

Emerging environmental stressors and oxidative pathways in marine organisms: Current knowledge on regulation mechanisms and functional effects

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Abstract: Oxidative stress is a critical condition derived from the imbalance between the generation of reactive oxygen species and the sophisticated network of antioxidant mechanisms. Several pollutants and environmental factors can affect this system through connected mechanisms, indirect relationships, and cascade effects from pre-transcriptional to catalytic level, by either enhancing intracellular ROS formation or impairing antioxidant defenses. This review summarizes the current knowledge on the pro-oxidant challenges from emerging environmental stressors threatening marine organisms, such as pharmaceuticals, microplastics and climate-related ocean changes. Emphasis will be placed on oxidative pathways, including signaling proteins and transcription factors involved in regulation of antioxidant responsiveness. Mechanistic insights and lack of knowledge will be pointed out by presenting single and combined effects of multiple stressors, unravelling questions to be addressed by future research in marine ecotoxicology.

Abbreviations:

| | | | |
|-----------|---|-------------------|---|
| (API): | Activator protein 1 | (NPs): | Nanoplastics |
| (APIs): | Active Pharmaceutical Ingredients | (Nrf2): | NF-E2-related factor 2 |
| (ATP): | adenosine triphosphate | (NF- κ B): | Nuclear factor kappa B |
| (CAT): | Catalase | (NSAIDs): | Non-Steroidal Anti-Inflammatory drug |
| (CBZ): | Carbamazepine | (PA): | polyamide |
| (CYP450): | Cytochrome P450 | (PAH): | Polycyclic aromatic hydrocarbon |
| (DIC): | Diclofenac | (PAR): | paroxetine |
| (ERK): | extracellular signal-regulated kinase | (PE): | polyethylene |
| (FLU): | Fluoxetine | (PET): | polyethylene terephthalate |
| (GCLC): | Glutamate-Cysteine Ligase Catalytic Subunit | (PLHC-1): | <i>Poeciliopsis lucida</i> hepatocellular carcinoma |
| (GPx): | Glutathione peroxidases | (PP): | polypropylene |
| (GR): | Glutathione reductase | (PS): | polystyrene |
| (GSH): | Glutathione | (PVC): | polyvinylchloride |
| (GST): | Glutathione S-transferases | (RCS): | reactive carbonate species |
| (JNK): | c-Jun N-terminal kinase | (RNS): | Reactive nitrogen species |
| (Keap1): | Kelch Like ECH Associated Protein 1 | (ROS): | Reactive oxygen species |
| (MAPK): | Mitogen-activated protein kinases | (SAF-1): | <i>Sparus aurata</i> Fibroblast-1 |
| (MPs): | Microplastics | (SOD): | Superoxide dismutase |
| (NADPH): | Nicotinamide adenine dinucleotide phosphate | (SSRIs): | Selective Serotonin Reuptake Inhibitors |
| | | (Trx2): | Thioredoxin 2 |
| | | (TrxR): | Thioredoxin reductases |

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Introduction

The maintenance of redox status is crucial for aerobic organisms, which are exposed to intracellular fluctuations of ROS, derived either from their own metabolism or from the external stimuli. Main sources of ROS formation include electron transport chain of mitochondria, peroxisomal and lysosomal functions, Fenton's and Haber-Weiss's reactions, and activities of specific oxido-reductase enzymes like monoamine oxidase, NADPH oxidase, xanthine oxido-reductase, arachidonic acid and cytochrome P450 oxidase, as well as inactivation of antioxidant enzymes and depletion of free radical scavengers (Regoli and Giuliani, 2014; Halliwell and Gutteridge, 2015). ROS have detrimental effects on cellular molecules and structures, resulting in lipid peroxidation, protein oxidation, DNA damage and unbalance of intracellular redox status. On the other side, ROS also act as signaling molecules, which trigger cytoprotective and antioxidant responses to protect the cellular components from oxidative damage and minimize their damaging effects (Halliwell and Gutteridge, 2015; Sachdev et al., 2021). Antioxidants include enzymes and nonenzymatic molecules that neutralize ROS and other oxidant molecules (Tab. 1).

The generation of ROS is a mechanism common to many environmental contaminants (e.g. trace metals, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, halogenated compounds, dioxins) that, in addition, can also inhibit the proper functioning of antioxidant system (Benedetti et al., 2015). In this respect, investigations on oxidative metabolism are largely used to examine the health status of marine organisms, and their susceptibility toward

environmental conditions (Benedetti et al., 2015). Few information is available on oxidative effects of emerging stressors in the marine environment, which are attracting great concern in the scientific community. Among these, Active Pharmaceutical Ingredient, microplastics/nanoplastics and CO₂-related changes (ocean warming and acidification) have a prevalent role.

The increasing occurrence of APIs in marine environment is strictly related to the development of the global pharmaceutical market and future projections suggest this increment will continue given the human population aging and growth (IQVIA, 2019). Despite being characterized by different environmental sources and distribution pathways, wastewater treatment plants have been identified as a major route for APIs release in aquatic systems (Ojemaye and Petrik, 2019). Moreover, the numerous uses of plastic and its low degradation rates have led to the accumulation of various sizes of plastic in the marine environment (Sorensen and Jovanović, 2021). The release into the sea occurs through a variety of pathways, that include deliberate or accidental direct inputs from land-and-sea-based sources and indirect inputs from land via rivers, drainage, sewage systems, atmosphere (UNEP, 2016). Nonetheless, APIs and plastics are released in oceans that are facing deep physical and chemical changes driven by the continuous emissions of anthropogenic CO₂ in the atmosphere: since the beginning of the industrial revolution, oceans have warmed by 0.7°C and seawater pH decreased of 0.1 units on a global scale, due to the absorption of almost 30% of anthropogenic CO₂ (IPCC, 2013); during this century, these changes are projected to continue and intensify.

TABLE 1

Function of the main non-enzymatic and enzymatic antioxidants

| Non-enzymatic antioxidants | |
|---|---|
| Reduced glutathione | Cytosolic scavenging of ROS (¹ O ₂ , O ₂ ⁻ , HO•) and reactive nitrogen species; Cofactor of glutathione-dependent antioxidant enzymes |
| Ascorbic acid, vit. C | Cytosolic scavenging of ROS (H ₂ O ₂ , O ₂ ⁻ , HO•, lipid peroxides) |
| α-Tocopherol, vit. E Carotenoids | Membrane-bound scavenging of ROS (¹ O ₂ , peroxides), protection of cell membrane from lipid peroxidation |
| Antioxidant and antioxidant-related enzymes | |
| Superoxide dismutase | Conversion of superoxide anion (O ₂ ⁻) to hydrogen peroxide (H ₂ O ₂) |
| Catalase | Reduction of H ₂ O ₂ to H ₂ O |
| Glutathione peroxidases | Reduction of H ₂ O ₂ and lipid peroxides |
| Peroxiredoxins | Reduction of H ₂ O ₂ , lipid peroxides and peroxyxynitrite |
| Thioredoxin | Reduction of oxidized cysteine residues |
| Thioredoxin reductase | Regeneration of reduced thioredoxin |
| Glutathione reductase | Reduction of oxidized glutathione (GSSG) to reduced form (GSH) |
| Glutathione S-transferases | Conjugation of GSH to organic chemicals, reduction of lipid peroxides |
| NAD(P)H:quinone oxidoreductase | Reduction of quinones |
| Heme oxygenase | Degradation of heme, reduction of O ₂ ⁻ and other ROS |
| Glutamate cysteine ligase | First step of glutathione synthesis |
| Glutathione synthetase | Second step of glutathione synthesis |

Despite the subtle occurrence of these emerging stressors, they impact the environment on a global scale, modulating, alone or interacting with other stressors, several biological processes. This review summarizes the current knowledge on the oxidative effects of such new challenges in marine organisms, with particular focus on signaling pathways, regulation mechanisms and interactions between different stressors.

Redox Metabolism Modulation by Residues of Active Pharmaceutical Ingredients

The widespread occurrence of APIs in marine and coastal areas represents a serious environmental issue due to the potential long-term deleterious consequences on non-target species (Almeida *et al.*, 2020; Mezzelani *et al.*, 2018a; Shi *et al.*, 2019; Zhang *et al.*, 2020). These heterogenous chemicals (~4000 compounds) are specifically designed to be active on living cells at very low concentrations. Since biological targets of APIs (e.g., transporters, receptors or enzymes) are evolutionarily and functionally conserved across the animal kingdom, marine organisms are exposed to the risk of these new typology of environmental stressors (Almeida *et al.*, 2020; Zhang *et al.*, 2020). Antibiotics, synthetic steroids, antiinflammatories, antidepressants, antiepileptics, cardiovascular and lipid regulating agents are considered as the most environmentally relevant APIs. Indeed, field investigations have documented their ubiquitous presence in water column, sediments and also in tissues of marine species (Martínez-Morcillo *et al.*, 2020; Mezzelani *et al.*, 2020; Moreno-González *et al.*, 2016; Ojemaye and Petrik, 2019; Wolecki *et al.*, 2019). Laboratory experiments contributed to demonstrate their role as promoters of molecular and biochemical changes, which might finally affect organismal physiological health status (Almeida *et al.*, 2020; Bebianno and Gonzalez-Rey, 2015; Kovalakova *et al.*, 2020; Mezzelani *et al.*, 2018a,b; Zhang *et al.*, 2020). APIs can act as enhancers of ROS production through the direct induction of the biotransformation pathway of CYP450, involved in the oxidative metabolism of numerous endogenous and exogenous compounds including several typologies of pharmaceuticals (Burkina *et al.*, 2015; Mezzelani *et al.*, 2018a,b; 2021). Under basal condition the ROS are generated *in situ* when CYP450 reacts with the substrate. However, excessive ROS production during induction results in uncoupling of the CYP450 cycle, leading to the increase of oxidative pressure (Ghosh *et al.*, 2015). The exposure of liver slices of Atlantic cod (*Gadus morhua*, Linnaeus, 1758) to the synthetic steroid 17 α -ethinylestradiol EE2 enhanced *cyp1a* gene transcription (reviewed by Burkina *et al.*, 2015), while *in vitro* studies on fish PLHC-1 cells exposed to the SSRIs, FLU, PAR and fluvoxamine revealed the increase of CYP450 activity demonstrating its responsiveness also at the catalytic level (Burkina *et al.*, 2015); similarly, more elevated CYP450 activity was measured in hepatic microsomes of *Dicentrarchus labrax* (Linnaeus, 1758) exposed to the NSAIDs DIC (Burkina *et al.*, 2015). Noteworthy, although the CYP450 biotransformation metabolism in marine invertebrates still needs to be fully elucidated, transcriptional changes of phase I-related genes were observed in bivalves

Mytilus galloprovincialis (Lamarck, 1819) and *Ruditapes philippinarum* (Adams & Reeve, 1850) exposed to environmental levels of NSAIDs (*cyp1a*) and to the antiepileptic CBZ (*cyp4f8*, *cyp3a2*, *cyp3a29*) (Mezzelani *et al.*, 2018a,b; 2021). The hypothesis that APIs can unbalance organismal redox homeostasis was further corroborated by a wide array of cellular damages measured in marine invertebrates: exposure to NSAIDs, SSRIs, CBZ and cardiovascular compounds was reflected by the significant increase of peroxidation products like lipofuscin and malondialdehyde in digestive gland of *M. galloprovincialis*, *R. philippinarum*, *Scrobicularia plana* (da Costa, 1778) and *Venerupis decussata* (Linnaeus, 1758) (Almeida *et al.*, 2020; Franzellitti *et al.*, 2014; Freitas *et al.*, 2016; Hampel *et al.*, 2017; Mezzelani *et al.*, 2018a, 2021; Munari *et al.*, 2014). Oxidative stress and ROS production lead to the activation of several signaling pathways involved in cell protection. In this respect, Nrf2-Keap1 modulates cytoprotective responses to oxidative stress, regulating the synthesis of antioxidant defenses to minimize oxidative damages (Espinosa-Diez *et al.*, 2015). Although detailed mechanistic studies in marine species are limited, various investigations demonstrate the activation of Nrf2-Keap1 pathway following APIs exposure (Almeida *et al.*, 2020; Bebianno and Gonzalez-Rey, 2015; Mezzelani *et al.*, 2018a,b; Ruiz *et al.*, 2020; Wang *et al.*, 2020a). In the fish species *Mugilogobius abei* (Jordan & Snyder, 1901), the widely used NSAIDs aspirin, caused a transient downregulation of Nrf2-Keap1-related genes expression (*nrf2*, *keap1*, *gclc*, *gst*, *sod*, *cat*, *trx2*, and *trxr*), followed by their induction throughout 7 days-exposure; at catalytic functional level a significant enhancement of related enzymatic activities (GPx, GST, SOD, CAT) and GSH levels were paralleled to the reduction of lipid peroxidation products (Wang *et al.*, 2020a). Conversely, limited variations in *nrf2*, *sod* and *cat* gene expression were observed in *Sparus aurata* (Linnaeus, 1758) cell line (SAF-1) exposed to CBZ (Ruiz *et al.*, 2020), although the complex relationships between transcriptional and catalytic levels of antioxidant defenses do not allow to exclude the modulation of such cytoprotective responses at functional level (Regoli and Giuliani, 2014). In this respect, variations of antioxidant enzymes were often measured in response to APIs, highlighting species-specific, dose and compound-dependent trends (Almeida *et al.*, 2020; Bebianno and Gonzalez-Rey, 2015; Mezzelani *et al.*, 2018a,b). Adults of *S. aurata* exposed to the antibiotic erythromycin, showed inhibited activities of GPx and induction of GR (Rodrigues *et al.*, 2019), while bivalve *S. plana* and the polychaete *Diopatra neapolitana* (Delle Chiaje, 1841) exhibited significant modulation of SOD, CAT and GST activity in response to CBZ (Freitas *et al.*, 2016). Induction of SOD, CAT, GST was reported in mussels *M. galloprovincialis* exposed to the antiinflammatory DIC (Almeida *et al.*, 2020; Bebianno and Gonzalez-Rey, 2015), while various bivalves species (*M. galloprovincialis*, *Perna perna*, Linnaeus, 1758 and *R. philippinarum*) highlighted biphasic variations of SOD, CAT, GR and GPx and the induction of GST in response to the antidepressant FLU (reviewed by Mezzelani *et al.*, 2018a). Among the large number of pathways regulating the perturbation of redox homeostasis (Fig. 1), the cooperation between NF-kB, API

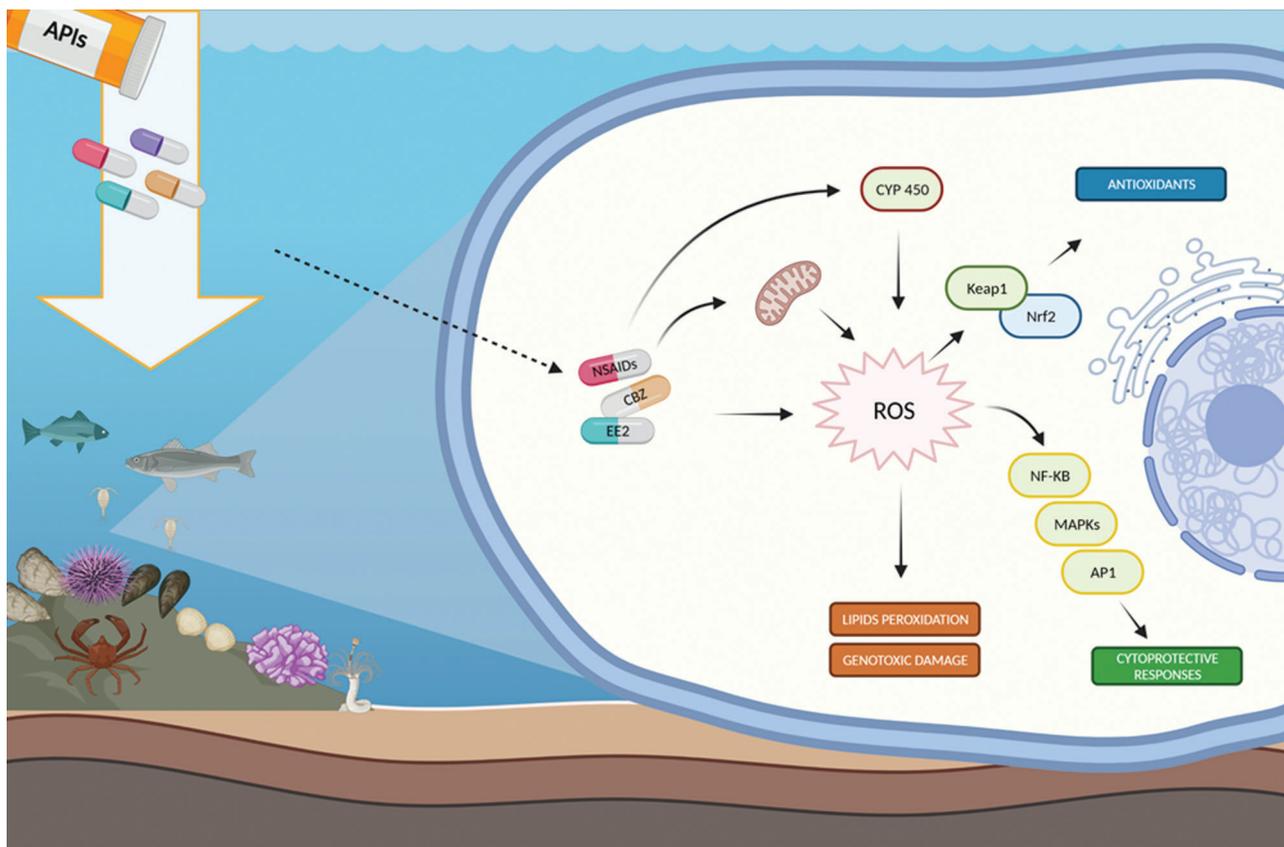


FIGURE 1. Main APIs-mediated ROS formation and scavenging pathways.

and MAPK cascade was shown to be an effective early cytoprotective response to oxidative stress (Espinosa-Diez *et al.*, 2015). Transcriptional responses revealed enhanced mRNA levels of *nf-kb* gene in *Mytilus spp.* treated with environmentally realistic concentrations of various NSAIDs, while NF-κB pathways was significantly suppressed in the bivalve *Tegillarca granosa* (Linnaeus, 1758) exposed to the antidepressant FLU (Shi *et al.*, 2019; Mezzelani *et al.*, 2018a).

Microplastics/Nanoplastics and Oxidative Stress in Marine Organisms

Over the last decade, field studies showed the constant presence of MPs in superficial seawater, along water column, in sediments, beaches and organisms worldwide (Cole *et al.*, 2011). MPs include any synthetic solid particle or polymeric matrix, consisting of items ranging from 1 μm to 5 mm that are manufactured to be of such microscopic dimensions, or deriving from the weathering and fragmentation of larger plastics (Bessa *et al.*, 2019). With the advent of modern analytical techniques and detection methods, most recent studies have observed and reported plastic debris to the nano-scale (Alimba and Faggio, 2019). MPs/NPs in the oceans exist in a variety of dimensions, shapes (e.g., fragments, films, sphere, fibers), colors and polymers with different density, being PE, PS, PVC, PP, PET and PA the most frequently found (Paul-Pont *et al.*, 2018). MPs/NPs affect all marine taxa and life stages, and observed interactions can occur via adhesion, absorption, ventilation and specially ingestion, that lead to accumulation and translocation within tissues and cells (Lusher, 2015). From an ecotoxicological perspective, MPs

have the peculiar characteristic to combine a physical stress with a chemical challenge (Pittura *et al.*, 2018). The chemical impact is mostly related to additives present in the plastic from manufacturing, as well as, to the environmental contaminants which can be adsorbed by the hydrophobic nature and high surface-to-volume ratio of MPs/NPs (Atugoda *et al.*, 2021). Pollutant-plastic interaction depends on properties of both MPs/NPs and chemical contaminants and can be modulated by the surrounding environmental conditions of pH, salinity and temperature (Menéndez-Pedriz and Jaumot, 2020). There is an active debate regarding the relevance of adsorption of pollutants on MPs/NPs and their possible transfer to marine organisms due to the variability of experimental results (Elizalde-Velázquez *et al.*, 2020).

Although the ecotoxicological effects of MPs/NPs are complex to be elucidated, several studies suggested oxidative stress as an important mechanism underneath microplastics toxicity (Hu and Palić, 2020). The first evidence of disturbance in redox homeostasis was the increase of intracellular ROS levels observed in rotifers (*Brachionus koreanus*, Hwang, Dahms, Park & Lee, 2013) (Jeong *et al.*, 2016), crustaceans (*Tigriopus japonicus*, Mori, 1938 and *Artemia salina*, Linnaeus, 1758) (Choi *et al.*, 2020; Suman *et al.*, 2020), bivalves (*Mytilus spp.* and *T. granosa*) (Paul-Pont *et al.*, 2016; Shi *et al.*, 2020), and fishes (*Oryzias melastigma*, McClelland, 1839) (Kang *et al.*, 2021) exposed to commercial PS-spheres, and in the sea urchin *Paracentrotus lividus* (Lamarck, 1816) exposed via diet to PET-MPs of irregular shape and size (Parolini *et al.*, 2020). Given the evidence that MPs/NPs can pose an oxidative challenge to marine organisms, main mechanisms can be supposed (Fig. 2).

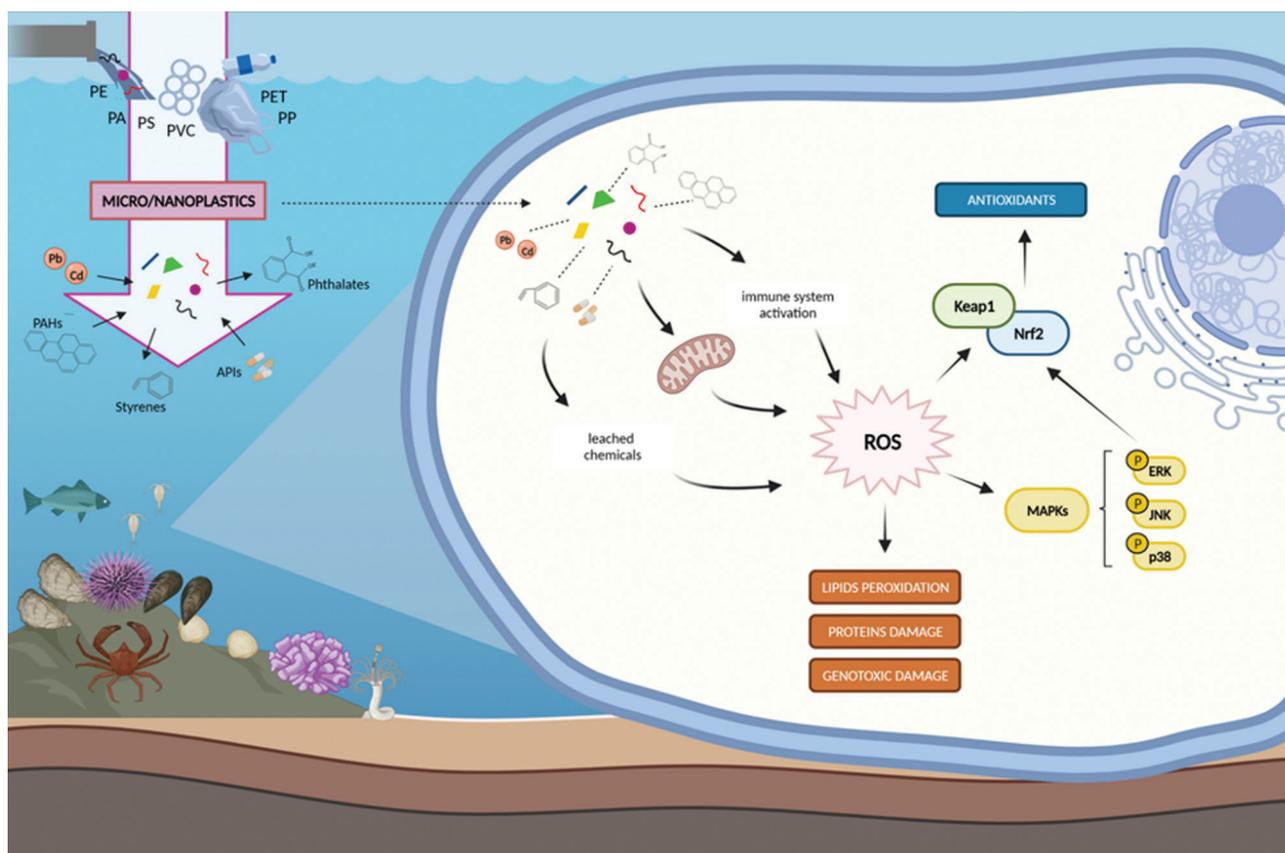


FIGURE 2. Main MPs- and NPs-mediated ROS formation and scavenging pathways.

Similar to other environmental stressors, MPs/NPs can trigger ROS production through damage to mitochondria (Yang *et al.*, 2020), or increasing responses of the immune system during attempts of the cell to neutralize potentially infectious foreign particles (Hu and Palić, 2020; Pittura *et al.*, 2018; Tang *et al.*, 2018, 2020; Sikdokur *et al.*, 2020). In addition, chemical additives leaching from plastics can further contribute to ROS formation (Hu and Palić, 2020; Yang *et al.*, 2020). Oxidative challenge of MPs/NPs exposure was extensively supported by significant changes in antioxidant defenses (i.e., CAT, SOD, GST, GPx and GSH) both at catalytic and transcriptional level and by the onset of oxidative damages to lipids, proteins and DNA (reviewed in Trestrail *et al.*, 2020, Kim *et al.*, 2021, Gonçalves and Bebianno, 2021), that was even observed after exposures to environmentally realistic concentrations of MPs (Hariharan *et al.*, 2021) and persistent also after a period of depuration (Hariharan *et al.*, 2021; Capó *et al.*, 2021). These effects suggest that MPs/NPs, as other pro-oxidant stimuli, can activate expression of antioxidant genes via the Nrf2-dependent mechanism. The up-regulation of *nrf2* was measured in the head-kidney leucocytes isolated from the gilthead seabream *S. aurata* after exposure to PE- or PVC-MPs (Espinosa *et al.*, 2018). Since no responses of antioxidant system occurred at biochemical level, the authors hypothesized a limited oxidative challenge on Nrf2 of seabream. In the copepod *Paracyclopsina nana* (Smirnov, 1935) a positive correlation was observed between intracellular ROS generation and phosphorylation of ERK and p38 kinase after the exposure to PS-microbeads,

supporting a defense mechanism against microplastic-induced oxidative stress via the MAPK/Nrf2 pathway (Jeong *et al.*, 2017). An increased phosphorylation of kinases, in particular p38 and JNK, after exposure to PS-microbeads was also shown in the monogonont rotifer *B. koreanus*, along with the induction of antioxidant enzymes, further confirming that MAPK-activating proteins are involved in signal transduction modulating the oxidative stress response (Jeong *et al.*, 2016). In both *P. nana* and *B. koreanus*, the activation of MAPK pathway was influenced by the particle size, and the nanosized PS-beads caused higher phosphorylation of p38 MAPKs when compared to 6 μm particles (Hu and Palić, 2020). The transcriptomic signal of JNK pathway was activated also in the scleractinian coral *Pocillopora damicornis* (Linnaeus, 1758), along with increased activities of CAT and SOD enzymes, in response to acute exposure to elevated concentrations of 1 μm PS-MPs (Tang *et al.*, 2018). Based on the limited available information, MAPKs pathways might play a synergistic role with Nrf2-Keap1 in the response to oxidative stress induced by MPs/NPs in marine organisms.

Ocean Changes as Sources of Oxidative Imbalance

Ongoing ocean changes, caused by the increasing anthropogenic CO₂ emissions, can represent a source of oxidative imbalance for marine organisms (Fig. 3). As alteration of environmental characteristics reaches or even exceeds the limits of homeostatic response, a number of cellular processes can reflect organisms stress-response

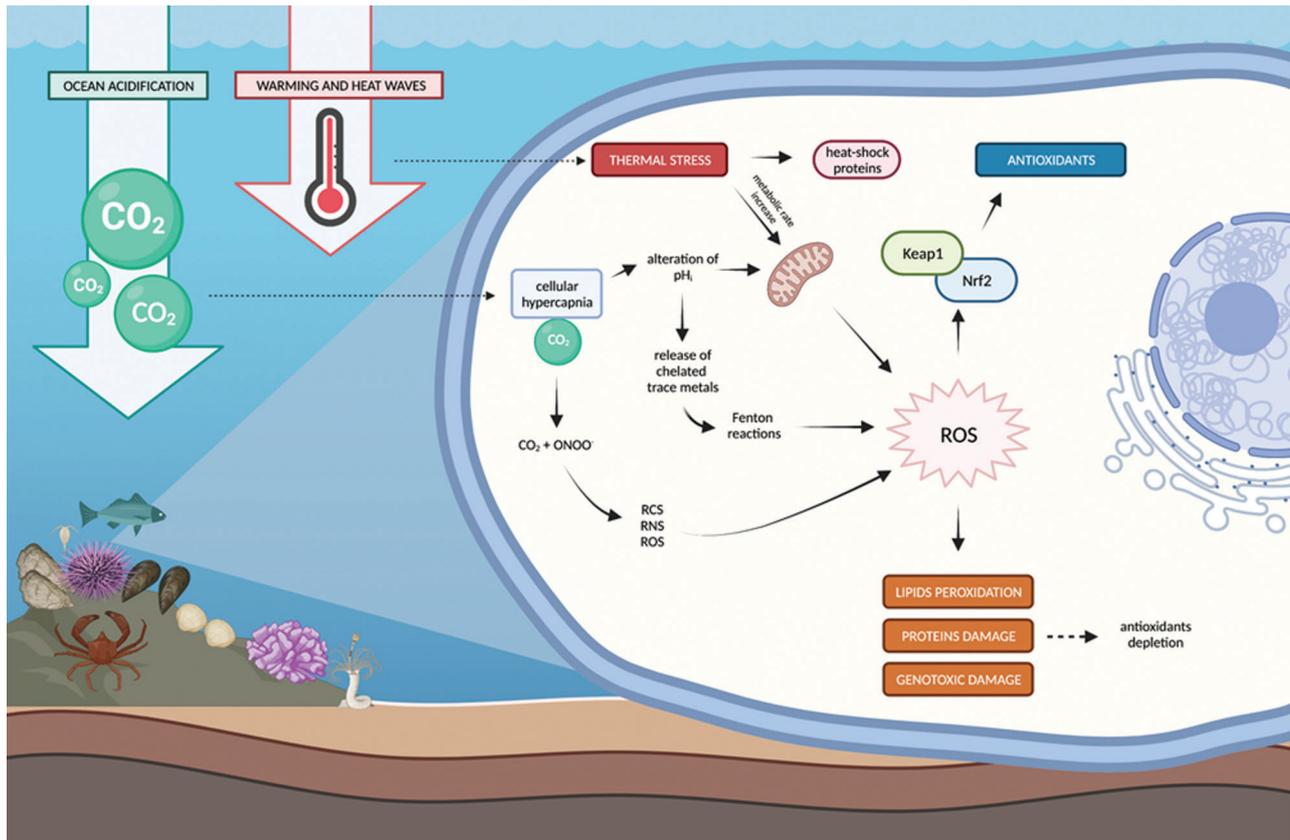


FIGURE 3. Main temperature- and pH/CO₂-mediated ROS formation and scavenging pathways.

(Tomanek, 2015). Detailing the extensive network of relationships between environmental changes, oxidative stress and individual components of the antioxidant network is beyond the scope of this review. However, the current trends of ocean warming and the frequent occurrence of extreme-temperature events (marine heatwaves) are certainly oxidative challenges for marine species. Thermal stress can directly boost the production of ROS at cellular level through increased metabolic rate and progressive mitochondrial uncoupling (Pörtner et al., 1999; Thorat et al., 2021): a positive relationship between temperature and ROS production has been demonstrated in either isolated mitochondria or *in vivo* studies on marine invertebrates and vertebrates (Abele et al., 2002; Heise et al., 2003; Keller et al., 2004; Nash et al., 2019; Okoye et al., 2019; Paital and Chainy, 2014). Oxidative challenge due to thermal stress in bivalves and fishes has been further evidenced by the Nrf2-dependent increase of antioxidants such as SOD, CAT, GPx, GR, GST, GSH, and the onset of oxidative damages as lipids peroxidation, loss of DNA integrity, nuclear abnormalities (Feidantsis et al., 2020a; Han et al., 2020 and references therein; Matozzo et al., 2013; Velez et al., 2017). The thermal range of each species plays a fundamental role in determining whether increased temperature elicits the activation of antioxidant responses, possibly hampered by overwhelming heat-mediated protein damage at temperatures close or above the tolerated limit (Madeira et al., 2013, 2016; Tomanek, 2015 and references therein). The proteome of two *Mytilus* congeners differentially adapted to thermal stress showed diverse responsiveness toward acute heat stress (Tomanek, 2014):

the less tolerant species reduced aerobic metabolic pathways to overcome the limited chaperones levels and antioxidant responsiveness compared to the more tolerant species. Despite the common mechanism of regulation through the Nrf2-Keap1 mediated pathway, antioxidant defenses often exhibit asynchronous responses to thermal stress (Han et al., 2020; Klein et al., 2017; Madeira et al., 2013, 2016; Nardi et al., 2017; Nardi et al., 2018b). Several factors contribute to oxidative regulation, including additional protective and/or compensative mechanisms: proteomic studies highlighted depression of arachidonic acid metabolism, decreased abundance of mitochondrial complexes, increased heat-shock proteins, upregulation of Toll-like receptor signalling pathway in response to temperature-mediated oxidative stress (Li et al., 2016; Tomanek, 2015 and references therein; Zheng et al., 2019). Tissue-specific effects and seasonal-related sensitivity toward thermal stress have also been highlighted: in *M. galloprovincialis* the effects of increased temperature at transcriptional and catalytic levels differed between digestive and respiratory tissues and showed diverse magnitude and thresholds of activation between summer and winter (Feidantsis et al., 2020b; Giuliani et al., 2020; Nardi et al., 2017; 2018b). Only a few studies focused on the role of Nrf2-Keap1 pathway in the antioxidant responsiveness toward thermal stress: *nrf2* and antioxidant genes transcription was not altered in *Trematopus bernacchii* (Boulenger, 1902) adults after 14 days acclimation to higher temperature (Giuliani et al., 2021) and a correlation between thermal stress and *nrf2* transcription was not evidenced during any stage of development in embryos of *G. morhua* (Skjærven et al., 2013). Mechanistic

studies are thus still needed to unravel the main actors and regulators of cellular responsiveness toward thermal stress and transfer this knowledge to whole organisms physiology.

Ocean acidification is another well recognized challenge (Pörtner, 2008; Tomanek, 2014) which, beside the effects on shell formation-dissolution in marine calcifiers, can affect several biological processes of cellular homeostasis (Tomanek, 2014): onset of cellular hypercapnia and acidosis (decrease of pH) can impact oxygen transport, ion-exchange rates and mitochondrial functioning, leading to increased oxidative stress conditions (Haider *et al.*, 2016; Pörtner, 2008; Tomanek, 2015; Wang *et al.*, 2020b). Three main mechanisms have been hypothesized by Tomanek *et al.* (2011) to explain acidification-mediated pro-oxidant challenge in marine organisms: i) reactions between cellular CO₂ and ONOO⁻ can generate secondary radicals, such as reactive carbonate, oxygen and nitrogen species; ii) in organisms with limited capacity of acid-base regulation, the lowering of intracellular pH will negatively affect the mitochondrial electron transport chain, resulting in increased electron slip and ROS production; iii) altered intracellular pH may also facilitate the release of chelated trace metals, like iron and copper which catalyse Fenton reaction and hydroxyl radical production. Experimental studies highlighted increased ROS production under hypercapnic stress (Haider *et al.*, 2016; Wang *et al.*, 2020b), and pro-oxidant effects have been confirmed by several laboratory and field conditions with down- or up-regulation of antioxidant defenses at transcriptional, proteomic and functional level (Cao *et al.*, 2018; de Marchi *et al.*, 2019; Matoo *et al.*, 2013; Munari *et al.*, 2018; Nardi *et al.*, 2018a; Ricevuto *et al.*, 2015; Tomanek *et al.*, 2011). Similarly to what is described for temperature-mediated oxidative pressure, the responsiveness of antioxidant defenses toward reduced-pH/high-CO₂ is highly influenced by other factors, including species-specific sensitivity and onset of compensation mechanisms, at least within a limited range of acidification: early increase of ROS in *Crassostrea gigas* (Thunberg, 1793) exposed to reduced pH were counteracted in long-term exposure by physiological adjustments supported by the up-regulation of calcium binding proteins and calmodulins (Wang *et al.*, 2020b). Also in *M. galloprovincialis* long-term exposure to acidification determined up-regulation of genes related to calcium homeostasis, calmodulins and calcium signalling pathways, causing a lower efficiency of antioxidant enzymes and accumulation of lipid peroxidation products (Mezzelani *et al.*, 2021). Changes of acid-base balance in *Hyas araneus* (Linnaeus, 1758) were coupled with higher metabolism, increase of antioxidant defenses, and more pronounced responsiveness toward moderate rather than high hypercapnia (Harms *et al.*, 2014); the explanation for these shifts was hypothesized to support indirect oxidative pressure due to high CO₂, causing energy imbalance and species-specific limits of stress tolerance. As already described for thermal stress, sensitivity toward high-CO₂/reduced-pH was demonstrated to vary between investigated tissues and seasons, with non-synchronous effects at transcriptional and catalytic levels (Giuliani *et al.*, 2020; Nardi *et al.*, 2017, 2018b). Antioxidant defenses regulation mechanisms in response to CO₂-mediated oxidative stress still need to be

fully elucidated and integrated within a physiological perspective of whole organism fitness.

In this context, since ocean warming and acidification are concomitant changes driven by the same cause, the respective interactions and influence on biological processes are of outmost relevance to understand the implications for organisms health. Overall, it has been extensively suggested that the reduction of seawater pH could narrow the thermal window of organisms reducing their capability to cope with thermal stress, especially in lower marine invertebrates that lack acid-base regulation systems (Pörtner *et al.*, 2017; Pörtner, 2008): thus the onset of oxidative disturbance due to thermal stress could be disclosed earlier. Despite this general assumption, meta-analysis studies on the effects of interactions between temperature and pH and on the nature of these interactions, revealed that the interplay between thermal and pH stress is rather than linear and easily depictable, but constrained by physiological aspects regarding tested life-stages and considered taxas (Kroeker *et al.*, 2013; Lefevre, 2016; Przeslawski *et al.*, 2015). Nonetheless, previous studies from our laboratory suggested that the tolerance of marine organisms to concomitant acidification and warming may be subjected to either additive or antagonistic effects of the two stressors, depending on the level of biological organization considered and on the physiological function of the analysed organ (Giuliani *et al.*, 2021; Nardi *et al.*, 2017, 2018a,b; Benedetti *et al.*, 2016). As a corollary, as already demanded for single stressors, mechanistic investigations of the interactive effects of ocean warming and acidification on oxidative pressure and antioxidant responsiveness would deeply increase our knowledge and would be very relevant in the context of finding a unifying principle.

Combined Oxidative Challenge from Emerging Multiple Stressors

Challenges for marine organisms typically occur and act in a multi-stressors context which may result in a plethora of unexplored additive, synergistic or antagonistic effects (Horton and Barnes, 2020). From a biological and environmentally realistic perspective, an even limited disturbance directly exerted by a single stressor may indirectly alter the susceptibility toward a secondary stressor (Kroeker *et al.*, 2017). In this respect, effects of APIs have been frequently modulated in marine organisms under projected ocean changes scenarios (among others Freitas *et al.*, 2016, 2019; Almeida *et al.*, 2018, 2021; Munari *et al.*, 2018; Mezzelani *et al.*, 2021). Lipid peroxidation due to DIC-exposure was enhanced in mussels *M. galloprovincialis* exposed at higher temperature despite the activation of antioxidant defenses (Freitas *et al.*, 2019), while this damage was not observed after the induction of antioxidant enzymes in *R. philippinarum* co-exposed to CBZ and temperature stress (Almeida *et al.*, 2021). On the other hand, CBZ and reduced pH inhibited CAT activity and interactively increased lipid peroxidation in *S. plana*, along with negative effects on electron transport activity (Freitas *et al.*, 2016). Under a similar exposure scenario, a synergistic increment of lipofuscin was observed in *M. galloprovincialis*

(Mezzelani *et al.*, 2021), in which, despite the lack of antioxidants variations, transcriptomic analyses revealed a conspicuous modulation of several pathways possibly contributing or related to oxidative stress (i.e., ATP generation, energy derivation by oxidation of organic compounds, apoptotic processes and calcium-mediated signalling). Changes in water pH and/or temperature have also the potential to influence the impact of MPs on organisms, modifying both the intrinsic toxicity of polymers and the bioavailability of chemicals adsorbed on MPs, like pharmaceuticals (Horton and Barnes, 2020; Menéndez-Pedriza and Jaumot, 2020). Interactions between MPs, temperature/pH and APIs were mostly investigated in freshwater organisms (Jaikumar *et al.*, 2018; Kratina *et al.*, 2019; Weber *et al.*, 2020; Guilhermino *et al.*, 2018; Zhang *et al.*, 2019; Schmiege *et al.*, 2020), while little is known for marine species concerning the combined modulation of oxidative pathways.

The effects of PE-MPs on the redox homeostasis of the marine fish *Pomatoschistus microps* (Kroyer, 1838) were influenced by temperature elevation, with a significant reduction of GST activity and slight effects on lipid peroxidation under temperature increase from 20°C to 25°C (Ferreira *et al.*, 2016; Fonte *et al.*, 2016). Limited interactive effects of PS-MPs and acidification were reported on antioxidant enzymes of *Mytilus coruscus* (Gould, 1861) by Wang *et al.* (2020c), while PET-MPs and acidification co-modulated antioxidant enzymes and lipid peroxidation in *M. galloprovincialis* (Provenza *et al.*, 2020). Interactions between microplastics and pharmaceuticals have been mainly investigated on the sorption/desorption processes under various environmental conditions (Atugoda *et al.*, 2021; Vieira *et al.*, 2021), whereas the possible role of MPs on APIs bioaccumulation, metabolization, and toxicity in marine organisms is poorly explored (Santos *et al.*, 2021). The impact of MPs-antidepressant co-exposure on the blood clam *T. granosa*, revealed a synergistic effect of sertaline and 30 µm PE-microbeads on haemocytes ROS production and lipid peroxidation (Shi *et al.*, 2020). The presence of MPs may facilitate the internalisation of APIs through the “Trojan horse” effect, leading to aggravated toxicity (Zhang and Xu, 2020). The interactive effect on oxidative stress in *T. granosa* was further exacerbated by nanoscale 500 nm PE-beads with a synergistic immuno-toxic effect, highlighting a size-dependent interaction between plastic and sertaline (Shi *et al.*, 2020). Similarly, *M. galloprovincialis* treated with PS-NPs in combination to the anticonvulsant CBZ revealed synergistic effects on biomarkers of neurotoxicity, carbohydrate metabolism, immune responses and DNA damage, and a slight impairment of oxidative metabolism (total oxidant status, total antioxidant capacity and levels of peroxidation products) (Brandts *et al.*, 2018). The increased toxicity of NPs-APIs compared to MPs-APIs may also arise from a higher amount of pollutants carried and delivered into the organisms due to the larger specific surface area of NPs compared to MPs (Brandts *et al.*, 2018; Shi *et al.*, 2019). Ultimately, short-term exposure of *P. microps* juveniles to PE-microspheres, antibiotic cefalexine and temperature-stress revealed significant interactions on redox homeostasis, as highlighted by the onset of lipid peroxidation (Fonte *et al.*, 2016).

Since oxidative balance can be altered by emerging stressors, acting either alone or in combination, a relevant challenge for marine ecotoxicology is to clarify mechanistic pathways of interaction behind such functional effects, to predict and prevent adverse outcomes affecting higher levels of biological organization.

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