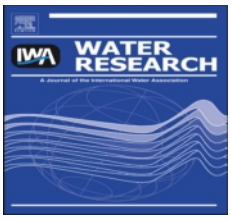

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Do suspended sediments modulate the effects of octylphenol on rainbow trout?

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Abstract

A system was devised which allows particles to remain in suspension in a conventional 60 L aquarium without undue disturbance to resident fish. Using this system, juvenile rainbow trout were exposed for one week to 4-tert-octylphenol (OP, 10-1000 µg/L) with or without the presence of suspended sediments (10-20 mg/L of natural suspended sediments from the River Calder, UK). About 8 % of the added OP partitioned to the solid phase. Vitellogenin levels were determined in the plasma of the exposed rainbow trout and showed a dose-dependent increase with regards to OP exposure concentration. Considerable variation in the vitellogenin response was observed between separate runs with the same OP concentration. There was no statistically significant (at $P<0.05$) difference in plasma VTG levels between the OP treatments with or without suspended sediments. This suggests that the dissolved concentration is the key factor and natural suspended sediment neither protects against, nor exacerbates, the endocrine disrupting effect of OP on fish.

Keywords: alkylphenols; suspended sediments; rainbow trout; octylphenol; endocrine disrupters; partition coefficient

Introduction

Most experimental studies of endocrine disruption in aquatic organisms have involved the exposure of animals to single pure chemicals dissolved in water. But in the real world, the aquatic environment consists not only of mixtures of chemicals, but also of a natural suspension of particles in the aqueous medium, at concentrations extending into the grams per litre range depending upon hydrological conditions (Wass and Leeks, 1999). Thus, there is the potential for suspended solids to modulate the effects of endocrine disruptors on susceptible organisms.

Although, after the restrictions on the use of alkylphenol polyethoxylate surfactants in the EU and some other countries (Directive 2003/53/EC, 2003, Soares *et al.*, 2008), much of the focus on endocrine disrupting chemicals in the aquatic environment has now turned to the ubiquitous steroid estrogens (Desbrow *et al.*, 1998, Sheahan *et al.*, 2002a), there are still rivers where alkylphenols from the degradation of their ethoxylates play a significant role in particular with respect to estrogenic chemicals associated with solid matter (Fenet *et al.*, 2003, Petrovic *et al.*, 2002, Sheahan *et al.*, 2002b). It is frequently found that while estrogenic activity measured in the water phase of sewage effluents, or receiving waters, is mostly caused by steroid estrogens, a large part of the estrogenicity extracted from solids, such as sediments and sewage sludge can be explained by alkylphenols (Bolz *et al.*, 2002, Fenet *et al.*, 2003). Furthermore, since in lakes with stratified sediment deposition, the highest NP concentrations were found in bed-sediments from the 70s or 80s (Giger and Alder, 2002, Isobe *et al.*, 2001), re-suspension of historic bed-sediments may provide a secondary source of alkylphenol contaminated solids. The most common akylphenols nonylphenol and octylphenol have octanol water partitioning coefficients (Log K_{ow}) in the range of 4-5 (Ahel and Giger, 1993, McLeese *et al.*, 1981), which is somewhat

higher than the steroid estrogens, which have Log K_{ow} values between 2.5 and 4 (Hansch, 1995, Holthaus *et al.*, 2002). Furthermore, distribution coefficients (K_d) observed in the field are often higher than those measured in laboratory experiments or predicted from log K_{ow} data (Isobe *et al.*, 2001, Patrolecco *et al.*, 2006).

In environmental samples, a significant proportion of alkylphenols may be bound to suspended sediments. For example, in the Tokyo area Isobe *et al* (2001) found an average of 85 % of nonylphenol (NP) and 61 % of OP in primary sewage effluent and 23% of NP and 8% of OP in river water was bound to suspended sediments. The proportion of NP on suspended particles was up to 86% in treated sewage and water from the river Aire (Blackburn and Waldock, 1995, Sheahan *et al.*, 2002a), 12-58% in water samples from a range of other UK rivers (Blackburn *et al.*, 1999), 6-25% and 2-42% in samples from the Italian rivers Lambro and Tiber respectively (Patrolecco *et al.*, 2006, Polesello *et al.*, 2003). In a Korean lake and its tributing creeks, Li et al. (2004) found between 5 and 78% of the measured NP in the suspended particles and this proportion tended to be higher in winter than in summer. Thus, despite the relatively low K_{ow}, a significant proportion of alkylphenols may sometimes be found on suspended sediments in a real environmental situation.

In this work, OP rather than the more ubiquitous NP was chosen, because unlike NP it exists as a single isomer rather than a mixture. There are, however, less data available for OP because it is less widely used than NP and therefore often not analysed or its concentration is below the detection limit. Earlier work (Johnson *et al.*, 1998) suggested that a significant proportion of OP could bind to suspended sediments, thus reducing the concentration in the aqueous phase, but Isobe *et al.* (2001) found only an average of 8 % of the OP in rivers to be bound to suspended sediments. It is important to note that the suspended sediments in a river do not remain of a consistent

composition during the year. For example, in slow flowing areas in summer a large proportion of suspended sediments may consist of algae, whereas rainstorms can flush out high concentrations of clay particles and at other times, the most significant contribution may be from decaying detritus.

For an aquatic organism, sorption of alkylphenols to suspended sediments could mean a reduced exposure relative to inputs unless the chemical is taken up from the particles, for example by accidental or deliberate ingestion. Ra *et al.* (2008) have shown that the sorption of OP and pentachlorophenol to (artificial) suspended particles did indeed reduce the toxic effects on *Daphnia magna* and *Vibrio fischeri*, but because the toxicity tests were carried out after the contaminated particles were removed from the system by filtration, uptake of chemicals from the particles was precluded. Uptake from sediments may be important because, at least with NP contaminated bed-sediments, about 95% of the chemical on the solids appeared to be bioavailable (de Weert *et al.*, 2008). It is well known that the uptake of (xeno-) estrogens *via* food can have endocrine effects on fish: increased vitellogenin concentrations in immature rainbow trout were, for example, measured after dietary exposure to E2 (Carlson and Williams, 1999) and OP (Pedersen *et al.*, 2003). In the present study, the contaminated particles were not removed, so the animals were exposed to OP and suspended sediments at the same time.

The question posed in this study was therefore: What effect, if any, does the partitioning of OP to suspended sediments have? Does it *reduce* the chemical's effect on fish by reducing the bioavailable aqueous concentration or does it *increase* its effect through ingestion of suspended sediments, which contain a higher concentration of the chemical than the water?

Material and Methods

Design of aquarium agitator

In order to study the effect of suspended sediments, they must be maintained in suspension within the water column without harming or unduly stressing the animals involved in the study. Several systems to expose animals to suspended sediment have been described in the literature. Generally the volume of water available to the animals is very limited compared to the size of the whole apparatus, which makes these designs either only suitable for small animals, such as invertebrates or fry (eg. Chilton, 1991, Schmidt-Dallmier *et al.*, 1992, Schrap and Opperhuizen, 1989, Servizi and Martens, 1991) or the apparatus requires considerable space, limiting the practicability of the design (Cope *et al.*, 1996). Some authors have suggested a flow through system with serial dilution of suspended sediments (eg. Sved and Roberts, 1995), a design which suffers from the practical problem of collecting sufficient amounts of natural suspended sediments to sustain such a setup. Therefore, a new compact apparatus was developed which fits into a conventional glass aquarium (l: 60 cm, w: 30 cm, h: 40 cm) filled with 60 L Windermere lake water with the space available to the fish within the mesh basket being approximately 35 L (figure 1). The device consists of a motor-driven bar that runs laterally back and forth along the base of the aquarium at a speed of about 8 cm/s, combined with a wire mesh basket within which the experimental animals were held. Preliminary tests of this equipment showed that OP sorption to glass and steel was negligible (less than 1 % in the entire aquarium setup), so all parts of the stirring mechanism to be immersed were constructed from stainless steel. However, there was a significant loss of chemical due to sorption to the silicone sealant used in aquaria; this was monitored in each

experiment by including control aquaria containing no fish. Although the device created sufficient motion to prevent particulate material from settling, the suspended sediments started to stick to the surfaces (even vertical surfaces), of both the aquarium itself and the mesh basket. These particles were re-suspended once each day by disconnecting the stirrer bar from the motor and manually moving it along the aquarium at increased speed three to four times. Care was taken to do this in the same way for all aquaria, including the ones without suspended sediments, to ensure that the disturbance experienced by the confined fish due to unexpected movement did not differ between treatments.

Collection of suspended sediments

Natural suspended sediments (table 1) were collected with a continuous flow centrifuge (Alfa-Laval SediSamp System II, model WSB/103-ENV) from the River Calder in Yorkshire at Methley Bridge (National Grid Reference SE 408 257). On one occasion (14/15-5-01) the suspended sediments was taken about 13 km further upstream at Stanley Ferry (National Grid Reference SE 355 231), because the original sampling site at Methley Bridge was not accessible. It was expected that the suspended sediments would not differ significantly between the two sites even though there is a small sewage works about 2 km upstream of Methley Bridge. At each of the sampling occasions, water samples were also taken to determine the ambient suspended sediment concentration as dry weight retained on a 0.45µm cellulose nitrate filter (Whatman, Maidstone, Kent, U.K.). The Stanley Ferry sample was also used by Holthaus *et al.* (2002) where the surface area is given as 5.5 m^2/g and particulate organic carbon as about 14%. The concentrated suspended sediment slurry was stored at 4°C up to three weeks prior to use in the fish exposure experiments.

Determination of a sorption distribution coefficient for the suspended sediments

A sub-sample of the concentrated suspended sediment slurry was used to determine a sorption distribution coefficient. A 5 ml aliquot of suspended sediment slurry was added to PTFE centrifuge tubes and ^{14}C-labelled OP was added to duplicate tubes to make final concentrations of 2,4,6,8, and 10 µg/L with the exception of the last experiment (River Calder, Stanley Ferry sample, May 2001) which was done at 10 fold higher concentrations to increase the accuracy. The tubes were then put on an orbital shaker (90 rpm) for 1 hr, after which they were centrifuged (15 min, 4750 g) and the aqueous phase was sampled, by transferring 1 ml into scintillation vials and mixing it with 5 ml scintillant (Ultima Gold, Packard Biosciences, Groningen, The Netherlands). The vials were then placed in a liquid scintillation counter (Beckmann LS 6500, Beckman instruments, Fullerton, CA, USA) and counted for 5 min. The counts were compared with those obtained from standards of radiolabelled OP in de-ionized water and the amount sorbed was calculated by difference. The slope of a best-fit line through the data for solid phase versus aqueous phase concentration yielded the distribution coefficient (K_d). To test the influence of suspended sediment concentration on sorption, this was repeated with suspended sediment samples (River Calder, Methley Bridge sample, January 2001), which were rediluted 1:2 or 1:10 with river water collected at the same time and place.

Testing for potential loss of OP through microbial degradation during experimental setup

To test whether there was the potential for a significant loss of chemical due to microbial degradation, two experiments were carried out.

The potential for OP degradation in natural river water was tested by spiking water collected from two sites (River Thames at Iffley Lock, Oxford, UK and Wallingford, Oxfordshire, UK, July 1999) with OP at a nominal concentration of 100 µg/L in triplicate and incubating at 10°C in the dark. Autoclaved (20 min, 121 °C) river water served as a sterile control. At regular intervals, sub-samples were taken and analysed as described below.

To examine whether the rapid decline of octylphenol concentrations in the aquaria was due to uptake by fish or to enhanced biodegradation due to fish waste products stimulating microbioal activity, degradation rates were compared in water samples with and without waste products. Triplicate water samples were taken from the unspiked aquaria (with and without suspended sediments) after an experiment, i.e. the fish had spent 3 days in this water and it therefore contained shedded mucus etc., but no OP. These were compared to fresh water and to fresh water with added suspended sediments. All samples were spiked with OP at a nominal concentration of 500 µg/L and analysed for OP immediately and after 3 and 6 days at 10°C.

Water quality parameters

Dissolved oxygen and pH meters (Jenway Instruments) were used to monitor these parameters in all experiments. In the 100 µg/L OP and 500 µg/L OP experiments NH_4/NH_3^+ was measured with a kit based on the indophenol method (ammonia test kit for fresh and salt water, HAGEN, Canada) and NO_2^-, and NO_3^- were measured only in the last (500 µg/L OP) experiment (Tetratest, Germany, kits for NO_2 and NO_3). In the 500 µg/L OP experiment the accuracy of these tests was improved by measuring the absorption at 650 nm for NH_4/NH_3^+ and 540 nm for NO_2^- and NO_3^- and comparing it to suitable standards (NH_4Cl, $NaNO_2$, $NaNO_3$ respectively), instead of visual comparison with the supplied colour charts.

OP concentration in water and suspended sediments

A 250 ml water sample was removed from the aquaria and filtered (glass fibre GF/C, Whatman, Maidstone, UK). The amount of OP sorbed to suspended sediments was determined by immediately extracting the damp filter with 20 ml methanol for at least 24 hrs and, where necessary, concentrating the methanol extract by rotary evaporation (50°C, approximately 600 mm Hg vacuum) to 2 ml. This sample was then diluted with an equal amount of de-ionized water and the OP concentration measured by HPLC/MS in electrospray negative mode (sometimes after further dilution). The concentration of OP in the aqueous phase was determined by withdrawing 1 ml into a syringe that already contained 1 ml methanol, mixing and then filtering the mixture through a 0.45 µm-PTFE filter (Gelman Sciences, Ann Arbor, MI, USA) into a HPLC vial. The presence of methanol prevents sorption to the filter or glass vial and inhibits degradation during storage. The vials were sealed with PTFE/silicone septa and stored in the dark at 4°C until analysis. To determine low concentrations of OP, 250 ml filtered sample was passed through a C18-solid phase extraction cartridge (Varian Bond Elut 500 mg, 3 cc, Varian, Inc. Palo Alto, CA, USA), which was then extracted with 1 ml methanol and the extract was diluted with 1 ml water before analysis by HPLC/MS.

Plasma vitellogenin and cortisol concentrations

At the end of the one week exposure period, fish were anaesthetised by immersion in a 2-phenoxyethanol (Sigma-Aldrich) solution (ca. 1:2000) and about 500 µL blood was withdrawn from the caudal vessel using a heparinized syringe. The blood was centrifuged (10 min, 3600 rpm) and the supernatant plasma divided into sub-samples which were stored frozen (-18°C) until analysis with previously validated

radioimmunoassays for vitellogenin (Sumpter, 1985) as a measure of estrogenic effects, or cortisol as a measure of general stress (Pottinger and Carrick, 2001). To control for overall effects of the experimental manipulations five to six fish each were also sampled at the beginning and end of each experiment directly from the stock population holding ponds.

Exposure of fish to OP with or without suspended sediments present

Experimental setup

Immature (10-18 months old) rainbow trout from commercial suppliers (Hawkshead Trout Farm, Cumbria, UK) were used in all experiments. In order to reduce problems caused by faecal material, food was withheld from the fish for at least three days (normally 1 week) before the start of each experiment and they were not fed during the one week exposure period. The temperature was kept at 10°C and an artificial light regime of 12 hours light/12 hours darkness was used.

Suspended sediments were added to the aquaria from concentrated slurry and OP was added from stock solutions dissolved in ethanol (up to 1 ml ethanol per tank); control tanks received the same amount of pure ethanol. Then the water was mixed for two hours, using the agitator described above, before water and suspended sediment samples were collected and the fish were added. To reduce problems due to deteriorating water quality, especially ammonia build-up, the fish were transferred to an identical set of aquaria, prepared with the same concentrations of OP and suspended sediments, after 4 days. Further water and suspended sediment samples were taken from both aquaria immediately after the fish were removed.

This semi-static rather than a flow through system had to be used, because of the difficulties of collecting sufficient amounts of natural suspended sediments, especially during low flow conditions and the difficulties of maintaining a relatively constant sediment concentration in a flow through system. Low flow is more interesting in connection with alkylphenols because their concentrations are likely to be higher during periods with little dilution.

Statistical analysis

With exception of the range finding experiment, which had small differences between the two runs (see below), the experiments consisted of duplicate runs with identical setup. A two sample t-test, assuming unequal variances was carried out on the log-transformed vitellogenin and cortisol data to test for significant differences between the two runs of the same treatment and between the different treatments. Where there was no significant difference ($P>0.05$) between the two runs, all fish from both runs were treated as one group, but where there was a significant difference between the repeat runs, we compared each of the two runs separately to the other treatments and only assigned significance where the difference was in the same direction and significant for both runs.

Range finding experiment

To establish a suitable OP concentration for the experiments a range finding study was carried out. This consisted of four aquaria with 20 mg/L suspended sediment and 0, 10, 100 and 1000 µg/L OP, together with one clean water control and a "chemical control" with suspended sediments and 100 µg/L OP, but no fish. Five juvenile female rainbow trout (average weight 59 g, range 37-83 g) were added to each aquarium. Accidentally, the 10 and 100 µg/L aquaria received only 2 mg/L suspended

sediments at the start of the experiment. When the error was discovered after one day, the remaining suspended sediment was added. This experiment differed from the standard setup in that there was only 55 L water, the fish were not transferred to a second set of aquaria, and water and suspended sediment samples were taken more frequently.

This experiment was repeated with the following alterations. (i) To reduce water quality problems, especially with regard to ammonia from the fish waste products, a complete water change was introduced after four days, by moving all fish into a second set of aquaria which had been prepared to contain the same initial concentrations of OP and suspended sediments as the first one. (ii) As there was not sufficient suspended sediment slurry remaining to provide the two aquaria needed for each treatment with 20 mg/L, the suspended sediment concentration was reduced to 10 mg/L. The average weight of the fish was 73 g (range 49-91g).

In the 1000 µg/L treatment of the second run, three out of the five fish showed signs of toxic effects one day after they were moved to the second aquarium, it was therefore decided to sample the fish in this treatment immediately (i.e. after a total of 5 days).

100 µg/L OP experiment

Following the range finding experiment, an experiment was designed with a nominal OP concentration of 100 µg/L. Suspended sediment slurry (River Calder, Methley Bridge, January 2001) was added to 60 L water to give a final solid concentration of 10 mg/L, which is a typical ambient suspended sediment concentration at this sampling site (Wass and Leeks, 1999). Six rainbow trout (mixed sex, about 10 months old, average weight 82g (50-111g)) were added to each aquarium.

The following replicated treatments were set up: clean water; water with 10 mg/L suspended sediments; water with 100 µg/L OP; water with 10 mg/L suspended sediments and 100 µg/L OP; two "chemical controls" without fish comprising: water with 100 µg/L OP and water with 100 µg/L OP and 10 mg/L suspended sediments.

500 µg/L OP experiment

The experiment above was repeated with a 500 µg/L concentration of OP and 20 mg/L suspended sediments (River Calder, Stanley Ferry, May 2001). To reduce variability and allow comparison to the previous experiments when the fish were a few months younger, relatively small fish (mixed sex, about 15 months old, average weight 60 g (41-79g)) were selected from the stock.

Results

Preliminary tests

Loss of OP through microbial degradation

The batch degradation experiment of OP in river water at 10°C gave estimated half-lives of 27 and 28 days for the samples from Wallingford and Iffley, respectively. In similar experiments with a range of river samples, half-lives between one and ten weeks were found for the higher incubation temperature of 20°C (Johnson *et al.*, 2000). Bearing in mind that a temperature reduction from 20°C to 10°C is generally expected to halve the biodegradation rate, losses from biodegradation were expected to be small in the fish exposure experiments, which ran for seven days at 10°C.

When testing whether the fish waste products increased the rate of degradation of OP, no loss of chemical was observed over six days in any of the four treatments (fresh water, fresh water with suspended sediments, water from aquarium, water from

aquarium with added suspended sediments). This confirmed that greater losses of chemical in the aquaria with fish compared to the chemical control (without fish) were due to uptake by the fish, not enhanced degradation.

Sorption distribution coefficients (K_d)

The sorption distribution coefficients (Kd) were about 400-1,500 L/kg (table 1). Assuming a linear sorption isotherm to extrapolate to low solids to liquid ratios, no more than 3 % would be expected to sorb to any of the suspended sediment samples at the concentration of 10-20 mg/L used in the aquaria

Fish exposure experiments

Water Quality

The concentrations of NO_2 and NO_3 were low with 0-0.1 mg/L and 0-12 mg/L respectively, total ammonia was <3.7 mg/L (100 µg/L OP experiment, from colour chart) or 0-2.0 mg/L in 500 µg/L OP experiment, (photometric) with pH 7.6-7.9, indicating that water quality was starting to deteriorate at the time when the water was changed, but was still within an acceptable range.

Exposure of fish to OP with or without sediments present

The **OP concentrations** measured in the aquaria, before fish were added, were close to the nominal values and then declined rapidly during the exposure period (figure 2 and table 2). The decline is believed to be largely due to fish uptake because, as mentioned above, the biodegradation rate was slow and although OP concentrations were also decreasing in the absence of fish, the decline was much faster when fish were present. The proportion of OP initially partitioning into the suspended sediments was between 3% and 15% (average 8%) and therefore somewhat higher than the value

expected from the K_d experiments (see above) which were done at a much higher solids to liquid ratio. The load of OP on the suspended sediments also reduced over time, but whether the fish took it up directly from the sediments, or whether it repartitioned into the water first, cannot be fully ascertained from these data. Some re-partitioning into the water phase was evident because the OP concentrations measured in the water at the end of the exposure period were higher in the treatments with suspended sediments than in the ones without. Small amounts of OP were measured even in the treatments where it had not been added. It is not clear, whether this was due to background contamination of the water or suspended sediments or to experimental artefacts such as cross contamination during sample preparation and analysis.

Cortisol data are only available for the 100 µg/L experiment. In none of the treatments were the cortisol levels of male fish significantly different from the clean water controls, but those of the females were significantly lower ($P<0.01$) in the pond control and higher ($P<0.01$) in the treatment with octylphenol but no suspended sediments.

Plasma vitellogenin concentrations after one week of exposure to different OP concentrations (range finding experiment) are shown in figure 3. Although the protocol of the two runs of the experiment differed slightly (First run: no water change, 20 mg/L suspended sediments; second run: one water change, 10 mg/L suspended sediments), the results were very similar and are therefore reported together. High OP concentrations produced, as expected, a dose dependent increase in vitellogenin levels. From these results it was concluded that 100 µg/L OP was a suitable concentration for assessing the effects of OP with or without suspended sediments (see below), as it gave a significant ($P<0.01$) vitellogenin increase without

obvious signs of toxic effects. The treatments with suspended sediments and no OP or 10 µg/L OP had lower VTG levels than both the pre-treatment controls from the ponds and the clean water control, but the difference was only significant ($P<0.05$) for the 10 µg/L OP + suspended sediment treatment. In both other experiments (figure 4 and figure 5) the females also showed a lower vitellogenin response in the treatment with suspended sediments but no chemical, compared to the clean water and pond controls. Due to the low number of individuals involved and the high variability between individuals, the differences were not significant in the 100 µg/L experiment though (figure 4).

Following from the range finding experiment it was expected that exposure of juvenile rainbow trout to a nominal concentration of 100 µg/L would give a clear vitellogenin response. However, in this experiment, the results were inconclusive with no significant differences in the vitellogenin levels between the controls and the exposed fish (figure 4). Therefore, the experiment was repeated at the higher concentration of 500 µg/L (figure 5). In the 500 µg/L experiment, no mortalities were observed and, despite the high OP concentrations, the fish showed no obvious signs of toxic effects. The nominal concentration of 500 µg/L OP led to a strong vitellogenin response in all the exposed fish compared to the clean water and pond controls and compared to the fish that had only been exposed to suspended sediments (figure 5). The vitellogenin levels of the fish exposed to OP in the presence of suspended sediments were over all slightly higher than in their absence in both experiments, but this difference was not statistically significant (at the $P<0.05$ level, figure 4 and figure 5) once the difference between the repeat runs was taken into account.

In both mixed sex experiments, males always had lower average vitellogenin levels than females apart from the groups exposed to 500 µg/L.

Discussion

1) OP and suspended sediments

There are few studies of OP and suspended sediments, but Isobe *et al.* (2001) found an average of 8%±8% of OP compared to 23%±15% of NP bound to suspended sediments in river waters in the Tokyo area. This difference between those two alkylphenols would be expected because NP is slightly more hydrophobic than OP. In the fish exposure experiments in the present paper, on average also about 8% (range 3-15%) of OP was found on the suspended sediments.

When the distribution coefficients from batch sorption experiments with high solids to liquid ratios were used to predict OP sorption in the fish experiments, the proportion of OP on the solids was expected to be lower than this, at around 3%, but the use of different solids to liquid ratios in the Jan 2001 sample showed that a high ratio can lead to underestimation of the sorption at realistic solids concentrations (table 1). Despite these difficulties of accurately measuring K_d, the experiments show that only a small proportion (less than 15%) of OP partitioned into these suspended sediments.

In these batch experiments, the suspended sediments had two effects on the OP concentration in the water phase, initially reducing them by a small amount but re-releasing some OP into solution once the aqueous concentrations had been sufficiently reduced through uptake by the fish. Because of this, the OP concentrations in the water phase at the end of each exposure period were consistently higher in the treatments with suspended sediments than in those without. Some of the OP that was initially removed from the water was returned, reducing the overall effect the suspended sediments had on water concentrations. This higher final concentration may explain the slightly higher (although not significant at $P<0.05$) vitellogenin levels

observed after exposure to OP with suspended sediments compared to that without. In the environment, alkylphenols would be constantly replenished so long as the source (eg. contaminated sewage treatment works effluents) remained, therefore the exposure would be relatively constant and not declining rapidly as in the present batch experiments.

2) OP and VTG

The production and depuration of vitellogenin is relatively slow: Serum vitellogenin concentrations in rainbow trout have been reported to reach peak values 3 weeks after the fish were injected with E2 and did not return to basal levels until five months later (Elliott *et al.*, 1979, quoted from Panter et al 2000). Therefore, the VTG levels we measured immediately after the one week exposure may not represent the maximum.

Care must also be taken when comparing the vitellogenin response to a particular nominal concentration of OP in the present study with those of flow through studies, where the water concentration is kept constant, because in this batch setup the OP concentration declined rapidly, mostly due to uptake by the fish (figure 2). This may explain the difference between the results of Servos (1999), who quotes acute LC 50 for OP and rainbow trout of 450 µg/L over 24 hours or 120 µg/L over 14 days, and the present study where a nominal concentration as high as 500 µg/L produced no obvious toxic effects, and even at 1000 µg/L toxic effects became only apparent after the water was changed and therefore the fish were exposed to a second spike of OP. The average concentration in the present setup is perhaps about 20% of the nominal, but this value cannot be directly used for comparison either because research by Panter *et*

al. (2000) showed that the maximum concentration may be sometimes more important than the average.

In all but the highest (500 µg/L) OP concentration, average vitellogenin levels of the male fish were consistently lower than those of the females in the same treatment. This confirms previous reports that even immature females produce more vitellogenin than males of the same age, but that this difference can disappear after exposure to high levels of (xeno)estrogens eg. dietary E2 (Carlson and Williams, 1999) or dietary OP (Pedersen *et al.*, 2003).

3) Sediments only and VTG

In the treatments with suspended sediments but no chemicals, vitellogenin levels in the females were overall slightly lower than in the clean water controls, although the difference was only significant in the range-finding experiment. Possible causes of this effect can be chemicals on the suspended sediments acting as anti-estrogens or the suspended sediments indirectly influencing the VTG production, for example if the turbidity was a cause of stress that in turn led to lower vitellogenin production. Plasma cortisol levels were determined in the 100 µg/L experiment, in order to assess whether the experimental setup was inherently stressful to the fish, but the results showed no increase in cortisol in the treatments with suspended sediments (figure 4). There was also no obvious relation between vitellogenin and cortisol levels of individual fish in any of the treatments (data not shown), suggesting that stress was probably not the explanation of these differences. However, as for vitellogenin, the cortisol levels at the time of sampling may not be wholly representative of those earlier in the study.

4) OP sediments and VTG

The vitellogenin levels of the OP exposed fish were not significantly reduced by the presence of suspended sediments. Although the amount of OP sorbed to the suspended sediments was only small in absolute terms, the K_ds show that the concentration on the suspended sediments is about 1000 times as high as that in the water (table 1). Therefore, the accidental ingestion of even quite small amounts of suspended sediments could contribute significantly to the uptake of alkylphenols by exposed fish. However, theoretical considerations and experimental data suggest, that for chemicals with a moderate log K_{ow} of 4-5 (log K_{ow} of OP is 4.1) the uptake via food would be less than 10% of the total if the fish and their prey were exposed to the same aqueous concentrations (Qiao *et al.*, 2000, Randall *et al.*, 1998). In addition, it has been shown that the estrogenic response is stronger if fish are exposed to alkylphenols via water than if the same amount enters their body via food (Pickford *et al.*, 2003) and there is only limited opportunity for bioaccumulation, which might otherwise increase the exposure via food, because alkylphenols are relatively rapidly biodegraded at least under aerobic conditions (Johnson *et al.*, 2000). The uptake via suspended sediments would be even less than that via food, because the suspended sediments are not deliberately ingested.

Conclusions

In summary, even with relatively high concentrations of suspended sediments from an urbanised lowland river the majority of the xenobiotic EDC OP is still substantially partitioned into the water phase. These data suggest that OP bound to suspended sediments does not increase the ED risk to the fish. We conclude that disruption of

the reproductive endocrine system arising from exposure to OP and perhaps other oestrogenic chemicals with log K_{ow} <5 is therefore likely to be due primarily to uptake of the dissolved chemical across the gill epithelium rather than via the ingestion of suspended sediments, or interaction of sediment particles with the gill surface.

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Tables

Table 1 K_d values for the suspended sediments from the River Calder in Yorkshire.

The sorption in the fish exposure experiments is predicted using the equation: (fraction sorbed) = (Kd x conc. of suspended sediments)/(1+ Kd x conc. of suspended sediments)

place, date	ss conc. in river (mg/L)	ss conc. in K_d expt	K_d (L/Kg)	predicted sorption to 10 mg/L susp. sed.	predicted sorption to 20 mg/L susp. sed.
Methley Bridge (Jul 2000)	9.6	concentrated (6.5 g/L)	1491	1.5%	2.9%
Methley Bridge (Jan 2001)	2.2	concentrated (2.7 g/L)	387	0.4%	0.8%
		re-diluted 1:2 (1.35 g/L)	882	0.9%	1.7%
		re-diluted 1:10 (0.27 g/L)	1427	1.4%	2.8%
Stanley Ferry (May 2001)[a]	day 1: 14 day 2: 40	concentrated (14 g/L)	562	0.6%	1.1%

[a] in the experiments a mixture of suspended sediments from the two days was used, with 60% of the solids originating from day 1 and 40% from day 2

Table 2 nominal and measured 4-tert-octylpenol (OP) concentrations immediately before fish were added to each tank and after the fish were removed. The fish were moved from tank 1 into tank 2 after 4 days to provide a complete water change in order to reduce build-up of ammonia and other waste products.

			measured: tank 1				measured: tank 2			
			at start		after 4 days		at start[a]		after 3 more days[b]	
Experiment description	**nominal OP [µg/L]**	**susp. sed. (mg/L)**	**OP in water [µg/L]**	**OP on susp. sed. [µg/L][c]**	**OP in water [µg/L]**	**OP on susp. sed. [µg/L][c]**	**OP in water [µg/L]**	**OP on susp. sed. [µg/L][c]**	**OP in water [µg/L]**	**OP on susp. sed. [µg/L][c]**
range finding (Jul/Aug 00)										
part 1: no change	0	0	0.10	n.a.[d]	n.a.	n.a.	no water change[e]		0.12	1.1
of water,	0	20	0.12	0.05	0.15	0.03			0.08	0.10
6 females	10	20	9.0	0.64	0.87	0.08			0.50	0.10
(average 59g)	100	20	112	6.48	7.2	0.83			4.8	0.90
per 55 L tank	1000	20	800	139	74	9.7			68	12.2
part 2: change of	0	0	0.08	0.35	0.33	0.32	0.48	n.a.	n.a.	0.19
water after 4 d,	0	10	0.07	0.18	0.31	0.22	0.22	0.16	0.2	0.06
6 females	10	10	7.5	0.51	0.47	0.07	9.0	0.52	1.04	0.12
(average 73 g)	100	10	103	4.8	3.5	1.2	82	5.7	4.66	0.58
per 60 L tank	1000	10	630[f]	303[f]	47	4.1	860	118	108	8.4
100 µg/L experiment (Jan/Feb 01): 6 fish mixed sex (average 82 g) per 60 L tank										
1. run	0	0	n.a.	n.a.	n.a.	0.10	n.a.	0.03	n.a.	0.02
	0	10	n.a.	<0.02	n.a.	0.06	n.a.	0.03	n.a.	0.02
	100	0	87	2.5	10	0.13	103	0.97	2	0.11
	100	10	77	6.03	n.a.	0.11	129	3.98	9	0.33
2. run	0	0	n.a.	0.04	n.a.	0.03	n.a.	0.03	n.a.	n.a.
	0	10	n.a.	0.01	n.a.	0.03	n.a.	0.03	n.a.	n.a.
	100	0	117	1.82	n.a.	0.09	113	1.28	n.a.	0.18
	100	0	94	1.43	7	0.16	91	1.26	4	0.09
	100	10	106	5.02	n.a.	0.22	112	3.44	15	0.46
500 µg/L experiment (May/Jun 01): 6 fish mixed sex (average 60 g) per 60 L tank										
1. run	0	0	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	0	20	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.6
	500	0	604	12	14	n.a.	464	11	60	0.6
	500	20	438	42	74	5.1	388	59	81	15
2. run	0	0	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	0	20	n.a.	n.a.	n.a.	n.a.	n.a.	0.2	n.a.	n.a.
	500	0	491	11	16	0.6	502	12	12	1.0
	500	20	398	42	60	6.7	500	57	42	n.a.

[a] just before the fish are transferred into this tank after 4 days in tank 1
[b] i.e. 7 days total
[c] Amount of OP extracted from the glass fibre filter
[d] n.a.: not available
[e] As there was no water change, the 7 day values for tank 1 are given instead of values for tank 2
[f] The high apparent sorption in this case is probably due to incompletely dissolved OP

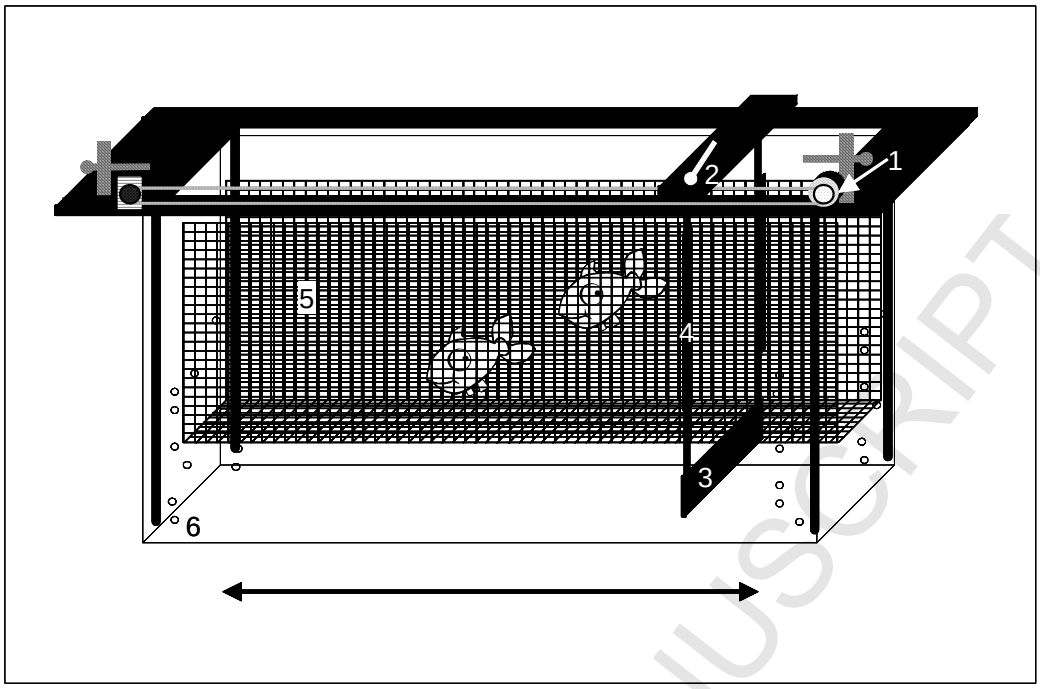


Figure 1 Aquarium agitator: A small electrical motor (1), which reverses by a mechanical switch (2), uses a pulley system to move a stainless steel bar (3) near the bottom of the aquarium at a speed of about 8 cm/s. Small bars at the sides (4) create additional turbulence to prevent suspended sediments settling on the cage (¼" x ¼" welded stainless steel wire mesh with 0.8 mm wire diameter) (5) which keeps the fish safe from moving parts. Aeration is through steel tubes (5 mm i.d., ends flattened to create a number of small openings) in the four corners (6).

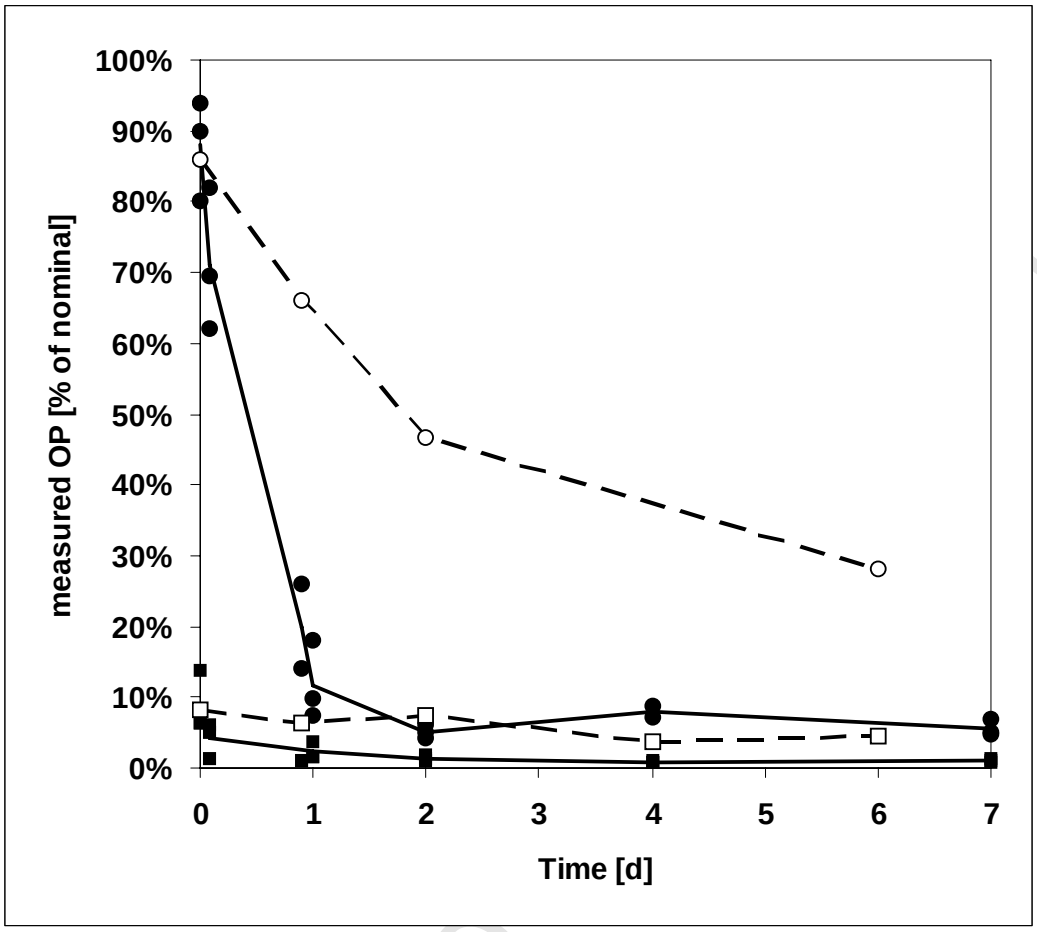


Figure 2 Measured OP concentrations in 55 L aquaria with 5 trout (average weight 59g, filled symbols) in water (circles) and on sediment (squares) expressed as % of nominal concentration added (10, 100 and 1000 µg/L) compared with an aquarium without fish (open symbols) The decline in OP concentration in the aquarium without fish is mainly due to losses to the silicone sealant, whereas in the aquarium with fish the majority of the loss is from uptake by the fish.

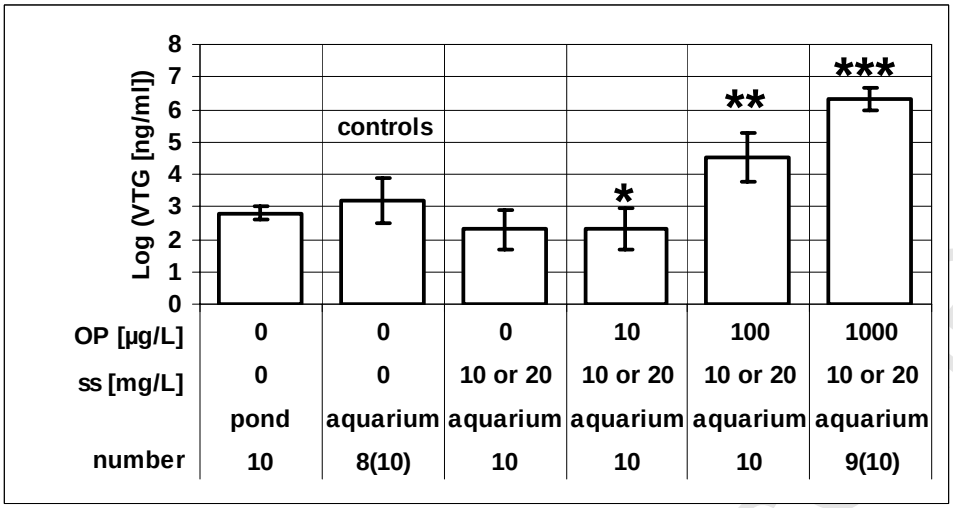
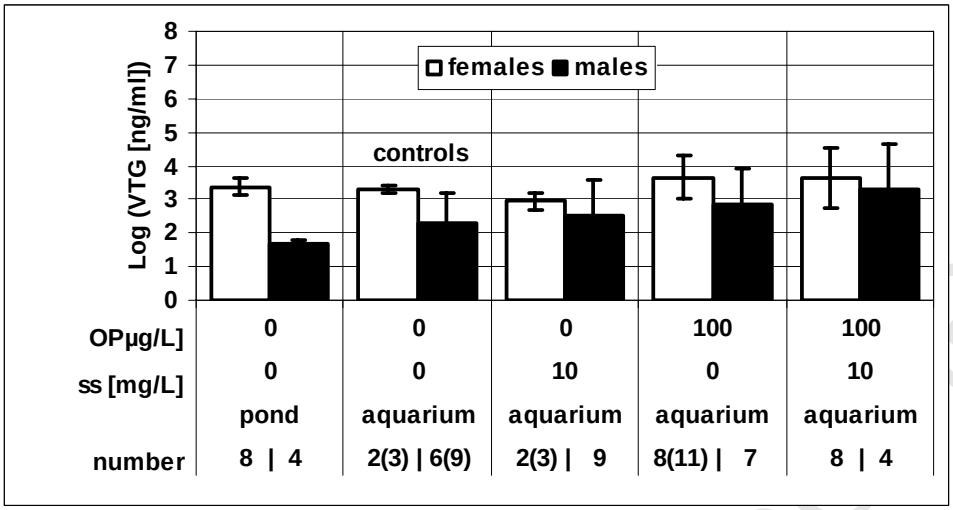


Figure 3 Log transformed plasma vitellogenin concentration in female trout after one week exposure to OP in the presence of 10 or 20 mg/L natural suspended sediments (ss). Concentrations are nominal concentrations at the start of the experiment or immediately after the water change. "Pond" refers to control fish taken directly from the outdoors holding pond, while "aquarium" refers to the fish from the aquaria in which the experiments were carried out. The average and standard deviation of all surviving individuals from two separate runs with five females per aquarium is given. In those treatments that had mortalities due to aggressive behaviour the original number of fish is given in brackets.

1. run: average weight 59 g, 55 L water, 20 mg/L ss, no change of water

2. run: average weight 73 g, 60 L water, 10 mg/L ss, complete change of water after 4 days

Stars denote significant difference to the controls at the $P < 0.05$ (*), 0.01 (**) or 0.001 (***) level.



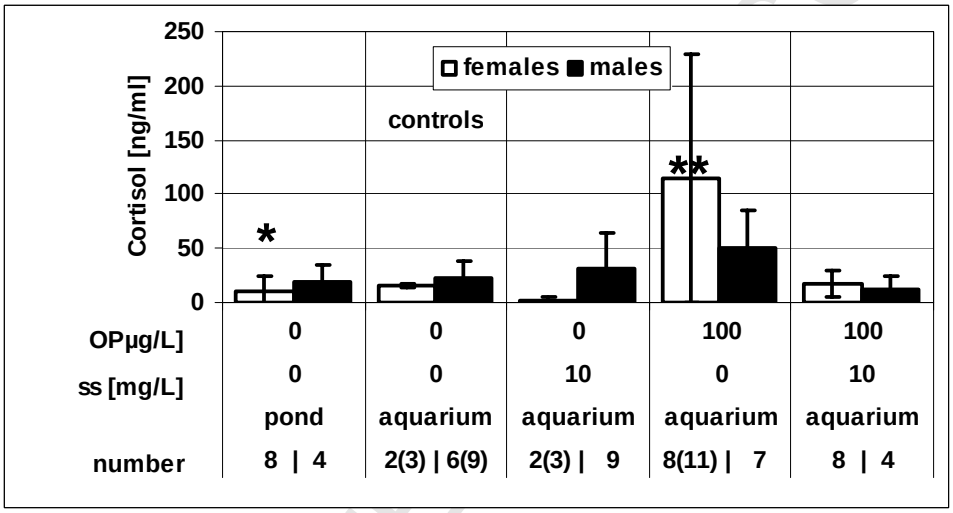


Figure 4 Log transformed plasma vitellogenin and cortisol concentrations in female and male trout after one week exposure to 100 µg/L OP and/or 10 mg/L natural suspended sediments (ss). Concentrations are nominal concentrations at the start of the experiment and immediately after the water change after 4 days. "Pond" refers to control fish taken directly from the outdoors holding pond, while "aquarium" refers to the fish from the aquaria in which the experiments were carried out. Average and standard deviation of all surviving individuals from two or three separate runs (6 fish per 60 L aquarium, the number in brackets is the total number per treatment including mortalities due to aggressive behaviour). Stars denote significant difference of the log transformed cortisol values to the controls at the $P< 0.05$ (*) or 0.01 (**) level. Vitellogenin levels were not significantly (at $P<0.05$) different between treatments.

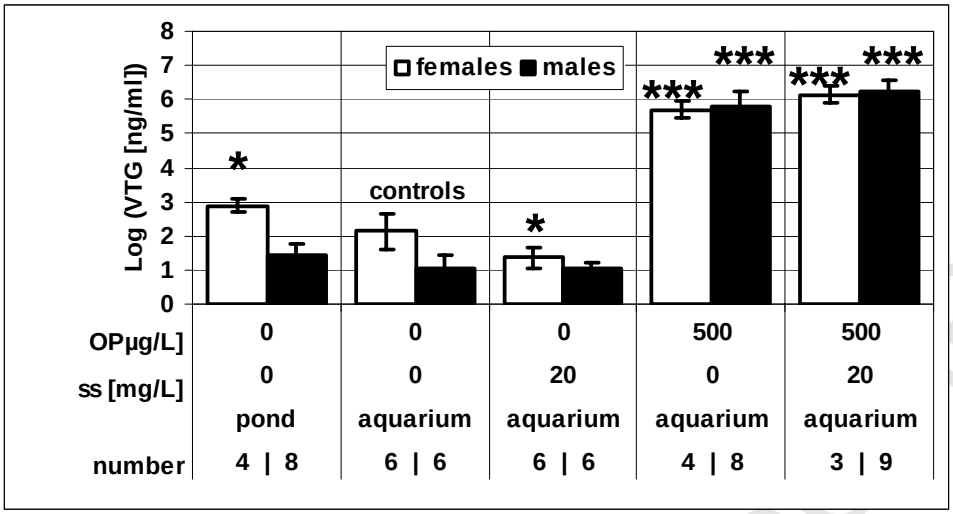


Figure 5 Log transformed plasma vitellogenin concentration in female and male trout after one week exposure to octylphenol (OP) and/or suspended sediment (ss). Concentrations are nominal concentrations at the start of the experiment or immediately after the water change after 4 days. "Pond" refers to control fish taken directly from the outdoors holding pond, while "aquarium" refers to the fish from the aquaria in which the experiments were carried out. Average and standard deviation of all individuals from two separate runs (6 fish per 60L aquarium, there were no mortalities in this run). Stars denote significant difference to the controls at the P<5% (*), 1% (**) or 0.1% (***) level**.** In both treatments with OP there is no significant difference (at the P<0.05 level) between males and females and the difference between the two treatments with OP with or without suspended sediments is not significant at the P<5% level when the tank effect is taken into account.