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Silver nanoparticles disrupt olfaction in Crucian carp (*Carassius carassius*) and Eurasian perch (*Perca fluviatilis*)

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ABSTRACT

The present study investigates the effect of silver nanoparticles on olfaction in Crucian carp (*Carassius carassius*) and Eurasian perch (*Perca fluviatilis*). The electro-olfactogram (EOG) signal was recorded by stimulating the olfactory epithelium with pulses of the odorant L-alanine during the pre-exposure, silver exposure and recovery periods, respectively. The nanosilver suspension concentrations applied were 0.00, 0.45 and 45 $\mu g\,L^{-1}$, respectively. Secondly, to compare the toxicity of silver nanoparticles with silver ions, perch were exposed to ionic silver. During exposure to nanosilver suspension, the olfactory epithelium rapidly hyperpolarized, which was not found after exposure to silver ion solution. Exposure to 0.45 $\mu g\,L^{-1}$ nanosilver suspension led to enhanced EOG responses, whereas exposure to 45 $\mu g\,L^{-1}$ silver nanoparticle suspension and silver ion solution resulted in suppressed EOG signals. The EOG signals partly recovered in silver-free water. The silver nanoparticle olfactory toxicity is believed to be a combination of silver particles and released silver ions.

1. Introduction

Olfaction plays important biological roles in fish. Odours, such as amino acids, sex pheromones and bile salts are essential signals ensuring that fish perform appropriate foraging, reproduction and social behaviours (Hara, 2006; Hamdani and Døving, 2007). The olfactory receptor cells are directly and constantly in contact with the ambient water and thereby to pollutants. Discharge of silver to the aquatic environment is inevitable due to the increasing use of nanosilver in numerous consumer products, e.g. textiles, toothpaste, nursing bottles, pillows, and food containers (Lee and Jeong, 2005; Benn and Westerhoff, 2008; Vandevelde et al., 2009). Currently, the nanosilver concentration in the environment is unknown (Tiede et al., 2009). A modelling study predicted that the silver nanoparticle concentration in the aquatic environment, derived from consumer products, is about 10 $ng\,L^{-1}$ (Tiede et al., 2009).

The general accepted definition is that nanoparticles have two or three dimensions between 1 and 100 nm. Metal nanoparticles possess unique properties due to their size, shape, surface structure, aggregation characteristics and chemical composition (Oberdörster et al., 2005; Nel et al., 2006) that differ from the bulk material and ions. Silver nanoparticles can release silver ions. The surface charge of the nanoparticle can vary from highly negative to highly positive depending on coating and the source of coating.

The olfactory organ in fish consists of three types of odour receptor cells, all with different functions and connections to the olfactory bulb. One receptor cell type detects food odours, e.g. L-alanine, a second detects sex pheromones, e.g. 17,20 P and the third detects social cues such as taurocholic acid (Døving and Selset, 1980; Stacey and Kyle, 1983; Sørensen et al., 1991; Hamdani et al., 2001).

The olfactory organ in fish has been demonstrated to be sensitive to metal ion exposure, e.g. silver, mercury and copper resulting in impaired olfaction (Hara et al., 1976; Brown et al., 1982; Baatrup et al., 1990; Winberg et al., 1992; Sandahl et al., 2006; Tierney et al., 2010). To our knowledge, the effect of nanoparticles on fish olfaction still needs to be investigated.

The aim of the present study was to measure the impact of silver nanoparticle exposure on the responses of olfactory receptor neurones in Crucian carp (*Carassius carassius*) and Eurasian perch (*Perca fluviatilis*) to the amino acid L-alanine, a potent olfactory stimulus. Secondly, the effect of silver ions on the olfactory receptor neurones in the Eurasian perch was investigated to establish if ionic silver affects olfaction differently from nanosilver. The olfactory receptor potentials were measured by electro-olfactogram (EOG)

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recordings during pre-exposure, a short-term nanosilver or silver ion exposures and during recovery in clean water. Finally, it was determined whether silver nanoparticles affected the three types of odour receptor cells differently by stimulating the epithelium with L-alanine, taurocholic acid (TLC) and 17α,20β-dihydroxy-4-pregnen-3-one (17,20 P), respectively.

2. Methods

Crucian carp (*Carassius carassius*) were caught in Lake Tjernsrud, outside Oslo City and brought to University of Oslo where they were acclimated for at least two weeks to 9–10 °C and a photoperiod of 8:14 h (light:dark) prior to experimentation. In total 18 fish, 14 males and 6 females, were examined with a standard length of 10.5 ± 0.2 cm and a body weight of 32.2 ± 2.2 g.

Eurasian perch (*Perca fluviatilis*) were obtained from Bornholm, Denmark, and held in aerated 17–20 °C tap water at Aarhus University for at least two weeks prior to experimentation. The photoperiod was 12 h:12 h (light:dark). Experiments were performed on total 12 perch, 5 males and 7 females, with a standard length of 25.3 ± 0.5 cm and a body weight of 348.4 ± 24.7 g.

2.1. Test chemicals

Two groups of six Crucian carp were exposed to a silver nanoparticle suspension with nominal concentrations of 0.45 or 45 μg L^{-1} by mass of silver. In addition, a third group of six unexposed fish served as controls. One group of four perch were exposed to a silver nanoparticle suspension with a nominal concentration of 45 μg L^{-1} by mass of silver. To investigate the effect of nanosilver dissolution, four perch were exposed to 35 μg L^{-1} silver ions, administrated as silver nitrate solution. Finally, a group of four unexposed fish served as controls.

2.2. Preparation of silver nanoparticle suspension and silver ion solution

Silver nanoparticle powder (stated by the manufacturer to be spherical 30–40 nm particles), coated with 0.2% of the stabilising agent PVP (polyvinyl pyrrolidone) and a purity of 99.5%, was purchased from NanoAmor (Houston, USA). A water dispersion of silver nanoparticles was prepared by suspending 0.5 g silver nanoparticle powder in 100 ml ELGA–water, followed by immediate ultrasonication (Ultrasonic Homogenizer, BioLogics, Inc., Virginia, USA). The sonicator was mounted with a solid titanium tip with a diameter of 9.5 mm. The suspension was placed on a magnetic stirrer and sonicated four times of 15 min over a 2 h time span with pulses of half a second duration. The power output was 100 W and the output frequency was 20 kHz. Following sonication, the suspension was centrifuged at 1000 × *g* for 2 h (Sigma 3k 30, Struers KEBO lab., Demark), and finally filtered through a 0.2 μm mixed cellulose ester filter (Frisenette ApS, Denmark). The colour stable stock nanosilver suspension with a clear yellow colour was stored in darkness at 6 °C until use. Two freshly prepared stock suspensions were used in the carp and perch studies, respectively. To determine the actual concentration of silver in the stock nanosilver suspension, equal volumes of the suspension and 69% HNO_3 were mixed resulting in dissolution of the nanosilver. This solution was then diluted with water until the silver concentration was within the linear measuring range of the Atomic Absorption Spectrophotometer (AAS). The concentrations of silver were determined using a Perkin-Elmer AAnalyst 300 AAS (Perkin-Elmer, Denmark) mounted with a silver lumina hollow cathode lamp (Perkin-Elmer, Hvidovre, Denmark).

Silver nitrate ($AgNO_3$) pellets (SigmaAldrich, Steinheim, Germany) with a purity > 99.5% were dissolved in Milli-Q water.

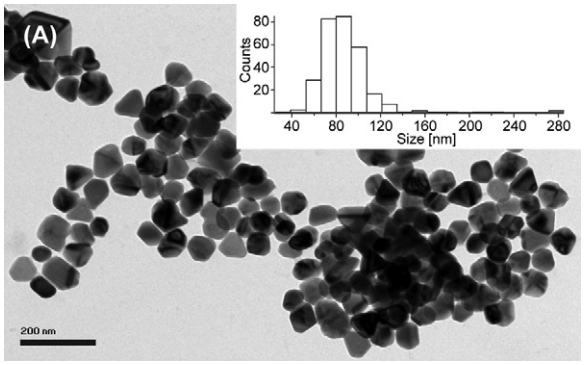

Fig. 1. Representative transmission electron microscopy (TEM) image of stock nanosilver suspensions and the corresponding size distribution profile (insert).

Nanosilver stock suspensions and ionic silver stock solution were diluted to the respective concentrations in artificial fresh water containing in μg L^{-1}: NaCl 29.0; KCl 3.7; $CaCl_2$ 58.0 and $NaHCO_3$ 16.0 at pH 7.9. Water hardness was 0.0029 dH (German degrees) indicating soft water. The content of DOC was negligible in the test water.

2.3. Characterisation of silver nanoparticles

Transmission electron microscopy (TEM), using a Phillips CM20 revealed that most of the nanoparticles had a slightly elliptical or multifaceted shape, but that some were triangular. 3D nanoparticle topography was investigated using a Nanoscope V MultiMode atomic force microscopy (AFM) showing clusters or single particles. The mean primary particle size of the nanosilver stock suspension was 81 ± 2 nm with an aspect ratio (length/breadth) of 1.2 ± 0.2 nm (Fig. 1). Powder X-ray diffraction (PXRD), using a STOE STAPI P (STOE & Cie GmbH, Darmstadt, Germany) powder diffractometer emitting Cu $K\alpha_1$ (λ = 0.15405 nm) radiation, equipped with a curved 1D-PSD detector, of the silver nanoparticle powder revealed a volume-weighted crystallite size of ~78.1 nm determined by the Scherrer formula (Langford and Louër, 1996) after a linear background subtraction and instrumental profile correction (Hald et al., 2006). The diffraction profile was consistent with a simple cubic crystalline orientation, and showed no sign of oxidation or other crystalline phases present. Dynamic light scattering, using a Malvern Zetasizer Nano (Malvern Instruments Ltd., Worcestershire, UK), showed that the zeta potential (reflects the charge on the nanosilver surface) of the nanosilver stock suspension was −32.6 ± 0.49 mV. The stability of the nanosilver suspension was monitored by UV–VIS-NIR spectrophotometer (UV-3600, Shimadzu, Japan). The nanosilver suspension demonstrated an absorption band at ~419 nm which is the typical absorbance band of silver nanoparticles due to their surface plasmon (Creighton et al., 1979).

The silver concentration in the nanosilver stock suspensions measured with AAS were 17.87 mg L^{-1} (carp study) and 17.70 mg L^{-1} (perch study), respectively.

Dissolution of silver nanoparticles in the stock suspensions was determined by a silver ion-selective electrode (Hach Lange APS, Brønshøj, Denmark). The calibration curve was constructed from diluting $AgNO_3$ solutions in the silver ion range 0.1–1 mg L^{-1}. Triple determination estimated that in the nanosilver suspensions, approximately 50% of the silver was in the form of ions. This emphasizes that silver ions are an important issue when examine the environmental toxicity of nanoparticle suspensions.

2.4. Odour stimulants

The primary odour stimulant was the amino acid L-alanine at 10^{-5} M for Crucian carp and 10^{-4} M for perch, respectively (purity 99%, Aldrich, Germany). Crucian carp were additionally stimulated with 10^{-8} M of the sex pheromone 17α,20β-dihydroxy-4-pregnen-3-one (17,20 P) and 10^{-8} M of the bile salt taurocholic acid (TLC). Stimulants were diluted to the respective concentrations in artificial fresh water.

2.5. Setup

Each fish was selected randomly from the stock aquarium, regardless of sex. Fish were anaesthetised in a benzocaine (45 μg kg^{-1}) solution and the skin covering the left olfactory organ was removed prior to the experiment in order to avoid the influence of alarm substances on the EOG response. Prior to the EOG measurements, the Crucian carp was anaesthetised intraperitoneally with alfaxan (12.5 μg kg^{-1}) and perch were anaesthetised by a benzocaine solution (0.033 g L^{-1}) during the experiment. The fish were then wrapped in wet tissue, placed in a fish holder and fixed belly down with non-chlorinated tap water continuously irrigating the gills. The fish were grounded electrically by an electrode attached to the fish side. A polyethylene tube was placed above the left olfactory rosette, continuously irrigating it with artificial freshwater at approximately 3 ml min^{-1}. Without changing the flow rate, the artificial fresh water could be replaced by solutions of toxicants and stimulants using miniature valves connected to the tube.

A glass microelectrode, filled with Ringer solution, was placed in the centre of the olfactory rosette close to the base of the olfactory filaments, while the reference electrode was positioned under the skin of the head or in the tail. The Ringer solution contained in mg L^{-1}: NaCl 8415; KCl 298; $CaCl_2 \cdot 2H_2O$ 294; $MgCl_2 \cdot 6H_2O$ 203.

2.6. Experimental procedure

For the Crucian carp recordings, the olfactory rosette was exposed to artificial fresh water for 10 min (pre-exposure period), secondly to the nanosilver suspension for 30 min (exposure period) and finally again to artificial fresh water for 30 min (recovery period). Prior to the pre-exposure period and following the termination of the recovery period the olfactory organ was additionally stimulated three times with L-alanine, 17,20 P and TLC separately. In the perch experiment, the olfactory rosette was exposed to artificial fresh water for 10 min (pre-exposure period), secondly to silver nanoparticle suspension or silver ion solution for 15 min (exposure period) and finally again to artificial fresh water for 15 min (recovery period).

During the entire experimental period, the olfactory organ was stimulated every second min with a 10 s pulse of L-alanine. The 2 min between the stimuli assured that the stimulating substance was washed away from the olfactory rosette and allowed time for the olfactory activity to return to baseline before the next stimulation. To ensure constant exposure, silver nitrate solution or nanosilver suspensions was added to the L-alanine solution used during the silver exposure period. All exposure suspensions were prepared immediately before use. After termination of the experiment, the fish were weighed, killed and sex determined.

2.7. Electro-olfactogram measurements

The summated receptor potential represents an excitatory response generated by many receptor neurons in response to olfactory stimuli. Different electrophysiological setups were used in the two fish species. In the Crucian carp study (conducted at Oslo University), the voltage change from the olfactory epithelium was recorded through a differential amplifier and displayed on an oscilloscope. The nervous activity was digitalized with an A/D converter and analysed by the Spike 2 software measuring the peak amplitude of the responses. Further details on the EOG recording are provided by Lastein et al. (2008). In the perch experiment (conducted at Aarhus University), the voltage potential of the olfactory epithelium was amplified by an Electro 705 amplifier (World Precision Instruments, Stevenage, UK) and digitized at 500 Hz, using a National Instruments multifunction card (NI USB-6008) with custom written software (written in LabVIEW, National Instruments). The data were low-pass filtered off-line (Butterworth filter, 0.1 Hz −3 dB frequency, 8 poles) and down-sampled to 10 Hz. A separate software application was written in LabVIEW to automate the data analysis. Here, the peak EOG response around the base line was determined by subtracting a 1st order regression line between the potential 0.5 s prior to stimulation and 20 s post stimulus and then measuring the difference between the regression line and the recorded level in that interval. In some rare cases when the potential was changing rapidly, a shorter analysis interval was used.

The amplitude and shape of the nervous activity vary with the position of the microelectrode, but were never less than 1 mV. The amplitudes of the EOG response during the pre-exposure, exposure and recovery periods were measured and expressed as percentage of the mean value of the first five stimulations during the 10 min pre-exposure period. In a few cases, during the silver exposure and recovery periods, silver totally abolished the EOG signal. In these cases, the response was set to zero, which was also used when calculating the average.

2.8. Statistics

Data were analysed for normality and homogeneity of variance. The EOG signals in the pre-exposure period were evaluated by a collective comparison of the five pre-exposure EOG measurements of the exposed groups and control group using ANOVA *repeated measures* statistics, interaction component (Roy's Largest Root) (comparing the interaction of the treatments) and between subjects effect (comparing the level of the treatments). The statistical tests were also used to examine the effects of the nanoparticle suspension and the silver ion solution on the EOG signal. To evaluate the changing exposure condition, the last data point from the control period was included in the analysis of the exposure period and the last data point from the exposure period was included in the analysis of the recovery period. ANOVA was used to examine whether the effect of previous exposure to nanosilver suspension differed between L-alanine, 17,20 P and TLC odour receptor cells, respectively. The statistical analyses were performed in SPSS version 13.0 (SPSS InC. Chicago, Il, USA) and the level of significance was 0.05. All data are presented as mean ± standard error (SE).

3. Results

The EOG signals showed peak responses upon stimulation in the range of 1–20 mV for all odours. In all cases, the pre-exposure (control) stimulations caused a depolarization of the olfactory epithelium. At cessation of stimulation, repolarisation of the same magnitude took place, returning to baseline level. Examples of control EOG responses from a perch are shown in Fig. 2A. Compared to the perch, the Crucian carp EOG signals were weaker, rarely exceeding 4 mV in peak amplitude (data not shown). Also, in most cases it was not possible to distinguish between the "peak" and the "sustained" responses of the EOG signal, previously described for Atlantic salmon (*Salmo salar*) (Baatrup et al., 1990; Winberg et al., 1992). In the Crucian carp and perch control group, the amplitudes of the EOG-responses dropped on average 23% and

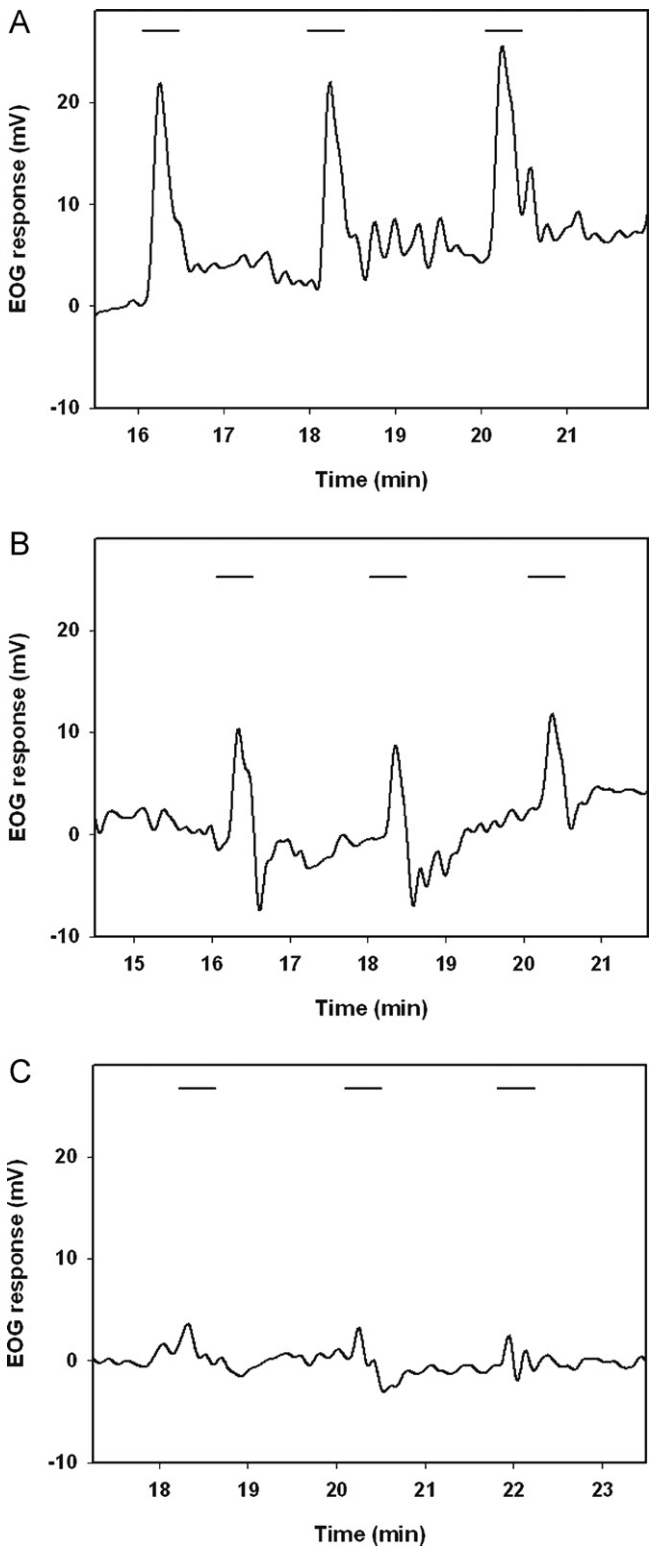

Fig. 2. Examples of electro-olfactogram (EOG) recordings from the olfactory epithelium in Eurasian perch (*Perca fluviatilis*). The signals were evoked by stimulation with L-alanine. Solids bars indicate the application of L-alanine. (A) Control perch. (B) Exposure to 45 $\mu g\,L^{-1}$ silver nanoparticle supsension. (C) Exposure to 35 $\mu g\,L^{-1}$ silver ion solution (administrated as silver nitrate).

21%, respectively, during the entire recording period, indicating some instability in the recording conditions. During exposure to both silver nanoparticle suspension and silver ion solution, L-alanine stimulation resulted in diphasic EOG-responses. First, a depolarization occurred followed by a hyperpolarization relative to baseline. After cessation of the stimulus, the EOG response returned to baseline (Fig. 2B and C). The amplitude of the EOG-response (depolarization) was depressed by both nanosilver and silver ions.

The baseline remained relatively stable during the recording period in the control groups although some instability was observed in the perch (Fig. 3A), whereas it became unstable during nanosilver suspension exposure. This instability was least pronounced in the Crucian carp experiment, where the baseline fluctuated about 1 mV during exposure (data not shown). Further, after addition of the high nanosilver suspension concentration of 45 $\mu g\,L^{-1}$, the olfactory epithelium rapidly hyperpolarized in both fish species, but partly returned to the original polarisation level during the exposure period (Fig. 3B). At the low nanosilver suspension concentration of 0.45 $\mu g\,L^{-1}$, this negative displacement of the baseline was less pronounced and was only observed in half of the recordings. In the cases of exposure to silver ion solution, no apparent hyperpolarisation of the olfactory epithelium took place (Fig. 3C).

3.1. Perch exposed to silver nanoparticles

A comparison of all five control measuring periods (*repeated measures statistics*) found no significant differences between the control group and the pre-exposure period in the nanosilver suspension exposed group (interaction component, $p = 0.186$).

During nanosilver suspension exposure, the relative EOG responses were suppressed on average 53%, which however was not statistically significant from the control EOG responses (interaction component, $p = 0.135$; between-subject effect, $p = 0.156$) due to the high variation in responses (Fig. 4). During the 15 min recovery period, the amplitude of the olfactory responses continued to decline (Fig. 4), but within one hour, the amplitudes rose again (data not shown). As evident from Fig. 4, the EOG-signals were significantly reduced in the nanosilver suspension exposed group during recovery when compared with the control group (between subjects, $p = 0.001$). In contrast, the development of the EOG-amplitudes during the 15 min recovery period were similar for the two groups (interaction component, $p = 0.384$). The silver ion solution clearly inhibited the olfactory receptor signal, resulting in a significant (82%) decrease in signal amplitude (between subjects effect, $p = 0.022$) (Fig. 4). Again, due to a high variation, the course of the decrease (interaction component) was statistically insignificant ($p > 0.257$). During the subsequent recovery period, the EOG amplitudes continued to decline significantly below the amplitudes of the control group (between subjects, $p = 0.0001$; interaction component, $p = 0.789$) (Fig. 4). Within 1 h after termination of the recovery period, the EOG signal increased again (data not shown).

The nanosilver suspension and the ionic silver solution affected the EOG signals similarly with no significant difference in amplitudes between the two groups during the exposure and recovery periods (*repeated measures*; interaction component, $p > 0.05$ and between-subject effect, $p > 0.05$).

3.2. Crucian carp exposed to silver nanoparticles

The sense of smell was also affected in the Crucian carp. During the initial pre-exposure period, no significant differences were found between the control group and the exposed groups (interaction component, $p = 0.773$).

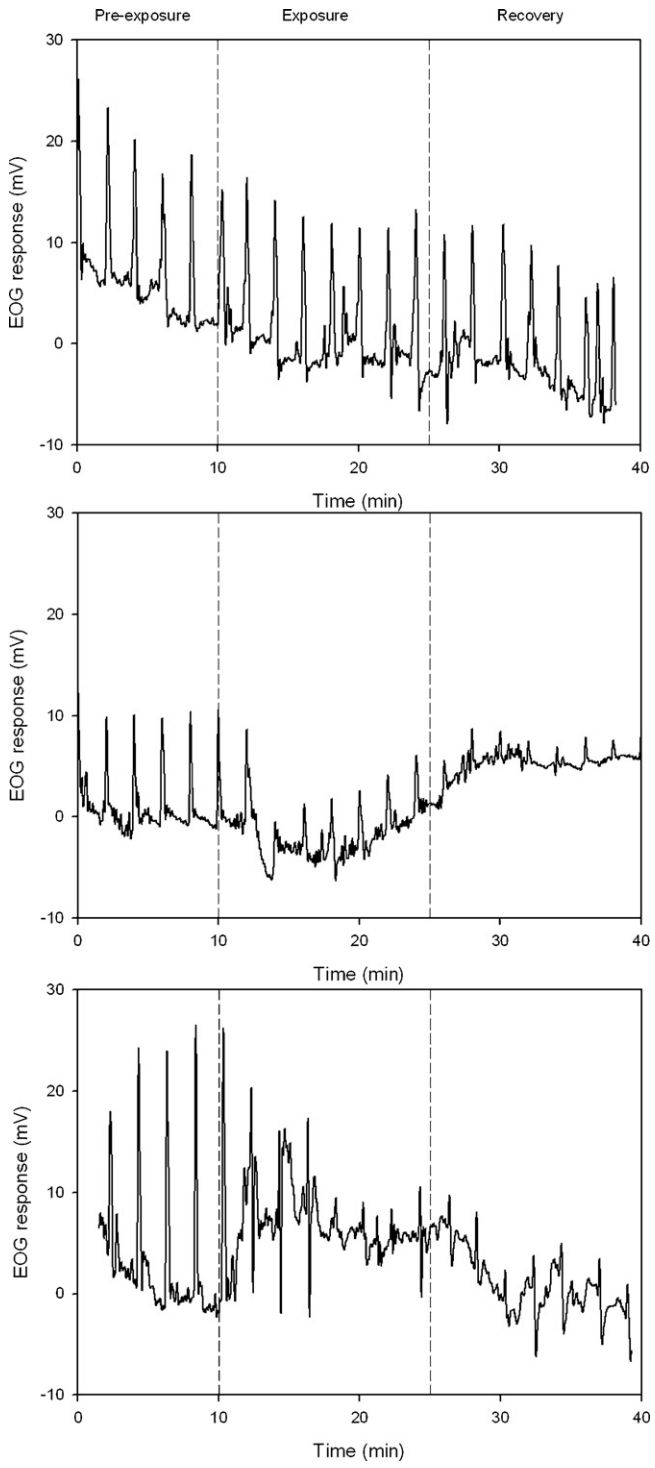

Fig. 3. Examples of electro-olfactogram (EOG) signals from the olfactory epithelium in Eurasian perch (*Perca fluviatilis*) during the 40 min recording period; pre-exposure (0–10 min), silver exposure (10–25 min) and recovery (25–40 min). The signals were evoked by stimulation with L-alanine. The vertical dotted lines represent start and end of the silver exposure. (A) Control perch. (B) Exposure to 45 μg L^{-1} silver nanoparticle suspension. (C) Exposure to 35 μg L^{-1} silver ion solution.

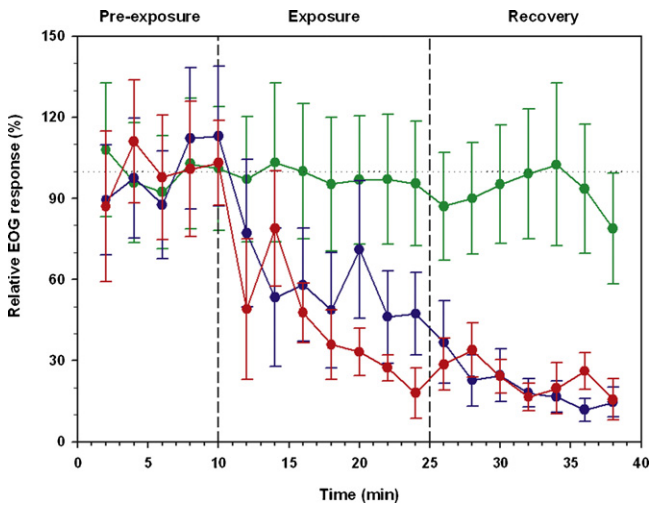

Fig. 4. Electro-olfactogram (EOG) responses of Eurasian perch (*Perca fluviatilis*) during pre-exposure (0–10 min), silver exposure (10–25 min) and recovery (25–40 min). The vertical dotted lines represent start and end of the silver exposure. The horizontal dotted line represents the relative EOG response at 100% Green circles represent the control group, red circles the group exposed to 35 μg L^{-1} silver ions and blue circles the group exposed to 45 μg L^{-1} nanosilver suspension. Individual amplitudes are expressed as the percentage of the mean amplitude of the first five responses (0–10 min). Entries are represented as mean ± SE. In each group $n = 4$.

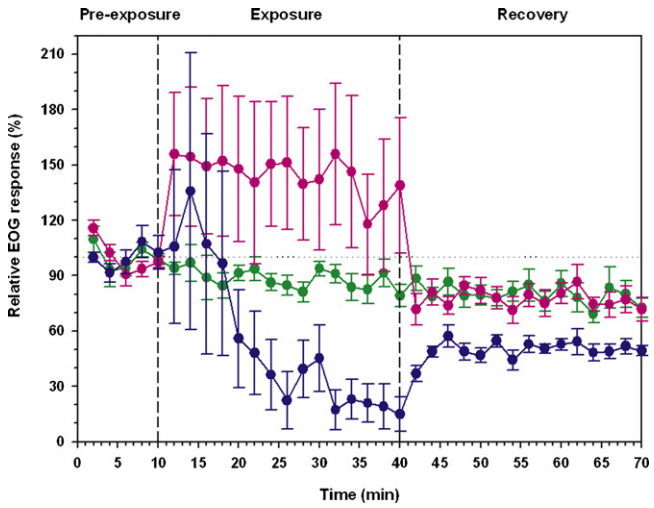

Fig. 5. Electro-olfactogram (EOG) responses of Crucian carp (*Carassius carassius*) pre-exposure (0–10 min), nanosilver suspension exposure (10–40 min) and recovery (40–70 min). The vertical dotted lines represent start and end of the silver exposure. The horizontal dotted line represents the relative EOG response at 100%. Green circles represent the control group, pink circles the group exposed to 0.45 μg L^{-1} nanosilver suspension and blue circles the group exposed to 45 μg L^{-1} nanosilver suspension. Individual amplitudes are expressed as the percentage of the mean amplitude of the first five responses (0–10 min). Entries are represented as mean ± SE. In each group $n = 6$.

Exposure to both nanosilver suspension concentrations resulted in irregular EOG responses (Fig. 5). The elevated EOG signals obtained during exposure to the 0.45 μg L^{-1} silver nanoparticle suspension did not differ significantly from the control group (interaction component, $p = 0.190$ and between-subject effect, $p = 0.171$). Exposure to 45 μg L^{-1} nanosilver suspension suppressed the EOG signals by 85% with the interaction component differing significantly from the control group ($p = 0.040$). The level effect of the two curves in Fig. 5 did not differ significantly (between-subject effect, $p = 0.196$). Again, the statistical insignificance is due to the large variation in the EOG responses.

During the recovery period, where the olfactory rosette again was irrigated with clean artificial fresh water, the EOG amplitudes of the 0.45 μg L^{-1} group rapidly stabilized around the level of the control group (interaction component, $p > 0.05$; between subjects, $p = 0.358$), while the EOG-amplitudes of the 45 μg L^{-1} group were approximately 30% smaller than the control recov-

ery amplitudes (interaction component, $p > 0.05$; between subjects, $p = 0.0001$) (Fig. 5).

3.3. Nanosilver's effects on three olfactory receptor types

In Crucian carp, the EOG-responses elicited by L-alanine (10^{-5} M), 17,20 P (10^{-8} M) and TLC (10^{-8} M), respectively, were measured before and after nanosilver suspension treatment. After the 30 min recovery period, the relative amplitudes of the three receptor types were similar following exposure to the 0.45 $\mu g\,L^{-1}$ ($n = 6$) (ANOVA, $p = 0.784$) or 45 $\mu g\,L^{-1}$ ($n = 6$) (ANOVA, $p = 0.528$) silver nanoparticle suspension, respectively (data not shown).

4. Discussion

To our knowledge, this is the first study treating the effects of silver nanoparticles on fish olfaction. It is demonstrated that even brief exposures to 45 $\mu g\,L^{-1}$ silver nanoparticle suspension suppress the olfactory response in both Crucian carp and perch. The lower suspension concentration of 0.45 $\mu g\,L^{-1}$ nanosilver was not significant, however a clear trend showed an increased EOG signal in Crucian carp. The olfactory epithelium rapidly hyperpolarized upon exposure to nanosilver suspensions, but not when exposed to ionic silver solution. During the recovery period of irrigation with clean freshwater, the EOG-signals slowly regained their pre-exposure amplitude demonstrating that the nanosilver suspension effect is reversible. The three olfactory receptor types of food (represented by L-alanine), sex pheromone (represented by 17,20 P) and conspecific (represented by the bile salt TLC) were all affected equally by the nanosilver suspension.

A paramount issue to consider is whether the measured toxicity is caused by the nanoparticles proper or released silver ions. Surprisingly, we found that up to 50% of the silver by mass existed as ionic silver in the nanosilver suspension, underlining the importance of establishing the ionic metal concentration in nanoparticle suspensions. Most likely, silver ions contributed to the toxicity of the nanoparticle suspension by reaction with the thiol (–SH) groups in the olfactory receptor proteins (Menevse et al., 1978; Chen and Schluesener, 2008) and causing functional disruption of the odour receptors. On the other hand, the nanoparticles undoubtedly have an inherent toxicity. For example, hyperpolarization of the olfactory epithelium only occurred in the presence of silver nanoparticles and the suppression of the EOG-signal was similar for the nanosilver suspension and the silver ion solution having almost the same total silver content.

Besides releasing toxic ions, nanosilver may also interfere with receptors in the epithelia or interfere with detection of odours by forming non-stimulating nanosilver–odour complexes that prevent binding of odours to the olfactory receptors as suggested by Sutterlin (1974) and Klaprat et al. (1992).

The nanosilver toxicity may at least be partially acting on the epithelia surface, because the EOG response of Crucian carp exposed to 0.45 $\mu g\,L^{-1}$ nanosilver suspension immediately regained the EOG control amplitude upon irrigation with clean freshwater. In contrast, the 45 $\mu g\,L^{-1}$ nanosilver suspension had a more sustained effect because the EOG responses were suppressed 30% during the entire 30 min recovery period. Even though the inhibition of the EOG response was even more severe in perch, the EOG signal again began to increase following 1½ h rinse with artificial freshwater. Partial olfactory recovery has previously been demonstrated after short-term exposure to copper ions in coho salmon (*Oncorhynchus kisutch*) (Baldwin et al., 2003). Another study showed that the effect of 30 min ionic copper exposure was reversible within one day in chum salmon (*Oncorhynchus keta*) (Sandahl et al., 2006). Injured olfactory receptor cells are constantly replaced (Julliard et al., 1993), likely within days to weeks (Hansen et al., 1999). If the recovery period in the present study had been extended, the olfactory sense would most likely have fully recovered. In the present study, the relative EOG amplitudes in the recovery period differed in the two examined fish species, demonstrating a potential species-specific difference in nanosilver suspension toxicity and reversibility.

The enhancement of the relative L-alanine EOG signal during exposure to 0.45 $\mu g\,L^{-1}$ nanosilver suspension in Crucian carp was to some extent similar to the odour enhancement in rats exposed to 5 mM silver nanoparticles for 30 min (Viswaprakash et al., 2009). Viswaprakash et al. (2009) demonstrated that 5 mM zinc nanoparticles, delivered with an odour, strongly enhanced the signal in rats, presumably by facilitating the odorant response by increasing the number of receptor-G_{olf} couplings. Such facilitated responses may not be true for nanosilver because the present study demonstrates that a higher concentration of the nanosilver suspension suppressed the EOG amplitude.

The uptake of metal ions such as cadmium, mercury, manganese, nickel and zinc have been demonstrated in olfactory neurons (Tjälve et al., 1986; Gottofrey and Tjalve, 1991; Persson et al., 2003), but whether this is also true for metal nanoparticles is questionable. Viswaprakash et al. (2009) stated that electrically neutral zinc nanoparticles can easily cross the olfactory membrane in rats. Also, 30–100 nm C_{60} aggregates have been proposed to enter the brain of largemouth bass (*Micropterus salmoides*) via the olfactory system (Oberdörster, 2004). Membrane diffusion of lipophilic nanoparticles cannot be excluded, but charged particles are unlikely to diffuse through the receptor membrane (Handy et al., 2008a). In contrast, endocytosis is a possible mechanism whereby nanoparticles may enter the olfactory system. Fifty nm silver-coated colloidal gold particles placed intranasally in squirrel monkey (*Saimiri sciureus*) have been detected in the olfactory bulbs (De Lorenzo, 1970). The particles are suggested to be taken up into 40–120 nm endocytotic vacuoles (De Lorenzo, 1970). Furthermore, silver has been detected in the olfactory bulb in Sprague–Dawley rats after inhalation of 1.98–64.9 nm silver nanoparticles (rats are obligatory nose breathers) (Ji et al., 2007) and after acute exposure to 14.6 nm elemental silver nanoparticles (Takenaka et al., 2001). Alternatively, depending on the charge of the nanoparticles, they may enter the olfactory receptor neurons when the cyclonucleotide gated channels are opening.

Fish are probably unable to detect nanosilver by olfaction. Viswaprakash et al. (2009) found that zinc nanoparticles did not produce odour responses in rats. The hyperpolarization of the olfactory epithelium during exposure to nanosilver is thus not a prolonged receptor response, but is hypothesized to be caused by accumulation of nanosilver on the surface on the olfactory rosette. It is difficult to measure the net surface charge of nanoparticles, but the zeta potential gives a voltage reflection of the effects of surface charge and flow dynamics near the particle surface (Handy et al., 2008b). In the present study, the zeta potential is negative and nanosilver accumulation may disrupt the voltage gradient across the epithelium by counterbalancing the positive charge outside the olfactory receptor cells.

Exposure to nanosilver may result in an increased olfactory mucus production. Pesticide exposure induced olfactory mucus production in salmon (Tierney et al., 2006), and silver nanoparticles exposure lead to gill mucus hypersecretion in perch (Bilberg et al., 2010) and zebrafish (*Danio rerio*) (personal communication). Olfactory mucus provides a protective layer over the receptor membrane (Klaprat et al., 1992). If the mucus layer increases in response to nanosilver exposure, the distance over which silver and odours will

have to diffuse to encounter receptor proteins on the olfactory neurons will increase correspondingly. Julliard et al. (1993) observed an increase in the number of goblet cells (mucus releasing cells) in the olfactory epithelium of trout after exposure to ionic copper. The number and size of goblet cells containing neutral mucins was also found to increase in rats exposed to silver nanoparticles in an inhalation chamber (Hyun et al., 2008). Both nanosilver accumulation and elevated mucus secretion can explain the unstable epithelium voltage and the suppressed and irregular EOG amplitudes found in fish exposed to nanosilver suspensions.

An inhibitory effect of ionic copper has been demonstrated on bile salt and amino acid olfactory receptor neurons in coho salmon (Baldwin et al., 2003). Similarly, the present study showed that amino acid, sex pheromone and bile salt olfactory receptor EOG responses were equally reduced in Crucian carp after exposure to 45 $\mu g\,L^{-1}$ nanosilver suspension. Consequently, most likely olfactory receptors associated with a variety of fish behaviours are impaired by nanosilver.

The consequences of the reduced capacity to smell are that fish may perform insufficient olfactory mediated behaviour such as foraging, reproduction and predator avoidance. For instance, social interactions has been affected in rainbow trout after cadmium exposure (Sloman et al., 2003), predator avoidance has been impaired in Coho salmon (Sandahl et al., 2007) and migration has been impaired in Atlantic salmon smolts after exposure to a pesticide (Moore et al., 2007).

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