<td>Formula</td>
<td>C₁₅H₂₄O</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>220 0</td>
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<tr>
<td>Freezing point</td>
<td>-10° C</td>
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<tr>
<td>Vapor pressure at</td>
<td>25°C(Pa)</td>
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<td>0.3</td>
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<tr>
<td>Log Kₒₓ</td>
<td>4.48</td>
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<tr>
<td>Log Kₒ∞</td>
<td>4.7</td>
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<tr>
<td>BCF in trout</td>
<td>24-98</td>
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<tr>
<td>BAF in lab. invertebrates</td>
<td>1-3400</td>
</tr>
<tr>
<td>Water solubility (mg/l)</td>
<td>4,6, 6,24, 11,9</td>
</tr>
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</table>

The stock solution was prepared in dimethylsulfoxide (DMSO) at a concentration of 4mg/ml, and after the addition of 1ml to the aquaria the concentration of 4-n-NP was calculated to be 100 µg/l and the solvent concentration was 0.000025 % DMSO. We added the same amount of DMSO to the control snails. The concentration of 4-n-NP in the experiment was chosen to be within the range found in surface water as referred to in the introduction.

The test organism was sexually mature (four months old) *Marisa cornuarietis* obtained from a laboratory breeding stock at Roskilde University under supervision of Prof. Valery Forbes. We used 32 pairs where 16 were used for the control aquaria and 16 in the 4-n-NP aquaria giving a total of eight aquaria. The
animals were fed three times a week with fresh Danish lettuce (*Sativa Lactuca* var. capitella) and the fish feed Tropi-granulate (Poppi Group, Køge, DK).

Arrangement of the eight aquaria, seen from above:

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<th>4-pairs</th>
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Test conditions and procedure
The duration of the experiment was 34 days. The experiment was performed as a static-renewal test. Thus during the experiment water was changed on day 20 and 27, when we changed half the water and all the water, respectively. We collected water samples of a volume of 150 ml on day: 1, 2, 3, 4, 5, 8, 11, 13, 15, 18, 20 and 22 and froze them for extraction of 4-n-NP. We added 4-n-NP on the following days: 1, 13, 20 and 27. The concentration of the added amount of 4-n-NP was for all times 4 mg/ml DMSO. On day 1 and day 13, 1 ml of the solution was added, on day 20 ½ of ml was added and on day 27 1 ml was added. Temperature and pH were checked two times during the experiment. Eggs were collected and counted on the following days: 4, 6, 8, 11, 13, 15, 18, 20, 22, 24, 27, 29, 32 and 34 (See Appendix 4). For the snail extraction 8 exposed snails were chosen after the end of the experiment (34 days) – 4 of the snails from aquarium 1 and 4 snails from aquarium 4. It was not possible to determine the sex of the snails at the time, because they had all withdrawn into their shells.

An optimization of an analytical method for determination the concentration of 4-n-NP is described in Appendix 1.

**Solid Phase Extraction procedure**
100 mL of the water-sample was used for the extraction. 1 mL of internal standard and 10 µg/ml 4-octylphenol in dichloromethane, were added to 100 mL of the water sample before extracting.
The extraction was performed using C18(EC) cartridges from Microlab A/S and a vacuum manifold.
The column was prewashed with 10 mL dichloromethane and then prepared with 10 mL 1:1 methanol:water. The sample was slowly passed through the cartridge and vacuumed until most of the water had been removed. To extract the sample 10 mL of 7:3 dichloromethane:methanol was used. The sample was collected in a glass vial.

This was followed by evaporation of the sample to dryness using a gentle stream of nitrogen and gentle heating. The sample was then re-diluted in 1 mL of acetonitrile.
Before running the sample on the GC-MS 100µl of silylate-mixture BSA (N,O-bis-(trimethylsilyl)acetamid, Fluka) was added and the extracted sample was heated in a water bath for one hour at 50°C.

Standard row: A stock solution of a concentration of 1mg 4-n-NP per ml acetonitrile was prepared. 4 standard concentrations were prepared from this stock solution; a) 9µg/mL 4-n-NP and 10µg/mL octylphenol, (b): 900ng/mL 4-n-NP and 10µg/mL octylphenol, (c): 80ng/mL 4-n-NP and 10µg/mL octylphenol, (d): 20ng/mL 4-n-NP and 10µg/mL octylphenol.

**Extraction from snail tissue**
The snail was crushed with scissors, the shell removed and the snail body was rinsed and weighed. Before transferring it to a test tube it was cut into smaller pieces with a scalpel to ease the following homogenizing process. 4 mL of 2:1 chloroform/methanol was added to the test tube now containing the sliced snail,
and the snail tissue was homogenized using a homogenizer (Tissues Tearor, model 98 5370, Biospec Products Inc). 2 mL of the homogenate was transferred to a pyrex tube for determination of lipid and 2mL was transferred to a pyrex tube for further extraction. The latter was sonicated for 10 minutes and then centrifuged at 2500 rpm for 10 minutes. The water phase was removed, the organic phase was transferred to a separate pyrex tube and 2 mL of chloroform was added to the remaining tissue followed by sonication and centrifugation. The organic phase was transferred to the previous pyrex tube containing the first removed organic phase. The extract was then filtered through a funnel containing glass wool and sodium sulfate and then evaporated completely using a rotation evaporator. After evaporation 1 mL of acetonitrile and 100µl BSA were added, and the samples were silylated in a water bath at 50 degrees for an hour. Samples were run on the GC-MS.

**Lipid determination**

1) 1 mL chloroform was added to the 1 mL homogenate prepared in the previous section.
2) The sample was whirl mixed for 30 seconds and then sonicated for 10 minutes.
3) 1 ml chloroform and 1 mL water were added to the test tube and it was whirl mixed for 30 seconds.
4) The sample was centrifuged at 500xg for 5 minutes and the chloroform phase was transferred to another test tube.
5) 1 mL of methanol, 0.5 mL chloroform and 0.4 mL water were added to the tissue pellet and the test tube was whirl mixed for 30 seconds.
6) The test tube was sonicated for 10 minutes and then 0.5 ml chloroform and 0.5 mL water were added followed by a whirl mixing for 30 seconds. The sample was centrifuged at 500xg for 5 minutes and the chloroform phase was added to the test tube from (4). Steps 5) – 6) were repeated. The chloroform phases were evaporated with nitrogen until a volume of 0.5 ml was left. The concentrated chloroform phase was transferred to a pre weighed tube, the sample was evaporated until dryness, and the tube was reweighed.

**Endpoints**

To study the effect of 4-n-NP we investigated the two endpoints fecundity and fertility/hatchability. Three days a week we collected clutches (see appendix 4) for fecundity data and in the end of the experiment the data were analysed and expressed as follows. Fecundity: median egg production per animal per day.
Some of the collected clutches were used for the hatchability test, we used 6 control clutches and 7 exposed clutches. The hatchability test was carried out as follows. After hatching had begun, the number of hatched eggs was recorded every day. After a week the test was stopped.
Hatched eggs were defined from the following principle; number of eggs that had not hatched in the test week was defined as not hatched. The fertility/hatchability was expressed as median percentage of successfully hatched eggs per total eggs.

**Statistical analysis**

Egg production, hatching efficiency and ammonium concentration among treatment groups were analysed by the nonparametric Kruskal-Wallis-test as a consequence of variances being non-homogeneous - thus not fulfilling the ANOVA assumptions. All statistical analyses were based on a 5% significance level.
As a consequence of the pseudo replicates (four pairs in each of the eight aquaria) in this experiment we compared the pairs of each of the four control aquariums and compared the pairs of each of the four exposed aquaria and found no significant difference. Thus we considered the 16 pairs in the control aquaria for being replicates and likewise the 16 pairs in the exposed aquaria.
Results

Physical conditions

PH was 6 for all aquariums during the entire experiment. As seen in figure 1 the ammonium concentration in all aquariums was over the threshold limit on 0.3 mg/l [Valery Forbes, pers. Comm.] during the entire experiment. Samples were taken before and after change of water on day 20 and 27 and before ending the experiment at day 34. The median concentration of ammonium at day 20 before changing the water in the exposed aquaria was significantly higher (\(p=0.039\)), 10.1 mg/l, than the median concentration of the control aquaria which was 2.6 mg/l at same day. After changing half the water the medium ammonium concentration of the exposed aquaria drops with 7.67 mg/l where as the median ammonium concentration in the control aquaria drops with 0.56 mg/l. During the next seven days the median ammonium concentration of the exposed aquaria increases with 3.65 mg/l and the median ammonium concentration of the control aquaria increases with 0.63 mg/l. A significant difference (\(p=0.043\)) between the median ammonium concentration of the exposed and control aquaria was also found at day 27. After changing the water the same day the median concentration of the exposed aquaria drops with 4.18 mg/l and the median concentration of the control aquaria drops with 0.915 mg/l. During the next seven days the median concentration of ammonium in the exposed aquariums further decreases with 0.21 mg/l and with 0.802 mg/l in the exposed aquaria.

![Ammonium concentration at selected days](image)

Fig.1. Median ammonium concentrations (\(n=4, \pm SD\)) among treatment groups at day 20, 27 and 34. A significant difference was found between the old samples of the water from the controls and the exposed at day 20 (\(p=0.039\)) and day 27(\(p=0.043\)).

All temperatures in aquaria containing test snails were between 22 and 24°C. In the aquarium with juveniles from control parents the temperature was found to be 25.6°C on day 14 and 26.0°C on day 20 of the hatching experiment and in the aquarium with juveniles from exposed parents the temperature was found to be 25.4°C on day 12 and 25.0 °C on day 18 of the hatching experiment. pH values, ammonium and calcium concentrations were not measured in the latter.

*Marisa cornuarietis*

All snails survived throughout the experiment. The snails had a tendency to crawl to the neighbouring snail pairs but were placed back to their original location each time we were in the climate room.
Fecundity
Over a 34 day period the fecundity for *M. cornuarietis* without 4-n-NP was 11.787 median no. of egg/animal/day and the fecundity for *M. cornuarietis* with 4-n-NP was 11.425 median no. of egg/animal/day. There were no significant differences in fecundity between exposed and control *M. cornuarietis* (p=1.0). The results are shown in Fig. 2.

Egg production
Mean egg production/animal/week for exposed and unexposed *M. cornuarietis* are shown in Fig. 3. The mean egg production per control snail in week one was 61.9 where as the mean egg production per exposed snail was 45.53 the same week. In the second week the mean egg production per control snail decreases with 61.4 to almost zero where the mean egg production per exposed snail is readily
consistent compared with the previous week with a value of 37.13. In the third week the mean egg production per snail in the exposed and the controls is 15.15 and 17.29, respectively and in the fourth week they both increase – the mean egg production per exposed snail being 31.58 and 41.27 per control snail. In week 5 the mean egg production per exposed snail further increases to 49.69 and the mean egg production per control decreases to a value of 22.9.

**Fertility**

The hatching efficiency for the unexposed and exposed snails at the 8th day after the first hatching day is shown in Fig. 4. The hatching success for the control snails was calculated to 97% and 51% for the exposed. There was a significant difference (p=0.015) in hatching efficiency between the eggs from the controls (n=6) and the exposed (n=7) snails. The hatching development during the 8 days after first hatching day is shown in Fig. 5. The hatching efficiency of eggs from both types increases evenly throughout the first four hatching days – the hatching efficiency of the eggs from unexposed snails increasing from 27% at day 1 to 57% at day 4 and the hatching efficiency of eggs from exposed snails increasing from 2% at day 1 to 17% at day 4. From day 5 the hatching efficiency of the eggs from the unexposed snails continues to increase from 74% to 97% at day 8 whereas the hatching efficiency of the eggs from the exposed snails within the same period stagnates – the hatching efficiency remaining at a percentage of 50 from day 5 to day 8. A significant difference was found at the first day of hatching (p=0.032) and on the sixth (p=0.049), the seventh (p=0.045) and on the eighth day of hatching (p=0.015).

**Concentration of 4-n-NP**

The calculations of concentrations of 4-n-NP are shown in Appendix 3. The concentration of 4-n-NP in two exposed aquaria over time is shown in Fig. 6.
The nominal concentration of 4-n-NP was at day 1 calculated to be 100µg/l but was in aquarium 1 measured to be 475µg/l and in aquarium 2 measured to be 87.9µg/l. At day 2 the concentration in aquarium 1 has decreased to 4.3µg/l and the concentration in aquarium 2 has increased to 517µg/l. At day 3 the concentration in aquarium 1 has decreased to 68.1µg/l whereas the concentration in aquarium 2 slightly has increased to 27.2µg/l. At day 5 the concentration in aquarium 1 is 60.6µg/l and the concentration in aquarium 2 is decreased to 2.4µg/l. At day 8 the concentration in aquarium 1 increases to 475µg/l and in aquarium 2 increases to 4.3µg/l. At day 13
where 1 ml of 4-n-NP is added the concentration in aquarium 1 is measured to be 51.7µg/l and in aquarium 2 to be 20µg/l. From day 13 to 19 the concentration in aquarium 1 increases to 86.3µg/l and from day 13 to 20 the concentration in aquarium 2 decreases to 15.67µg/l. At day 20 half the water is changed and ½ ml 4-n-NP is added. The concentration in aquarium 2 slightly increases from day 20 to 22 where it is measured to be 28.3 µg/l. Because of the great fluctuations within our measured concentrations we stopped measuring the concentrations after day 22.

The median concentration of 4-n-NP over time is shown in figure 7. At day 2 and 8 the median concentration is found to be greater than 100µg/l and at day 1, 3, 5, 13, 20 and 22 the median concentration is measured to be below 100µg/l. At day 1, 2 and 8 we see a great variability within the replicates.

The median concentration of 4-n-NP measured in 8 snails after 34 days of exposure is shown in Fig. 8. The median concentration of the four snails from aquarium 1 is measured to be 15.26ng/g and the median concentration in the 4 snails from aquarium 4 is measured to be 1.12ng/g.

The median lipid content of snails exposed to 4-n-NP after 34 days is shown in figure 9. The median lipid content of 4 snails from aquarium 1 is found to be 33.64% and the median lipid content of 4 snails from aquarium 4 is found to be 27.65%.

The BCF value was calculated as mean concentration of 4-n-NP in the eight snails divided by the nominal concentration. The calculated value was 0.08.
Discussion

The fecundity of the exposed snail were not significantly affected (p=1.0) by the presence of 4-n-NP although this was expected. In an experiment performed by Czech et al. in 2001 with the hermaphroditic gastropod Lymnaea stagnalis and 4-NP, no effect on fecundity was found in a seven-eight week experiment at concentrations of 1, 10 and 100 µg/l was found. But in a twelve-week experiment they detected a significantly lower fecundity in exposed compared to controls at a concentration of 100 µg/l 4-NP. Even though they used another isomere (4-NP) in their experiment, the short duration of our experiment (4.8 weeks) could explain the fact that we didn’t find a significant difference in fecundity.

Another experiment which was performed by Oehlmann et al. in 2000 with M. cornuarietis and the two xeno-estrogens octylphenol (OP) and bisphenol A (BPA), measuring fecundity at the concentrations of 1, 25 and 100 µg/l. The results showed for both of the xeno-estrogens that they induced a complex syndrome of alterations in female of M. cornuarietis referred to as “superfemales” at the lowest concentrations in exposure periods of both five and twelve months. These super females had a high increase in oocyte and spawning mass production, for example at 25 µg/l OP the number of eggs per female

Fig 8. The figure shows the median concentration (n=4, ±SD) of 4-n-NP in exposed snails from the aquarium 1 and aquarium 4 at the end of the experiment

Fig 9. The figure shows the median content of lipid content for aq. 1 (n=3, ±SD) and for aq. 4 (n=4, ±SD) of lipid in snails exposed to 4-n-NP.
was increased by 400% and then decreased to 150% at a concentration of 100 μg/l. It is difficult to compare the experiment mentioned with the present since our experiment had a much shorter duration. Even though the compounds OP and BPA both are phenols like 4-n-NP they act by a different mechanism compared to 4-n-NP.

In the egg production over time we expected to see a change in egg production for the snails exposed to 4-n-NP and a constant value for the egg production of the control snails. When analysing the graph you can conclude that there has been no effect of 4-n-NP on the egg production over time compared with the control. The graph shows a rapid decline in control egg production reaching zero on day 11. Egg production from exposed snails also reach zero but only once and not until day 22.

After half the water was changed on day 20 we saw an increase in the egg production of the controls and after we changed all the water on day 27, it further increased. The same pattern is the case in the egg production for the exposed snails, all though this increase was delayed and smaller compared to the controls.

We detected a significant difference in the hatchability between the eggs from the exposed parents and unexposed parents (p=0.015). The percentage of hatched eggs was 97 % for controls and 51 % for eggs from exposed parents. The difference between the two percentages is 46% indicating that 46% off the eggs coming from the exposed parents had been disturbed in some way. They may have been sterile, unfertilized or been on another developmental stage.

In the experiment involving Lymnaea Stagnalis performed by Czech et al. in 2001 no significant differences in hatchability were observed with the highest concentration of 4-NP (100μg/L). One could expect to see the same pattern when exposing M. cornuarietis to 4-n-NP because 4-NP and 4-n-NP are isomers.

We expected the snails to have a high bioconcentration of 4-n-NP due to their high content of lipid, compared to lipid contents found in other animals. Here can be mentioned a content of 3.6 % in Crepidula fornicata [Herbreteau et al., 1994] and 4.35 % in Lymnaea Stagnalis [Lalah et al., 2003]. Concentrations of 4-n-NP in the snails from the two different aquaria were 15.20ng/g and 1.12ng/g respectively which was smaller than expected.

Moreover the concentration of 4-n-NP seemed to be very small taking the concentration in the surrounding water into account – unless of course the actual concentrations were much lower than calculated - and the BCF value was therefore extremely low, 0.08, compared with other chemicals that have similar log Kow values. For example flouranthene (log Kow=4.90) has a BCF value on 3,200 and pyren (log Kow=4.88) has a BCF value on 6,100. Low bioavailability can be a cause of the low content of 4-n-NP found in the snail.

As mentioned in the introduction a survey of molluscs in Italy found NP concentrations ranging from 67 to 696ng/g [Porter & Hayden, 2002]. Our concentrations are not within that range.

The concentration of 4-n-NP in the water shows enormous fluctuations within the duration of this experiment. Some days the concentration was found to be unrealistically high – for example day 2 where the concentration was found to be above 500 μg/l – and some days the concentration was found to be unexpectedly low. These fluctuations can either be due to unreliable standard-curves on whose slope our calculations so critically depend, or - where the concentration is found to be lower than expected - errors in the extraction method, loss of 4-n-NP through volatilization or/and adsorption during collection and transfer of water sample or that 4-n-NP and/or 4-OP had not been silylated properly.
The fecundity was not significantly different between the exposed and unexposed snails just like we did not see a change in the production of egg over time of the exposed snails. This is contrary to our hypothesis where we expected to see an effect in this parameter. In contrast the fertility was significantly ($p = 0.015$) lower for the exposed than for the unexposed snails. Despite of a high lipid content and nominal water concentration the BCF value was extremely low when considering the high log $K_{ow}$ of the compound. This can maybe be explained from that of the low bioavailability of the compound.

We expected to be able to measure water concentrations of 4-n-NP but unfortunately these were not reliable compared to our nominal concentration.

**Additional considerations**

According to Oehlmann et al. 2000, *M. cornuarietis* is a candidate to be a test organism in future investigations regarding endocrine disruptors. This makes it necessary to know more about optimal requirements for having *M. cornuarietis* in culture in the laboratory. Several of these parameters such as temperature, diet and water chemistry are still not completely revealed. The lack of information leads to uncertainty of endpoints such as survival, reproduction and development of the organism [Forbes et al. 2002]. Especially parameters regarding reproduction and mortality are important to know, since they can affect the organism at the population level [Porter & Hayden, 2002]. Thus an increase or decrease in mortality or fecundity or fertility can cause the number of individuals in a population to rise or fall. Husbandry requirements are therefore important to know before *M. cornuarietis* can be used as an official test organism.

The consideration on whether *M. cornuarietis* is a good test organism must also be based on how easy the snail is to work with in an experiment. As we see it, in this case several problems are present. Keeping *M. cornuarietis* in aquaria requires large amounts of water creating practical difficulties regarding water replacement where the use of a flow-through set-up would be more convenient. Moreover when toxic chemicals are added, contamination of the environment of the control aquaria is a possibility. Another problem is that excretory products from the snails increase the ammonium concentration in the water [Valery Forbes, pers.comm., 2005]. Although excretory products at the bottom of the aquarium can be partly removed, the ammonium concentration can be difficult to hold below the threshold limit unless coal is applied to the filter in the pump. The reason why this procedure has not been followed in this experiment, is that coal has the ability to remove compounds like 4-n-NP from the water. Another way to control the ammonium concentration is by using a flow-through set-up instead of the static-renewal system used in the experiment.

As mentioned earlier the threshold value of ammonium is 0.3 mg/l, a value that has been exceeded in this experiment. However ammonium is known to be relatively non-poisonous compared to its product ammonia, which can be very toxic towards aquatic organisms. As long as the pH in the water does not exceed 7.0, ammonia is only present in small amounts [Wurts, 2003]. PH values in this project are 6.0 meaning that the toxic ammonia may assumed to be present in very small amounts in the water, which then almost only contains ammonium. However, whether ammonium has an effect on *M. cornuarietis* has not been investigated yet.

Furthermore it must be considered that testing one chemical on an organism might not show the same effect as if the animal were to meet the same chemical in nature. First of all chemicals in nature are represented in many different concentrations depending on kinds of ecosystems, of landscapes and of water.
relations [Porter & Hayden, 2002]. Secondly when being in its natural habitat the animal not only meets one chemical but a complex mixture of compounds at the same time. Since these can also interact with each other, there is no guarantee that a specific chemical will have the same effect on the animal in the laboratory as observed in situ [Sumpter, 2003].

Silva et al. (2002) found that when eight weakly estrogenic compounds were combined, estrogenic effects were seen even though each of the component compounds were present at concentrations that were individually too low to cause any observable effects. This result confirms that the risk posed by endocrine disruptors should account for the additive effects of these types of chemicals in the environment since the combination of many low concentration contaminants could prove hazardous [Porter & Hayden, 2002].

Quantifying low concentrations of NP in environmental samples can be difficult and requires specialized handling and processing techniques. Specifically, care must be taken to prevent loss of NP through volatilization or adsorption during collection and transfer. At present, there are no standard methods for sampling, storage and analysis of NP in environmental samples. Solid phase extraction is often used to concentrate NP from aqueous samples, and there are often recovery issues with this technique. In contrast, carryover from one analytical sample to the next could be a source of error in NP analysis. Because of the complications mentioned above, measuring nominal concentrations of 4-n-NP in this experiment has not been successful.

The problems mentioned about *M. cornuarietis* also sets a question mark whether anything in this experiment could have been done differently. Clearly dealing with the large amounts of water would have required more space than was actually available in the laboratory. Furthermore working with chemicals right next to the control aquaria is not optimal. Instead the controls and the exposed aquaria should have been placed further apart from each other though still in the same room to maintain the same conditions.

When doing statistical analysis of fecundity in this experiment, single pairs of snails were each counted as n. This is problematic since many snails (especially males) are very active and in some way or another capable of crawling to the neighbouring pair of snails. This creates a statistical uncertainty since a female can reach copulation with different males, while another female may not reach copulation at all. To avoid snails crawling across barriers to other individuals, another technique should have been used regarding the partition walls between the snail pairs, securing that crossing barriers could not happen. However under the being condition terms such improvements was not possible. Another source of error was the fact that the 4-n-NP adsorbs to the glass walls, to the water pump and to any organic material present in the aquaria (like food) or partly evaporates and therefore might not be bioavailable to the snail.

References


