Aquatic Toxicology 101 (2011) 31–37

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Differential expression of ribosomal protein gene, gonadotrophin releasing hormone gene and Balbiani ring protein gene in silver nanoparticles exposed *Chironomus riparius*

Prakash M. Gopalakrishnan Nair, Sun Young Park, Si-Won Lee, Jinhee Choi*

School of Environmental Engineering and Graduate School of Urban Sciences, University of Seoul, 90 Jeonnong-dong, Dongdaemun-gu, Seoul 130-743, Republic of Korea

ARTICLE INFO

Article history:
Received 29 April 2010
Received in revised form 18 August 2010
Accepted 26 August 2010

Keywords:
Chironomus riparius
Silver nanoparticles
Differential gene expression
Ecotoxicity
Genotoxicity

ABSTRACT

The eco- and genotoxicity of silver nanoparticles (AgNPs) was investigated in the fourth instar larvae of the aquatic midge, *Chironomus riparius*. AgNPs did not have acute toxicity in *C. riparius*, but did exhibited chronic toxicity on development (pupation and emergence failure) and reproduction. Genotoxicity also occurred in AgNPs exposed *C. riparius*. Differential Display PCR (DD-PCR), based on the Annealing Control Primer (ACP) technique, was conducted to investigate the underlying toxic mechanism, which identified altered gene expression in *C. riparius* after treatment with AgNPs. The possible toxicity mechanism of AgNPs in *C. riparius* involves the down regulation of the ribosomal protein gene (CrL15) affecting the ribosomal assembly and consequently, protein synthesis. Up regulation of the gonadotrophin releasing hormone gene (CrGnRH1) might lead to the activation of gonadotrophin releasing hormone mediated signal transduction pathways and reproductive failure. Up regulation of the Balbiani ring protein gene (CrBR2.2) may be an indication of the organism's protection mechanism against the AgNPs. The overall results suggest that the toxicity of AgNPs towards aquatic organisms should be thoroughly investigated to allow for their safe use, as they seem to exhibit important toxicity towards *C. riparius*.

1. Introduction

There has recently been great scientific concern about the possible adverse effects associated with manufactured nanomaterials because of the rapid development of commercialized nanoproducts (Perelshtein et al., 2008; Arora et al., 2008; Li et al., 2008; Chen and Schluesener, 2008). Of these nanomaterials, due to their effective antimicrobial property, silver nanoparticles (AgNPs) are extensively used in many commercial products, surgical instruments, water purification products and paints, as well as in various house hold items, and could end up in the environment during disposal (Maynard et al., 2006). The release of AgNPs into aquatic environments occurs through various means, and is becoming of great concern due to their high surface area and mobility (Benn and Westerhoff, 2008).

Despite the recent increase in research on the ecotoxicity of nanoparticles (NPs), limited investigations have been conducted on the toxicity of AgNPs on aquatic organisms (Griffitt et al., 2009; Asharani et al., 2008). Moreover, most of the ecotoxicity studies on aquatic organism have been conducted using organism level endpoints; such as mortality and growth, (Kahl et al., 1997; Watts and Pascoe, 1998; Crane et al., 2002), with only a few having been performed that include genotoxic endpoints. However, the presence of genotoxic and potentially carcinogenic compounds in aquatic environments is of major concern with respect to the health of aquatic media biota; therefore, the genotoxicity of NPs need to be identified before their widespread release to the aquatic environment.

In the present study, the eco- and genotoxicities of AgNPs were investigated on the aquatic larvae of nonbiting midges, (Chironomidae, Diptera) *Chironomus riparius*, the most abundant group of insects found in freshwater ecosystems, which hold an important position in the aquatic food chain (Cranston, 1995). Taking into account the ecological importance of *Chironomus* larvae in freshwater, studying the effects of AgNPs on *C. riparius* could contribute to a better understanding of the aquatic toxicity of AgNPs. Pupation and adult emergence were used as development descriptors, with the number of egg masses per treatment and number of eggs per egg mass as parameters for reproduction. Genotoxicity was investigated by measuring tail moments using the Comet assay.

Gene expression analysis has also been increasingly used in aquatic ecotoxicology, as it offers high sensitivity and mechanistic values in the diagnosis of environmental contamination (Ankley et al., 2006) and several studies have focused on the responses to chemical stressors at the molecular level in aquatic invertebrates

* Corresponding author. Tel.: +82 2 2210 5622; fax: +82 2 2244 2245.
E-mail address: jinhchoi@uos.ac.kr (J. Choi).

0166-445X/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquatox.2010.08.013

(Lee et al., 2006; Ha and Choi, 2009; Park et al., 2010). However, integrating gene expression profiling into an ecotoxicological study using organisms without sequenced genomes is a difficult and challenging task (Snell et al., 2003). Since genomic information on *C. riparius* is limited, the genes that are differentially expressed due to AgNPs toxicity need to be studied and sequenced to predict their possible impacts. Therefore, to gain an insight into the mechanisms of the eco- and genotoxicities of AgNPs in *C. riparius*, a differential display technique (Liang and Pardee, 1992) based on annealing control primers (ACPs) was used to identify Differentially Expressed Genes (DEGs) whose expressions were affected by AgNPs exposure. The toxicity mechanisms of AgNPs toward *C. riparius* are discussed on the basis of the experimental evidence obtained.

2. Materials and methods

2.1. Organism culture and exposure to AgNPs

Using an original strain provided by the Korea Institute of Toxicology (Daejeon, Korea), *C. riparius* larvae were obtained from adults reared in our laboratory, as described previously (Lee et al., 2008). Briefly, *C. riparius* larvae were reared in a 2 l glass chamber, containing dechlorinated tap water (800 ml) and acid washed sand (60 g), fed with fish flake food (Tetramin, Tetrawerke, Melle, Germany), with aeration under a 16–8 h light–dark photoperiod at $20 \pm 2\,^{\circ}C$.

AgNPs (size < 100 nm, Sigma–Aldrich Chemical, St. Louis, MO, USA) were homogenously dispersed in deionized water by sonication for 13 h (Branson-5210 sonicator, Branson Inc., Danbury, CT) and stirring for 7 d, and then filtered through a cellulose membrane (pore size 100 nm, Advantec, Toyo Toshi Kaisha, Japan) to remove nanoparticle aggregations. To determine the size and shape of the AgNPs, 20 μl of the particle suspension were dried onto a 400 mesh carbon-coated copper grid and imaged with a Transmission Electron Microscope (TEM), LIBRA 120 (Carl Zeiss, Oberkochen, Baden-Wrttemberg, Germany) at 80–120 kV. The size distribution of the AgNPs was evaluated using a Photal dynamic light scattering (DLS) spectrometer, DLS-7000 (Otsuka Electronics Co., Inc., Osaka, Japan).

2.2. Exposure analysis of silver in water and sediment

Exposure tanks containing dechlorinated water (800 ml) and sand (60 g) were spiked with 1 mg/l AgNPs and fifty-fourth instar larvae were added to each chamber. Water samples (10 ml) were taken from the middle of the water column after 15 min and different days (1, 2, 5, 20 and 25) of exposure and filtered through 0.2 μm filters, for analysis of dissolved silver in water. To check the total silver content in sediment, 1.5 g sand was collected after 15 min and 1, 2, 5, 20 and 25 d and digested with aqua-regia extraction procedure (ISO, 1995). All samples were frozen immediately after collection until analysis. The metal content in water and sediment was determined using Inductively Coupled Plasma-Mass Spectrometer (ICP-MS, Varian 820-MS, Palo Alto, CA, USA).

2.3. Acute and chronic ecotoxicity

For the acute toxicity test, groups of 10 larvae were exposed to four concentrations (0.5, 1, 2 and 4 mg/l) of AgNPs, with another group made the control. After 24 h of exposure, the number of individuals died was determined.

For the chronic ecotoxicity study, fifty-fourth instar larvae were introduced at the beginning of the experiment, and exposed to different concentrations (0, 0.2, 0.5 and 1 mg/l) of AgNPs until the end of the experiments (25 d). The emerging adults were retained with steel wire mesh until the emergence in the control and experimental aquaria were complete. The numbers of pupae and emerged adults from each vessel were counted and their sexes were determined. The two sexes were easily distinguished by the form and length of their antennae and abdominal terminalia. Additionally, the dead pupae were counted and the time to their complete emergence also investigated. For the reproduction parameters, the numbers of egg masses oviposited by the emerged adults and the numbers of eggs per egg mass in the control and AgNPs-treated vessels were counted. Every 2 d, 50 mg of Tetramin fish food flakes was supplied to each aquarium. The test solutions were not renewed. All the data were recorded at daily intervals.

2.4. Comet assay

An alkaline Comet assay was performed, as described previously (Park and Choi, 2007). Briefly, a total of 10 fourth instar larvae of *C. riparius* were collected 24 h after treatment with AgNPs (0, 0.2, 0.5 and 1 mg/l) from both experimental and control tanks, and pooled for a Comet assay. Treated organisms were placed in 1 ml of phosphate-buffered saline (PBS), containing 20 mM ethylene diamine tetra acetic acid (EDTA) and 10% dimethyl sulfoxide (DMSO), and disintegrated mechanically by mincing. The cell suspension was precipitated by vortexing, and then immediately mixed with 100 μl of 1% low-melting-point (LMP) agarose for use in the Comet assay. To prepare slides, 100 μl of 1% LMP agarose was spread onto a normal agarose pre-coated microscope slide and incubated at 4 °C for 5 min to allow for solidification. The cells were lysed in high salt and detergent (10 mM Tris, 100 mM EDTA, 2.5 M NaCl, 10% DMSO, 10% Triton X-100, pH 10), and subsequently exposed to alkaline conditions (300 mM NaOH, 1 mM EDTA, pH > 13) for 20 min at 4 °C to allow for DNA unwinding and expression of alkali-liable sites. For electrophoresis, an electric current of 300 mA (25 V) was applied for 20 min. After the electrophoresis, the slides were neutralized and dehydrated in 70% ethanol. The slides were stored in a dry place until the image analysis. Before analysis, the slides were stained with 50 μl ethidium bromide (5 μg/ml), then analyzed at 400× magnification using a fluorescence microscope, equipped with an excitation filter of BP 546/12 nm and barrier filter of 590 nm. Three slides were prepared per treatment and 50 cells per slide were examined. DNA damage was expressed as the tail moment (tail length × tail % DNA/100) using an image analysis computerized method (Komet 5.5, Kinetic Imaging Limited, Nottingham, UK).

2.5. Differentially expressed gene analysis

Based on the eco- and genotoxicity tests, fifty-fourth instar larvae were treated with 1 mg/l of AgNPs for 24 h to study the AgNPs induced DEGs. Total RNAs from treated and control larvae were isolated using Trizol (GibcoBRL), according to the manufacturer's protocol, and purified using an RNeasy mini kit (Qiagen, Valencia, CA, USA) suspended in 30 μl of RNase free water, was then quantified using a spectrophotometer (Thermospectronic, Rochester, NY, USA). Samples were stored at −80 °C until analyzed. Three replicates were maintained for each treatment.

GeneFishing™ DEG kits were used for screening DEGs, using the ACP based method, with 80 different arbitrary ACPs, as per the manufacturer's instructions (Seegene™, Seoul, South Korea). The DNA fragments produced by PCR were separated by electrophoresis on a 2% agarose gel and extracted from the agarose gel using the Geneclean® II kit (Q-BIOgene, Cambridge, UK). The DNA sequencing was performed by Macrogen (Seoul, South Korea). Sequence data were identified by comparing with the GenBank database through the BlastX program of the NCBI

(http://www.ncbi.nlm.nih.gov/BLAST/), with sequence alignments performed using the ClustalW program (Thompson et al., 1997).

Selected genes were reamplified, with the mRNA levels reconfirmed by quantitative real time PCR using cDNA made by reverse transcribing 1 μg of total RNA with oligo dT_{18} primer and RT-Premix (Bioneer, South Korea), in a 20 μl mix, following the manufacturer's instructions. The real time RT-PCR was performed using a SYBR supermix kit (Bio-Rad), initially running at 95 °C for 7 min followed by 44 cycles at 95 °C for 15 s and 55 °C for 1 min, with a final extension at 72 °C for 5 min, using a Chromo4 Real-Time PCR detection system (Bio-Rad) and gene specific primers and actin as the control (Supplementary Table 1, Primer list used for real-time PCR). The mRNA level of each gene was normalized to that of the actin mRNA, with the data shown as the mean ± SE of three replicates.

2.6. Data analysis

Statistical differences between the control and treated larvae were examined with the aid of a parametric *t*-test, using SPSS 12.0 KO (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Characterization of AgNPs in test media

Prior to the main experiment, the AgNPs employed in the *C. riparius* toxicity studies were characterized using TEM and DLS methods in the test medium. The TEM image showed that AgNPs existed as single particles and were homogeneously distributed in the test medium (Fig. 1A). The calculated size distribution histogram from DLS spectroscopy measurements confirmed that the sizes of the AgNPs in the test medium were mainly distributed in the range 40–70 nm (Fig. 1B).

3.2. Analysis of silver contents in water and sediment

The dissolution of AgNPs in water samples was very low and the maximum dissolution was noticed after 1 d (0.154 mg/l) and after that the amount of soluble silver decreased overtime (Fig. 2). Analysis of the sediment samples revealed that the rate of sedimentation of AgNPs increased overtime. After 15 min, 0.105 mg/kg of AgNPs were sedimented to the exposure medium and measurements at the end of the exposure showed 6.069 mg/kg of silver content in sediment (Fig. 2).

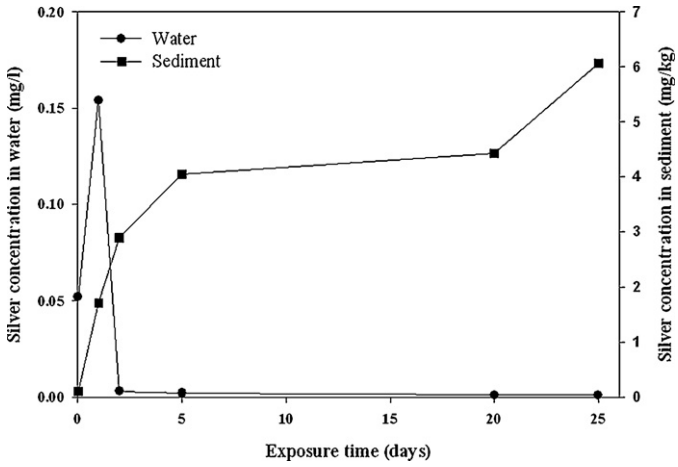

Fig. 2. Analysis of silver contents in water and in sediment using inductively coupled plasma-mass spectrometer.

3.3. Ecotoxicity of AgNPs exposure

To find an exposure concentrations range for the chronic test, an acute toxicity study was preliminary conducted using mortality as an endpoint. However, the 24 h median lethal concentration (LC_{50}) could not be estimated, because up to 2 mg/l of AgNPs caused no *C. riparius* larvae mortality. Therefore, a chronic ecotoxicity study was conducted at three concentrations: 0.2, 0.5 and 1 mg/l, where no short-term mortality was observed.

The chronic ecotoxicity of AgNPs towards *C. riparius* was investigated using pupation, adult emergence and reproduction as the endpoints. Pupation and adult emergence were significantly affected at all three concentrations. The rates of decreased pupation and adult emergence were about 64 and 50% with exposure to 1 mg/l AgNPs in *C. riparius* compared to control groups, respectively (Fig. 3A). Moreover, among the emerged adults, the average number of males was less than that of females, showing an alteration in the sex ratio relative to the control groups (Fig. 3B). Reproduction was

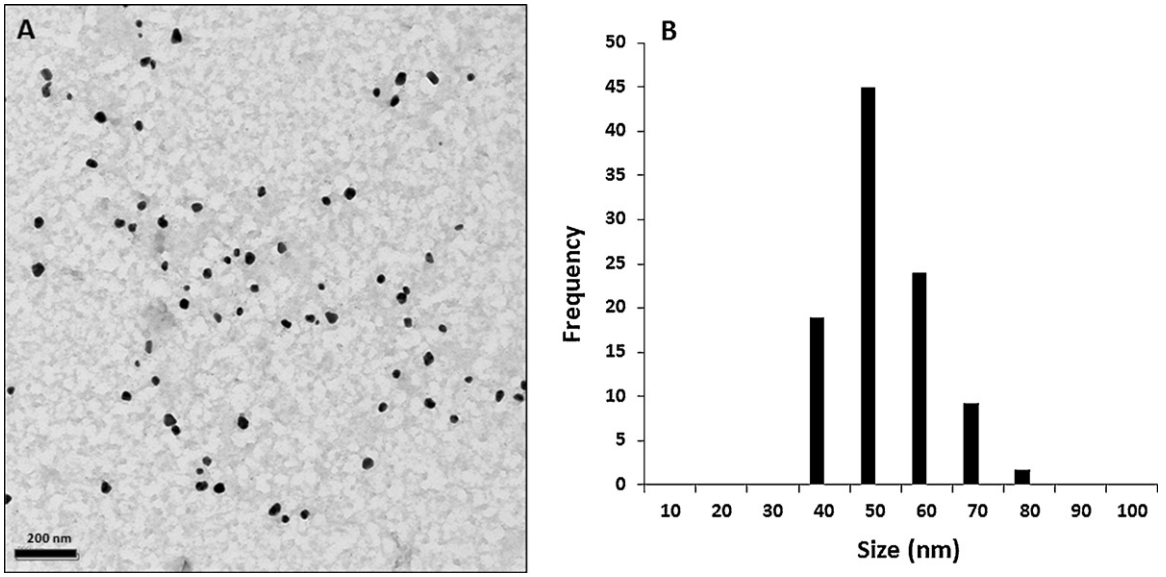

Fig. 1. Characterization of AgNPs in test media using (A) transmission electron microscopy and (B) dynamic light scattering spectrometer.

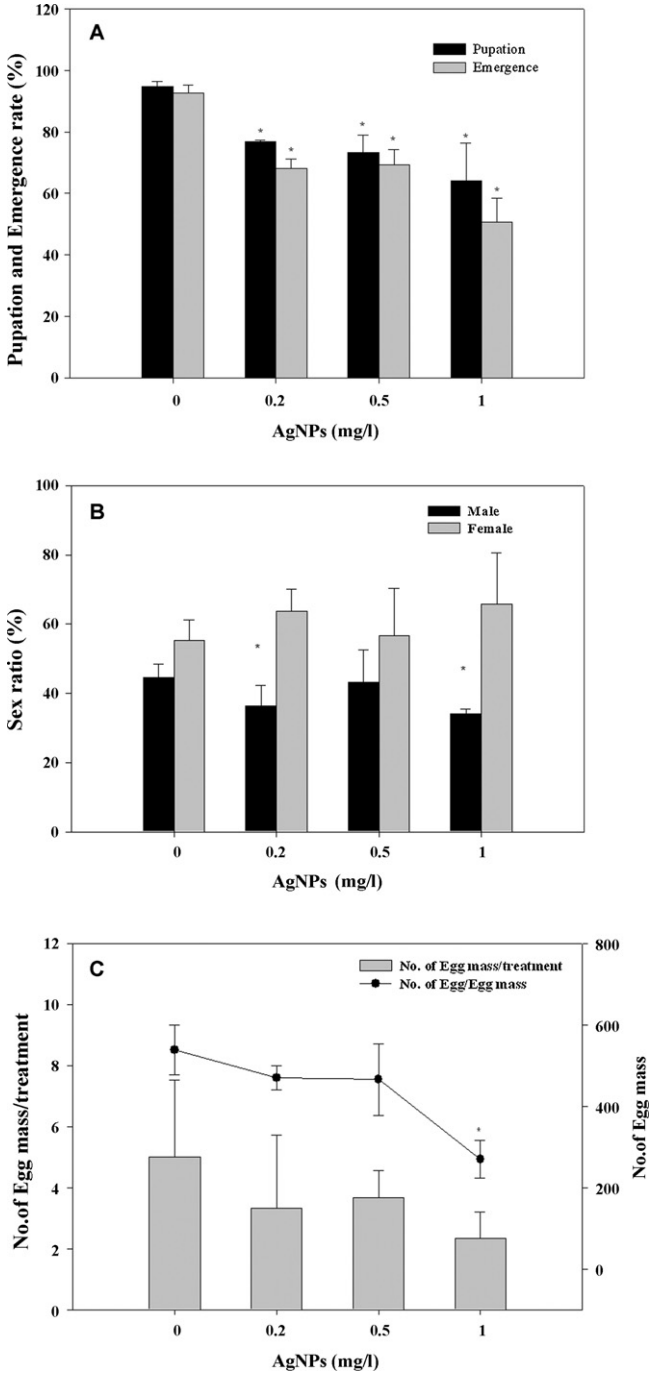

Fig. 3. Development and reproduction parameters investigated in silver nanoparticles (AgNPs) exposed 4th instar larvae of *C. riparius*: (A) pupation and emergence rate (%) (B) emergence of male and female adults ratio (%) and (C) total number of eggs and number of eggs per egg mass ($n = 3$, mean ± SE, $^*p < 0.05$).

studied by counting the number of egg masses oviposited by the emerged adults and the number of eggs per egg mass. The reproduction potential also seemed to be affected by AgNPs exposure, as the number of eggs per egg mass was significantly decreased on exposure to 1 mg/l of AgNPs (Fig. 3C).

3.4. Genotoxicity of AgNPs exposure

Genotoxicity was investigated in AgNPs exposed *C. riparius* using the Comet assay, and a dose dependent increase in DNA damage was observed after treatment of *C. riparius* with AgNPs

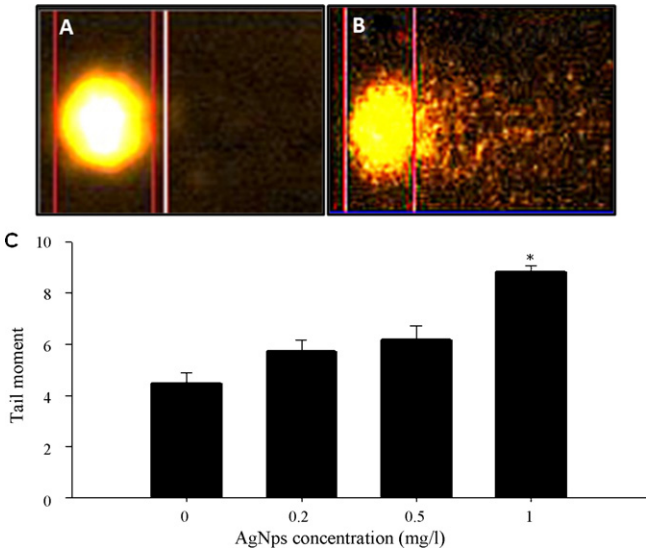

Fig. 4. Comet analysis: (A) untreated and (B) AgNPs treated *C. riparius* stained by ethidium bromide (5 μg/ml). (C) The tail moments of DNA (μm). The results were expressed as tail moment obtained by comet assay ($n = 3$, mean ± SE). Asterisks (*) indicate statistically significant differences between treatments and the corresponding control group, $p < 0.05$.

(Fig. 4A–C). The increase in the tail moments due to DNA damage after AgNPs treatment indicated the extent of DNA damage compared to cells from the control, which was statistically significant on larvae treated with 1 mg/l of AgNPs.

3.5. Differentially expressed genes (DEGs) by AgNPs exposure

As exposure to 1 mg/l of AgNPs resulted in serious ecotoxicity (alteration on development and on reproduction) and genotoxicity towards *Chironomus*, differentially expressed genes were investigated in *C. riparius* exposed to 1 mg/l of AgNPs to gain an insight of the underlying mechanism of the observed toxicity. An ACP RT-PCR analysis was conducted, using 80 arbitrary primers and two anchored oligo (dT) primers, dT-ACP1 and dT-ACP2, as per the manufacturer's instructions. The differentially expressed genes were marked in the gels as both up-regulated bands (bands more intense in treated material compared to the control) and as down-regulated bands (bands more intense in the control material compared to the treated) (Supplementary Fig. 1). For convenience, a specific name was given to each selected DEG amplicon viz. CrACP7, CrACP17, CrCP18, CrACP23 and CrACP40 for up-regulated and CrACP2 and CrACP8 for two down-regulated. The first two letters in the name indicate the organisms: Cr – *C. riparius,* and the letters or numbers that follows the first two letters describes the primer and number used. The eluted bands were sequenced directly, with a BlastX search performed for sequence similarity. Six DEGs exhibited significantly higher sequence similarity with the coding regions of known genes (Supplementary Table 2). Multiple sequence alignment was conducted on three selective sequences with corresponding genes from the NCBI data base (Supplementary Figs. 1–4), which showed that *C. riparius* genes possessed well conserved residues. Consequently, CrACP17, CrACP18 and CrACP8 were named as the CrGnRH1, CrBR2.2 and CrL15 genes, respectively. The expression levels of the two up-regulated genes coding for the gonadotropin releasing hormone gene (CrGnRH1) and Balbiani repeat gene 2.2 (CrBR2.2), and the one down-regulated gene, the Ribosomal protein gene L15 (CrL15), were reconfirmed with real-time PCR (Fig. 5). All genes identified in the present study were submitted to GenBank and assigned the following accession

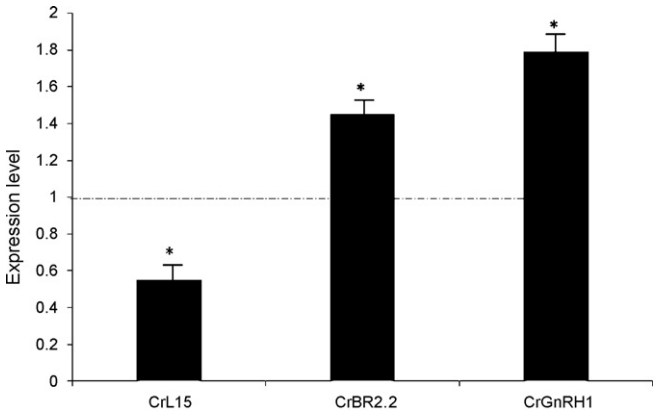

Fig. 5. Real Time PCR confirmation of selected differentially expressed genes by AgNPs in *C. riparius* after exposing to 1 mg/l AgNPs for 24 h. Densitometric values of CrL15, CrBR2.2 and CrGnRH1 gene expression were normalized using Actin mRNA. Asterisks (*) indicate statistically significant differences between treatments and the corresponding control group, $p < 0.05$ ($n = 3$, mean ± SE).

numbers GW836480 (CrACP2), GW836481 (CrACP7), GW836482 (CrACP8), GW836483 (CrACP17), GW836486 (CrCP18), GW836484 (CrACP23), and GW836485 (CrACP40C).

4. Discussion

In this study, the eco-and genotoxicities of widely used NPs, AgNPs, were investigated on the aquatic organism, *C. riparius*, with the AgNPs-induced gene expression also studied to find a potential mechanism of AgNPs exposure toxicity in *C. riparius*. Our exposure characterization study revealed that the dissolution of AgNPs in water is very slow. Elzey and Grassian (2010) also observed high stability and low dissolution rate (0.5%) of silver nanoparticles in water. In a previous report, Griffitt et al. (2008) also reported that the dissolution of silver nanoparticles is very slow; however they also reported the sedimentation of silver nanoparticles out of the water column. Based on previous reports the dissolution of silver nanoparticle does not seem to be increasing the toxicity caused by silver nanoparticles. Griffitt et al. (2008), found a negative correlation between the dissolution and toxicity of nanosilver in zebrafish and *D. pulex*. Asharani et al. (2008) also reported that silver nanoparticles were more toxic than soluble silver in their experiments with zebrafish.

The constantly changing exposure parameters that differ between test systems make it difficult to standardize the exposure parameters accurately (Griffitt et al., 2008). It has been reported that 50–90% of the initial mass of nanometal added to an exposure were lost through sedimentation from the water column (Griffitt et al., 2009). In our experiment also we have found that the silver nanoparticles were sedimented out of the water column. In this study, the exposure medium contained both water and sand and the presence of silver was detected both in water and sand showing that the *C. riparius* were constantly exposed to AgNPs throughout the developmental period.

The results of a 24 h lethality test suggest that AgNPs have low acute toxic potential to this species; however, the long-lasting environmental impact of AgNPs needs to be investigated, as AgNPs are likely to be continuously introduced to the aquatic environment, because of its use in several products (Perelshtein et al., 2008; Arora et al., 2008; Li et al., 2008; Chen and Schluesener, 2008; Benn and Westerhoff, 2008). Indeed, chronic exposure of AgNPs leads to alterations in the development and reproduction of *C. riparius*, such as failure in pupation and emergence (Fig. 3A). The decreases in the pupation and emergence rates, indicators of animal development at the highest AgNPs concentration, suggest that the alteration of these parameters may be considered as a consequence of a serious progression of the toxic effect.

One of the most notable results of the present ecotoxicity study was AgNPs-induced disturbance of the male/female adult ratio (Fig. 3B). Changes in the sex ratio due to chemical exposure has been reported by many groups (Egami, 1954; Gimeno et al., 1997; Galbreath et al., 2003; Parrott et al., 2003), and has been used by our group as an indicator for chemical toxicity among emerged adults in *C. riparius* exposed to organic compounds, such as nonylphenol (Lee and Choi, 2006) and octachlorostyrene (Lee et al., 2008). Although the broad environmental relevance of the AgNPs induced disruption of the neuroendocrine function on the reproductive health of wildlife is uncertain, it is widely known that chemical-induced sex ratio alteration might be due to its toxic potential to disturb hormonal processes (Klein et al., 1994; Bogart, 1987; De Loof and Huybrechts, 1998; Hahn et al., 2001; Eldridge et al., 2008). To further understand this phenomenon, more experimental evidence, such as the results of a hormone analysis, will be needed. A significant disturbance of the male/female adult ratio due to AgNPs exposure suggests that AgNPs may have serious consequences in *Chironomus* reproduction at the population level. Indeed, reduced numbers of eggs in egg masses upon AgNPs exposure was observed (Fig. 3C), which may provide experimental evidences to support this hypothesis.

Genotoxicity is considered one of the most important toxic endpoints in most chemical toxicity testing and risk assessment; however, little is known about the genotoxicity of NPs, especially towards aquatic organisms. The results of the Comet assay suggested that AgNPs may provoke genotoxicity in *C. riparius* (Fig. 4). Previously, the geno and ecotoxicities of CeO_2, SiO_2 and TiO_2 NPs on the aquatic sentinel species, *Daphnia* and *Chironomus*, were investigated, and CeO_2 NPs found to be potentially genotoxic to these aquatic organisms (Lee et al., 2009); AgNPs were also found to provoke genotoxicity toward *Daphnia magna* (Park and Choi, 2010). However, it is unclear how the genotoxicity due to NPs occurs in those organisms, but it is probable they might have been deposited inside the cell, where they interact with DNA and lead to DNA damage. The present data, as well as data from our previous genotoxicity studies, may contribute to the knowledge of the nanoecotoxicity on aquatic ecosystems, which suggest that the potential genotoxic effects of emerging nanomaterials on aquatic systems should be thoroughly identified to allow for their safe use.

To identify a plausible mechanism for the observed alterations in development and reproduction, as well as the genotoxicity, an AgNPs-induced gene expression analysis was conducted in *C. riparius*, as the expression of certain genes may be linked to short-term toxicological responses that impact on individual fitness. The basic premise that changes in gene expression can be harnessed to diagnose exposure to and the effects of environmental chemicals is currently receiving significant attention. Several genes of ecotoxicological relevance have been isolated from many aquatic species, such as the identification of genes induced by pollution in *Mya arenaria* (Rhodes and Van Beneden, 1996), *Donax trunculus* (Sultan et al., 2000) and the isolation of vitellogenin and estrogen receptor genes in *Cyprinodon variegatus* (Denslow et al., 2001a, b). The altered gene expressions can be used to sensitively diagnose the effects of stressors in aquatic organisms (Sultan et al., 2000; Snell et al., 2003; Poynton et al., 2007). Recent sequencing of the entire genome in environmentally relevant aquatic organisms (i.e., *Daphnia pulex*) has heightened the use of gene expression as indicators in ecotoxicity testing. However, despite its ecotoxicological importance, the genome of *C. riparius* has not been intensively investigated. Therefore, most ecotoxicity studies using *C. riparius* have focused on organism/population and biochemical level endpoints, and only small numbers of specific biomarker genes, such

as heat shock proteins or hemoglobin genes, have been studied as ecotoxicity indicators (Perkins et al., 2004; Lee et al., 2006, 2008). Several techniques have been designed to identify DEGs in cells or tissues under various experimental conditions, including differential display PCR (DD-PCR) (Liang and Pardee, 1992). DD-PCR and its modifications have been successfully used to isolate a number of differentially expressed genes from animals, whose genomes have not been sequenced (Snell et al., 2003).

In this study, a differential display technique based on ACPs was used to identify AgNPs-induced DEGs. Seven genes whose mRNA levels were altered were identified using an ACP-based PCR system, with the effect of AgNPs discussed based on the three most significant DEGs, CrL15, CrBR2.2 and CrGnRH1. Over 80 ribosomal protein genes have been identified in eukaryotes, and have various secondary functions apart from protein synthesis, such as regulation of development and malignant transformation (Wool, 1996; Chen and Ioannou, 1999). Mutations in ribosomal protein genes have identified genes that encode ribosomal proteins as cancer genes in *Danio rerio* (Amsterdam et al., 2004). The ribosomal protein L15 plays a dominant role in the total reconstitution of active 50S subunits from *E. coli* ribosomes, causing the reconstitution of an active peptidyltransferase center, with the incorporation of 5S rRNA into the 50S particles (Franceschi and Nierhaus, 1990; Giorginis and Chen, 1977). Ribosomal protein gene L15 is differentially expressed in mammalian cancer cells (Chen and Ioannou, 1999). It has also been reported that the human ribosomal protein gene L15 is over expressed in some esophageal tumors compared to normal matched tissue and is also associated with cell proliferation in gastric cancer (Wang et al., 2006). In a previous report, a proteomic analysis of *E. coli* treated with Ag ions showed a reduction in the expression of the ribosomal subunit gene S2, which impaired the synthesis of other proteins (Yamanaka et al., 2005). In our study, the expression level of the ribosomal protein gene CrL15 was also found to be down-regulated as a result of AgNPs exposure, which in turn may affect the ribosome assembly and synthesis of other proteins. An anti-proliferative effect of AgNPs was also found from our cytotoxicity study with AgNPs in human jurkat cell lines, with the anti-proliferative activity of AgNPs in cancer cells assumed be due to its toxic effect on the ribosomal protein gene L15, which in turn affects the ribosomal assembly and protein synthesis.

The differential regulation of GnRH gene expression is a crucial regulatory mechanism in reproductive physiology, and is involved in the neuropeptide pathways of signal transduction to environmental stimuli and growth-factor-induced mitogenic signal transduction (Sundaresan et al., 1996). A previous study suggested that a variety of environmental chemicals, such as heavy metals and pesticides, interfere with the neuroendocrine pathways controlling reproduction in mammals (Uphouse, 1985). The mechanism by which AgNPs modulate the level of GnRH mRNA is not well understood, but the mechanism could be partially explained based on previous studies with other heavy metals. In juvenile *Oncorhynchus mykiss*, cadmium exposure for 48 h increased two isoforms of GnRH (sGnRH 1 and sGnRH 2) mRNA expression in a dose-dependent manner (Vetillard and Bailhache, 2005). In female *Carassius gibelio*, there was a significant elevation of plasma luteinizing hormone (Lh) with high doses of dietary Cd (Szczerbik et al., 2006) and in pituitaries treated with Cd in vitro; aquatic exposure showed an increase in Lh release in *Micropogonias undulates* (Thomas, 1993). In yet another study, the gonadotrophin release in rats was differentially modulated in a dose-dependent manner on Cd exposure (Lafuente et al., 2003). In *Oryzias latipes*, Cd exposure affected sex steroid production, and changes in GnRH mRNA may be due to alterations in circulating steroid hormones (Tilton et al., 2003). Lead induced impairment of reproduction in male rats has been shown to involve an increase in the levels of GnRH mRNA (Klein et al., 1994). Additional studies are needed to fully explore the relationship between GnRH synthesis and regulation induced by AgNPs. GnRH has also been shown to exert a direct anti-proliferative effect on ovarian cancer cells (Choi et al., 2001; Gründker and Emons, 2003). Genes coding for gonadotropin releasing hormones have been reported for many species, but to our knowledge, no such genes have been identified in *C. riparius*. In the present study, the CrGnRH1 gene was found to be up-regulated on exposure to AgNPs. This identified the CrGnRH1, with significant homology to human GnRH1, for the first time, which is very important for the study of molecular endocrinology of reproduction in *C. riparius*. Based on our results, AgNPs were assumed to act on GnRH signal transduction pathways controlling many cellular functions, and the increased expression of this gene may be involved in the altered reproduction potential after treatment with AgNPs (Fig. 3C).

The expression of the BR gene family undergoes correlated changes depending on the environment. The expression of Balbiani ring genes in *Chironomus* offers unique possibilities to visualize the assembly and nucleocytoplasmic transport of a specific transcription product, and for the study of gene expression in situ (Bauren and Wieslander, 1994; Daneholt, 2001; Kiesler et al., 2002). Moreover, BR genes in polytene chromosomes of *Chironomus* salivary glands code for secretary proteins (Rydlander and Edström, 1980) used to create the larval tube for the feeding and protection of aquatic larva. In this study, the up-regulation of the CrBR2.2 gene upon treatment with AgNPs may be due to its protective role towards the adverse effects of AgNPs in *C. riparius*, which may be useful as a molecular indicator for AgNPs toxicity.

5. Conclusion

In conclusion, it was found that exposure to AgNPs could cause developmental and reproductive failure, as well as genotoxicity in *C. riparius*. The possible mechanism of AgNPs toxicity towards *C. riparius*, involving the down regulation of the CrL15 gene affecting the ribosomal assembly and; in turn, protein synthesis, and the up-regulation of the CrGnRH1 gene leading to GnRH mediated signal transduction pathways, causing developmental and reproductive failure, and the up regulation of the CrBR2.2 gene may be an indication of the organisms protection mechanisms against AgNPs.

Acknowledgement

This work was supported by Mid-career Researcher Program through the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (No.2009-0084685) and the International Research & Development Program of the National Research Foundation of Korea(NRF) funded by the Ministry of Education, Science and Technology(MEST) of Korea (Grant number: K20912000002-09B1300-00210).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2010.08.013.

References

Amsterdam, A., Sadler, K.C., Lai, K., Farrington, S., Bronson, R.T., Lees, J.A., Hopkins, N., 2004. Many ribosomal protein genes are cancer genes in zebrafish. PLoS Biol. 2, 690–698.

Ankley, G.T., Daston, G.P., Degitz, S.J., Denslow, N.D., Hoke, R.A., Kennedy, S.W., Miracle, A.L., Perkins, E.J., Snape, J., Tillitt, D.E., Tyler, C.R., Versteeg, D., 2006. Toxicogenomics in regulatory ecotoxicology. Environ. Sci. Technol. 40, 4055–4065.

Arora, S., Jain, J., Rajwade, J.M., Paknikar, K.M., 2008. Cellular responses induced by silver nanoparticles: in vitro studies. Toxicol. Lett. 179, 93–100.

Asharani, P.V., Wu, Y.L., Gong, Z., Valliyaveettil, S., 2008. Toxicity of silver nanoparticles in zebrafish models. Nanotechnology 19, 255102–255109.

Bauren, G., Wieslander, L., 1994. Splicing of Balbiani ring 1 gene pre-mRNA occurs simultaneously with transcription. Cell 14, 183–192.

Benn, T.M., Westerhoff, P., 2008. Nanoparticle silver released into water from commercially available sock fabrics. Environ. Sci. Technol. 42, 4133–4139.
Bogart, M.H., 1987. Sex determination: a hypothesis based on steroid ratios. J. Theor. Biol. 128, 349–357.
Chen, F.W., Ioannou, Y.A., 1999. Ribosomal proteins in cell proliferation and apoptosis. Int. Rev. Immunol. 18, 429–448.
Chen, X., Schluesener, H.J., 2008. Nanosilver: a nanoproduct in medical application. Toxicol. Lett. 176, 1–12.
Choi, K.C., Auersperg, N., Leung, P.C., 2001. Expression and antiproliferative effect of a second form of gonadotropin-releasing hormone in normal and neoplastic ovarian surface epithelial cells. J. Clin. Endocrinol. Metab. 86, 5075–5078.
Crane, M., Sildanchandra, W., Kheir, R., Callaghan, A., 2002. Relationship between biomarker activity and developmental endpoints in *Chironomus riparius* Meigen exposed to an organophosphate insecticide. Ecotoxicol. Environ. Safe 53, 361–369.
Cranston, P.S., 1995. The Chironomidae—The Biology and Ecology of Non-bitting Midges. Chapman & Hall, London, UK.
Daneholt, B., 2001. Assembly and transport of a pre-messenger RNP particle. Proc. Natl. Acad. Sci. U.S.A. 98, 7012–7017.
De Loof, A., Huybrechts, R., 1998. Insects do not have sex hormone: a myth? Gen. Comp. Endocrinol. 111, 245–260.
Denslow, N.D., Bowman, C.J., Ferguson, R.J., Lee, H.S., Hemmer, M.J., Folmar, L.C., 2001a. Induction of gene expression in sheepshead minnows (*Cyprinodon variegatus*) treated with 17B-estradiol, diethylstilbestrol, or ethinylestradiol: the use of mRNA fingerprints as an indicator of gene regulation. Gen. Comp. Endocrinol. 121, 250–260.
Denslow, N.D., Lee, H.S., Bowman, C.J., Hemmer, M.J., Folmar, L.C., 2001b. Multiple responses in gene expression in fish treated with estrogen. Comp. Biochem. Physiol. 129, 277–282.
Egami, N., 1954. Appearance of the male character in the regenerating and transplanted rays of the fish, *Oryzias latipes*, following treatment with methyldihydrotesterone. J. Fac. Sci. 7, 271–280.
Eldridge, J.C., Stevens, J.T., Breckenridge, C.B., 2008. Atrazine interaction with estrogen expression systems. Rev. Environ. Contam. Toxicol. 196, 147–160.
Elzey, S., Grassian, V.H., 2010. Agglomeration, isolation and dissolution of commercially manufactured silver nanoparticles in aqueous environments. J. Naopart. Res. 12, 1945–1958.
Franceschi, F.J., Nierhaus, K.H., 1990. Ribosomal proteins L15 and L16 are mere late assembly proteins of the large ribosomal subunit. Analysis of an *E. coli* mutant lacking L15. J. Biol. Chem. 265, 16676–16682.
Galbreath, P.F., Adams, N.D., Sherrill III, L.W., 2003. Successful sex reversal of brook trout with 17 alpha methyl dihydrotestosterone treatments. North Am. J. Aquacult. 65, 235–239.
Gimeno, S., Komen, H., Venderbrosch, P.W.M., Bowmer, T., 1997. Disruption of sexual differentiation in genetic male common carp (*Cyprinus carpio*) exposed to an alkylphenol during different life stages. Environ. Sci. Toxicol. 31, 2884–2890.
Giorginis, S., Chen, R., 1977. The primary structure of protein L15 located at the peptidyl-transferase center of *Escherichia coli* ribosomes. FEBS Lett. 84, 347–350.
Griffitt, R.J., Luo, J., Gao, J., Bonzongo, J.C., Barber, D.S., 2008. Effects of particle composition and species on toxicity of metallic nanomaterials in aquatic organisms. Environ. Toxicol. Chem. 27, 1972–1978.
Griffitt, R.J., Hyndman, K., Denslow, N.D., Barber, D.S., 2009. Comparison of molecular and histological changes in zebrafish gills exposed to metallic nanoparticles. Toxicol. Sci. 107, 404–415.
Gründker, C., Emons, G., 2003. Role of gonadotropin-releasing hormone (GnRH) in ovarian cancer. Reprod. Biol. Endocrinol. 1, 65–72.
Ha, M.H., Choi, J.H., 2009. Effects of environmental contaminants on hemoglobin gene expression in *Daphnia magna*: a potential biomarker for freshwater quality monitoring. Arch. Environ. Contam. Toxicol. 57, 330–337.
Hahn, T., Liess, M., Schulz, R., 2001. Effects of the hormone mimetic insecticide tebufenozide on *Chironomus riparius* larvae in two different exposure setups. Ecotoxicol. Environ. Safe 49, 171–178.
ISO standard 11466, 1995. ISO (International Organization for Standardization) 11466, Soil quality, Extraction of Trace Elements Soluble in Aqua Regia.
Kahl, M.D., Makynen, E.A., Kosian, P.A., Ankley, G.T., 1997. Toxicity of 4-nonylphenol in a life-cycle test with the midge *Chironomus tentans*. Ecotoxicol. Environ. Safe 38, 155–160.
Kiesler, E., Miralles, F., Visa, N., 2002. HEL/UAP56 binds co transcriptionally to the Balbiani ring pre-mRNA in an intron independent manner and accompanies the BR mRNP to the nuclear pore. Curr. Biol. 12, 859–862.
Klein, D., Wan, Y.Y., Kamyab, S., Okuda, H., Sokol, R.Z., 1994. Effects of toxic levels of lead on gene regulation in the male axis: increase in messenger ribonucleic acid and intracellular stores of gonadotrophs within the central nervous system. Biol. Reprod. 50, 802–811.
Lafuente, A., Cano, P., Esquifino, A., 2003. Are cadmium effects on plasma gonadotropins, prolactin ACTH, GH and TSH levels, dose-dependent? Biometals 16, 243–250.
Lee, S.B., Choi, J.J., 2006. Multilevel evaluation of nonylphenol toxicity in fourth instar larvae of *Chironomus riparius* (Diptera Chironomidae). Environ. Toxicol. Chem. 25, 3006–3014.
Lee, S.M., Lee, S.B., Park, C.H., Choi, J., 2006. Expression of heat shock protein and hemoglobin genes in *Chironomus tentans* (Diptera, Chironomidae) larvae exposed to various environmental pollutants: a potential biomarker of freshwater monitoring. Chemosphere 65, 1074–1081.
Lee, S.W., Kim, S.M., Choi, J., 2009. Genotoxicity and ecotoxicity assays using the freshwater crustacean *Daphnia magna* and the larva of the aquatic midge *Chironomus riparius* to screen the ecological risks of nanoparticle exposure. Environ. Toxicol. Pharmacol. 28, 86–91.
Lee, S.W., Park, K., Hong, J., Choi, J., 2008. Ecotoxicological evaluation of octachlorostyrene in fourth instar larvae of *Chironomus riparius* (Diptera Chironomidae). Environ. Toxicol. Chem. 27, 1118–1127.
Li, Q., Mahendra, S., Lyon, D.Y., Brunet, L., Liga, M.V., Li, D., Alvarez, P.J.J., 2008. Antimicrobial nanomaterials for water disinfection and microbial control: potential applications and implications. Water Res. 42, 4591–4602.
Liang, P., Pardee, A.B., 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 257, 967–971.
Maynard, A.D., Aitken, R.J., Butz, T., Colvin, V., Donaldson, K., Oberdorster, G., Philbert, M., Ryan, J., Seaton, A., Stone, V., Tinkle, S.S., Tran, L., Walker, N.J., Warheit, D.B., 2006. Safe handling of nanotechnology. Nature 444, 267–269.
Park, K., Park, J., Kim, J., Kwak, I.S., 2010. Biological and molecular responses of *Chironomus riparius* (Diptera Chironomidae) to herbicide 2, 4-D (2,4-dichlorophenoxyacetic acid). Comp. Biochem. Physiol. Part C: Toxicol. Pharmacol. 151, 439–446.
Park, S.Y., Choi, J., 2007. Cytotoxicity, genotoxicity and ecotoxicity assay using human cell and environmental species for the screening of the risk from pollutant exposure. Environ. Int. 33, 817–822.
Park, S.Y., Choi, J., 2010. Geno- and ecotoxicity evaluation of silver nanoparticles in freshwater crustacean *Daphnia magna*. Environ. Eng. Res. 15, 23–27.
Parrott, J.L., Wood, C.S., Boutot, P., Dunn, S., 2003. Changes in growth and secondary sex characteristics of fathead minnows exposed to bleached sulfite mill effluent. Enivron. Toxicol. Chem. 22, 2908–2915.
Perelshtein, I., Applerot, G., Perkas, N., Guibert, G., Mikhailov, S., Gedanken, A., 2008. Sonochemical coating of silver nanoparticles on textile fabrics (nylon, polyester and cotton) and their antibacterial activity. Nanotechnology 19, 245705-1–1245705-6.
Perkins, E.J., Furey, J., Davis, E., 2004. The potential of screening for agents of toxicity using gene expression fingerprinting in *Chironomus tentans*. Aquat. Ecosys. Health Manage. 7, 399–405.
Poynton, H.C., Varshavsky, J.R., Chang, B., Cavigiolio, G., Chan, S., Holman, P.S., Loguinov, A.V., Bauer, D.J., Komachi, K., Theil, J.E., Perkins, E.J., Hughes, O., Vulpe, C.D., 2007. *Daphnia magna* ecotoxicogenomics provides mechanistic insights into metal toxicity. Environ. Sci. Technol. 413, 1044–1050.
Rhodes, L.E., Van Beneden, R.J., 1996. Gene expression analysis in aquatic animals using differential display polymerase chain reaction. In: Ostrander, G.K. (Ed.), Techniques in Aquatic Toxicology. CRC Lewis Publishers, Boca Raton, FL, pp. 161–183.
Rydlander, L., Edström, J.E., 1980. Large sized nascent protein as dominating component during protein synthesis in *Chironomus* salivary glands. Chromosoma 81, 85–99.
Snell, T.W., Brogdon, S.E., Morgan, M.B., 2003. Gene expression profiling in ecotoxicology. Ecotoxicology 12, 475–483.
Sultan, A., Abelson, A., Bresler, V., Fishelson, L., Mokady, O., 2000. Biomonitoring marine environmental quality at the level of gene expression-testing the feasibility of a new approach. Water Sci. Tech. 42, 269–274.
Sundaresan, S., Colin, I.M., Pestell, R.G., Jameson, J.L., 1996. Stimulation of mitogen-activated protein kinase by gonadotropin-releasing hormone: evidence for the involvement of protein kinase C. Endocrinology 137, 304–311.
Szczerbik, P., Mikołajczyk, T., Sokołowska-Mikołajczyk, M., Socha, M., Chyb, J., Epler, P., 2006. Influence of long-term exposure to dietary cadmium on growth, maturation and reproduction of goldfish (subspecies: Prussian carp *Carassius auratus* gibelio B.). Aquat. Toxicol. 77, 126–135.
Thomas, P., 1993. Effects of cadmium on gonadotropin secretion from Atlantic croaker pituitaries incubated in vitro. Mar. Environ. Res. 35, 141–145.
Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL-X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl. Acids Res. 25, 4876–4882.
Tilton, S.C., Foran, C.M., Benson, W.H., 2003. Effects of cadmium on the reproductive axis of Japanese medaka (*Oryzias latipes*). Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 136, 265–276.
Uphouse, L., 1985. Effects of chlordecone on neuroendocrine function of female rats. Neurotoxicology 6, 191–210.
Vetillard, A., Bailhache, T., 2005. Cadmium: an endocrine disrupter that affects gene expression in the liver and brain of juvenile rainbow trout. Biol. Reprod. 72, 119–126.
Wang, H., Zhao, L.N., Li, K.Z., Ling, R., Li, X.J., Wang, L., 2006. Overexpression of ribosomal protein gene L15 is associated with cell proliferation in gastric cancer. BMC Cancer 6, 91–99.
Watts, M.M., Pascoe, D., 1998. Selection of an appropriate life cycle stage of *Chironomus riparius* meigen for use in chronic sediment toxicity testing. Chemosphere 36, 1405–1413.
Wool, I.G., 1996. Extraribosomal functions of ribosomal proteins. Trends Biochem. Sci. 21, 164–165.
Yamanaka, M., Hara, K., Kudo, J., 2005. Bactericidal actions of a silver ion solution on *Escherichia coli*, studied by energy-filtering transmission electron microscopy and proteomic analysis. Appl. Environ. Microbiol. 71, 7589–7593.