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Contaminant-induced oxidative stress in fish: a mechanistic approach

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Abstract The presence of reactive oxygen species (ROS) in living organisms was described more than 60 years ago and virtually immediately it was suggested that ROS were involved in various pathological processes and aging. The state when ROS generation exceeds elimination leading to an increased steady-state ROS level has been called "oxidative stress." Although ROS association with many pathological states in animals is well established, the question of ROS responsibility for the development of these states is still open. Fish represent the largest group of vertebrates and they inhabit a broad range of ecosystems where they are subjected to many different aquatic contaminants. In many cases, the deleterious effects of contaminants have been connected to induction of oxidative stress. Therefore, deciphering of molecular mechanisms leading to such contaminant effects and organisms' response may let prevent or minimize deleterious impacts of oxidative stress. This review describes general aspects of ROS homeostasis, in particular highlighting its basic aspects, modification of cellular constituents, operation of defense systems and ROS-based signaling with an emphasis on fish systems. A brief introduction to oxidative stress theory is accompanied by the description of a recently developed classification system for oxidative stress based on its intensity and time course. Specific information on contaminant-induced oxidative stress in fish is covered in sections devoted to such pollutants as metal ions (particularly iron, copper, chromium, mercury, arsenic, nickel, etc.), pesticides (insecticides, herbicides, and fungicides) and oil with accompanying pollutants. In the last section, certain problems and perspectives in studies of oxidative stress in fish are described.

Abbreviations

ACh	Acetylcholine
AMT	3-Amino-1,2,4-triazole (aminotriazole)
2,4-D	2,4-Dichlorophenoxyacetate
DDC	Diethyldithiocarbamate
DPXs	DNA–protein cross-links
DTT	Dithiothreitol
ETC	Electron-transport chain
GPx	Glutathione peroxidase
GSH, GSSG	Reduced and oxidized glutathione, respectively
GST	Glutathione-S-transferase
HCB	Hexachlorobenzene
MDA	Malonic dialdehyde
NAC	N-Acetylcysteine

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OP	Organophosphate pesticides
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid-reactive substances

Introduction

Free radicals were first described in chemical systems by Moses Gomberg more than a century ago (Gomberg 1900), and about 40 years later Leonor Michaelis suggested that free radicals were involved in all oxidation reactions (Michaelis 1939). Although the ideas of Michaelis were generally wrong, they stimulated interest in free radicals, particularly their role in biological systems. In 1950s, free radicals were identified in biological systems (Commoner et al. 1954) and were quickly proposed to be involved in various pathological processes (Gerschman et al. 1954) and aging (Harman 1956, 2009). Furthermore, it was discovered that many stresses, particularly those induced by diverse physical and chemical factors in the environment were accompanied by enhanced levels of free radicals. Most research on free radicals has concentrated on radicals of oxygen, which together with some non-radical active oxygen forms are collectively called reactive oxygen species (ROS). A main question in ROS research quickly arose: Do living organisms possess regulated enzymatic systems to defend against ROS? This was first positively answered by McCord and Fridovich (1969) who discovered the enzyme superoxide dismutase and clearly demonstrated that living organisms possessed protective systems against ROS. Over time, this was supported by continuing discoveries of other so-called antioxidant enzymes and proteins as well as low molecular mass antioxidants. Later, in 1970s–1980s ROS were shown to be responsible for combating pathogens by immune system (Babior et al. 1973, 1975; Rossi et al. 1985; Britigan et al. 1987). In the 1980s, the endothelium-derived relaxing factor in blood vessels was identified as being free radical nitric oxide ($^{\cdot}$NO), and this started studies of the biochemistry of reactive nitrogen species (RNS) and in particular the roles of $^{\cdot}$NO in metabolic signaling (Furchgott and Zawadzki 1980; Palmer et al. 1987, 1988; Furchgott and Vanhoutte 1989). Finally,

ROS were found to play signaling roles not only in ROS-related processes, but in many basic functions such as fertilization, growth, and differentiation (Christman et al. 1985; Morgan et al. 1986; Tartaglia et al. 1989; Scandalios 2005; Semchyshyn et al. 2005). Further ROS participation was discovered in many well-known hormonal signal transduction pathways such as insulin signaling in animals (Spagnoli et al. 1995) and jasmonic acid and ethylene-based signaling in plants (Lushchak 2011a).

Three decades ago, Helmut Sies (1985) first proposed definition of oxidative stress as "imbalance between oxidants and antioxidants in favor of the oxidants, potentially leading to damage." The definition of oxidative stress can be made even more specific: The situation when the steady-state ROS concentration is acutely or chronically increased leading to oxidative modification of cellular constituents and resulting in disturbance of cellular metabolism and regulatory pathways. Now, we clearly understand that virtually all extensive stresses are accompanied by oxidative stress, i.e., oxidative stress is a component of many extensive stresses. Different physical, chemical and biological environmental factors have been found to induce oxidative stress. Actually, oxidative stress may be a general feature of most stresses, and ROS-modified substances may play integrating roles in coordination of an organism's response to the stresses. Recognizing the primary interests of this journal "Fish Physiology and Biochemistry" and my long experience in the field of oxidative stress and antioxidant defenses in fish, the present review focuses on the responses of fish to environmental pollutants that induce oxidative stress.

Fish represent the largest group of vertebrates with about 20,000 species known. They live in a wide range of ecosystems: from Antarctic cold seas to hot springs, from oxygen oversaturated mountain rivers to highly hypoxic stagnant bodies of water, from salinities lower than fresh water to higher than oceanic salinity, etc. To survive in such diverse environments, fish need high plasticity and adaptive potential. Although aquatic conditions are generally more stable than terrestrial, environmental conditions can still change dramatically in many cases demanding adequate responses from fish. If fish adaptive potential is overwhelmed by external factors, organisms may enter stress conditions. The most studied environmental stress conditions in aquatic environments include changes in

salinity, ion composition, temperature and oxygen availability, as well as exposure to pollutants due to natural processes or human activity. Many studies have found that environmental stress induce oxidative stress in fish. This review uses a mechanistic approach devoted to analysis of oxidative stress induction in fish by diverse pollutants emerging in the environment mainly due to human activity. The established mechanisms will be given and if not known, they will be suggested with special attention to the effects on fish.

Homeostasis of reactive oxygen species

General aspects of ROS homeostasis

In many cases, the terms "free radicals" and "reactive oxygen species" are used interchangeably which is not always correct. Since it is important to understand the proper meaning of these terms, I briefly will highlight the principal points. The processes of ROS generation, interconversion and elimination are schematically presented in Fig. 1. In eukaryotes, under aerobic conditions more than 90 % of oxygen consumed is reduced directly to water by cytochrome oxidase in the mitochondrial electron-transport chain (ETC) using four-electron mechanisms without ROS release (Ott et al. 2007; Skulachev 2012). Although over 90 % of oxygen consumed by organisms is used to produce energy in the form of ATP (via coupling of the ETC with oxidative phosphorylation), much less than 10 % of oxygen consumed is reduced via one-electron successive pathways that begin with the conversion of molecular oxygen to the superoxide anion radical ($O_2^{\cdot-}$) (Fig. 1). The latter may be further reduced via a one-electron mechanism to successively form hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\cdot}$) and finally water. Of these intermediates, $O_2^{\cdot-}$ and $HO^{\cdot}$ possess unpaired electrons in external electron orbitals and hence are termed free radicals, whereas H_2O_2 does not possess such electrons and is not a free radical. All three species $O_2^{\cdot-}$, H_2O_2 and $HO^{\cdot}$ are more active than molecular oxygen and collectively are called reactive oxygen species (ROS). It is well known that ROS reactivity is reduced in the order: $HO^{\cdot} > O_2^{\cdot-}, > H_2O_2$ (Halliwell and Gutteridge 1989). Most ROS are produced as a result of electrons escaping from the mitochondrial ETC to molecular oxygen. These electrons interact with molecular oxygen to produce
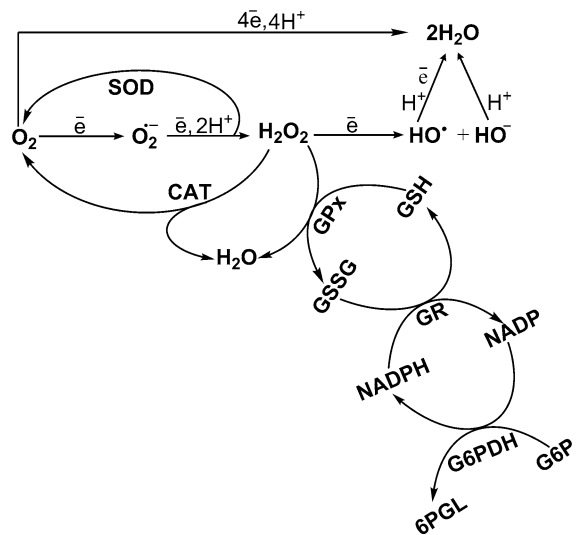

Fig. 1 One-electron and four-electron reduction of molecular oxygen and the first- and the second-line antioxidant enzymes. The addition of one electron to an oxygen molecule leads to the formation of the superoxide anion radical ($O_2^{\cdot-}$). Further addition of one electron to $O_2^{\cdot-}$ leads to the formation of hydrogen peroxide (H_2O_2). One-electron reduction of H_2O_2 forms a hydroxyl radical ($HO^{\cdot}$) and hydroxyl anion. Finally, the addition of one electron and proton to $HO^{\cdot}$ yields a water molecule. Two of the abovementioned partially reduced oxygen species, namely $O_2^{\cdot-}$ and $HO^{\cdot}$, are radicals, whereas H_2O_2 is not a radical. Transformation of $O_2^{\cdot-}$ and H_2O_2 is substantially accelerated by certain enzymes, called primary antioxidant enzymes. The first enzyme in the process is superoxide dismutase (SOD) which carries out the redox reaction with the participation of two molecules of $O_2^{\cdot-}$ dismutating them to produce molecular oxygen and hydrogen peroxide. Next is the one-electron reduction of H_2O_2 that is transformed to a less harmful species by several specific enzymes and a big group of unspecific ones. Specific enzymes include catalase that catalyzes the conversion of H_2O_2 to molecular oxygen and water, whereas glutathione-dependent peroxidase (GPx) uses the cofactor glutathione to reduce H_2O_2 to water. There is no information on specific enzymatic systems that can destroy hydroxyl radical due to which it is widely believed that prevention of $HO^{\cdot}$ production is the best way to avoid harmful impacts. The restoration of oxidized glutathione (GSSG) to its reduced form (GSH) is catalyzed by glutathione reductase (GR) using NADPH provided mainly by the glucose-6-phosphate dehydrogenase reaction (G6PDH)

$O_2^{\cdot-}$ with further spontaneous or enzymatic conversion to H_2O_2 and $HO^{\cdot}$. The amount of electrons escaping the ETC depends on the physiological state of the organism and oxygen availability, and the total amount of ROS produced by mitochondria seems to be poorly controlled by the cell. Certain ROS amounts are produced also in ETC located in/at endoplasmic

reticulum (ER), plasmatic and nuclear membranes. Different oxidases such as those that can act on selected carbohydrates, amino acids, heterocyclic and other compounds also produce ROS (Yeldandi et al. 2000; Bartosz 2009). In particular, the role of xanthine oxidase in ROS production, especially under hypoxic conditions, has been widely discussed (Griguer et al. 2006; Nanduri et al. 2013). If ROS generation by the abovementioned pathways is poorly controlled by the organisms, NADPH oxidase does it in finely controlled manner. The enzyme was discovered in late 1970s (Briggs et al. 1977; Yamaguchi et al. 1980) and was found to be responsible for defending animals against bacteria and other pathogens by the immune system. In the latter, ROS operate in concert with RNS such as $^{.}NO$ and peroxynitrite ($ONOO^-$), and reactive forms of halogens to attack invading pathogens. Interestingly, NADPH oxidase was also found in non-immune cells and here it seems also to be responsible for controlled ROS production. In some cases, autoxidation of certain endogenic and exogenic small molecules, particularly xenobiotics, may provide substantial ROS amounts (Miller et al. 1996; Saller et al. 2012; Michail et al. 2013).

Control over ROS steady-state levels is provided not only via their production, but also via elimination. Living organisms possess multilevel and complicated antioxidant systems operating to prevent ROS formation, eliminate ROS and ROS-modified molecules, or minimize their negative effects. There are several approaches to classify these systems, and here, we will use the mostly appreciated one based on molecular masses. Hence, antioxidants are generally placed in two groups: low molecular mass antioxidants (usually with molecular masses below one kilodalton) and high molecular mass antioxidants (with molecular mass higher than one or actually higher than ten kilodaltons). The group of low molecular mass antioxidants includes a variety of different chemical compounds including vitamins C (ascorbic acid) and E (tocopherol), carotenoids, anthocyanins, polyphenols, uric acid and glutathione. For fish, most of these are acquired from their diet but the tripeptide glutathione (γ-glutamyl-cysteinyl-glycine, GSH) is synthesized by most aerobic organisms and used to control ROS levels either via direct interaction with them or by serving as a cofactor for ROS-detoxifying enzymes (Marí et al. 2009; Lushchak 2012). The GSH level is finely adjusted by organisms to specific physiological or environmental conditions via several regulatory pathways, but is mainly regulated at the level of biosynthesis.

However, most research is focused on the operation of high molecular mass antioxidants that include primary antioxidant enzymes that directly detoxify ROS and associated enzymes that support their function. Primary antioxidant enzymes include superoxide dismutase (SOD, EC 1.15.1.1) that converts $O_2^{.-}$ to H_2O_2 and molecular oxygen, catalase (EC 1.11.1.6) that destroys H_2O_2 and peroxidases such as glutathione-dependent peroxidase (GPx, EC 1.11.1.9). The latter may reduce lipid peroxides as well as other peroxides. The glutathione pool is maintained in reduced state by glutathione reductase (GR, EC 1.6.4.2) which reduces oxidized glutathione GSSG to GSH using NADPH as a reductant. Finally, NADPH pool is maintained via reduction of $NADP^+$ by several enzymes such as glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49), 6-phosphogluconate dehydrogenase (6PGDH, EC 1.1.1.43), malate dehydrogenase (oxaloacetate-decarboxylating $NADP^+$, EC 1.1.1.40) or NADP-malic enzyme (NADP-ME), and $NADP^+$-dependent isocitrate dehydrogenase [IDH, threo-DS-isocitrate:$NADP^{(+)}$ oxidoreductase (decarboxylating, EC 1.1.1.42)] (Lushchak 2011a, 2014a).

Interconversion of ROS is schematically depicted at Fig. 1 where the upper part of the figure demonstrates routes of ROS production, conversion and destruction, whereas the bottom part shows the relationship among the enzymes responsible for ROS elimination. Reduction of molecular oxygen can occur via a four-electron scheme that results in water formation without ROS generation (as conducted by cytochrome C oxidase), whereas the one-electron reduction leads to the successive formation of $O_2^{.-}$, H_2O_2, and $HO^{.}$. As mentioned above, SOD dismutates $O_2^{.-}$ to form molecular oxygen and H_2O_2 and the later is sequentially reduced to form $HO^{.}$ and water. The enzymes shown in Fig. 1, namely SOD, catalase and GPx, form the first line of defense against ROS, whereas GR and G6PDH form the second defense line. Enzymes of the first line of defense (primary) are those that utilise ROS as substrates, whereas the second line (also called associated or secondary) antioxidant enzymes assist the first line ones. The second-line antioxidant enzymes provide reducing equivalents (GSH, NADPH) needed for the operation of the primary ones. Resources for the operation of secondary

antioxidant enzymes come from intermediary metabolic pathways, for example, the primary source of NADPH-reducing power comes from the pentose phosphate cycle.

It has to be emphasized that due to their high reactivity ROS disappear very fast (for example, the hydroxyl radical has a half-life in vivo of approximately 10^{-9} s), and therefore, it is not correct to say that "under some conditions ROS are accumulated." Since they are continuously produced and degraded, one must discuss their dynamics in terms of steady-state (stationary) levels or concentrations.

The consequences of ROS interaction with components of biological systems depends on their nature, the type of molecules or macromolecules that they interact with, and the microenvironmental conditions. Since ROS can interact with diverse cellular components, there can be many effects and consequences of ROS action. Some of the main ones are: (i) "non-specific" damage to cellular components, (ii) defense of organisms against pathogens, and (iii) signaling. Certainly, this division is very relative, because at the molecular level the effects are caused by ROS interactions with diverse endogenic cellular or foreign compounds. The specificity is determined by the ROS type, target attacked and microenvironment. Relatively low specificity of the reactions that ROS take part in is increased by the specificity of targets via different mechanisms. For example, signaling systems that demonstrate very high sensitivity and specificity to ROS are frequently based on the participation of specific groups in regulatory proteins. Two distinct types of such specificity are probably best exemplified by bacterial OxyR and SoxR sensors possessing thiol (–SH) and [4Fe,4S] cluster sensor centers that respond to the presence of H_2O_2 and O_2^-, respectively (Lushchak 2001). Specificity of the regulatory systems may be further enhanced by the existence of certain microenvironment and existence of highly specific downstream components.

Damage to cell constituents

It is well established that ROS can interact with virtually all cellular components, namely lipids, carbohydrates, proteins, nucleic acids, etc. Modified by ROS, lipids and carbohydrates may be degraded or transformed in toxic end products. A similar situation takes place with proteins with several exceptions when

oxidized proteins are reduced by specific systems (Lushchak 2007). ROS-modified RNA also is degraded, but ROS-modified DNA, if not catastrophic, is fixed by specific reparation systems.

The first studies on oxygen-promoted lipid oxidation (later called ROS-induced lipid peroxidation or autoxidation) were carried out in the 1930s (Olcott and Mattill 1931a, b; Monaghan and Schmitt 1932). Interestingly, since that time fish oil has been used as classic substance to investigate processes of lipid oxidation or peroxidation (Olcott and Mattill 1931a, b). Lipid peroxidation (LPO) was first studied extensively in relation to food deterioration and the stability of the polyunsaturated fatty acids in fish oil (Fritsche and Johnston 1988; Gonzalez et al. 1992; Niki 2000). Later investigations revealed that LPO products could be formed in living organisms (Hermes-Lima et al. 1995; Storey 1996; Lushchak 2011a, b; Lushchak 2012). With increased evidence of the physiological significance of the process, LPO received renewed attention in biochemistry and medicine.

Measurement of the products of lipid peroxidation is a popular approach to evaluating ROS-induced effects on biological systems. Several products of ROS-promoted lipid peroxidation are commonly analyzed, and the evaluation of malonic dialdehyde (MDA) levels being a chief one. Frequently, MDA is measured due to its reaction with thiobarbituric acid (TBA) to form thiobarbituric acid-reactive substances (TBARS). However, this method is rather non-specific and should be used with many precautions which is true for works with fish (Talas et al. 2008; Zhang et al. 2008; Falfushynska and Stolyar 2009). Recently, an HPLC technique was introduced to measure MDA, and since it is more specific, it may be recommended for use whenever possible (Fedotcheva et al. 2008). Lipid peroxides may be measured by several techniques but our experience shows that the iron/xylenol method (Hermes-Lima et al. 1995) is very successful, particularly as applied to monitor oxidative damage to lipids in fish (Lushchak and Bagnyukova 2006b; Lushchak et al. 2009a, b). Some products of lipids peroxidation such as MDA and 4-hydroxy-nonenal (4-HNE) conjugate with proteins and can then be measured by immunologic techniques (Brannian et al. 1997). In some cases, the so-called lipofuscins are considered to be used as markers of aging and pollutant effects, particularly in fish (Viarengo et al. 2007; Boscolo Papo et al. 2014; Passantino et al.

2014). Lipofuscins are a heterogenic group of compounds with uncertain composition believed to be formed by the condensation of products of lipid peroxidation with proteins, carbohydrates and nucleic acids. Due to such heterogeneity, no reliable methods to measure their general levels are available and virtually all applied ones provide semiquantitative data.

Measurement of the levels of ROS-modified proteins is another very popular approach among researchers. Usually oxidatively modified proteins are degraded, but in some cases they can be accumulated, and moreover, some may even become ROS producers. The level of oxidatively modified proteins is a commonly used marker of oxidative stress [for discussion see (Lushchak 2007; Lamarre et al. 2009)]. The most convenient and broadly used approach (including in studies with fish) is to measure the ROS-induced formation of additional carbonyl groups in proteins via reaction with dinitrophenylhydrazine.

Oxidation of DNA is another effect of ROS presence in the cell. This type of damage is critically important for cell functions, because it can result in mutations. ROS-induced DNA modification is more extensive in mitochondria than in the nucleus, and, as mentioned above, is commonly repaired by specific systems. The 8-oxoguanine formed due to DNA attack by ROS is the most frequently evaluated marker of DNA damage and can be measured by HPLC (Olinski et al. 2006) or immune (Ohno et al. 2009) techniques. Unfortunately, to our best knowledge, very few studies in this field have been carried out at fish (Malek et al. 2004; Grygoryev et al. 2008). On the other hand, the so-called Comet assay has been frequently applied to monitor damage to DNA in aquatic organisms and interested readers should refer to works of A. Jha et al. (Jha 2008; Vevers and Jha 2008).

Modification of specific proteins

Reactive oxygen species can modify virtually all cellular components. However, in certain cases such modifications seem to be rather specific due to peculiarities of the molecules modified. In many cases, such molecules are ROS sensors. The [4Fe-4S] cluster of the SoxR protein in *Escherichia coli*, and the thiol groups of bacterial OxyR, yeast Yap1, and animal Keap1 proteins are the best studied examples of this. These systems will not be described here in details, but interested readers may find information in many review papers (Demple 1991; Demple and Amabile-Cuevas 1991; Kuge and Jones 1994; Toone and Jones 1999; Itoh et al. 1999; Després et al. 2000; Lushchak 2001, 2010, 2011a, 2012; Scandalios 2005; Sies 2015). Interestingly, in some cases ROS-promoted modification of proteins leads to changes in their functions. For example, iron ions may be released during oxidation of the cytosolic form of aconitase, a [4Fe-4S] cluster-containing enzyme (Lushchak et al. 2014). The resulting [3Fe-4S]-containing protein cannot catalyze the conversion of citrate to isocitrate, but becomes a protein that regulates iron metabolism (IRP). This conversion was first described in yeast (Narahari et al. 2000) and further found in mammals (Rouault 2006), but information on its operation in fish is scarce (Andersen et al. 1998; Zhao et al. 2014).

Defense systems

The respiratory burst was discovered in 1933 by Baldridge and Gerard when they realized that phagocytosis was linked with elevated oxygen consumption (Baldridge and Gerard 1933). However, it was completely ignored for the next 30 years. Interest in the burst was rekindled in the 1960s when it was found that the oxidative burst was not blocked by inhibitors of mitochondrial oxidative phosphorylation (Mürer 1968). Further studies indicated that the burst did not provide energy for phagocytosis, but instead was used to produce lethal levels of oxidants to kill microbes (Drath and Karnovsky 1975; Curnutte et al. 1979; Babior 1984). In the 1970s, several groups described an enormous stimulation of oxygen consumption by leukocytes under bacterial attack which was termed "oxidative burst" (Muenzer et al. 1975; Drath and Karnovsky 1975). In response to certain stimuli, phagocytes (neutrophils, eosinophils, and mononuclear phagocytes) substantially change their metabolism: Their rates of oxygen uptake increase greatly, sometimes by more than 50-fold, they produce large amounts of O_2^- and H_2O_2, and at the same time they start to metabolize large quantities of glucose via the hexose monophosphate shunt to produce NADPH. The "respiration" associated with oxidative burst, however, has nothing to do with energy production. Rather it is used to generate powerful antimicrobial agents by the partial reduction of oxygen.

Due to its potential applications in biomedicine and its possible involvement in the immune response, studies of the oxidative burst attracted many researchers and led to the discovery of specific systems for reducing molecular oxygen via the one-electron scheme. The system was shown to be an integral part of the leukocyte plasma membrane and needed NADPH for operation. Therefore, it was called "NADPH oxidase." The system catalyzes one-electron reduction of molecular oxygen yielding superoxide anion radical which is further converted either spontaneously or enzymatically into H_2O_2 and $HO^{\cdot}$. These ROS are believed to be responsible for fighting pathogens by immune system cells. Leukocytes were later found to also possess NO synthase (NOS), which collaborates with NADPH oxidase. The rationale for this is that the combination of $^{\cdot}NO$ with $O_2^{\cdot-}$ produces a very powerful oxidant, peroxynitrite. The latter one at disproportionation (a chemical reaction, typically a redox reaction, where a molecule is split into two or more different products) gives $HO^{\cdot}$ among other products.

Primarily described in mammals, the system also operates in fish. For example, fish leukocytes possess both, NADPH oxidase and NO synthase (Alvarez-Pellitero 2008). They are actively involved in defense against many infections. Interestingly, NADPH oxidase and NO synthase have also been described in many other cell types. This system is rather well studied in many organisms and is still of high interest due to its potential importance in aquaculture where highly effective antimicrobial mechanisms would be a bonus for fish raised, for example, in dense populations.

ROS-based signaling

In the early 1990s, several groups found that in bacteria certain specific systems are involved in ROS-induced up-regulation of antioxidant and some other enzymes (Demple 1991; Demple and Amabile-Cuevas 1991; Storz and Imlay 1999; Lushchak 2001). Somewhat later, similar systems were described in yeast (Kuge and Jones 1994; Toone and Jones 1999; Lushchak 2010) and higher eukaryotes (Itoh et al. 1999; Després et al. 2000). In most cases, operation of these systems is based on the reversible oxidation of cysteine residues of specific proteins (Toledano et al. 2007; Lushchak 2011a, 2012). However, while in bacteria these proteins may serve as both sensors and regulators of cellular

responses, such as certain transcription regulators (for example, SoxR and OxyR), in eukaryotes the regulatory pathways are much more complicated. Commonly, a sensor molecule is localized in the cytoplasm and after signal reception it either diffuses into the nucleus to directly stimulate gene expression or the sensor transduces the signal to the transcription factor machinery to activate certain transcription factors that then mediate gene expression. In animals, particularly in fish, several systems regulated by ROS have been described. Among them, the Nrf2/Keap1 system seems to attract most attention (Mukhopadhyay et al. 2015). Although ROS-induced signaling was primarily found to regulate cellular ROS defense systems, it has now become clear that it regulates many other cellular processes such as development, apoptosis and necrosis. This is a field of interest for many research groups, and there is no doubt that it will gain even great attention in the future.

Oxidative stress theory and classifications of oxidative stresses

There are many definitions of oxidative stress, but, to date, this term has no rigorous meaning and none of the definitions are fully satisfactory. Intuitively, it is accepted that oxidative stress is the situation when oxidative damage is increased, which is explained as an imbalance between ROS production and elimination in the favor of the first. First defined by H. Sies in 1985, later the definition was partially modified to "oxidative stress results from imbalance in this pro-oxidant–antioxidant equilibrium in favor of the pro-oxidants" (Sies 1991). Halliwell and Gutteridge (1989) defined oxidative stress as "in essence a serious imbalance between production of ROS/RNS and antioxidant defense." All these definitions lack a very important element—they seem to ignore the dynamics of ROS production and elimination, i.e., steady-state ROS level should be referred to. The multiple ROS roles should also be mirrored in the definition to also reflect their signaling function. Therefore, I propose one more definition: "Oxidative stress is a transient or chronic increase in steady-state ROS levels, disturbing cellular core and signaling pathways, including ROS-based ones, leading to oxidative modification of cellular constituents which may culminate in cell death via necrosis or apoptosis." Not pretending to be ideal or full, the definition

accounts for the information gained in the last few decades.

Oxidative stress can arise in several ways: (i) increased ROS production, (ii) decreased ROS elimination and (iii) a mismatched combination of production versus elimination. Although it is difficult to demonstrate that oxidative stress can directly lead to disease, there is much evidence to demonstrate a strong relationship between oxidative stress and many pathologies as well as aging. In various cases, the application of different antioxidants has been shown to provide prophylaxis, cures or at least a reduction in disease symptoms. According to ROS homeostasis theory, the steady-state concentration of oxidants and antioxidants is maintained within a limited range (Lushchak 2011a, 2014a, b; Ayer et al. 2014). This may explain why antioxidant therapy is generally found to be ineffective.

Ideologically similar to the concept of oxidative stress, but with opposite logic, is the challenge to organisms from reductants. A decrease in the ROS steady-state concentration may be called "reductive stress"—i.e., either exposure to elevated levels of reductants or shift of intacellular redox status to reduced state. Although this term is not commonly used, this situation can affect aquatic organisms particularly fish. For example, deep water in the Black sea contains high concentrations of hydrogen sulfide that are very toxic to most living organisms. Surface or midwater organisms can be harmed if exposed to benthic waters and bottom mud with high H_2S levels. However, various fish and other inhabitants of these biotopes have developed different strategies to resist environmental reductive conditions successfully (Rudneva et al. 2010). Nonetheless, we feel that it is of interest to explore "reductive stress" hypothesis.

Although the first definition of oxidative stress was proposed by H. Sies in 1985, until now it was difficult to differentiate categories of oxidative stress in terms of time or intensity. Therefore, recently, I proposed such classifications (Lushchak 2014a, b) that can be described in two ways: (a) analyzing the time course of metabolic perturbations under steady-state ROS levels (Fig. 2a) and (b) analyzing the intensity of oxidative stress developed due to application of different doses or concentrations of an oxidative stress inducer
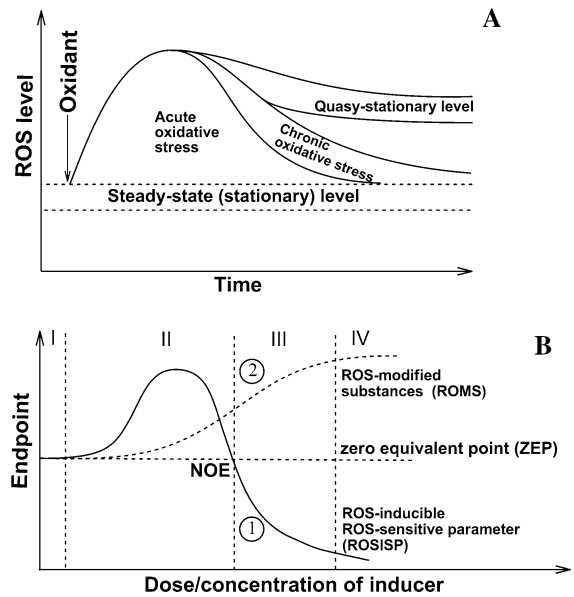

Fig. 2 Schematic presentation of time course (**a**) and intensity-based (**b**) classifications of oxidative stresses. Normally, ROS concentration is maintained within a certain steady-state (stationary) range and fluctuates similarly to other parameters in the organism according to homeostasis theory. However, under certain circumstances the concentration may leave this corridor due to increased production of ROS or decreased elimination. **a** A state when ROS levels are increased for a short time is referred to acute oxidative stress, whereas prolonged elevation of ROS is called chronic oxidative stress. In some cases, ROS levels do not return into the initial corridor or close to it and stabilize at a higher steady-state level called quasi-stationary. Both acute and chronic oxidative stresses may differently affect organisms and cause more or less injury to tissues that, if not brought under control, can culminate in cell death via apoptosis or necrosis mechanisms. **b** Curve 1 shows the effects of increasing levels of an inducer on ROS-induced ROS-sensitive cellular functions, for example, the activity of an antioxidant enzyme, whereas Curve 2 shows the level of and ROS-modified (or damaged) cellular component. Actually, curve 2 also shows the relationship between the dose/concentration of oxidative stress inducer and various commonly used endpoint parameters that can be experimentally measured. Zone I—no observable effects are registered due to very low intensity oxidative stress (basal intensity oxidative stress—BOS); zone II—low-intensity (mild) oxidative stress (LOS) with a slightly enhanced level of oxidatively modified molecules and enhanced activity of antioxidant enzymes in response to oxidative stress; zone III—intermediate-intensity oxidative stress (IOS); and zone IV—high-intensity (strong) oxidative stress (HOS). Abbreviations: *NOE* no observable effect point, *ZEP* zero equivalent point—the level of components of interest corresponding to the initial (basic) level seen in the absence of an inducer of oxidative stress. (Modified from Lushchak 2014a, b)

(Fig. 2b). Examining the time course-based classification (Fig. 2a), we can see that, under normal conditions, steady-state ROS concentration would fluctuate within some range that reflects the balance between ROS generation and elimination in the cell/tissue. However, under circumstances of oxidative challenge, the steady-state ROS concentration can increase beyond the steady-state range. If the antioxidant potential of the cell/tissue is powerful enough, ROS levels would return into previous steady-state range quickly and probably without any serious consequences for the organism; this is the pattern for an acute oxidative stress. However, if the antioxidant potential is not high enough to cope with the enhanced ROS level, the organism may need to increase its antioxidant potential before it can effectively decrease ROS levels. This could have at least two consequences: (i) the ROS steady-state concentration would return into the initial steady-state range or close to it (chronic oxidative stress); or (ii) a new steady-state ROS level can be established, a so-called quasi-stationary one. Chronic oxidative stress is usually related to the up-regulation of expression of many genes particularly those related to the stress response. This stress may not have serious consequences for the cell, but in some cases it can result in the development of certain pathologies. In other words, stabilization of increased ROS steady-state levels can be deleterious for the organism and related in many cases with chronic inflammation. This scheme may be of interest to describe the dynamics of ROS levels under normal conditions and oxidative insults.

Oxidative stress also may be classified due to its intensity (Fig. 2b). In this case, oxidative stresses are categorized due to the registered response of the organism to different intensities of oxidative challenge as a result of the dose or concentration of the inducer. The theoretic classification of oxidative stress in this system differentiates four zones: (a) Zone I: basal oxidative stress (BOS) when no observable effects of changing inducer concentrations are seen; (b) Zone II: low-intensity (mild) oxidative stress (LOS) where slightly enhanced levels of oxidatively modified molecules occur and stimulate enhanced activity of antioxidant enzymes in response to oxidative stress; (c) Zone III: intermediate-intensity oxidative stress (IOS) when the level of ROS-modified components is higher that under LOS, but the activity of antioxidant enzymes is lower than that in the control situation; and

finally (d) Zone IV: high-intensity (strong) oxidative stress (HOS) when the levels of ROS-modified components tend toward a maximum and the activities of antioxidant enzymes tend to a minimum (Fig. 2b). However, in practice, probably the most useful classification discriminates two zones: (1) mild oxidative stress (MOS) when the levels of ROS-modified components and the activities of antioxidant enzymes are higher than those in control, and (2) strong oxidative stress (SOS) when the level of ROS-modified components is higher than in MOS, whereas the activities of antioxidant enzymes is lower than under control conditions. Interested readers can consult the original papers (Lushchak 2014a, b) where these descriptions are discussed in detail along with an analysis of the molecular mechanisms underlying such states.

Concerning oxidative stress development in fish, generally it does not differ from other animals. Therefore, all the basic things described above are applicable to fish. However, there are some situations that are peculiar to the aquatic environment including fluctuations in oxygen levels, environmental pH, routes of exposure and uptake of oxidants, etc.

Environmental pollutants as oxidative stress inducers in fish

In nature, animals may experience oxidative stress as a result of multiple factors (Storey 1996; Lushchak 2011a, b), but the present paper focuses on contaminant-induced stress resulting from human activity. Environmental pollutants can induce oxidative stress in fish in two ways: directly via effect on the animals and indirectly via effects due to modification of environmental conditions. Another definition of direct and indirect oxidative stress may be related to the mechanisms leading to its development. That is, direct oxidative stress is caused by diverse substances directly entering the cellular redox cycles such as metal ions with changeable valences, quinones and others. Indirect oxidative stress may be caused by substances having a stable redox state, but which, for example, can interfere with providing energy and substrate resources, substitute for essential metals in their regular places, have mechanistic effects (nanoparticles) or affect the operation of transcriptional factors. Actually, we will not always follow

these classifications and in each specific case will describe either the established or suggested mechanisms involved.

Mediated (non-direct) pollutant-induced oxidative stress

Oxygen availability is one of the critical environmental parameters that is responsible for fish distribution. Induction of oxidative stress in fish by oversaturation of water with oxygen (hyperoxia) or, alternatively, reduced (hypoxia) or full (anoxia) oxygen deprivation can all occur and the effects of all of these on oxidative stress and antioxidant response have been analyzed by my laboratory (Lushchak and Bagnyukova 2006a). It is logical to expect hyperoxia could induce oxidative stress in fish (Olsvik et al. 2005, 2006; Lushchak et al. 2005a). The mechanism involved could be enhanced ROS production due to an elevated probability of electrons escaping the mitochondrial ETC to interact with oxygen molecules, which are abundant in this case. In aquatic ecosystems, an increase in environmental oxygen may be related to human activities such as bubbling at purification stations either with air or ozone. The latter at low concentrations may be beneficial for the organisms, while higher levels are deleterious (Bocci et al. 2009). Although high ozone concentrations in nature are not supposed to exist, fish may be exposed to them during some technological processes like water purification and ultraviolet irradiation. In model experiments, red blood cells (RBC) of rainbow trout (*Oncorhynchus mykiss*) were exposed to ozone which induced hemolysis, formation of methemoglobin and RBC membrane lipid peroxidation (Fukunaga et al. 1999). The incubation with ozone enhanced H_2O_2 generation which was proposed to play a critical role in mediating of RBC damage. It was proposed that neither ozone nor its derivatives directly attacked the cell from the outside, but ozone that penetrated through the membrane stimulated ROS generation by intracellular hemoglobin. The effects of ozone on rainbow trout (*O. mykiss*) could be also connected with the stimulation of ROS production (Hébert et al. 2008). In natural and artificial water bodies, oversaturation by oxygen can take place due to extensive photosynthesis associated with eutrophication, typically resulting from an excess of nutrients, like nitrogen and phosphorous, derived from runoff

from surrounding agricultural lands. Algae and photosynthetic oxygenic bacteria grown extensively under solarization produce oxygen which further affects fish. But, oxygen consumption by all organisms during the night may reverse this daytime hyperoxia so that it may not be a chronic problem. However, oxygen deficiency (hypoxia or anoxia) can also induce oxidative stress (Lushchak and Bagnyukova 2006a). Unfortunately, to date, there is no information on what levels of ROS in water are likely to be dangerous to fish and on the extent of ROS penetration across fish gills and other tissues from the external environment.

Logically, environmental hypoxia could be expected to decrease the risk of oxidative stress development due to a reduced probability of ROS generation due to oxygen lack. In the European glass eel (*Anguilla anguilla*), hypoxia decreased the expression of genes encoding respiratory chain proteins and those involved in defense against ROS (Pierron et al. 2007) which generally corresponded to expectations. However, goldfish exposure to anoxia resulted in signs of oxidative stress development (Lushchak et al. 2001). Although the mechanisms involved are not known to date, it can be suggested that the described changes in the activities of antioxidant enzymes might be related to transient oxidative stress during the transition from normoxia via hypoxia to anoxia over the experimental course. Hypoxia also increased the activities of SOD and catalase in liver of common carp (*Cyprinus carpio*) (Lushchak et al. 2005b) and studies with another fish model, the rotan (*Percottus glenii*), also demonstrated the induction of oxidative stress under hypoxic conditions (Lushchak and Bagnyukova 2007). Some signatures of hypoxia-induced oxidative stress were also found in the North Sea eelpout (*Zoarces viviparous*) which paralleled with HIF-1 activation (Abele 2005). At least two mechanisms that could be responsible for hypoxia-induced oxidative stress development may be proposed. They are: (i) an enhanced reduction state of electron carriers in the mitochondrial ETC could lead to increased electron escape and the consequent reduction of molecular oxygen via the one-electron mechanism (Fig. 1), and (ii) proteolytic conversion of xanthine reductase to xanthine oxidase and ROS production by the latter. There is no experimental information which may help either confirm these, or propose other mechanisms of hypoxia-induced oxidative stress in fish or other organisms.

Interestingly, fish responses to hypoxia may also be combined with responses to highly reduced environments where oxygen lack interplays with high reductive power such as that found in the benthic waters of the Black sea (Di Giulio and Hinton 2008; Rudneva et al. 2010; Rudneva 2013).

Theoretically, other types of human-induced activity which may indirectly induce environmental oxidative stress in fish may be related to changes in environmental temperature and illumination. Recently, Johannsson et al. (2014) found that patterns of oxygen saturation could change from virtually zero at night to daytime highs of 400 % saturation; hydrogen peroxide levels could similarly vary in the range from zero to 8 μM.

Direct contaminant-induced oxidative stress

There are three principal mechanisms of oxidative stress induction by environmental contaminants: (i) enhanced ROS generation, (ii) weakening of ROS elimination systems and/or (iii) various combinations of the first two. Actually, classification of environmental stressors for natural and human-produced ones is rather artificial. We do that only to differentiate some main types of inducers of oxidative stress in fish. Human-derived pollution of the environment is now a critically important factor affecting aquatic ecosystems, and in many cases it is connected with contaminant-induced generation of reactive species in hydrobionts including fish. Here, I will describe several groups of contaminants which are known to induce oxidative stress in fish. These groups include the following: metal ions, pesticides, oil products and chlorinated hydrocarbons.

Metal and metalloid ions

These substances may be found in aquatic systems due to natural processes and human activity. Depending on their chemical properties, they can induce oxidative stress via different mechanisms. From the point of view of ROS generation, metal ions can be divided in two groups: (i) ions with changeable valence and (ii) ions with constant valence state. Therefore, the mechanisms implicated may differ, although may have some common features. Ions with constant valence states may interfere generally with metabolic pathways. For example, bivalent magnesium,

strontium and barium ions may affect calcium- and zinc-related processes, compromising the latter ones, such as, energy production or regulation of gene expression. They can either interfere with Ca^{2+}- or Zn^{2+}-dependent processes or substitute for these ions in enzymes or regulatory proteins. However, more critical events are associated with the substitution of ions with changeable valence, like iron and copper, in certain proteins/enzymes. This may lead to the stimulation of ROS production due to their involvement in the Fenton reaction. The so-called heavy metal ions usually have high affinity for thiol groups and in this way affect many cellular processes, among them, leading to enhanced ROS levels.

Principally, ions with changeable valence are among the most frequently and well-studied inducers of oxidative stress. They may undergo free radical processes directly due to their ability to accept/donate electrons. Among this group, ions of iron, copper, manganese and chromium are the best investigated particularly due to their substantial biological importance and capacity to stimulate ROS production via participation in the Haber–Weiss reaction (1). Hydrogen peroxide cleavage due to acceptance of one electron from metal ions with changeable valence is usually mechanistically described by the scheme:

$$H_2O_2 + Me^{n+} \rightarrow HO^{\cdot} + OH^{-} + Me^{(n+1)+}, \qquad (1)$$
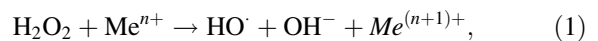
where Me means any metal ion with a changeable valence. Oxidized metal ions may be further reduced in a reaction with O_2^{-} (or other electron donors) to form molecular oxygen according with equation:

$$O_2^{-} + Me^{(n+1)+} \rightarrow O_2 + Me^{n+} \qquad (2)$$

A combination of these reactions (1) and (2) gives the net Fenton reaction:

$$H_2O_2 + O_2^{-} \rightarrow HO^{\cdot} + OH^{-} + O_2 \qquad (3)$$

Many ions are constitutive components of living organisms, and under normal conditions their involvement in free radical processes is tightly controlled by the cell. However, under some circumstances this control may be disturbed leading to the development of oxidative stress. In the aquatic environment, the concentrations of ions may be enhanced for many reasons and organisms must cope with them. There are several ways to prevent oxidative stress development induced by enhanced environmental ion levels:

(i) prevention of their entrance into the organism, (ii) tight binding of them by specific and non-specific substances, (iii) enhancement of antioxidant potential and (iv) release of absorbed ions into the environment. The toxicity of metal ions associated with free radical processes in animals and humans was reviewed by Valko et al. (Valko et al. 2007), for aquatic organisms (Lushchak 2011b) and for fish (van der Oost et al. 2003; Lushchak 2008).

Much information is available on the induction of oxidative stress by metal ions in fish, but it has not yet been systematized. Because of this, I will briefly analyze available information for fish in order to highlight potential mechanisms responsible for the induction of oxidative stress taking into account their possible valence states and redox properties. The analysis concentrates mainly on the best studied and/or biologically important metals such as iron, copper, chromium, mercury, arsenic and nickel. The behavior of ions in solution differs substantially from their behavior in nanoparticle form, and information on the influence of nanosized particles on living organisms is still scarce. Moreover, the level of nanomaterials in the aquatic environment is usually extremely low and the particles themselves are usually in the form of aggregates that are poorly available uptake/absorption by living organisms. Hence, although the effects of man-made nanomaterials on biological systems are a growing concern, the current review will not delve into the potential effects of nanomaterials on fish. General ideas on the molecular mechanisms responsible for metal-catalyzed ROS generation in living organisms along with their biological effects are shown at Fig. 3.

Iron The electronic structure of iron (*ferrum*) results in its capacity to drive one-electron reactions, and together with its relatively high content of this element in cells, this makes iron one of the major elements responsible for the production and metabolism of free radicals in biological systems, particularly in fish. Under physiological conditions, especially at neutral pH, Fe^{3+} precipitates in the form of oxyhydroxide polymers, whereas Fe^{2+} remains soluble. However, Fe^{2+} is unstable in physiological solutions and tends to be oxidized, particularly by molecular oxygen, to yield superoxide anion.

It is assumed that iron metabolism and function in teleost fish species is similar to that in other vertebrates. Fish obtain iron ions from water by uptake across the gill epithelium and by intestinal uptake from food (Bury et al. 2003). Iron metabolism in rainbow trout with normal and iron-deficient diets has been studied in detail (Walker and Fromm 1976; Carriquiriborde et al. 2004). Iron is an essential element as a constituent of many proteins (e.g., hemoglobin, cytochromes) where its ability to undergo redox cycling is key to its function, but this ability also means that iron can initiate and propagate non-specific free radical processes that are damaging to cells. Although much is known about the iron-mediated free radical processes in mammals, information on this for fish is scarce.

Ingestion of a higher dietary iron ration by African catfish *Clarias gariepinus* over five weeks suppressed fish growth indicating that the metal was supplied at sublethal, but toxic levels (Baker et al. 1997). Tissue iron concentrations were unaffected by the dietary regimen, but the concentration of malonic dialdehyde in liver and heart increased in proportion to the dietary iron dose. At the same time, the fat-soluble antioxidant α-tocopherol (vitamin E) was significantly depleted in liver of fish fed the high iron diet. The dynamics of the last two parameters indicates the development of oxidative stress induced by the high iron level in food (Baker et al. 1997).

The effects of waterborne iron on free radical processes in goldfish *Carassius auratus* liver and kidney were studied by my laboratory (Bagnyukova et al. 2006). Fish were exposed to 20 or 500 μM ferrous sulfate added to their water for 7 days, and then, effects on multiple tissue parameters were assessed. The treatment increased the levels of protein carbonyl groups, but reduced the concentration of lipid peroxides. At the same time, the concentrations of TBARS increased in liver and kidney of fish treated with 500 μM of ferrous sulfate. Changes in these levels of markers of oxidative damage all demonstrate the development of mild oxidative stress (Lushchak 2014a, b) in goldfish tissues. Fish exposure to high environmental iron concentrations also affected antioxidant enzymes in goldfish tissues. The activity of superoxide dismutase did not change, but catalase activity was strongly reduced. The activity of glutathione-*S*-transferase was also decreased under high iron concentrations, but glutathione reductase activity was suppressed only in kidney of goldfish treated by
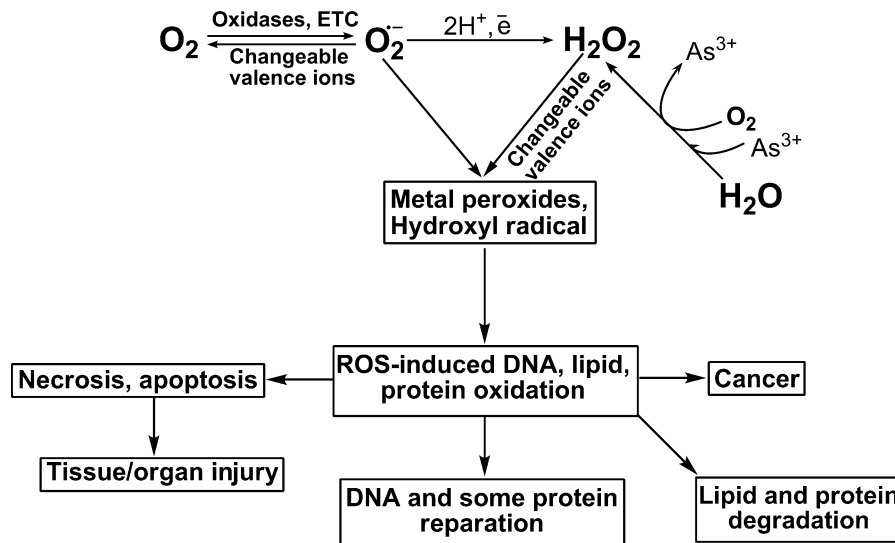
Fig. 3 Involvement of metals ions with changeable valence in the metabolism of free radicals in biological systems: generation, interconversion and functional consequences. Superoxide anion radical may be produced as a side-product of the electron transport chain and by some oxidases and can be further processed either by oxidization to molecular oxygen by the donation of one electron or reduced by accepting one electron and two protons to give hydrogen peroxide. Hydrogen peroxide may be also produced by some oxidases, as well as from the oxidation of arsenic (III) to arsenic (V) in the presence of oxygen. The net result of metal ion catalyzed processing of superoxide and hydrogen peroxide (Fenton reaction) leads to the formation of highly reactive hydroxyl radicals that along with singlet oxygen and peroxides of metals may interact with virtually any constituents of living organisms. Oxidative damage to DNA, proteins and lipids can have many negative effects on metabolism, causing cancer and other diseases, and frequently leading to apoptosis or necrosis. Systems for the repair of damaged DNA are well characterized and are critically important for cell survival. However, most ROS-modified proteins or lipids are degraded

20 µM ferrous sulfate. This treatment did not affect glucose-6-phosphate dehydrogenase activity in liver, but decreased it in kidney of the animals exposed to 20 µM of ferrous ions. A strong positive correlation between levels of lipid peroxidation products and the activities of catalase in the liver, and glutathione reductase in kidney indicated possible up-regulation of the enzymes by these products. Coordinated response of antioxidant systems to mild oxidative stress induced by high concentrations of environmental iron ions in goldfish tissues was proposed (Bagnyukova et al. 2006).

Molecular biology approaches provide substantial insight into our understanding of the mechanisms responsible for the protection of organisms against ROS promoted by many contaminants including iron. In a recent study, the molecular properties and expression of the ferritin M-like subunit (RbFerM) were described for both pathogen- and mitogen-stimulated rock bream (*Oplegnathus fasciatus*) (Elvitigala et al. 2013). RbFerM showed features similar to both mammalian ferritin subunits, H and L. Interestingly, fish exposure to lipopolysaccharides (LPS) from *Edwardsiella tarda and Streptococcus iniae*, as well as rock bream irido virus (RBIV) significantly enhanced transcription of RbFerM in liver. The purified recombinant RbFerM protein possessed detectable iron chelating activity in a temperature-sensitive manner that was probably responsible for protection against DNA damage induced by Fe^{2+} and H_2O_2 (Elvitigala et al. 2013).

One may conclude that despite iron abundance in the environment, its toxicity for fish (mediated at least partially via Fenton chemistry) is limited due to three mechanisms: (1) fast autoxidation of Fe^{2+} to Fe^{3+} and its subsequent precipitation thereby limiting iron bioavailability; (2) the presence of efficient antioxidant mechanisms preventing development of negative effects; and (3) the existence of mechanisms of iron sequestration in the tissues preventing its participation in ROS-mediated processes.

Copper Copper (*cuprum*) is an essential element for most living organisms. The possibility to exist in two valence states creates a Cu^{2+}/Cu^+ redox pair. This provides basis for the transfer of electrons in biological systems because of the high redox potential of this pair. Cupric ions (Cu^{2+}) in the presence of biological reductants such as GSH or ascorbic acid can be reduced to cuprous ion (Cu^+), which is able to catalyze the formation of HO˙ during the decomposition of hydrogen peroxide via the Fenton reaction (1).

Several studies have examined the effects of fish exposure to copper ions administered in either water-borne, injected or dietary formats. Injection of gilthead seabream (*Sparus aurata*) with $CuCl_2$ increased concentrations of TBARS (Pedrajas et al. 1995). The treatment also enhanced SOD activity and resulted in appearance of two new Cu,Zn-SOD isoforms. Similar to the results in vivo, the new SOD isoforms were also generated in vitro by incubation of cell-free extracts with systems that generated superoxide anion or hydrogen peroxide. These data were interpreted as showing that the new SOD isoforms were generated due to copper-mediated oxidative modification of the original SOD forms (Pedrajas et al. 1995). The results lead to the conclusion that in gilthead seabream tissues copper injection induced oxidative stress resulting in oxidative modification of both lipids and proteins.

A 40-day exposure of goldfish *C. auratus* to copper ions at different concentrations in the water altered the activities of antioxidant enzymes: The activities of catalase (at 0.005–0.025 mg/l exposure) and selenium-dependent glutathione peroxidase (at 0.05–0.05 mg/l) were reduced whereas glutathione-5-transferase activity increased (at 0.0025–0.01 mg/l) (Liu et al. 2006). The modification of the activities of these antioxidant enzymes implies that copper ions induced oxidative stress in goldfish tissues. In a warm water fish, African walking catfish (*C. gariepinus*), dietary copper exposure elevated copper concentrations in the intestine, liver and gills in tissue-specific manner (Hoyle et al. 2007). This treatment also significantly increased TBARS concentrations in gills and intestine, and total glutathione content in intestine doubled. The liver also showed glycogen depletion consistent with reduced food intake, but no pathological changes were found in the gills, liver or intestine (Hoyle et al. 2007).

Hansen et al. (2006a, b) investigated the effects of water contamination on the activities and steady-state concentrations of mRNA of certain antioxidant enzymes and their activities in brown trout tissues. The authors concluded that (i) fish exposure to metal ions, particularly to copper, enhanced the activities/levels of primary (SOD, catalase, glutathione peroxidase) and secondary (glutathione reductase, metallothionein) antioxidant enzymes/proteins; (ii) mRNA levels did not always correlate with respective protein levels, and (iii) metallothioneins were not necessary up-regulated by the addition of metal ions like copper.

Copper is a component of several antioxidant systems due to which its deficiency may also lead to oxidative stress. For example, copper ions are integral parts of cytochrome oxidase, ETC carriers and Cu,Zn-containing SOD. In such situations, copper ions are clearly antioxidants. However, either reduced uptake or disrupted cellular metabolism of copper could theoretically increase the steady-state ROS concentration due to copper ion redox cycling. Elevation of copper concentration can result in saturation of the cell with this micronutrient and excessive amounts may be damaging. Therefore, the concentration of free intracellular copper is typically held at a low level. When increased copper level saturates the intracellular all possible binding sites, new molecules possessing binding sites are produced to lower the concentrations of free copper ions. Metallothioneins and chaperones play a crucial role in binding copper when the ions are in excess. Under these conditions, the production and degradation of ROS are well balanced and only negligible (basal) oxidative stress takes place (Lushchak 2014a, b). The situation is substantially changed when the cell is not able to bind all of the excess copper ions. In this case, copper can catalyze the Fenton reaction resulting in enhanced generation of hydroxyl radicals.

It should also be mentioned here that the bioavailability of copper ions may be affected by certain compounds. For example, as will be described below in the section on pesticides, several studies of the effects of thiocarbamates on fish indicate that they can induce oxidative stress via extraction of copper ions from active sites of Cu,Zn-SOD (Lushchak et al. 2007). This example clearly demonstrates that only a systematic approach, i.e., the analysis of plural processes induced by copper as well as other effectors, may give a broad picture of fish responses to environmental challenges.

The environmental relevance of model experiments is always under question. Therefore, recent work from a Portuguese group is especially valuable (Nunes et al.

2015). They studied effects of short- and long-term, i.e., acute and chronic, exposure to copper ions at ecologically relevant concentrations on ROS-related processes in gills and liver of eastern mosquitofish (*Gambusia holbrooki*). They modeled conditions close to actual conditions of exposure in the wild. The biomarkers tested clearly demonstrated that both acute and chronic challenges perturbed ROS-related processes resulting in oxidative stress (Nunes et al. 2015). Since copper ions exist in the environment as cations, ion composition (particularly salinity) is an important factor which can affect its toxicity. For example, the intensity of oxidative stress induced by exposure of the estuarine guppy (*Poecilia vivipara*) to copper at environmentally relevant concentrations (5, 9 and 20 μg l^{-1}) showed concentration-dependent induction of oxidative stress (Machado et al. 2013). The data on the effects of changing salinity on the induction of oxidative stress by copper ions are contradictory and may depend upon species tested. With zebra fish (*Danio rerio*), Craig et al. (2007) clearly found protective effects of increased waterborne Ca^{2+} and Na^+ on acute copper toxicity which was related to attenuation of oxidative damage in this model tropical species. However, although the results with adult killifish (*Fundulus heteroclitus*) potentially demonstrated the possibility that increased salinity could decrease the intensity of oxidative stress, the signatures were not absolutely convincing (Ransberry et al. 2015). Since salinity and copper load were generally inversely related in fish bodies, one might suggest that protective effects of inorganic cations could be connected with competition between copper and other ions for entrance routes.

Recent work sheds light on the molecular mechanisms underlying copper toxicity (Sappal et al. 2014). In vitro experiments with mitochondria isolated from rainbow trout (*O. mykiss*) demonstrated that maximum respiration was monotonically inhibited by copper exposure whereas copper ions at low and high concentrations stimulated and inhibited the basal respiration and proton leak, respectively. Copper ions also inhibited complex I and dissipated mitochondrial membrane potential (MMP), whereas antioxidants N-acetyl cysteine and vitamin E partially prevented deleterious copper effects. The latter suggests ROS involvement in these responses. It was concluded that copper ions impaired oxidative phosphorylation in part by inhibiting the ETC, stimulating proton leak,

inducing the mitochondrial permeability transition pore (MPTP) and dissipating MMP (Sappal et al. 2014).

Interestingly, exposure of *C. auratus* to copper ions induced oxidative stress as expected and also increased the level of metallothioneins, but the fish taken from polluted areas demonstrated weaker responses as compared to those seen in an area where the water was more pure (Falfushynska et al. 2011). This effect was not due to compromised antioxidant responses in fish from polluted waters, but rather was traced to significantly higher initial levels of antioxidants in fish from the polluted area compared with the more pure area. This work, directly shows how important it is to be careful in the selection of experimental animals and that depuration and/or acclimation to laboratory conditions may be a required in some cases before experiments begin in order to be able to correctly interpret the results. Again, if we want to approximate experimental conditions to environmental ones, pollution of the places of fish origin should be taken into consideration.

Interspecies peculiarities of fish exposure to copper ions were analyzed by a Belgian team (Eyckmans et al. 2011). They found that some fish species rely more on glutathione as a first line of defense against exposure to this metal, whereas others rely more on metallothionein in combination with antioxidant enzymes.

Finally, the question of potential regulatory pathways which may attenuate Cu-induced oxidative damage is important in order to identify molecular targets that could be manipulated to reduce the deleterious effects of fish exposure to this ion. In recent years, an involvement of several signaling pathways in the response to copper exposure was identified. Firstly, although copper stimulated adaptive increases in the expression of some antioxidant enzyme genes via the Nrf2/ARE signaling pathway in goldfish brain, the treatment also induced oxidation, inhibition and depletion of most antioxidant enzymes and GSH content due to enhanced ROS production (Jiang et al. 2014). Secondly, in gill of young grass carp (*Ctenopharyngodon idella*) NF-*k*B, TOR and Nrf2 signaling pathways were found to be involved in responses to copper exposure (Wang et al. 2015). Finally, within muscle of Jian carp (*C. carpio* var. jian), copper exposure was found to increase ROS levels associated with toxicity (Jiang et al. 2015). This was connected with decreased antioxidant enzyme

activities probably via down-regulation of the expression of genes related to the disruption of the Nrf2/ARE signaling. The latter could be partially caused by caspase-3-regulated DNA fragmentation (Jiang et al. 2015). So, the last three papers clearly agreed that the Nrf2/Keap1 system was involved in response of fish to copper exposure, but other signaling systems may be involved also.

Chromium Chromium (*chromium*) occurs in the workplace and the environment predominantly in two valence states: hexavalent Cr^{6+} and trivalent Cr^{3+}. Hexavalent chromium compounds are used widely in diverse industries, and trivalent chromium salts, for example, chromium picolinate, chromium chloride and niacin-bound chromium are used as micronutrients and dietary supplements. Like other metal ions, chromium may be toxic and is not biodegradable, remaining in ecosystems and only changing forms (Kubrak and Lushchak 2011). The direct or indirect bioaccumulation of chromium in organisms exposed to contaminated waters may be significant, and therefore, it can affect not only individual organisms, but also all ecosystems.

Two aspects should be noted: (i) cellular chromium reduction is needed for HO· generation and (ii) chromium may play a role as a catalyst by being able to enter reversible oxidation. Therefore, it is commonly accepted that biological effects of chromium, at least partially, are connected with ROS generation.

Chromium has both beneficial and deleterious effects in organisms being an essential trace element involved in the regulation of a broad array of biological processes, particularly in glucose metabolism. An insulin-mimetic effect of chromium has been demonstrated in mammals and *Drosophila* (Hua et al. 2012; Perkhulyn et al. 2015), but direct studies with fish are not available. In experiments with guppies (*P. reticulata*) Perez-Benito (2006) found that Cr^{6+} at low concentrations ($<10^{-4}$ M) extended maximum life span in both males and females. The toxic effects of chromate were substantially decreased when it was used in combination with the antioxidant D-mannitol which could reflect ROS involvement in the mechanisms of chromium action (Perez-Benito 2006).

Potassium dichromate clearly induced oxidative stress in gills and kidney of the European eel (*A. anguilla* L.) (Ahmad et al. 2006). In gills, dichromate

at concentration 1 mM did not affect catalase and glutathione-*S*-transferase activities, but increased glutathione peroxidase activity and decreased GSH concentrations. In kidney, lipid peroxidation was stimulated, but no other signatures of chromate toxicity were found in this tissue. DNA integrity, evaluated as DNA strand breaks, was higher in both tissues of dichromate-treated animals (Ahmad et al. 2006).

Activation of free radical processes under fish exposure to chromium compounds may change many physiological and metabolic parameters. For example, chromium exposure activated lipid peroxidation in tissues of Chinook salmon (*O. tshawytscha*) and high chromium concentrations significantly impaired fish health (Farag et al. 2006). Kidney was the target organ during chromium exposure: It showed gross and microscopic lesions (e.g., necrosis of cells lining kidney tubules) and lipid peroxidation products were elevated. These changes were associated with increased chromium concentrations in the kidney, and reduced fish growth and survival. Chromium accumulation was proposed to stimulate lipid peroxidation and DNA damage resulting in cell death and tissue damage (Farag et al. 2006).

Another approach to evaluate ROS-induced DNA damage by chromium was used by Kuykendall et al. (2006). They studied the formation of DNA–protein cross-links (DPXs) in erythrocytes of largemouth bass (*Microproterus salmonoides*), and fathead minnows (*Pimephales promelas*) exposed to waterborne and dietary hexavalent chromium. Exposure of fathead minnows to 2 mg/l Cr^{6+} led to significant DPX formation in erythrocytes, with 140–200 % increases above background occurring in just 3–4 days. Similar exposure of largemouth bass to chromium led to a 62 % increase in DPX levels after 4 days of exposure. Furthermore, when largemouth bass were fed a diet of minnows injected with 20 mM Cr^{6+} for 5 days, a significant increase in DPXs in bass erythrocytes was observed. Therefore, it was concluded that both waterborne and high-dose dietary exposure to Cr^{6+} could result in DPX formation in erythrocytes of predatory fish species such as bass (Kuykendall et al. 2006). Genotoxicity of hexavalent chromium and induction of oxidative stress was recently confirmed with common carp (*C. carpio*) under in vivo exposure (Kumar et al. 2013). Genotoxicity of chromium was also found with goldfish where kidney appeared to be

more vulnerable and sensitive to Cr-induced toxicity than the liver (Velma and Tchounwou 2013).

The effect of Cr^{6+} and Cr^{3+} on ROS-related processes in goldfish and other fish were summarized in detail in recently published work from my laboratory (Lushchak et al. 2008, 2009a, b; Kubrak et al. 2010; Vasylkiv et al. 2010; Kubrak and Lushchak 2011). In general, our data showing Cr^{6+}-induced oxidative stress in goldfish tissues, such as statistically significant increases in the activities of SOD, GPx, along metallothionein (MT) expression, were independently supported by other authors (Velma and Tchounwou 2013). Interested readers can find more details about Cr^{6+} action in fish in these publications and those cited therein. Here, we will focus mainly on a comparison of the effects of both chromium ions on some aspects of free radical metabolism in goldfish tissues. Being an element with changeable valence, chromium undergoes redox transformations in organisms and is involved in Haber–Weiss reactions resulting in the formation of hydroxyl radicals, which along with other ROS has broad damaging biological effects (Shi and Dalal 1990). The toxic effects of chromium are widely believed to be associated with the stimulation of free radical processes (Valko et al. 2005; Ahmad et al. 2006; Lushchak 2008, 2011b; Kubrak and Lushchak 2011). However, other effects are known, such as disruption of metal ion-regulated processes and interference with the regulation of metabolism, particularly carbohydrate metabolism (Opperman et al. 2008; Stout et al. 2009). It is interesting to note that fish mucus can reduce Cr^{6+} and Cr^{3+}, and this mechanism is implicated in the detoxification of Cr^{6+} by fish skin (Arillo and Melodia 1990). Because the glutathione system actively responded to goldfish exposure to Cr^{6+}, we suggested that glutathione could be involved in its detoxification (Lushchak et al. 2008; Lushchak 2011b, 2012).

Electron paramagnetic resonance (EPR) was used to monitor the conversion of Cr^{6+} in liver of the European eel (A. anguilla) (Pacheco et al. 2013). This showed that in fish tissue Cr^{6+} was reduced to Cr^{3+}, but a substantial amount of Cr^{5+} was also found. Despite induction of antioxidant defenses under exposure to Cr^{6+}, the oxidative deterioration of lipids was not prevented. The authors proposed that Cr^{5+}, as a short-lived species, was not directly or primarily responsible for the cellular damage observed. In my opinion, reversible shuttling between different oxidative states drives Fenton chemistry with concomitant ROS generation resulting in oxidative damage to cellular constituents.

In most cases, it is appropriate to study the effects of in vivo exposure to environmentally relevant concentrations of toxic compounds, but in some cases in vitro studies with tissue extracts may potentially produce valuable insights. Work carried out by Czech researchers who exposed rainbow trout (O. mykiss) to Cr^{6+} both in vitro and in vivo (Li et al. 2011) showed that in vitro tests with tissue homogenates were more sensitive than in vivo ones for detecting and evaluating toxic effects. In reality the best result may be reached by the wise combination of two mentioned approaches.

Prevention of ROS-induced damage to tissues is a very important goal in many investigations. Up-regulation of genes encoding antioxidant enzymes was shown to be a potential mechanism for augmentation of antioxidant potential in response to Cr^{6+} exposure of juvenile C. auratus (Li et al. 2013a). In some cases, application of individual compounds or their mixtures may shed light on the mechanisms that increase tolerance. For example, in their work Karaytug et al. (2014) sought to determine whether such antioxidants as taurine (TAU), alpha-lipoic acid (LA), curcumin (CUR) or N-acetylcysteine (NAC) could provide in vivo protection to liver and kidney tissues of common carp (C. carpio carpio L.) against chromium-induced toxicity. These data can be explained by the induction of weak oxidative stress which, due to its definition, is associated with an increase in the activity of first-line antioxidant enzymes (Lushchak 2014a, b). For example, curcumin could stimulate the Nrf2/Keap1 signaling pathway to augment expression of ARE-regulated genes including those encoding antioxidant enzymes (Lushchak 2011a, b; García-Niño and Pedraza-Chaverrí 2014). N-acetylcysteine was a very effective antioxidant agent, particularly probably owing to its metal-reducing activity as well as its effects on GSH redox status (Karaytug et al. 2014). We believe that NAC might be the most efficient because it may be a precursor for biosynthesis of the most powerful cellular antioxidant, glutathione (Lushchak 2012). Finally, simultaneous administration of propolis and Cr^{6+} ameliorated the functional state of C. carpio parameters (Yonar et al. 2014), suggesting that propolis might alleviate chromium-induced oxidative stress. Propolis is a very

complex and not standardized mixture from the sap on conifer trees that is collected by honey bees and further combined with their own excretions and beeswax to create a coating used to build their hives. Due to its poorly defined composition, there are difficulties in coming to general conclusions about its health benefits and the mechanisms involved. It is possible that certain polyphenols in propolis may act similarly to curcumin to stimulate the Nrf2/Keap1 signaling pathway to up-regulate expression of antioxidant enzymes and in this way counteract the deleterious effects of chromium-induced oxidative stress.

Mercury Mercury (*hydrargyrum*) exists as a cation with an oxidation state +1 (mercurous) or +2 (mercuric). In the environment, mercury may be found in the methylmercury form, produced mainly as the result of methylation of inorganic (mercuric) forms by microorganisms in soil and water (Valko et al. 2007). Environmental mercury is ubiquitous and consequently it is practically impossible to avoid exposure to it. Elemental inorganic and organic mercury forms exhibit neurotoxicity, nephrotoxicity and gastrointestinal toxicity with ulceration and hemorrhage. The biological effects of inorganic or organic mercury are related to their interactions with sulfhydryl-containing residues (Rooney 2007). Mercuric conjugates of cysteine and glutathione are transportable species at the site of the organic anion transporter.

Like other animals, fish may accumulate high levels of mercury in their tissues (Salonen et al. 1995; Guallar et al. 2002). High intake of mercury by freshwater fish and the subsequent accumulation of mercury in the bodies of humans that eat the fish are associated with increased risk of acute myocardial infarction and coronary heart disease. Therefore, it should be noted that although the usage of fish oil-derived fatty acids to reduce the risk of acute coronary events has a rational basis, high mercury content in fish could attenuate this protective effect (Valko et al. 2007).

Most studies of mercury effects on fish are related to regional brain pathology and behavior changes (Baatrup 1991; Ribeiro et al. 1995). In experiments with Atlantic salmon (*Salmo salar* L. parr) exposed for 4 months to mercuric chloride, methyl mercury accumulated significantly in brain, but did not cause mortality or growth reduction (Berntssen et al. 2003).

However, it resulted in the significant increase in the levels of lipid peroxidation products (evaluated as TBARS) and decrease in the activities of SOD and glutathione peroxidase. Mercury at high concentrations induced pathological damage (vacuolation and necrosis) and decreased monoamine oxidase activity. Compared with other organs, brain was particularly susceptible to dietary mercury, whereas kidney and liver were less sensitive. It should be also noted that low dietary concentrations of mercury induced protective redox defenses in the brain as evidenced by the induction of antioxidant enzyme SOD (Berntssen et al. 2003). Neurotoxic effects of methyl mercury (MeHg) on zebra fish embryos were recently confirmed by Ho et al. (2013). They proposed that MeHg disturbed the function of the CNS by disrupting cellular homeostasis. The disturbances were at least partially associated with induction of oxidative stress and affected the expression of genes that are involved in normal neuronal activity and learning.

Chronic exposure of medaka (*Oryzias melastigma*) to mercuric chloride (HgCl$_2$) at different concentrations for 60 days was analyzed by two-dimensional difference gel electrophoresis (2D-DIGE) of liver preparations (Wang et al. 2013). The study identified 33 proteins that were mainly involved in cytoskeleton assembly, oxidative stress response and energy production. Since several proteins with altered profiles were related to mitochondrial function (e.g., respiratory metabolism) in the treated hepatocytes, it was suggested that this organelle might be the primary target for mercury attack in the cells. Hepatotoxicity was proposed to be among the leading toxic mechanisms of mercury on fish and other aquatic organisms (Wang et al. 2013).

To further study mercury effects, a freshwater top predator, the wolf fish (*Hoplias malabaricus*) was fed with mercury-contaminated live juvenile matrinxã (*Brycon amazonicus*), as a prey vehicle (Monteiro et al. 2013). Inorganic mercury acquired from the prey fish enhanced the activities of superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase and glutathione reductase in wolf fish. Levels of reduced and oxidized glutathione also changed with a concomitant decrease in [GSH]/[GSSG] ratio, and levels of ROS-modified lipids and proteins also increased. Mercury accumulation was tissue-specific and to large extent was related to the intensity of oxidative stress.

The potential involvement of purinergic receptors, P2X7R, in mercury chloride toxicity was identified in experiments with zebra fish (*D. rerio*) larvae (Cruz et al. 2013). Induction of oxidative stress was modulated by ATP and an antagonist of these receptors, A740003. In addition, quantitative real-time PCR showed a decrease in P2X7R expression in larvae treated with HgCl$_2$, but general mechanisms responsible for the mercury toxicity were not only related to inhibition of P2X7R activation. Interestingly, mercury effects may be realized not only directly, but via induction of oxidative stress leading to injury and cell death, ATP release and consequently activation of the receptor.

Very few studies have examined potential protection mechanisms against mercury toxicity. Using Nile tilapia (*Oreochromis niloticus*), Elseady and Zahran (2013) investigated the effects of the antioxidant β-carotene on the antioxidant response and haematological parameters of mercury-treated fish. Interestingly, β-carotene was found to be useful as an immunostimulant and alleviated the immune-depressive effects under fish exposure to mercury.

Arsenic The most common oxidation numbers of metalloid arsenic (*arsenicum*) are +5, +3 and −3. It can form both inorganic and organic compounds in the environment and within cells. The oxidation of As^{3+} to As^{5+} under physiological conditions results in H$_2$O$_2$ formation (Valko et al. 2005; Jomova et al. 2011) (Fig. 3) by the following reaction:

$$H_3AsO_3 + H_2O + O_2 \rightarrow H_3AsO_4 + H_2O_2 \qquad (4)$$
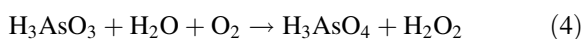
Therefore, arsenic compounds generating both ROS and RNS may be involved in oxidative processes, particularly oxidation of lipids, DNA and proteins.

Initial toxicological studies of different arsenic forms focused mainly on mammals, while fish studies were relatively rare. For example, arsenite significantly increased the concentrations of oxidized glutathione (GSSG) and TBARS in liver of rats, providing clear signatures of oxidative stress development (Kalia et al. 2007). In both whole fish and cell cultures, arsenic could also induce apoptosis (Wang et al. 2004; Datta et al. 2007). In two cell lines, TF (fin cells of *Therapon jarbua*) and TO-2 cells (ovary cells of tilapia), treatment with sodium arsenite resulted in time- and concentration-dependent cell death in a cell line-specific manner. DNA fragmentation analysis and flow cytometric analysis of cell cycle progression demonstrated that cells were mostly killed via apoptosis. Since antioxidants, *N*-acetyl-cysteine (NAC) and dithiothreitol (DTT), significantly prevented apoptosis in TF cells, ROS were suggested to be involved in the arsenite-induced apoptotic cell death (Wang et al. 2004). Arsenite also induced oxidative stress and apoptosis along with heat shock protein 70 (HSP70), a chaperone protein, in a zebra fish liver cell line and pretreatment with NAC or DTT before arsenite insult effectively protected cells (Seok et al. 2007). Schlenk et al. (1997) studied the effects of arsenite, arsenate and the herbicide monosodium methyl arsenate on hepatic metalloprotein expression and lipid peroxidation in channel catfish. They found a dose-dependent increase in metallothionein levels, whereas hepatic lipid peroxidation and glutathione concentrations were unaltered by any of the arsenical treatments. This demonstrates the lack of correlation between metallothionein expression and oxidative stress.

We also investigated the effects of arsenate treatment (10–200 μM) on the levels of ROS-modified proteins and lipids and the activities of antioxidant enzymes in goldfish liver (Bagnyukova et al. 2007). Treatment for 1–4 days did not affect the levels of TBARS and protein carbonyls, but increased the level of oxidized glutathione, total glutathione concentration and lipid peroxides, thereby clearly showing the development of oxidative stress. The activities of the primary antioxidant enzymes—SOD, catalase and GPx—were also enhanced reflecting an antioxidant response. These data demonstrated development of weak oxidative stress under goldfish exposure to arsenate (Lushchak 2014a, b).

Short-term acute exposure of the common carp (*C. carpio*) to As^{3+}- and As^{5+}-induced oxidative stress in gills and liver and the effects were tissue-specific (Ventura-Lima et al. 2009). Juvenile rockfish (*Sebastes schlegelii*) exposure to waterborne arsenic at different levels for 20 days increased the activities of antioxidant enzymes and glutathione concentrations, and the increase in these parameters correlated positively with the amounts of accumulated arsenite (Kim and Kang 2015). Neurotoxic effects related to induction of oxidative stress by arsenite compounds were also reported for zebra fish (de Castro et al. 2009; Sarkar et al. 2014). Neurological disturbances tightly correlated with increased levels of markers of

oxidative stress and the up-regulation of antioxidant enzymes. Moreover, enhanced mRNA levels of the transcription factor Nrf2 occurred in As_2O_3-exposed zebra fish indicating potential involvement of Nrf2 in the protective response under these conditions (Sarkar et al. 2014). It seems that Nrf2, a master regulator of adaptive and protective responses to insults inducing oxidative stress, is a key player in fish protection against environmental challenges. Xu et al. (2013) carried out a genome-wide analysis of liver gene responses to arsenic in zebra fish using RNA-SAGE (serial analysis of gene expression). Arsenic treatment disturbed specific processes: certain metabolic pathways, proteasome, oxidative phosphorylation, cancer, etc. These responses were coordinated mainly by Jun, Kras, APoE and Nrf2 transcription factors (Xu et al. 2013).

Taking into account the above effects of arsenic compounds on fish, it is easy to understand that this element may enter cellular metabolism and, in some cases, enhance ROS production. The latter can modify virtually all cellular constituents, including membranes. This may culminate in cell death via necrosis or apoptosis, and the discrimination between these death pathways depends on many circumstances.

Nickel Although nickel (*niccolum*) is thought to have no biological function in animals, its extensive use by industry leads to environmental pollution. The most common oxidation state of nickel is Ni^{2+}, but compounds of Ni^+ and Ni^{3+} are also well known. Due to its capacity to enter redox processes, nickel may take part in Fenton chemistry. This theoretical prediction was confirmed in experiments with salmon sperm DNA and pBluescript K^+ plasmid (Lloyd and Phillips 1999). DNA incubation with hydrogen peroxide and Ni^{2+} was found to result in site-specific formation of double-strand breaks and, to a lesser extent, 8-OHdG and putative intrastrand cross-links, whereas formation of single-strand breaks was proposed to result from the generation of hydroxyl radicals in solution.

Studies by my laboratory analyzed fish exposure to nickel. Goldfish exposed to Ni^{2+} concentrations of 10, 25 or 50 mg/l for 96 h showed decreased glycogen levels in white muscle and liver, but substantially increased blood glucose levels (Kubrak et al. 2012b). Ni^{2+} exposure induced very weak oxidative stress in these tissues, but gills (Kubrak et al. 2013a) and heart

and brain were efficiently protected against the development of Ni^{2+}-induced oxidative stress (Kubrak et al. 2014). Only kidney and spleen demonstrated induction of oxidative stress under goldfish exposure to waterborne Ni^{2+} (Kubrak et al. 2012c). Although in vitro studies showed that nickel had a good ability to enter Fenton reactions and damage DNA, and in vivo studies confirmed that goldfish can accumulate certain amounts of environmental nickel in their tissues, it appears that goldfish possess antioxidant systems efficient enough to prevent damage by this ion. These results were also supported by findings from Chinese researchers which showed that goldfish exposure to waterborne nickel induced apoptosis in liver via the JNK pathway (Zheng et al. 2014).

Interestingly, environmental cations partially decreased nickel accumulation in tissues of euryhaline killifish (*F. heteroclitus*) and, moreover, protected them and attenuated development of oxidative stress (Blewett and Wood 2015). Induction of oxidative stress by nickel ions in neotropical fish *Prochilodus lineatus* was accompanied by genotoxic effects (Palermo et al. 2015), whereas similar exposures of rainbow trout caused both oxidative stress and neurotoxic effects with visible histopathological changes (Topal et al. 2015).

Miscellaneous Several other transition metal ions not mentioned above may have selective biological significance and/or be toxic. Cobalt and vanadium must be mentioned here. These elements were identified in individual organisms and food chains, but information on their levels and effects in fish is scarce. For example, Soares et al. (2007) found that vanadium affected the levels of lipid peroxides and activities of certain antioxidant enzymes in heart of gilthead seabream (*S. aurata*). Vanadates were accumulated in mitochondria where they inhibited mitochondrial oxygen consumption, induced mitochondrial membrane depolarization and changed the redox steady state of complex III. Moreover, the effects depended on the form of vanadate used: oligomeric vanadium species were much more effective than monomeric. The authors concluded that mitochondria were toxicological targets for vanadate, but the net effects depended on vanadate oligomeric state (Soares et al. 2007). Clearly, any effect on mitochondrial operation might modify ROS metabolism. Zinc is another essential metal ion, but in

high concentrations it may be toxic for fish due to induction of oxidative stress (Loro et al. 2012).

In model experiments, the effects of individual metal ions are usually studied. However, in nature, animals are subjected to the combined effects of many factors, often making it very difficult to reveal the molecular mechanisms responsible for toxicity. Therefore, model experiments on the combined effects of selected environmental factors can be of help. Such an approach is exemplified by studies of the combined effects of heat stress and cadmium ions on the European bullhead *Cottus gobio* (Dorts et al. 2012). Fish exposed for 4 days to either 15 or 21 °C in the presence of 0.01 or 1 mg/l $CdCl_2$ showed no mortality in three of the temperature/Cd combinations, but fish exposed to 1 mg Cd/l at 21 °C for 4 days showed signs of toxicity indicating that temperature substantially influenced metal ion toxicity. Such exposures induced weak oxidative stress in liver and gills. The authors concluded that elevated temperature and cadmium exposure independently influenced the antioxidant defense system in bullhead with clear tissue-specific and stress-specific antioxidant responses (Dorts et al. 2012). Similarly, cellular thiol pools in rainbow trout responded differently to individual metals and metal mixtures, particularly cadmium and zinc (Lange et al. 2002).

In conclusion, in most cases, fish exposure to metal ions, either dietary or environmental, induces oxidative stress with similar effects. These are typically increased levels of ROS-modified cellular components (lipids, proteins, nucleic acids) and augmentation of antioxidant potential such as activities of antioxidant enzymes and levels of glutathione (Lushchak 2011b, 2012, 2014a). Although the exact molecular mechanisms responsible for enhancement of antioxidant potential are not fully established, it seems that up-regulation of the expression of certain genes is crucial. Several direct and a number of indirect indications show that such gene up-regulation may be coordinated by the Nrf2/Keap1 signaling system (Lushchak 2011a, 2012). Further studies of the effects of metal ions on fish are certainly needed.

Pesticides

Pesticides are physical, chemical or biological agents intended to kill undesirable plant or animal pests. Based on target organisms, they are divided for three major classes: insecticides, herbicides and fungicides. Virtually all pesticides that are used industrially are synthetic agents, products of chemical synthesis; hence, they are xenobiotics with respect to the environment and its organisms, including humans. This creates diverse problems for nontarget organisms, and therefore, their effects on biological systems are poorly predictable. In fact, it is impossible to classify pesticides systematically because of their great chemical diversity due not only to the wide variety of effective pesticide compounds, but also to the hundreds of different formulations on the market, many of which contain additional compounds used to improve their efficacy. Hence, the discussion below will classify pesticides based on their target organism, even though many of them may kill several different groups of organisms. Since this paper is devoted to fish, naturally the focus is on this animal group, keeping in mind that fish are rarely, if ever, the target organisms for pesticides.

Insecticides This group includes not only insecticides, but also acaricides, and they may be effective for other types of arthropods as well. Here, we will mainly concentrate on organophosphate pesticides (OP) used to control insects on crops, in the household or on stored products, and treat external parasitic infections of livestock, domestic animals and farmed fish. For example, dichlorvos has been used extensively to treat sea lice infestations by the copepod parasites *Lepeophtheirus salmonis* and *Caligus elongatus* on Atlantic salmon (*S. salar*) in aquaculture (Bron et al. 2006).

Several studies have described induction of oxidative stress in fish under treatment with OPs. For example, dichlorvos was found to induce oxidative stress in carp (*C. carpio*) and catfish (*Ictalurus nebulosus*) (Hai et al. 1997a), as well as in European eels (*A. anguilla*) (Peca-Llopis et al. 2003a). Other OPs have been shown to cause oxidative stress including trichlorfon in Nile tilapia (Thomaz et al. 2009), Folisuper 600 BR (methyl parathion) in freshwater characid fish matrinxã (*B. cephalus*) (Monteiro et al. 2009), fenthion in *Oreochromis niloticus* (Piner et al. 2007), and malathion in gilthead seabream (Rosety et al. 2005).

The mode of action of organophosphate insecticides on target organisms is well studied (Fig. 4, right side), but information on their effects on nontarget
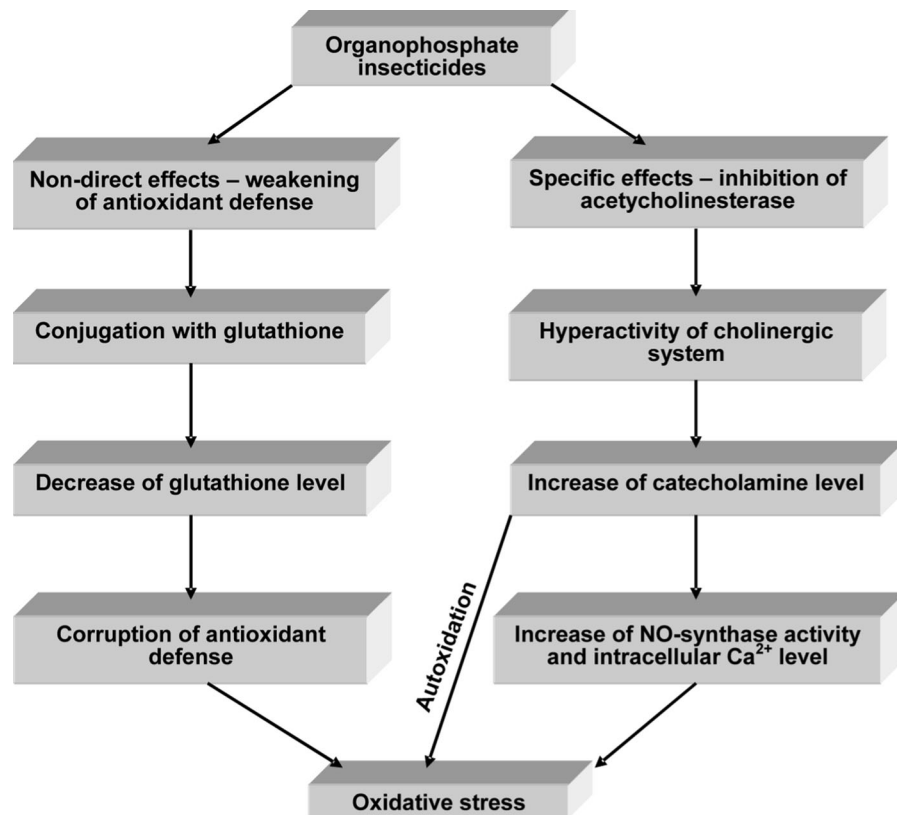
Fig. 4 Potential pathways leading to induction of oxidative stress by organophosphate insecticides (OPs). These pesticides may affect ROS-related processes at least in two ways. The first one is a non-specific effect related to a decrease in antioxidant potential, whereas the specific effect is connected with inhibition of acetyl cholinesterase (AChE). Inhibition of AChE, which is responsible for termination of the transmission of nerve impulses, is a primary effect of OPs in both invertebrates and vertebrates. OPs block the hydrolysis of the neurotransmitter acetylcholine (ACh) at both central and peripheral neuronal synapses, enhancing ACh levels and activating ACh receptors. The resulting overstimulation of cholinergic neurons initiates a process of hyperexcitation and convulsive activity that progresses rapidly to *status epilecticus*, leading to profound structural brain damage, respiratory distress, coma and ultimately the death of the organism (Shih and McDonough 1997). The toxic effects of OPs are believed to be largely due to the hyperactivity of the cholinergic system as a result of increased ACh levels at the synaptic cleft. OPs can also affect other neurotransmitter systems that become progressively more disrupted, releasing initially catecholamines and afterward excitatory amino acids (EAAs), such as glutamate and aspartate, which prolongs the convulsive activity. The break in neuronal and endocrine signaling leads to increased influx of Ca^{2+} into cells, triggering the activation of proteolytic enzymes, nitric oxide synthase and enhanced ROS generation (Beal 1995) resulting in oxidative stress. Since the metabolism of OPs (particularly, dichlorvos) in liver is connected with glutathione consumption, this may also initiate oxidative stress. Glutathione consumption decreases its concentration, thereby reducing the efficiency of the antioxidant system and disrupting the balance between ROS generation and elimination leading to oxidative stress

organisms is limited although potentially they can affect fish and other animals in similar ways to arthropods. However, there is a growing concern about their application especially when this is poorly controlled. Discrepancies between the information provided by OP producers and results of certain independent studies have led the call for more detailed investigations. There is a real need for studies to elucidate the mechanisms of OP action and consequences of long-term application on nontarget organisms. With regard to fish, this often involves pesticide runoff from agricultural fields into nearby bodies of water. Indirect effects of OPs and other similar pesticides are depicted at Fig. 4 (left side).

The mechanism of action of another insecticide group, chlorinated hydrocarbons such as lindane and

DDT (1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane), clearly include induction of oxidative stress as has been shown with cell lines from a skin tumor of carp *C. carpio* (Ruiz-Leal and George 2004) and hepatocytes from *H. malabaricus* (Filipak Neto et al. 2008). The mechanisms responsible for oxidative stress induction may be related to ROS generation at their metabolic activation along with P450 enzyme induction by DDT (Harada et al. 2003). Catabolism of these xenobiotics includes metabolic activation and products of such transformation along with formed intermediates, and initial compounds like DDT may be responsible for enhanced ROS production (as in Fig. 4). In concert, these compounds can be key factors in hepatocarcinogenesis and may play an important role in tumor promotion and progression (malignant transformation).

Herbicides Although there are many kinds of herbicides, the present discussion will focus on the effects of two well-known groups of herbicides: (1) those that are directly responsible for the enhancement of ROS generation, such as paraquat (N,N'-dimethyl-4,4′-bipyridinium dichloride), and that enter redox cycles accompanied by ROS generation via autoxidation, and (2) those that inactivate antioxidant enzymes like aminotriazole and dithiocarbamates (Fig. 5).

The mode of action of paraquat appears to be well established (Fig. 5). It is capable of entering reversible redox cycles and donating electrons to molecular oxygen giving rise to ROS. Paraquat (also known as viologen) was found to induce oxidative stress in *Channa punctata* (Parvez and Raisuddin 2006), zebra fish (Bretaud et al. 2004) and rainbow trout (Stephensen et al. 2002). Its close relative, diquat, also stimulated ROS production in fish, for example, in an established carp cell line (Wright et al. 2000) and in rainbow trout (*O. mykiss*) tissues (Hook et al. 2006). We proposed that the cyclic chlororganic herbicide 2,4-dichlorophenoxyacetate (2,4-D), that is chemically similar to paraquat and diquat, could affect fish similarly. Indeed, goldfish exposure to 2,4-D induced oxidative stress in all tissues studied, but with different results. A first analysis of vital biomarkers in blood showed diverse effects of 2,4-D exposure such as alterations in leukocyte types and numbers, increased alanine transaminase activity, and enhanced levels of ROS-oxidized proteins (Kubrak et al. 2013b).
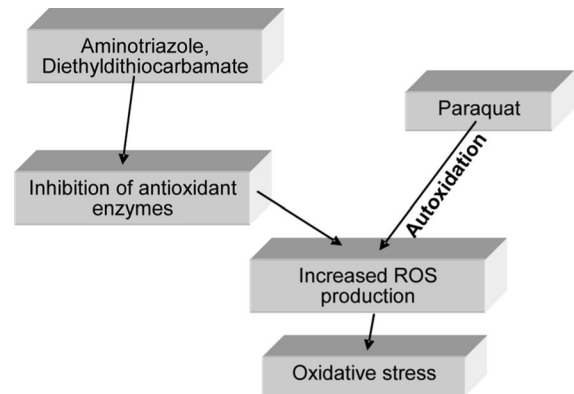

Fig. 5 Two suggested mechanisms of herbicide-induced oxidative stress. Herbicides of the first group, such as paraquat, are capable of entering redox cycles in living organisms. This can enhance ROS levels and cause oxidative stress. The second group of herbicides does not directly enter redox cycles. Rather they compromise the antioxidant system. For example, aminotriazole interacts with the active center of catalase and inactivates the enzyme. Similarly, diethyldithiocarbamate inactivates Cu,Zn-SOD via removing copper ions from the active site of the enzyme. Inactivation of both these enzymes enhances ROS steady-state level and if antioxidant potential is overwhelmed, oxidative stress may develop

Interestingly, a 96-h recovery period largely restored these parameters to their initial levels. The release of alanine transaminase into blood indicated that fish treatment with 2,4-D could damage liver and/or heart, and therefore, subsequent studies analyzed 2,4-D effects on specific tissues. We found development of oxidative stress in muscle, liver, brain and kidney and its intensity was tissue specific. In white muscle and gills, only weak oxidative stress was indicated (Atamaniuk et al. 2013; Kubrak et al. 2013c). Similarly mild oxidative stress was observed in goldfish brain and heart, whereas moderate oxidative stress occurred in liver and kidney in response to 2,4-D exposure of fish (Matviishyn et al. 2014). Again, given 96 h recovery in herbicide-free water, virtually all parameters either returned to their initial states or showed a clear trend to do so. Our systemic experiments on 2,4-D effects on goldfish showed several important things: (i) vital markers measured in blood may be used to predict whether herbicide treatment induces oxidative stress and/or damage to tissues; (ii) liver and kidney are typically the tissues that are most sensitive to animal treatment with waterborne environmental contaminants; (iii) fish possess efficient systems to prevent oxidative damage to brain and heart; and (iv)

since gills are in continuous contact with environmental water they possess very efficient defense systems against environmental toxicants.

The second group of herbicides, which will be systematically analyzed here, consists of several classes of compounds known to be mainly inhibitors of antioxidant enzymes, such as SOD and catalase (Fig. 5). Firstly, Babich et al. (1993) demonstrated that diethyldithiocarbamate (DDC), an inhibitor of superoxide dismutase, slightly increased the sensitivity of bluegill sunfish BF-2 fibroblasts toward hydrogen peroxide and paraquat, which was supposed to result from reduced antioxidant defense. Later Hai et al. (1997b) showed that although DDC modified pro/antioxidant systems in tissues of common carp, it could also serve as an antioxidant due to the presence of thiol groups in its structure. Finally, work in my laboratory (Lushchak et al. 2007) clearly demonstrated that DDC injection induced oxidative stress in goldfish tissues. The mechanism believed to be responsible for the induction of oxidative stress was connected with extraction of copper ions from the active center of Cu,Zn-containing SOD. Such a mechanism was also described by us for another eukaryotic model, baker's yeast *Saccharomyces cerevisiae* (Lushchak et al. 2005c), which suggested the universality of mechanisms responsible for DDC toxicity.

Another thiocarbamate herbicide, molinate, also induced oxidative stress in the European eel *A. anguilla* (Peca-Llopis et al. 2001). Molinate is detoxified by sulfoxidation via the endoplasmic reticulum oxygenase system followed by conjugation with glutathione that may lead to depletion of glutathione pool. This effect on glutathione may be the cause of molinate-induced oxidative stress when animals are exposed to this herbicide. Uncoupling of the mitochondrial ETC was proposed to be other potential mechanism that can increase ROS production under these conditions (Peca-Llopis et al. 2001). However, in our opinion, uncoupling could also reduce ROS generation due to a reduction of the transmembrane electrochemical potential at the inner mitochondrial membrane; hence, the net effect would depend on the balance two factors that could potentially increase or decrease ROS production.

Aminotriazole (AMT, 3-amino-1,2,4-triazole), a heterocyclic organic compound, is a broadly used non-selective systemic herbicide. It is a competitive inhibitor of imidazoleglycerol phosphate dehydratase, but also it inhibits catalases via binding to the iron atom in the active site of catalase (Bayliak et al. 2008). In vivo exposure of adrenocortical cells of rainbow trout (*O. mykiss*) to aminotriazole (Dorval and Hontela 2003) or intraperitoneal injection of the herbicide into goldfish (*C. auratus*) (Bagnyukova et al. 2005a, b) were found to induce oxidative stress. The role of catalase inhibition by aminotriazole in the induction of oxidative stress was assessed by us also in yeast (Bayliak et al. 2008) and frogs *Rana ridibunda* and *R. esculenta* (Lushchak et al. 2003).

Other herbicides may also exert their biological activity via induction of oxidative stress. For example, glyphosate (N-phosphoromethyl glycine) is a non-selective herbicide that inhibits plant growth through inhibition of the enzyme enolpyruvylshikimate phosphate synthase that is involved in the biosynthesis of essential aromatic amino acids in plants. This enzyme is responsible for the biosynthesis in plants of chorismate, a precursor of phenylalanine, tyrosine and tryptophan. When we exposed goldfish to Roundup, a glyphosate-based herbicide, an induction of weak oxidative stress was found (Lushchak et al. 2009c). That supported previous data on the induction of oxidative stress by Roundup in other fish species including pava (*Leporinus obtusidens*) (Glusczak et al. 2006) and silver catfish (*Rhamdia quelen*) (Glusczak et al. 2007).

Recently, we carried out a set of experiments to evaluate the potential toxic effects of the metribuzin-containing herbicide, Sencor, and determine whether those are related to the induction of oxidative stress. Triazines (a six-membered ring containing three carbon and three nitrogen atoms) have been used extensively for weed control, and their herbicidal activity is believed to be mediated by the inhibition of photosynthesis. Goldfish exposure to Sencor resulted in enhanced haematocrit and altered counts of several classes of white blood cells and caused substantial histopathological changes in kidney and liver (Husak et al. 2014; Maksymiv et al. 2015). Based of this information showing tissue damage, we then checked to see whether that could result from ROS-induced process and showed that goldfish exposure to Sencor resulted in oxidative stress development which correlated with tissue injury.

In many cases, the herbicides found in bodies of water are not just individual compounds, but rather as

mixture of several ones. Therefore, it is very valuable to investigate their combined effects. Unfortunately, such studies are scarce. From this point of view, the work of a Belgian group provides important insight in this field (Fatima et al. 2007). For this reason, goldfish (*C. auratus*) were exposed to a mixture of herbicides, namely atrazine, simazine, diuron and isoproturon (ASDI), at a cumulative concentration 50 µg/l for 12 weeks. It was found that ASDI induced oxidative stress as evidenced by enhanced $O_2^{\cdot-}$ production in phagocytic cells of head kidney and spleen, and changes in the activities of antioxidant enzymes in spleen, kidney and liver. In addition, the authors evaluated impacts of goldfish exposure to ASDI on the operation of the goldfish immune system and clearly demonstrated that such treatment reduced resistance against pathogen invasion, although it enhanced lysozyme activity (Fatima et al. 2007). This study showed that although the simultaneous exposure of fish to a mixture of herbicides generally induced oxidative stress similar to the effects of the individual herbicides, the net effects might differ due to effects on selected fish cell/tissue systems via different mechanisms.

Since many herbicides affect target and nontarget organisms via induction of oxidative stress, one could expect that certain antioxidants protect nontarget organisms from deleterious herbicide effects. This was demonstrated when the antioxidant *N*-acetylcysteine was given to European eels (*A. anguilla*) exposed to the organophosphate dichlorvos: The antioxidant reduced toxicity of the pesticide probably due to increased antioxidant potential (Peca-Llopis et al. 2003b).

Fungicides Very few studies have been published on the induction of oxidative stress in fish by fungicides. Copper-containing compounds are traditionally used as fungicides, but because those were analyzed above, we will not deal with them in this section. Instead, the focus here is on induction of oxidative stress in fish by organic fungicides, although very little work is available on this topic. The chlorocarbon compound hexachlorobenzene (HCB), or perchlorobenzene, was found to induce oxidative stress in liver and brain of common carp (*C. carpio*) (Song et al. 2006). This compound is one of the side-products of some industrial technologies and was also used formerly as a fungicide to treat seeds, especially on wheat to control the fungal disease bunt. Due to the high stability of HCB in the environment and its high

toxicity to nontarget organisms, it has been banned globally under the Stockholm Convention on persistent organic pollutants. However, it is still one of the most widespread persistent organic pollutants, because it continues to be released into the environment as a by-product of several industrial processes and is frequently detected in inland waters with concentrations generally below 1 ng/l, but higher values have been reported in aquatic systems that receive industrial discharges and surface runoff. In sediments of Lake Ya-Er near a chemical plant in Hubei province, China, the reported concentrations of HCB reached up to 57 mg kg^{-1} (cited after Song et al. 2006). Exposure to HCB did not induce cytochrome P4501A in hepatocytes of carp (*C. carpio*) (Smeets et al. 1999), but was found to stimulate ROS production in carp brain (Song et al. 2006). Two potential mechanisms could be responsible for enhanced ROS generation under HCB exposure: (i) it may bind to cytochromes and, if not readily metabolized, can uncouple mono-oxygenase ETC favoring ROS production; and (ii) one of the major HCB metabolites called pentachlorophenol may enter reactions of autoxidation with ROS production.

The group of azole fungicides is widely used in agriculture around the world. One such compound, prochloraz [*N*-propyl-*N*-(2-(2,4,6-trichlorophenoxy)ethyl)imidazole-1-carboxamide], is a broad spectrum contact fungicide employed in Europe, South America, Asia and Australia to prevent mycoses of many plants. Similar to AMT and other imidazole compounds, it interacts strongly but non-specifically with the iron atom of cytochrome P450 and other iron-containing proteins. Due to this ability, prochloraz inhibits cytochrome P450-dependent 14α-demethylase, the enzyme which converts lanosterol to ergosterol. The latter is an essential component of fungal membranes, and therefore, prochloraz perturbs membrane assembly. Prochloraz may cause adverse effects to fish, particularly, modulating the activities of cytochrome P450 enzymes (Navas et al. 2003).

In studies with three-spined stickleback (*Gasterosteus aculeatus*), prochloraz affected the activities of phase II biotransformation enzymes such as GST, a well-known enzyme involved in protection against oxidative stress (Sanchez et al. 2008). In juvenile rainbow trout (*O. mykiss*), the triazole fungicide propiconazole also induced oxidative stress (Li et al. 2013b). The investigation of the effects of the chiral

triazole fungicide, difenoconazole, on zebra fish also found development of oxidative stress (Mu et al. 2015). Hydroxylation of these heterocyclic chlororganic compounds leads to the formation of metabolites capable of autoxidation due to which these may also increase ROS production, induce oxidative stress and injure fish tissues.

Recently, my laboratory (Kubrak et al. 2012a; Atamaniuk et al. 2014) and others (Falfushynska et al. 2012) studied the effects of the carbamate fungicide, Tattoo, which contains mancozeb [ethylenebis (dithiocarbamate)] as a main constituent, on fish with a focus on free radical processes. Mancozeb was registered several times by the US EPA (1987, 2005) and is extensively used as an active ingredient in fungicides applied worldwide as commercial preparations including dithane, fore, manzeb, nemispot, zimaneb and others. The wide use of mancozeb in agriculture is due to its reported low acute toxicity and scarce persistence in the environment (Maroni et al. 2000). It is known that mancozeb can exhibit both prooxidant and antioxidant properties (Rath et al. (2011). Goldfish exposure to Tattoo for 96 h resulted in moderate lymphopenia with a concomitant increase in both stab and segmented neutrophils, increased levels of protein carbonyl groups in blood and decreased levels of protein thiols and enhanced lipid peroxide concentrations in gills (Kubrak et al. 2012a). These data along with increases in the activities of antioxidant enzymes were interpreted as the induction of mild oxidative stress in blood and gills of C. auratus due to Tattoo exposure. Later, we found that mancozeb also induced oxidative stress in goldfish brain, liver, and kidney and the activities of key antioxidant enzymes were increased (Atamaniuk et al. 2014). Collectively the abovecited papers show that goldfish exposure to environmental Tattoo induces mild oxidative stress which increases antioxidant potential (Lushchak 2014a, b).

Oil and accompanying pollutants

More and more of the oil consumed globally is derived from offshore oil fields, and therefore, there is increasing concern about the potential for aquatic pollution either due to the technologies used or accidents arising from oil production or transportation. The main pollutants from this activity include polycyclic aromatic hydrocarbons (PAH), alkylphenols, hydrocarbons, etc. (Sturve et al. 2006). These may

affect fish in many ways, and induction of oxidative stress is one of the key elements of their toxicity. Several PAHs have been found to be cytotoxic, immunotoxic, mutagenic and/or carcinogenic (Vogelbein 2003) which can also be related to their capacity to enhance ROS production. PAH are primarily metabolized via hydroxylation and therefore can be detoxified by enzymes in the cytochrome P450 system, mainly CYP1A (Goksøyr and Förlin 1992). This route leads to the formation of compounds entering redox cycles. Interestingly, it was shown that CYP1A might be inhibited by alkylphenols in several fish species (Arukwe et al. 1997; Hasselberg et al. 2004a, b). Such effects may lead to uncoupling of ETCs with increased ROS production followed by induction of oxidative stress.

At least two more potential mechanisms of increasing ROS generation must be highlighted here. The first one is related to the conjugation of products of PAH metabolism with glutathione in phase 2 xenobiotic detoxification; this consumes glutathione while attenuating antioxidant defense. The second one is related to the production of cyclic metabolites capable of entering autoxidation and leading to ROS production. For example, benzo[a]pyrene can be metabolized by CYP1A to benzo[a]pyrene diones that have the ability to form ROS through the abovementioned process. Exposure to several PAHs induced oxidative stress in fish tissues (Winston and Di Giulio 1991; Livingstone 2001). In Atlantic cod (Gadus morhua), exposure to alkylphenols elevated glutathione reductase activities and total glutathione levels which might reflect induction of weak oxidative stress (Hasselberg et al. 2004a, b). Alkylphenols have also been suggested to cause oxidative stress in fish. Nonylphenol depleted GSH in Atlantic cod (Hasselberg et al. 2004a) and in largemouth bass (Micropterus salmoides) (Hughes and Gallagher 2004), whereas in rainbow trout (O. mykiss) it increased GST activity (Uguz et al. 2003). In an in vitro model of hepatic fragmented endoplasmic reticulum (frequently called microsomes) from flounder (Platichthys flesus) and perch (Perca fluviatilis), model compounds, viz. menadione (2-methyl-1,4-naphthoquinone) and nitrofurantoin (N-(5-nitro-2-furfurylidene)-1-aminohydantoin), and pollutant xenobiotics, viz. benzo[a]pyrene (BaP) diones (products of BaP metabolism in endoplasmic reticulum), duroquinone (tetramethyl-1,4-benzoquinone—present in pulp mill effluent) and the pesticide lindane (gamma-

hexachlorocyclohexane) were found to stimulate hydroxyl radical production in NAD(P)H-dependent manner (Lemaire et al. 1994). Depletion of total glutathione pool can result from excretion of its oxidized form or/and consumption for conjugation with xenobiotics in the second phase of detoxification, whereas an increase in oxidized glutathione levels could result from oxidative inactivation of glutathione reductase or its increased oxidation (DeLeve and Kaplowitz 1991; Lushchak 2012).

Although crude oil induced oxidative stress in post-juveniles of African sharptooth catfish (*C. gariepinus*) and it was believed that indices of oxidative stress could be used as markers of environmental pollution (Kayode et al. 2014), the data are of limited use due to poor understanding of real mechanisms involved.

One more aspect of oil toxicity should be also highlighted. Toxicity of the many components of crude oil may be enhanced by exposure to light, especially to ultraviolet (UV) radiation which has been called photo-induced toxicity or phototoxicity. Although UV light does not penetrate deeply into water, in surface waters or in shallow waters its effects cannot be ignored. Willis and Oris (2014) exposed zebra fish to selected polycyclic aromatic hydrocarbons (PAHs). The authors studied photo-induced toxicity of both single compounds and mixtures of PAHs in a sensitive larval life stage of zebra fish. They found that PAHs with phototoxic properties (anthracene and pyrene) and non-phototoxic ones (carbazole and phenanthrene) either as single compound or in mixtures in binary, tertiary and quaternary combinations differently affected zebra fish, and the acute toxicity of PAH mixtures were additive in phototoxic scenarios (Willis and Oris 2014).

The products of oil refining also may affect free radical metabolism in fish. For example, diesel oil induced transient oxidative stress in *Pagrosuma major* larvae (Yu et al. 2003), which clearly shows that the toxic effects of this oil might be arisen from oxidative damage.

Problems and perspectives in fish oxidative stress research

Although there are many problems in the evaluation of oxidative stress in fish and in elucidating the functional consequences of this stress, I would like to focus on several points that are critical to my opinion. They will be analyzed along with proposed approaches to solve the highlighted problems.

1. Work with fish always has problems related to the huge diversity of species and the large number of species that have been used in studies of oxidative stress effects on fish. However, in recent years, clear leaders have appeared as useful model systems mainly due to the sequencing of genomes. The first fish species with sequenced genomes were the zebra fish (*Brachidanio (Danio) rerio*) and fugu (*Fugu rubripes*), and these are now widely used as "model fish species." Indeed, zebra fish are widely used in biomedical and environmental studies; for example, in the PubMed database (accessed June 3, 2015) using the key words *D. rerio* 24330 papers were found, whereas with *F. rubripes* 949 papers were found. However, there are some limitations with these small fish such that many conventional methods to study biochemical parameters cannot easily be applied. Many of these limitations may be overcome with the use of larger species such as common carp (*C. carpio*) (11336 references in PubMed), rainbow trout (*O. mykiss*) (6920 references in PubMed) and goldfish *C. auratus* (6757 references in PubMed). Although the genomes of the last three species are not fully sequenced yet which complicates certain modern studies, the sequences of many genes are present in different databases. Their substantial economical importance combined with developed biochemical and molecular biology approaches to characterize the physiological states of *C. carpio*, *O. mykiss* and *C. auratus* make these species favorites for future studies.

2. Variation in the results produced by different laboratories in their studies of fish is an important problem. This is not uncommon when studies measure only one or two parameters of oxidative stress as the basis for their conclusions. This problem has been discussed in detail in earlier publications (Lushchak 2011a, b), and interested readers may examine these or other similar publications. For conclusive results that can be compared across species, I believe that several parameters of oxidative stress and/or antioxidant defense must always be assessed in order to

support any hypothesis. Recently introduced classifications of oxidative stress (Lushchak 2014a, b; Sies 2015) may also be used to help compare results from different laboratories.

3. In recent years, experiments on isolated organs and cells as well as cell cultures of fish tissues are being used more frequently to replace or supplement studies of whole animals. Such an approach can extend the experimental possibilities for controlling, manipulating and measuring the degree of oxidative stress imposed and the cell/tissue responses to such stress. This allows a closer examination of the molecular mechanisms of the induction and pathways coordinating adaptive responses or operations of rescue systems.

4. Although there is more or less a general consensus on the principal sources of ROS generation in animals, there is no clear understanding of the relative importance or actual mechanisms of ROS production by the various potential endogenous sources: ETCs in mitochondria or other membranous organelles, NADPH and other oxidases, or the input of autoxidation into net ROS production. Therefore, further investigations to identify ROS sources in fish and the regulation of their activities are expected to shed some light on these routes.

5. There are very few publications that reveal the role of specific regulatory pathways and transcription factors in coordinating the adaptive responses of fish to oxidative insults. Certainly, we know that many such pathways are conserved, at least among vertebrate animals, but there are always certain specific features in different species that experience unique stress conditions that can provide investigators with models that may disclose the operation of delicate regulatory pathways. For example, a principal regulator of animal responses to hypoxia, the hypoxia-inducible transcription factor 1-alpha (HIF-1α), was shown in fish to be rather sensitive to ROS (Nikinmaa et al. 2004). This differs substantially from the mammalian situation and may illustrate crucial taxon-specific regulatory mechanisms on HIF-1α activity. Indeed, to date, little is known about operation of such "classic" animal regulators of response to oxidative stress such as AP1, Nrf2/Keap 1 and

NF-kB in fish species and this direction awaits future investigation.

6. A systematic approach should be applied to evaluate the complex fish response to oxidative insults. In order to characterize development of oxidative stress, many markers should be evaluated in concert with the activities of antioxidant enzymes, levels of low molecular mass antioxidants such as glutathione, and steady-state levels of corresponding mRNA and proteins. Since the responses to oxidative challenge are a dynamic process, the inhibitors/activators of transcription, translation and degradation of RNA and proteins, certain genetic switches may be useful additional tools.

7. In most studies, the effects of individual contaminants on fish oxidative stress and/or antioxidant responses were investigated. However, in many cases, the use of certain mixtures of contaminants would be more environmentally realistic and this may open new avenues for understanding of their environmental effects. Moreover, combined effects of contaminants with physical factors (such as temperature, illumination) may add ecological relevance to model studies.

8. Practical needs cannot be ignored and it looks possible to develop the complex system for evaluation of fish physiological state using approaches from ROS research. Obviously, such an approach can be used in combination with traditionally applied health monitoring parameters. Vital markers in this case may be very attractive ones. It is critically important for biomonitoring purposes with fish to combine ROS-oriented studies with many other parameters such as bioaccumulation and biotransformation (van der Oost et al. 2003).

9. Many publications in the field do not provide a critical assessment of their environmental relevance. Model laboratory experiments frequently are carried out without tight association with conditions found in real bodies of water. Despite the fact that in many cases the data produced do not show dose dependency, the authors invariably conclude that their chosen endpoints are useful markers of oxidative stress for use as biomarkers of pollution. Such a simplified approach ultimately clutters the field studies that

are of limited use, and therefore, complicates the use of oxidative stress markers as true biomarkers for environmental toxicological studies. In some cases, a recently proposed classification system for oxidative stress based on its intensity (Lushchak 2014a) may provide some useful clues.

10. How important are environmental ROS levels which occur in natural and artificial waters (e.g., due to UV radiation effects or hyperoxia in the water) in inducing oxidative stress in fish? Can different external ROS species cross the gills and cause damage of intracellular components?

11. What is the time course for oxidative stress to develop, and for oxidative stress to be repaired in fish (seconds, minutes, hours, days, months)?

12. The information received and approaches developed in studies of oxidative stress in fish may be used for development of tools to decrease the deleterious impact of such stress on fish in industrial procedures, transportation, etc.

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