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# Endocrine and cellular stress effects of zinc oxide nanoparticles and nifedipine in marsh frogs *Pelophylax ridibundus*

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ABSTRACT

Freshwater organisms including amphibians experience increasing exposures to emerging pollutants such as nanoparticles and pharmaceuticals, which can affect their fitness and performance. We studied the effects of two common pollutants extensively used in industry, pharmaceutical and personal care products, nano-zinc oxide (nZnO) and a Ca-channel blocker nifedipine (Nfd), on endocrine status and cellular stress markers of the marsh frog *Pelophylax ridibundus*. Males were exposed for 14 days to nZnO (3.1 μM), $Zn^{2+}$ (3.1 μM, as a positive control for nZnO exposures), Nfd (10 μM), and combination of nZnO and Nfd (nZnO + Nfd). Exposure to nZnO and $Zn^{2+}$ led to an increase in Zn burdens, elevated concentrations of the metal-bound metallothioneins (MT-Me) in the liver and increased vitellogenin in the serum, whereas exposures to Nfd and nZnO + Nfd resulted in the metal release from MTs and a significant increase in the ratio of total to metal-bound MTs. This likely reflects oxidative stress caused by Nfd exposures as manifested in the elevated levels of oxyradical production, upregulation of superoxide dismutase activity (SOD) and increase in the total and oxidized glutathione concentrations in Nfd-exposed frogs. Zn-containing exposures upregulated activity of deiodinase (in nZnO and nZnO + Nfd exposures) and serum thyrotropin level (in the case of $Zn^{2+}$). All exposures caused an increase in DNA fragmentation, lipofuscin accumulation as well as upregulation of caspase-3 and CYP450 levels reflecting cytotoxicity of the studied compounds in the liver. Across all experimental treatments, nZnO exposures in the absence of Nfd had the least impact on the cellular stress traits or redox status in frogs. This indicates that at the low environmentally relevant levels of pollution, pharmaceuticals such as Nfd and free metals (such as $Zn^{2+}$) may represent a stronger threat to the health of the frogs than nZnO particles.



## 1. Introduction

In the past decades, the composition of pollutants in the surface waters have undergone a principal change as the chemical industrial pollution decreased and the environmental loads of complex, variable mixtures of pollutants from non-point municipal and agricultural wastes increased (Directive, 2013/39/EU; Burkart, 2007). Among the emerging pollutants, nanoparticles (Exbrayat et al., 2015) and pharmaceuticals (Schaider et al., 2016) are of particular concern due to their potentially high bioavailability and significant biological effects. These emerging pollutants also commonly co-occur in aquatic habitats thus presenting a potential for interaction. However, the effects of complex mixtures of pharmaceuticals and nanoparticles have not been well studied in aquatic organisms (Amin et al., 2014; Czech and Buda, 2015).

Nano zinc oxide (nZnO) are metal-based nanoparticles commonly used in electronics, personal care products (such as toothpaste, sunscreens and beauty products containing up to 20–25% of nZnO), and textiles to provide UV and antibacterial protection (Exbrayat et al., 2015). nZnO can enter surface waters from multiple non-point sources and accumulate in aquatic animals causing growing environmental and health concerns (Fabrega et al., 2012). The current level of nZnO in the surface waters of Europe is estimated at 100–500 ng/L in the industrial areas and predicted to increase in the future (NanoFATE 2010–2014; Dumont et al., 2015). nZnO can interact with pharmaceuticals and other organic pollutants enhancing degradation of photosensitive compounds in the water (Tan et al., 2011) and thereby affecting the composition of the

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wastewaters (Schaider et al., 2016). nZnO may also modulate the biological effects and toxicity of pharmaceuticals in aquatic organisms such as shown for nifedipine-nZnO interactions in freshwater bivalves (Falfushynska et al., 2015b). A cardiac drug nifedipine (Nfd) is a common pharmaceutical pollutant in surface waters. It is a calcium uptake inhibitor widely used as a cheap antianginal and antihypertensive medicine (Heberer, 2002; Kolpin et al., 2002). Nfd and its metabolite (dehydronifedipine) were detected in the effluents of the municipal sewage treatment plants and surface waters (Heberer, 2002; Kolpin et al., 2002). In a large urbanized watershed of New York City, environmental Nfd concentrations exceeded 1 $\mu g\,L^{-1}$ (2.9 nM) (Benotti and Brownawell, 2007). Nfd is photosensitive, and thus co-occurrence of Nfd with photoactive substances such as nZnO may modulate toxicity of these pollutants and potentially diminish of the individual activity of each of these substances (Tan et al., 2011).

Amphibians are excellent bioindicators of aquatic pollution and ecosystem health due to susceptibility of all life stages to dermal absorption of water-borne toxicants (Hermes-Lima and Storey, 1998). To date, the biological effects of metal-containing nanoparticles on amphibians have not been extensively studied, especially in the context of co-exposures in a complex environmentally-relevant mixtures of pollutants (Nations et al., 2011; Bacchetta et al., 2012; Zhang et al., 2012; Bour et al., 2015; Exbrayat et al., 2015; Falfushynska et al., 2016). In such mixtures, both direct and indirect (i.e. mediated by the release of Zn), toxicity of Zn-containing nanoparticles may be modulated by the presence of other pollutants, including pharmaceuticals. Pharmaceuticals such as Nfd and other Ca-channel blockers up-regulate antioxidant responses in different species (Ray et al., 2012; Velena et al., 2016). Oxidative stress pathways are also commonly involved in toxicity of free ionic metals and metal-containing nanoparticles thereby creating a molecular basis for interactions with Nfd. Furthermore, Nfd may affect metal toxicity by modulating metal transport (e.g. via blocking the Ca2+ channels) as well as due to the indirect, off-target effects in the groups distantly related to the target organisms (such as amphibians). Therefore, a comprehensive assessment of potential targets for combined toxicity is needed to understand the mechanisms of Nfd-nanoparticles interactions and their health effects in amphibians.

The present study aims to determine the molecular and cellular mechanisms of combined toxicity of nZnO and Nfd and assess the potential for Zn release and accumulation during single and combined exposures to nZnO and Nfd in a common marsh frog *Pelophylax ridibundus*. Based on the earlier studies that showed a strong oxidative stress response to nZnO exposure modulated by Nfd in freshwater bivalves with the involvement of metallothionein (Falfushynska et al., 2015b) and the susceptibility of amphibians to the endocrine-disrupting effects of environmental pollutants (Venturino et al., 2003; Hayes et al., 2006; Kloas et al., 2009; Falfushynska et al., 2016), we focused on the assessment of the parameters of Zn homeostasis (including the total Zn burdens and Zn binding to metallothioneins) as well redox and endocrine markers in frogs exposed to nZnO, Nfd and their mixtures. The oxidative stress response was evaluated from activity of a key antioxidant enzyme superoxide dismutase (SOD), the rates of oxyradical formation and levels of reduced and oxidized glutathione (GSH & GSSG). Oxidative lesions to membranes were determined by accumulation of the end-products of lipid peroxidation, malondialdehyde (assessed as the total concentration of the thiobarbituric acid-reactive substances, TBARS) and lipofuscin. Potential endocrine disruption due to nZnO and Nfd exposures was assessed by markers of thyroid function, levels of vitellogenin (Vtg) that serves as a Zn-carrier and a marker for xenoestrogen exposure, and cortisol as a general stress marker. Cytotoxicity was determined as the levels of hepatic cytochrome P450 oxidase (CYP450), DNA damage and activity of the apoptotic proteases caspase-3 and cathepsin D. This comprehensive assessment of biomarkers provides insights into the integrated cellular, molecular and endocrine responses of frogs to single and combined exposures to environmentally relevant levels of a common nanoparticle (nZnO) and pharmaceutical pollutant (Nfd) and the underlying toxicity mechanisms.

## 2. Materials and methods

### 2.1. Chemicals

5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), thiobarbituric acid (TBA), reduced glutathione (GSH), glutathione reductase from baker's yeast (*S. cerevisiae*), 2-vinylpyridine, quinine sulphate, dihydrorhodamine, salmon sperm DNA, Hoechst 33342, nitroblue tetrazolium (NBT), acetyl-Asp-Glu-Val-Asp *p*-nitroanilide, hemoglobin, serum albumin, phenazine methosulfate, phenylmethylsulfonyl fluoride (PMSF), chymotrypsinogen, cytochrome c, myoglobin, ubiquitin, insulin chain B oxidized, Sephadex G-50, β-mercaptoethanol, β-nicotinamide adenine dinucleotide oxidized ($NAD^+$), β-nicotinamide adenine dinucleotide phosphate reduced (NADPH), ethylenediaminetetraacetic acid (EDTA), and Triton *X-100* were purchased from Sigma (St. Louis, USA). nZnO particles (mean size 35 nm) were obtained from Sigma Chemical Company (St. Louis, USA). All other chemicals were obtained from the Synbias (Kyiv, Ukraine), Bayer (Kyiv, Ukraine) and Balkanpharma-Dupnitsa (Dupnitsa, Bulgaria) commercial suppliers. All reagents were of the analytical grade or higher.

### 2.2. Experimental exposures

The experiments were carried out in mid-September of 2013. Adult non-breeding males of marsh frog *Pelophylax ridibundus* (8–10 cm long) were collected from a pristine site in the upstream portion of river Seret (49°49′ N, 25°23′ E). Collections and experiments were performed in accordance with the national and institutional guidelines for the protection of animal welfare with permission of the Ministry of Ecology and Natural Resources of Ukraine, No 466/17.04.2013 and approval of the Committee on the Bio-Ethics at Ternopil National Pedagogical University (No 2/10.06.2013).

Frogs were transported to the laboratory in 60 L cages with aerated native water. The preliminary acclimation and experimental exposures were carried out in 40 L tanks filled with aerated, softened tap water. After seven days of preliminary acclimation, frogs were randomly distributed into five groups (15 individuals per group) and exposed for 14 days to one of the following conditions: 1) control (C), 2) nZnO (corresponding to 3.1 μM Zn), 3) Nfd (10 μM), 4) combination of nZnO (3.1 μM) and Nfd (10 μM), or 5) $Zn^{2+}$ (added as $ZnSO_4$, 3.1 μM) as a positive control for Zn effects. A static renewal design was used, with water changed and chemicals replenished every two days. Throughout the experiment, the frogs were fed with commercial sticks "Turtle menu" (21% of protein, Aquarius, Ukraine). These conditions of exposures were the same as used in an earlier study on bivalve mollusks and reflected the lowest observed effect concentrations in the study of mollusk (Falfushynska et al., 2015b). The utilized concentrations were the same as for the studied early mollusks that reflected the lowest observed effect concentrations in the study of mollusks (Falfushynska et al., 2015b). To the best of our knowledge, we don't know about other studies of the effect of waterborne Nfd on the aquatic animals. So we selected the biologically active concentration basing on the previous results (Falfushynska et al., 2015b) and on the range of concentrations in the studies with human cell cultures (from 100 nM to 100 μM) (Hinkle et al., 1987;