Ecotoxicology and Environmental Safety ■ (■■■■) ■■■–■■■

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

The impacts of neutralized acid mine drainage contaminated water on the expression of selected endocrine-linked genes in juvenile Mozambique tilapia *Oreochromis mossambicus* exposed *in vivo*

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ARTICLE INFO

Article history:
Received 9 September 2013
Received in revised form
6 November 2013
Accepted 8 November 2013

Keywords:
Treated acid mine drainage
Endocrine disruption
Fish
Gene expression
Metal ions

ABSTRACT

Acid mine drainage (AMD) is a global environmental concern due to detrimental impacts on river ecosystems. Little is however known regarding the biological impacts of neutralized AMD on aquatic vertebrates despite excessive discharge into watercourses. The aim of this investigation was to evaluate the endocrine modulatory potential of neutralized AMD, using molecular biomarkers in the teleost fish *Oreochromis mossambicus* in exposure studies. Surface water was collected from six locations downstream of a high density sludge (HDS) AMD treatment plant and a reference site unimpacted by AMD. The concentrations of 28 elements, including 22 metals, were quantified in the exposure water in order to identify potential links to altered gene expression. Relatively high concentrations of manganese (~ 10 mg/l), nickel (~ 0.1 mg/l) and cobalt (~ 0.03 mg/l) were detected downstream of the HDS plant. The expression of *thyroid receptor-α* (*trα*), *trβ*, *androgen receptor-1* (*ar1*), *ar2*, *glucocorticoid receptor-1* (*gr1*), *gr2*, *mineralocorticoid receptor* (*mr*) and *aromatase* (*cyp19a1b*) was quantified in juvenile fish after 48 h exposure. Slight but significant changes were observed in the expression of *gr1* and *mr* in fish exposed to water collected directly downstream of the HDS plant, consisting of approximately 95 percent neutralized AMD. The most pronounced alterations in gene expression (i.e. *trα*, *trβ*, *gr1*, *gr2*, *ar1* and *mr*) was associated with water collected further downstream at a location with no other apparent contamination vectors apart from the neutralized AMD. The altered gene expression associated with the "downstream" locality coincided with higher concentrations of certain metals relative to the locality adjacent to the HDS plant which may indicate a causative link. The current study provides evidence of endocrine disruptive activity associated with neutralized AMD contamination in regard to alterations in the expression of key genes linked to the thyroid, interrenal and gonadal endocrine axes of a teleost fish species.

1. Introduction

Acid mine drainage is one of the major environmental risks associated with the mining industry (Akcil and Koldas, 2006), disturbing natural ecosystems by lowering the pH and by releasing contaminants such as metals and other toxic substances into water bodies. In particular, AMD is generally characterized by high levels of manganese, nickel, cobalt, iron and aluminum and other metals known to be harmful to wildlife (Akcil and Koldas, 2006). A further negative impact of AMD is salinization and the high sulfate content (Tutu et al., 2008). Not surprisingly, numerous studies have demonstrated detrimental effects of AMD on wildlife at organismal as well as ecosystem level, including overall declines in macro-invertebrate diversity (Janssens de Bisthoven et al., 2006; van Dam et al., 2008). Moreover, highly AMD impacted waterways are reported to be devoid of fish and other organisms (Parsons, 1977).

In many cases, AMD cannot be prevented or controlled and has to be treated. The most common approach to AMD treatment is pH control, but although such remedying action is capable of increasing the pH to near-neutral and subsequent precipitation of a large fraction of elements from the solution, not all metals are removed (Cravotta and Trahan, 1999).

Certain metals have been identified as endocrine disrupting contaminants (EDCs) (reviewed in Iavicoli et al., 2009). For example, reference has been made to different metals including Al, As, Ba, Cd, Co, Cr (II), Cu, Hg, Ni, Pb, Sn, Sb as "metalloestrogens" (Darbre, 2006), whereas Cd has been implicated as an androgen receptor agonist *in vitro* (Martin et al., 2002). Moreover, certain

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0147-6513/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ecoenv.2013.11.005

metals have been shown to disrupt the teleost fish reproductive- (Kime, 2009), thyroid- (Carr and Patino, 2011) and interrenal (Hontela, 1998) endocrine systems. Treated AMD may therefore exhibit endocrine disruptive potency due to metal loads.

Although reports exists indicating that the treatment of acidic waters reduces or even eliminate the endocrine disruptive effects in certain fish (Brown et al., 1990; Sangalang et al., 1990), these works were performed on acidic river or lake water, in most cases acidified by atmospheric deposition and not AMD (Baker et al., 1991). Even though endocrine disruptive activity has been investigated in a number of water bodies, to our knowledge no study to date has explicitly investigated the endocrine disruptive potential of neutralized AMD contamination in fish. The biological activities of individual chemicals are known to be altered when present in mixtures of other chemicals or elements, an occurrence referred to as the "cocktail effect", wherein which inter-chemical interactive effects such as potentiation or antagonism may occur (Celander, 2011). Neutralized AMD present an opportunity to investigate the effects of complex contaminant mixtures, consisting of metals and other elements, on the vertebrate endocrine system, without the toxic acid mediated effects associated with untreated AMD.

Alterations in the expression of certain genes have been shown as sensitive biomarkers for endocrine disruption after exposure periods as short as 12 h (Helbing et al., 2008; Opitz et al., 2006). Although changes in mRNA transcript abundance will not necessarily result in physiological impairment, these toxicogenomic biomarkers can be applied as signposts prompting further investigation based on chronic testing including more concrete endpoints such as impaired fecundity or development (Hutchinson et al., 2006).

Acid mine drainage (AMD) is a concern in certain regions in South Africa, due to decades of intensive mining activity (McCarthy, 2011). The Western Basin of the Witwatersrand has been identified as one of the problem areas in relation to AMD in South Africa, where a number of streams are subject to contaminated water from abandoned gold mines (McCarthy, 2011). The objective of this investigation was to evaluate aspects of water quality and the subsequent endocrine disruptive potencies of surface water collected from a river catchment receiving high volumes of neutralized (treated) AMD, released by a high density sludge plant, in the Witwatersrand Western Basin, South Africa. The specific aims were first to collect water samples from six localities and a reference location uncontaminated by AMD, record basic water quality parameters and determine the concentrations of selected metals and other elements. Second to expose juvenile *Oreochromis mossambicus* for a short term (48 h) and quantify altered expression of *thyroid receptor-α* (*trα*), *trβ*, *androgen receptor-1* (*ar1*), *ar2*, *glucocorticoid receptor-1* (*gr1*), *gr2*, *mineralocorticoid receptor* and *aromatase* (*cyp19a1b*) as representative of the endocrine system.

2. Materials and methods

2.1. Study sites

Water was collected from seven localities in the Bloubank stream catchment near Krugersdorp, South Africa (Fig. 1). The Bloubank stream is a tributary of the Crocodile River, and at times receive AMD emanating from flooded defunct gold mines associated within the West Rand Gold Field. The Tweelopie stream originates in close proximity of the Randfontein Estates Gold Mine and represents the most direct route for AMD into the Bloubank drainage. A high density sludge AMD treatment plant discharges into the Tweelopie stream (approximately 18 ML/d at the time of sampling) and the majority of water in the headwaters of this stream consists of treated AMD.

Site 1 is located 800 m downstream of the point where neutralized AMD released from a HDS facility enters the Tweelopie stream (Fig. 1), and the water consisted of approximately 95 percent neutralized AMD when the current study was performed. Sites 2 and 3 are located downstream within the Tweelopie stream, whereas sites 4–6 are further downstream and in the Bloubank stream. Water at the latter sites are subject to further anthropogenic impacts including agricultural runoff and spray drift via the Riet stream and waste water treatment plant (WWTP) effluent via the Blougat stream (Fig. 1). A reference site uncontaminated by AMD was also investigated. The reference site is located within the Krugersdorp Game Reserve in a tributary of the Tweelopie stream with corresponding geology as all six the other sites (i.e. dolomitic strata from the Malmani Subgroup within the Transvaal Supergroup, Hobbs and Cobbing, 2007) (Fig. 1).

2.2. Water collection

Surface water samples were collected on the 6th of December 2012 in PTFE capped glass bottles and kept on ice packs or ice in the dark. The samples were filtered through 11 μm nitral cellulose stacked on 1.2 μm glass-microfibre filters (Munktell, DE). Fifty milliliter of each filtered sample including a filter blank was used for metal analyses.

2.3. Chemical analysis

The concentrations of Ca, K, Mg, Na, P, Rb and Si were measured using a Thermo ICAP 6300 ICP-AES (Thermo Scientific, USA), and Al, As, Ba, Cd, Co, Cr, Cu, Fe, Hg, Li, Mn, Mo, Ni, Pb, Sb, Se, Sn, Sr, Ti, V, Zn using an Agilent 7700× ICP-MS (Agilent Technologies, USA).

2.4. Fish exposure

Juvenile *O. mossambicus* (13 days post fertilization [dpf]) were obtained from a single breeding pair (Rivendell Hatchery, Grahmstown, RSA) prior to the exposure, and maintained in buffered reverse osmosis (RO) water (containing 250 mg iodine containing marine salt, 80 mg $NaHCO_3$ per litre) at 28 ± 1 °C subject to a 14 h:10 h light:dark cycle. Seven fish (30 dpf) were assigned per treatment group representing the seven localities as well as a buffered RO water (pH 7) negative control. The fish were exposed to 800 ml of liquid in 1 l glass containers for 48 h, without food. All the fish were acclimatized for at least 48 h prior to the exposure to similar containers and volumes as applied during exposures, being fed crushed tilapia pellets (AquaNutro, RSA) twice a day. The fish were euthanized in 0.1 percent benzocaine at exposure termination and either transferred to TriReagent (Sigma, GE) or snap frozen and stored at −80 °C. The current study was approved by the Stellenbosch University Committee for Animal Care and Use (Protocol No. SU-ACUM12-00036).

2.5. RNA isolation and cDNA synthesis

Whole body homogenates of juvenile fish were prepared in TriReagent (Sigma, DE) using an ultrasound sonicator (Omni-ruptor 400, Omni International Inc., USA). Total RNA was isolated according to the TriReagent technical bulletin. RNA integrity was assessed through argarose gel electrophoresis. The RNA was subsequently DNase I (Sigma, DE) treated, and complementary DNA (cDNA) was prepared from 4 μg of total RNA in 20 μl-, or 2 μg in 10 μl reaction volumes using Maxima H-minus cDNA synthesis kits (Thermo Scientific, USA) according to manufacturer's instructions.

2.6. RT-qPCR

Messenger RNA expression of thyroid receptor-α (trα), trβ, glucocorticoid receptor-1 (gr1), gr2, androgen receptor-1 (ar1), ar2, mineralocorticoid receptor (mr) and aromatase cyp19a1b with β-actin as reference gene was evaluated using real-time RT-qPCR. The PCRs were performed as 15 μl reactions containing 2 μl cDNA as template (200 ng cDNA per reaction for cyp19a1b and 20 ng/reaction for all the other genes), 7.5 μl Jumpstart® SYBRgreen mix (Sigma, DE), 0.33 μM of each primer and nuclease free water. The PCR programs for al primer pairs included an enzyme activation step at 95 °C (9 min), followed by 40 cycles of denaturing at 95 °C (15 s), annealing at 58–63.5 °C (30 s) and elongation at 72 °C (45 s). The primer sequences (in the 5′–3′ direction) and respective annealing temperatures (Ta) were as follows: trα forward GCTCAGGGCTCACAGTGGAA, reverse AACGACACGGGTGATGGC, Ta 63.5 °C (Shiao et al., 2008); trβ forward AATGTGTTATTGACAAAGTG, reverse GATCGGATGAAAGCAGGATA, Ta 63.5 °C (Shiao et al., 2008); gr1 forward TGTCTGGCTCTATCGCCTTCA, reverse TCCCTCGTACCCAAGTGCAT, Ta 63.5 °C (Aruna et al., 2012b); gr2 forward GCCCGAGTAGATGATCTCTGGTT, reverse CAGGAGCATGCCCCAACT, Ta 60 °C (Aruna et al., 2012b); mr forward TGGTACGCATGGTGAAATGG, reverse TCAGGGTGATTTGGTCCTCAAT, Ta 65 °C (Aruna et al., 2012b); ar1 forward CTATCAAGAGTGGGCCTTCGG, reverse GCGCCTTAAACTGCGATCTG, Ta 65 °C (Ijiri et al., 2008); ar2 forward AGGGTGAGGTCGGCGAAT, reverse TGGACTCAAACCTGGTGTCGT, Ta 58 °C (Ijiri et al., 2008; cyp19a1b forward GAGCGTCAGAAGTCACTGC, reverse GCTCAAAATCAGGGTCTCCC, Ta 60 (Esterhuyse et al., 2008); β-actin forward TGTGATGGTGGGTATGGG, reverse CTGTGGTGGTGAAGGAGTAG, Ta 63.5 °C (Esterhuyse et al., 2008). Each PCR plate contained an internal non-template control (no cDNA) as well as a five point two-fold serial dilution