BIOCELL
2023 47(5): 1177-1189

Tech Science Press

Fe-dependent cellular alterations of oxidative balance in aquatic organisms. Could be ferroptosis involved?

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Key words: Glutathione, Labile Fe pool, Lipid peroxidation, Oxidative stress

Abstract: The purpose of this review is to briefly summarize the central role of iron (Fe) in terms of cellular alterations of the oxidative/protective balance with special emphasis on its possible involvement in ferroptosis-dependent disruption in aquatic organisms. In ferroptotic cells or tissues, the intracellular Fe level increases; meanwhile the treatment with Fe chelators limits ferroptosis. Eukaryotic algae can assimilate Fe from the environment through several mechanisms, and aquatic animals incorporate dissolved Fe and Fe bound to both inorganic particles and organic matter. The central role of lipid peroxidation mediating ferroptosis was demonstrated in some algae where both low and high Fe concentrations could induce oxidative stress and programmed cell death. Aquatic animals have high levels of polyunsaturated fatty acids and numerous studies have analyzed Fe effects on the lipidic fraction which could be related to ferroptosis. The ferroptosis reaction can be regulated through the antioxidant defense system, in combination with the protein degradation structure, metabolism, and gene transcription. Early depletion of non-enzymatic antioxidants like reduced glutathione (GSH) in animals, and the reduction of both GSH and ascorbate in photosynthetic organisms, are characteristic features of ferroptosis. Therefore, ferroptosis can be prevented if Fe chelators, certain antioxidants, and specifically regulating genes are activated. Thus, the global scenario for the Fe role as a toxic component in biological systems seems to be even more complicated than it was previously understood. Much more research on this subject is needed to improve the life span and survival of aquatic organisms after exposure to natural and anthropogenic adverse conditions.

Introduction

Iron (Fe) is an essential element for the growth and life maintenance of heterotrophic and autotrophic aquatic organisms (González *et al.*, 2012a). Fe can be naturally abundant in certain regions or could be incorporated into water bodies by human actions. Eolian deposition of dust, river discharge, washout of particles in the atmosphere by rainfall, groundwater discharge, glacial melting, volcanic sediments, coastal erosion, and upwelling of Fe-rich deep waters over hydrothermal vents are among the natural Fe sources (Watson, 2001). Meanwhile, nanoparticles, chemical and mining industries, disposal of waste metal, ports, and eolian deposition of atmospheric dust from polluted areas are a few of such anthropogenic factors. Final Fe concentration in seawater depends on the area under consideration. The environmental conditions may act, adding potential stress to the aquatic biota since sediments can act either as a major reservoir (Caccia *et al.*, 2003) or as a source of Fe (Adams *et al.*, 1992) and other chemical elements.

Fe could act as a potent toxicant which has a wide spectrum of adverse effects generating adverse consequences in aquatic organisms. At the cellular physiological pH (7.0), both ferric (Fe^{3+}) and ferrous (Fe^{2+}) forms are present as complexes with low molecular weight compounds reported as the labile Fe pool (LIP). A permanent Fe flux from the extracellular medium to the cytoplasm is generated (González *et al.*, 2010). Terms such as "redox-active" or "chelatable Fe" have been also used when referring to LIP (Koppenol and Hider, 2019). Different ligands have been associated with cytosolic LIP. Glutathione (GSH), a molecule present in eukaryotic cells at relatively high concentrations, is considered a key Fe^{2+} ligand forming a 1:1 complex (Hider and Kong, 2011). Hider and Kong (2013) proposed that GSH is the major chelator to form "low molecular weight" Fe complexes in mitochondria. Fe concentration was estimated to be much higher than 1 μM

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Received: 18 October 2022; Accepted: 26 December 2022

Doi: 10.32604/biocell.2023.027107

inside the lysosome, due to the continued breakdown of ferritin (Ft) (Kurz *et al.*, 2007). The LIP is well known as an oxidative stress inducer through the increase in cellular levels of reactive oxygen species (ROS). The one-electron reduction of hydrogen peroxide (H_2O_2) by a Fe^{2+} complex in the Fenton reaction is an important contribution to the biological environment (Koppenol and Bounds, 2011). Fe can be involved in the initiation and propagation of free radical generation (Lushchak, 2011). These reactive species could trigger damage at the lipidic, DNA, and protein levels (Regoli *et al.*, 2004), enzyme inhibition, weakening of cell signaling, disruption of calcium homeostasis, and modifications in gene regulation (Stohs and Bagchi, 1995).

Xenobiotic uptake by phytoplankton is the first step in bioaccumulation processes in aquatic food webs. The water quality can depend on the type and number of algal species. Estévez *et al.* (2001) demonstrated the induction of oxidative stress due to an excess of Fe content in *Chlorella vulgaris* cultures. This response, coupled with other severe environmental conditions could affect cellular growth and have a negative biogeoimpact on phytoplankton. Fe uptake is strictly required for phytoplankton development since the photosynthetic complex contains numerous Fe loci. Ocean primary productivity may be limited by deficiency of this essential element in some regions (Martin and Fitzwater, 1988). On the other hand, Fe can control plankton blooms, which also affects the biogeochemical cycles of C, N, Si, and S, influencing the climate system of the earth (Boyd *et al.*, 2007). Zooplankton can aid in Fe mobilization by acidic digestion (Moore *et al.*, 1984), and grazing is an essential factor in recycling biogenic Fe, making it highly bioavailable (Hutchins *et al.*, 1999; Nuester *et al.*, 2014). The mobilized Fe can be released in a dissolved manner directly from krill, or via multiple pathways involving microbes, other zooplankton species, and krill predators. Then, substantial amounts of bioavailable Fe can be delivered to the water column contributing to the fertilization of coastal areas and the ocean itself. For example, Nuester *et al.* (2014) observed that diatoms take up 43- to 73-faster the regenerated Fe by copepods than the inorganic Fe. Copepods also release Fe-binding ligands when grazing on phytoplankton (Sato *et al.*, 2007). The ligands can form complexes with inorganic Fe and, thereby, increase Fe solubility. Filters and deposit feeders ingest Fe bound to dissolved and particulate matter in the water column and the sediment surface, but also dissolved Fe can be absorbed into respiratory surfaces (González *et al.*, 2015).

In recent years, a new form of Fe-dependent cell death, termed ferroptosis, was described in terrestrial animals and plants (Dixon *et al.*, 2012). This regulated cell death usually accompanies a large amount of Fe accumulation and lipid peroxidation (LP) (Li *et al.*, 2021). However, ferroptosis is poorly studied in aquatic environments and organisms. The purpose of this manuscript is to briefly summarize the central role of Fe in terms of cellular alterations of the oxidative/protective balance, with special emphasis on its possible involvement in ferroptosis disruption in aquatic organisms.

Fe and ferroptosis: General characteristics

Ferroptosis has been described as a complex mechanism involving (i) morphological, (ii) biochemical, (iii) genetic, and (iv) protein hallmarks (Chen *et al.*, 2021). Consequently, this kind of programmed cell death could have a great impact on growth, tissue homeostasis, and the development of diverse pathological conditions and diseases. The proliferation of the inducing signal occurs upstream of cell rupture and involves the spread of a cell swelling effect through cell populations (Riegman *et al.*, 2020). However, the main studies on this issue have been performed on vertebrates and have been focused on clinical consequences. The existence of these pathways in invertebrates and other aquatic organisms is still in the initial stages of being proved.

Biochemical hallmarks

The biochemical pathways involved in ferroptosis, a Fe-dependent regulated cell death (RCD), are the initial aspects that are studied or interpreted in aquatic organisms. These pathways are related to Fe cellular accessibility and accumulation, LP, and the dysfunction or decrease of the antioxidant ability of the defense system. Fe accumulation could be a biochemical sign of ferroptosis, mediating biochemical events (e.g., elevated Fenton reaction or activated Fe-containing enzymes) (Chen *et al.*, 2020). In ferroptotic cells or tissues, the intracellular or mitochondrial Fe^{2+} level is increased, and the treatment with Fe chelators, like deferoxamine, limits ferroptosis both, *in vitro* (Dixon *et al.*, 2012) and *in vivo* (Lu *et al.*, 2020).

Fe cellular availability

Fe is allocated in several subcellular organelles (e.g., mitochondria and lysosomes). Fe concentration in cells is regulated by a complex network that entails the absorption, storage, utilization, and discharge of the metal (Chen *et al.*, 2020). Specific molecular regulators, which are related to Fe homeostasis, control ferroptosis sensitivity. For example, Fe uptake facilitated by transferrin (TF; Gao *et al.*, 2015), lactotransferrin (LTF; Wang *et al.*, 2020), TF receptor (TFRC; Gao *et al.*, 2015), and the nuclear receptor coactivator 4 (NCOA4)-dependent Ft degradation (Hou *et al.*, 2016) promote ferroptotic cell death. According to Feng *et al.* (2020), TFRC is considered a biomarker of ferroptosis in cell cultures or tissues. Consequently, the presence of the anti-TFRC antibody (called 3F3-FMA) indicates ferroptotic cell death or damage. Studies of this nature have not yet been performed either in invertebrates or in photosynthetic aquatic organisms.

Eukaryotic algae can assimilate Fe from the environment through several mechanisms: release of siderophores, which are high affinity Fe-binding molecules (Krachler *et al.*, 2019), Fe^{3+} reduction by membrane ferric reductases, assimilation of photochemically reduced Fe^{3+} or Fe^{3+}–L (L = low affinity Fe-binding molecules; Shaked *et al.*, 2005), or by assimilation of bacteria and particulate minerals (Nodwell and Price, 2001). Uptake and use of Fe in metabolic activities by microalgae require a water-soluble form of Fe (Fe^{2+}) (Juneja *et al.*, 2013). The presence of the Fe^{2+} form is

needed in phytoplankton biomass growth and may facilitate the enhanced production of energy molecules, like lipids (Liu *et al.*, 2008; Ghafari *et al.*, 2018; Rana *et al.*, 2020). However, under Fe-limited circumstances, the rate of light absorption and light utilization in the electron transport system is imbalanced. For example, Fan *et al.* (2014) reported a 22.1% decrease in chlorophyll concentration under Fe-deficient conditions. Nevertheless, high Fe concentrations also have negative responses in microalgal cells decreasing their growth rate (Fan *et al.*, 2014).

During food ingestion, aquatic animals incorporate dissolved Fe and Fe bound to both inorganic particles and organic matter. In lobsters, Fe^{2+} is absorbed by the cells through a divalent cation exchanger (Chavez-Crooker *et al.*, 2001). In the digestive glands of the bivalve *Mya arenaria* the concentration of Fe^{3+} appears to be significantly lower than the concentration of Fe^{2+} in the LIP (González *et al.*, 2012b). Another study observed that the LIP represented 10% of the total Fe content in the digestive gland of the same bivalve species (González *et al.*, 2010), meanwhile, the LIP denoted a 3.5% of the total Fe content in the digestive gland of the bivalve *Laternula elliptica* (González and Puntarulo, 2011). Thus, a relatively more active Fe sequestration process can be suggested as a part of the safe cell compartments in *L. elliptica* than in *M. arenaria*. This information could indicate that *L. elliptica* has a better adaptation to an ecosystem with higher Fe natural marine content than *M. arenaria*.

Cellular injury and toxic effects related to Fe

Fe content in aquatic ecosystems could be a main stressor with a major role in LP in photosynthetic unicellular species and multicellular heterotrophic organisms, such as invertebrates and vertebrates (González *et al.*, 2012c). LP is defined as the oxidative damage of lipids with two or more instaurations (Hummel *et al.*, 2006). Polyunsaturated fatty acids (PUFA) are critical targets for powerful oxidizing species such as ROS. This mechanism of damage produces lipid hydroperoxides, one of the most significant hallmarks of ferroptosis (Dixon and Stockwell, 2014; Kuang *et al.*, 2020). Therefore, LP is widely accepted to play a key role in mediating ferroptosis (Yang and Stockwell, 2016).

Thamatrakoln *et al.* (2012) and Luo *et al.* (2014) demonstrated that in some microalgae, such as *Thalassiosira pseudonana* diatoms, both low and high Fe concentrations can lead to oxidative stress and the induction of RCD. Estévez and Puntarulo (2005) reported that at high Fe concentrations (500 μM Fe), oxidative damage was involved in the deleterious effects seen in *C. vulgaris*. However, at moderate concentrations (50 μM Fe), the metal administration not only improved the nutritional state but also increased the metabolic activity of the microalgae. *C. vulgaris* cells showed an increase of lipid radical ($LR^{\bullet}$) EPR signals in a Fe dose-dependent manner (Estévez *et al.*, 2001). Quantification of $LR^{\bullet}$ in algal supplemented with Fe exhibited a significantly increased in the $LR^{\bullet}$ content in the membranes at a higher tested dose of Fe (500 μM), as compared to those supplemented with 5 and 90 μM Fe. Robello *et al.* (2021) evaluated the supplementation of Fe at the latent growth phase in the marine diatom *Fragilaria* sp. In the presence of 50 μM Fe, the cells showed a significant reduction in the hydrophilic and lipophilic redox balance, as compared to control values. Significant changes in the $LR^{\bullet}$ content, generated as a result of growth or Fe supplementation with 0.3 and 50 μM Fe, were also observed. González *et al.* (2017) quantified the $LR^{\bullet}$-dependent EPR signals in two Antarctic macroalgae, *Gigartina skottsbergii* and *Himantothallus grandifolius*, from two different areas. They found that, in both species, $LR^{\bullet}$ content was significantly higher in the macroalgae inhabiting the Island D zone, heavily influenced by a glacier sediment inflow with high Fe input, as compared to samples isolated from specimens collected in Peñón de Pesca, a zone with low Fe presence. Even more, both algae showed changes in the lipophilic redox balance in environments with different Fe inputs (González *et al.*, 2017).

Other stressors, like temperature, could also stimulate LP-related ferroptosis. Aguilera *et al.* (2021) measured in the cyanobacteria *Synechocystis* sp. cytosolic ROS and LP accumulation after heat shock at 50°C heat. While the major level of cytosolic ROS was detected 1 h after heat shock, the accumulation of lipid peroxides reached a maximum of 3 h after the treatment. Both increases were suppressed by two ferroptotic inhibitors, the lipophilic antioxidant ferrostatin-1 and the membrane-permeable Fe chelator ciclopirox olamine, applied 24 h after the heat shock. These authors also observed a protective effect of D-linoleate (16 h) against the treatment in *Synechocystis* sp., suggesting that prokaryotic cells exposed to 50°C heat shock could lead to an oxidative Fe-dependent form of cell death with similar characteristics to ferroptosis in the eukaryotic cells (Bogacz and Krauth-Siegel, 2018; Dangol *et al.*, 2018).

Aquatic animals have high levels of PUFA to maintain cell membrane fluidity, especially those living in low-temperature environments (Abele and Puntarulo, 2004); therefore, they are prone to face LP when coping with high Fe concentrations. Numerous studies have analyzed Fe effects on the lipidic fraction of marine organisms, and they could be related to ferroptosis. Recently, Taze *et al.* (2016) exposed the bivalve *Mytilus galloprovincialis* to Fe oxide nanoparticles. They observed in the hemocytes a positive correlation between an increase in the DCFH-DA oxidation with LP (measured as thiobarbituric acid reactive substances, TBARS), DNA damage, and protein carbonylation. Also, blue mussel *Mytilus* sp. gills exposed to both, nano-Fe_2O_3 particles and a solution of the hydrated $FeCl_3$ for 12 h showed increased LP (measured as malondialdehyde, MDA) as compared to un-exposed animals (Kádár *et al.*, 2010). In another study, the gills and mantle of *M. arenaria* were analyzed after the exposure of the bivalve to elevated Fe levels (500 μM of Fe as Fe-EDTA) (González *et al.*, 2015). After 9 days of exposure, there was a significant increase in the total Fe and LIP content in the gills. Even more, LIP content represented 40% of the total Fe on day 17. After 17 days of exposure, the DCFH-DA oxidation rate was higher as compared to controls, and TBARS content increased by 1.9- and 3.7-fold over controls on days 9 and 17, respectively. Similar experiments were performed on the digestive glands and significant increases in total Fe, LIP, and TBARS contents were observed after

17 days of Fe incubation (González *et al.*, 2010). These results address the possibility of a ferroptosis disruption in *M. arenaria* after facing an excess of dissolved Fe. Similar effects were noticed in the mantle. Even more, González and Puntarulo (2016b) showed that the oxidative condition of the digestive gland of the Antarctic limpet *Nacella concinna* constantly covered by natural Fe-enriched waters (subtidal) was more altered than the intertidal population. Total Fe, LIP content, and lipid damage (assessed as TBARS content) were significantly higher in the subtidal, as compared to the intertidal population (1242 ± 367 and 491 ± 102 pmol/mg FW, respectively). Han *et al.* (2021) reported an increment in the intracellular ROS levels and a decrease in the neutral lipid content, in the sexual organs of the rotifer *Brachionus plicatilis* after exposure to 6 and 12 μg/mL of Fe.

The antioxidant defense system

Ferroptosis response can be regulated through the antioxidant defense system, together with metabolism, gene transcription, and protein degradation mechanisms (Chen *et al.*, 2021). Even though ferroptosis can be suppressed by the effect of Fe chelators and lipophilic antioxidants (Stockwell *et al.*, 2017), it can be initiated by a decrease in the antioxidant mechanisms. For example, the early depletion in non-enzymatic antioxidants such as GSH and ascorbate (AH^-), was characterized in ferroptotic conditions in plants and animals (Seiler *et al.*, 2008; Skouta *et al.*, 2014; Distéfano *et al.*, 2017). The GSH metabolism is the main pathway limiting ferroptosis. Biochemically, ferroptosis occurs mainly when there is an intracellular GSH depletion and a drop in the glutathione peroxidase 4 (GPx4) activity. Then, lipid peroxides cannot be metabolized by the GPx4-catalyzed reduction reaction, and Fe^{2+} oxidizes lipids through the Fenton reaction, generating a large amount of ROS (Yang and Stockwell, 2008; Friedmann Angeli *et al.*, 2014). GPx4 is a phospholipid selenium-dependent glutathione peroxidase (GPx) isozyme mainly present in mammalian tissues (Margis *et al.*, 2008). GPx4 is present in the nucleus, cytosol, and mitochondria and bound to membranes in cells (Herbette *et al.*, 2007). This enzyme is also known to be present in fishes and plants; but its connection to ferroptosis has not been established yet (Conrad *et al.*, 2018). Nevertheless, the Fe-dependent cell death process with similar pathways in plants during heat shock and other stressors has already been described. Invertebrates usually express cysteine (Cys)-containing homologs of GPx4 (Ingold and Conrad, 2018). Possible scenarios of ferroptosis in aquatic organisms exposed to Fe could be linked to some of the above-mentioned conditions.

Lipophilic antioxidants enhance the oxidative stability of the cell membranes owing to their ability to protect PUFA from peroxidation and to scavenge free radicals. Among the lipophilic antioxidant compounds, carotenoids, tocopherols, and ubiquinols are the most recognized. Carotenoids represent an important group of pigments in marine environments and terrestrial species (Galasso *et al.*, 2017). They are biosynthesized by autotrophic marine groups: bacteria and archaea, algae, and fungi. Some heterotrophic organisms contain carotenoids, but these are accumulated from food sources or are partly modified through cellular metabolic reactions (Cardoso *et al.*, 2017). Their lipophilic nature allows them to enter the cellular bilayer membrane. Therefore, they can: (i) chelate singlet molecular oxygen ($^1O_2$); (ii) change hydroperoxides into more stable compounds; (iii) inhibit free radical oxidation reactions; and (iv) act as Fe and Cu quenchers (Galasso *et al.*, 2017). Since these antioxidants reduce lipidic damage and trap Fe ions, their synthesis in algae and their incorporation in animals result crucial to avoid any scenario of ferroptosis, especially in cold water organisms.

Estévez *et al.* (2001) evaluated the content of lipophilic antioxidants, both α-tocopherol (α-T) and β-carotene (β-C) in *C. vulgaris* cells exposed to 5, 90, and 500 μM Fe. Although the content of β-C was not affected, α-T content showed a significant increment with the increase in cellular Fe availability. Robello *et al.* (2021) also reported a significant increase in the α-T and β-C contents in the lag phase of the development of *Fragilaria* sp. Cells supplemented with Fe, as compared to the values of controls. Considering lipophilic oxidative ratios, the Fe supplementation produced a significant reduction in the $LR^{\bullet}$/α-T and $LR^{\bullet}$/β-C ratios in the lag phase of growth. During the lag phase, the cells seemed well prepared to deal with an excess Fe also in the hydrophobic intracellular environment. Due to an increment in AH^- content, the ascorbyl ($A^{\bullet}$)/AH^- ratio, an indicator of the oxidative balance in the hydrophilic medium of the biological systems, was significantly increased in cells in the stationery and lag phases of growth. Antioxidant enzymes also showed significant changes in *Fragilaria* sp. exposed to Fe. A reduction in the superoxide dismutase (SOD, 36% as compared to controls) activity, catalase (CAT, 72% of control values) content, and nitric oxide generation rate (NO, 60% of control values) was reported in 50 μM Fe supplementation experiments in cells in the lag phase of development.

González *et al.* (2017) reported a significantly higher activity of the antioxidant enzymes CAT and glutathione transferase (GST) in the Antarctic macroalgae *G. skottsbergii* collected from Island D with high Fe input as compared to those values in the algae samples collected in Peñón de Pesca (the control area). No significant differences were observed in SOD activity and α-T content in the organisms from both locations. In contrast, the content of AH^- and β-C was lower in the macroalgae collected from Island D compared to those measured in Peñón de Pesca. The $LR^{\bullet}$/α-T, $LR^{\bullet}$/β-C, and $LR^{\bullet}$/(α-T + β-C) content ratios (indicators of the oxidative balance in the lipophilic medium of the biological systems) were increased by 2-, 6- and 4-fold, respectively in *G. skottsbergii* collected from the island, as compared to those algae from the control zone, suggesting that the damaging effect of excess Fe overwhelmed the protection in the macroalgae exposed to high Fe content.

Moreover, Aguilera *et al.* (2021) observed that the exposure of *Synechocystis* sp. cells to a temperature of 50°C induced a decrease in GSH and AH^- total content. The addition of ferroptosis inhibitors (Fer-1 or CPX) could not prevent the depletion of these antioxidants, suggesting that the reduction of GSH and AH^- might be an early outcome in this cell death pathway, as described for Distéfano *et al.*

TABLE 1

Rate of Fe release from ferritin (Ft) isolated from digestive glands of mollusk at 25°C using different AH^- concentrations as a reductant agent

Species	Rate of Fe release (pM Fe/min)		
	1 mM AH^-	5 mM AH^-	10 mM AH^-
Bivalves			
Laternula elliptica (67 μg Ft/mL)	52 ± 5	115 ± 12*	221 ± 22**
Limpets			
Nacella concinna intertidal (67 μg Ft/mL)	7.1 ± 0.4	13.0 ± 0.7*	nd
N. concinna subtidal (60 μg Ft/mL)	71 ± 4	168 ± 8*	264 ± 13**
N. deaurata (67 μg Ft/mL)	6.1 ± 0.3	12.1 ± 0.6*	27 ± 1**
N. magellanica (67 μg Ft/mL)	13.9 ± 0.7	33 ± 2*	66 ± 3**

Note: *Significantly different to 1 mM AH^-, ANOVA ($p < 0.05$). **Significantly different to 1 and 5 mM AH^-, ANOVA ($p < 0.05$). nd stands for not determined.

(2017) in plant ferroptosis. Moreover, cell death was prevented by preincubation with GSH and AH^-, suggesting that the decrease in these hydrophilic antioxidants is necessary for the heat shock-induced cell death in *Synechocystis* sp.

Studies in marine animal tissues, such as the Anguilla gills exposed to silica-coated Fe oxide nanoparticles functionalized with dithiocarbamate (Fe_3O_4@SiO_2/SiDTC, 100 nm; 2.5 mg/L) under *in vitro* conditions, showed a statistically significant reduction of the total GSH content of the cells after 2 to 72 h of exposure, as compared to control cells. Even more, LP, measured as TBARS, increased significantly after exposure to Fe nanoparticles. Therefore, the antioxidant defense system of the gill was stressed (Srikanth *et al.*, 2014). On the other hand, Li *et al.* (2009) experimented on exposed embryos and adult medaka *Oryzias latipes* to nano-Fe particles (average size 30 nm, at doses of 0.5, 5, and 50 μg/mL, actual nano-Fe concentrations of 0.48 ± 0.05, 4.7 ± 0.2, and 46.6 ± 2.7 μg/mL, respectively) and found that the embryos were more sensitive than the adults, regarding the alterations on levels of GSH and MDA. Thus, oxidative damage was only reported in the embryos. Even more, these authors could not find significant changes in the histopathological and morphological alterations or GSH and MDA content in the liver and brain of adult fish. These findings may support the idea that (i) ferroptosis is not developed when GSH levels are not affected by Fe exposure, (ii) immature organisms may be more susceptible to Fe deleterious effects, such as ferroptosis, and (iii) that different tissues may respond distinctly to Fe exposure.

In mammals and invertebrates, two specialized Fe-binding proteins: the extracellular Tf and the intracellular (extracellular in invertebrates) Ft (Winzerling and Law, 1997) provided defense against the toxic effect of Fe and O_2 mixtures. The Tf serves to sequester Fe^{3+} (Kd~10–22 M; Aisen *et al.*, 1978), protecting Fe^{3+} from hydrolysis at physiological pH, and rendering it unavailable for catalysis of superoxide anion (O_2^-) formation via Fenton reaction (Gutteridge, 1994). Fts are known as the main Fe storage proteins that can be also used as detoxifying cellular components. The bivalves *Hyridella depressa* and *Margaritifera* accumulate Fe in their lysosomes and in calcified concretions (granules) to contribute to the shell formation via Tf and Ft (Simon *et al.*, 2011). Ft has been also shown to function as a participant in shell biomineralization from the bivalve *Pinctada fucata* (Zhang *et al.*, 2003). *Mytilus edulis* hemocyte cells with the presence of Ft are considered the major Fe-storage cells in invertebrates (Winston *et al.*, 1996; Ahearn *et al.*, 2004). González and Puntarulo (2011) found that Fe, which is constantly absorbed by the bivalve *L. elliptica*, is gradually incorporated into Ft. This report also showed that there were non-significant differences in the total amount of Ft in the digestive gland from neither this bivalve nor *M. arenaria*. In contrast, the Ft isolated from the digestive gland of *L. elliptica* showed a significantly higher Fe content than the Ft isolated from *M. arenaria*. These findings suggest that the environmental Fe content is responsible for the Fe amount incorporated into the main cellular storage protein. Ft has also been identified in the oyster *Crassostrea gigas* (Durand *et al.*, 2002).

To be incorporated into Ft, Fe^{3+} is reduced to Fe^{2+}, where it is stored as Fe^{3+} (inert form); nevertheless, by redox reaction with other cellular components (i.e., O_2^-), Fe^{3+} from Ft could be reduced to Fe^{2+}, released to the cytoplasm and be incorporated into the LIP. Ft plays a dual role in LIP homeostasis regulating the amount of catalytically active Fe in the cytosol. At high intracellular Fe concentrations, Ft binds Fe to protect cells against its toxicity. In contrast, at low Fe concentrations, Ft releases the metal from its protein core to satisfy the cellular requirements. This release was already proved through *in vitro* experiments (Winzerling and Law, 1997). However, since Fe is stored as Fe^{3+}, appropriate reductants are required for the mobilization process (Funk *et al.*, 1985). Ft isolated from digestive glands from different mollusk species, a bivalve, and limpets, were exposed to three different AH^- (used as a Fe reductant) concentrations (Table 1). In the studied species, Fe was released from the Ft and the Fe liberation rate was significantly increased at higher AH^- content duplicating its release in every case and species, which suggests the importance of this mechanism for cellular Fe availability.

Genetic hallmarks of ferroptosis linked to Fe

Ferroptosis is a biological process regulated by multiple genes, including genetic changes in Fe homeostasis and LP. Genes coding for Tf and transferrin receptor (Tfr) control Fe content and modulate ferroptosis due to their capacity to import Fe into cells (Gao *et al.*, 2015); Fe-responsive element binding protein 2 (IREB2) a regulator of Fe metabolism (Dixon *et al.*, 2012); and the machinery for degradation of Ft, known as ferritinophagy (Mancias *et al.*, 2014; Gao *et al.*, 2016; Hou *et al.*, 2016; Wang *et al.*, 2016). Ft is recognized by NCOA4 (Mancias *et al.*, 2014), and this product also modulates ferroptosis sensitivity in some species. Finoshin *et al.* (2020) studied the Fe metabolic pathways in the processes of sponge plasticity and some known proteins of Fe metabolism, such as the light chain of Ft, IREB2, Zip14 (Fe^{2+} transporter), Dcytb and STEAP2/3 (membrane metal reductase transporters), ceruloplasmin (Cu-binding glycoprotein with ferroxidase activity), hephaestin (intestinal and ferroxidase), mitochondrial transporter ABCB10, hepcidin/hemochromatosis/hemojuvelin (systemic regulator of Fe metabolism and its regulators), and Tf and Tfr1/2 were not found.

Caspase-3/7 activities were not induced after heat shock (50°C treatment) in *Synechocystis* sp. In animals, the central executioners of apoptosis are the cysteine-dependent proteases named caspase families implicated in the regulation of several RCD signaling pathways (van Opdenbosch and Lamkanfi, 2019). Other multicellular and unicellular organisms lack true caspases but contain various protein homologs, such as metacaspases and orthocaspases (Klemenčič and Funk, 2018). Most of their functions and regulations have not yet been studied (Klemenčič *et al.*, 2019).

Aguilera *et al.* (2021) also examined the molecular mechanisms governing ferroptosis in the cyanobacteria *Synechocystis* sp. cells exposed to 50°C. The studied genes included cyanobacterial GSH synthesis (gshA and gshB) and GSH catabolism (ggt; Narainsamy *et al.*, 2016); and gpx1 and gpx2 are orthologues to human GPx4, essential in animals ferroptosis regulation (Friedmann Angeli *et al.*, 2014). While the three genes gshA, ggt, and gpx1, were upregulated after heat shock exposure, the gpx2 gene was downregulated. The involvement of a pseudo-orthocaspase (SyOC), a prokaryotic caspase-homolog lacking the p10 domain, in the oxidative stress of the cyanobacteria model *Synechocystis* sp. PCC 6803 was described by Aguilera *et al.* (2021). To study the *in vivo* impact of this pseudo-protease during oxidative stress, its gene expression at H_2O_2 exposure was monitored by real-time-quantitative polymerase chain reaction. Deletion of SyOC led cells with a higher tolerance towards oxidative stress, suggesting that this protein may be involved in a pro-death pathway.

NCOA4 is a nuclear receptor that can be found in different tissues. Arroyo Salazar (2019) studied the expression of NCOA4 in the gonads of the bivalve *Crassostrea virginica* faced to hydrocarbons exposition. NCOA4 genes displayed a higher significative expression in males than in females (Guévélou *et al.*, 2013). Even more, higher gene expression was found in organisms of both sexes exposed to hydrocarbons, as compared to control values. Even though up to now there are no studies that may link NCOA4 and Ft in mollusks, it is possible to presume a relationship between this gene activity with the oxidative stress generated by exposure to hydrocarbons (Arroyo Salazar, 2019).

Table 2 summarizes the biochemical and genetic hallmarks of ferroptosis in recent literature on mollusks. Much more work is required to understand this complex phenomenon.

TABLE 2

A summary of the reported data on biochemical and genetic ferroptosis hallmarks in different aquatic organisms

Species	Treatment or natural stressor	Fe content	LP	Antioxidants	References
Biochemical ferroptosis hallmarks					
Photosynthetic organisms					
Chlorella vulgaris	5 μM (C)	0.16 ± 0.01 nmol/10^6 cells	$LR^{\bullet}$	AH^-/α-T/β-C	Estévez *et al.* (2001)
(LOG phase)	90 μM	2.8 ± 0.4 nmol/10^6 cells	↑$LR^{\bullet}$	=AH^-/=α-T/=β-C	
	500 μM	8.4 ± 1 nmol/10^6 cells	↑↑$LR^{\bullet}$	↑AH^-/↑α-T/=β-C	
Fragilaria sp.	0.3 μM (C)	<5 pmol/10^6 cells	$LR^{\bullet}$	AH^-/α-T/β-C/SOD/CAT/NO	Robello *et al.* (2021)
(LAG phase)	50 μM	70–75 pmol/10^6 cells	=$LR^{\bullet}$	=AH^-/↑α-T/↑β-C/↓SOD/↓CAT/↓NO	
Gigartina skottsbergii	Peñon de Pesca (C)	1 ± 0.5 nmol/mg FW	$LR^{\bullet}$	AH^-/α-T/β-C/SOD/CAT/GST	González *et al.* (2017)
	Island D (Fe input)	2.9 ± 0.7 nmol/mg FW	↑↑$LR^{\bullet}$	↓AH^-/=α-T/↓β-C/=SOD/↑CAT/↑GST	

(Continued)

Table 2 (continued)

Species	Treatment or natural stressor	Fe content	LP	Antioxidants	References
Himantothallus grandifolius	Peñon de Pesca (C)	0.55 ± 0.05 nmol/mg FW	$LR^{\bullet}$	AH^{-}/α-T/β-C/SOD/CAT/GST	González *et al.* (2017)
	Island D (Fe input)	1.0 ± 0.2 nmol/mg FW	↑↑$LR^{\bullet}$	↓AH^{-}/=α-T/=β-C/↑SOD/=CAT/↑GST	
Invertebrates					
Mya arenaria	(C)	0.70 ± 0.08 nmol/mg FW	LP	AH^{-}/GSH/SOD/CAT/NO	González *et al.* (2010)
Digestive gland	500 μM (17 days)	1.88 ± 0.06 nmol/mg FW	↑LP	=AH^{-}/=GSH/↓SOD/=CAT/↓NO	
Mya arenaria	(C)	0.3 ± 0.1 nmol/mg FW	LP	CAT	González *et al.* (2015)
Gills	500 μM (9 days)	0.435 ± 0.005 nmol/mg FW	↑LP	↓CAT	
	500 μM (17 days)	0.44 ± 0.04 nmol/mg FW	↑↑LP	↓CAT	
Mytilus galloprovincialis	(C)		LP		Taze *et al.* (2016)
Hemocytes	50 mg/L Fe oxide (7 days)		↑LP		
Mytilus sp.	(C)		LP		Kádár *et al.* (2010)
Gills	nano-Fe_2O_3/$FeCl_3$ (12 h)		↑LP		
Nacella concinna	Intertidal (C)	2.0 ± 0.1 nmol/mg FW	LP	SOD/CAT/Ft/NO	González and Puntarulo (2016a)
Digestive gland	Subtidal (Fe input)	3.4 ± 0.4 nmol/mg FW	↑LP	=SOD/=CAT/=Ft/=NO	
Genetic ferroptosis hallmarks		**Genetic parameters**			
Synechocystis sp.	(C)	SyOC gene/gshA/gshB/ggt/gpX1/gpx2	LP	AH^{-}/GSH	Aguilera *et al.* (2021)
(LOG phase)					
	Heat shock (50°C)	↑SyOC/↑gshA/=gshB/↑ggt/↑gpX1/↓gpx2	↑↑LP	↓AH^{-}/↓GSH	

Note: (C) indicates basal conditions. = indicates no changes with respect to basal conditions. ↓ indicates a significant decrease in the content/activity/expression with respect to basal conditions. ↑ indicates significant increase in the content/activity/expression with respect to basal conditions. ↑↑ indicates a high increase in the content/activity with respect to basal condition.

Discussion and Conclusions

Interesting research on the Fe role has been done on aquatic organisms throughout the years; however, no direct relation with ferroptosis was clarified yet. A new approach to this LP-mediated effect is proposed since many of the biomarkers and parameters measured in these organisms (e.g., after exposure to high amounts of Fe, derived from natural or anthropogenic sources), could be directly implicated in triggering ferroptosis. Fe is a metal with a double-edge effect on cells especially depending on its concentration. At low levels, it acts as a micronutrient, often being the central ion in molecules of diverse biological functions, especially in electron transport systems. However, passing a certain threshold level may act as a catalyst for the generation of free radicals. This last Fe function in cells is now of interest to medical surveys, as a new form of cell death, by the triggering of ferroptosis. However, this impact could be also expanded to environmental organisms that face exposure to high Fe concentrations in the medium.

Phytoplankton often dies spontaneously in adverse environmental conditions. Cell death by lysis has been documented in field populations exceeding 50% of phytoplankton growth (Agustí *et al.*, 1998) The induction of autocatalytic programmed cell death (PCD) by biotic or abiotic stresses in prokaryotic and eukaryotic phytoplankton provides one of many reasons behind high lysis rates, independent of viral attack or grazing by heterotrophs (Bidle, 2016). These intrinsic molecular mechanisms could influence the microbial loop changing the flow of photosynthetically fixed organic matter, and associated elements, through the main ecosystem food web pathways, and serves to regulate ocean biogeochemistry (Kirchman, 1999; Bidle and Falkowski, 2004; Bidle, 2015).

On the other hand, RCD has been proposed to optimize differentiation, dynamics, and colony fitness in cyanobacteria

(Meeks and Elhai, 2002; Bar-Zeev *et al.*, 2013). Aguilera *et al.* (2021) found that in response to heat stress (50°C), *Synechocystis* sp. follows PCD exhibiting biochemical and morphological features that resemble eukaryotic ferroptosis. Moreover, the pathway in *Synechocystis* sp. PCC 6803 single orthocaspase is characterized by LP and an early decrease of the antioxidants GSH and AH^- content (Aguilera *et al.*, 2019). Reduction of GSH content could be a potential hallmark to distinguish ferroptosis-like from other types of PCD (Zhou *et al.*, 2020). As mentioned, this cell death is dependent on Fe availability, and LP, and is inhibited by canonical ferroptosis inhibitors. However, Aguilera *et al.* (2021) proved that inhibitors and the external addition of antioxidants could not suppress cell death under conditions of high temperature (77°C) or H_2O_2 (10 mM) exposure. This result suggests that both conditions led to accidental cell death. Still, additional components of the pathway involved in ferroptosis-like processes in cyanobacteria have not yet been identified even though genes related to GSH synthesis and Fe transport are induced after 50°C exposure (Aguilera *et al.*, 2021). The fact of endosymbiotic cyanobacterial origin of chloroplasts and the presence of this Fe-dependent oxidative PCD pathway in cyanobacteria, suggest the evolutionary origin of the role of chloroplast during plant ferroptosis (Distéfano *et al.*, 2017). This observation has not only provided information about the evolution of cell death in unicellular photoautotrophs, but also acknowledges the impact of PCD on the fate of natural phytoplankton assemblages and its role in aquatic biogeochemical cycles (Bidle, 2016).

Studies in biological systems suggest hormesis as an adaptive response of cells and organisms to a moderate stress condition. Hormetic responses involve signaling pathways typically leading to the alteration of the activities of enzymes, such as kinases and deacetylases, and transcription factors that trigger biologically beneficial effects. Fe is not only responsible of ferroptosis conditions, but it is also a hormetic agent in the low-dose range (Galleano *et al.*, 2011; Piloni *et al.*, 2018). Some aquatic organisms exposed to slight sublethal stress conditions trigger a protective response against greater successive challenges. Therefore, the level of stress can lead to a dose-response effect resulting in the so-called hormesis process (Southam and Ehrlich, 1943; Calabrese and Blain, 2011). Thus, hormesis is a biphasic dose-response relationship with contrasting effects of low (with positive/stimulatory responses) *vs.* high (with negative/inhibitory adverse responses) doses of stress (Agathokleous *et al.*, 2020). The stress conditions known may initiate hormesis comprise exposure to pollutants, metals, agrochemicals, toxins, natural products, ionizing radiation, caloric restriction, hypoxic conditions, ischemic pre-conditioning, and many other provocations (Schmitt *et al.*, 2002; Schulz *et al.*, 2007; Calabrese, 2013; Schmeisser *et al.*, 2013, Chamsi *et al.*, 2019). A temporary up-regulation of endogenous activities of antioxidant enzymes focusing on the improvement in reactive species detoxification has emerged as a feature for many aquatic organisms to tolerate, for example, hypoxia conditions (Oliveira *et al.*, 2018). Nowadays hormesis is fast emerging in plant science research and has large repercussions for risk assessment, stress biology, and agriculture (Agathokleous *et al.*, 2020). In this context, moderate increases in ROS, generated by Fe exposure, during a limited period may lead to gene expression regulation with consequently cytoprotective responses for exposure to subsequent noxious events (Dröge, 2002; Das and Das, 2008). Protective effects of Fe have been reported by Pagano *et al.* (1996) in sea urchin embryos from the species *Paracentrotus lividus* and *Psammechinus microtuberculatus* and embryos of the bivalve *M. galloprovincialis* exposed to ferric chloride [$FeCl_3 \bullet 6H_20$]. The authors showed that in *P. lividus* embryos, an addition of Fe^{3+} low-level concentration (10^{-8} to 10^{-7} M) improved larval quality, addressing it to a hormetic effect, as was

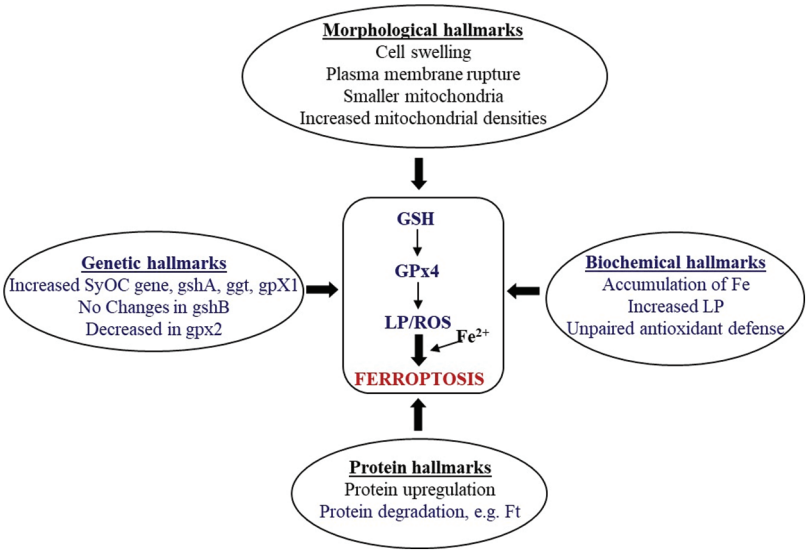

FIGURE 1. A summary of ferroptosis hallmarks in aquatic organisms. Writing in blue indicates reported information in aquatic organisms. LP, lipid peroxidation; ROS, reactive oxygen species; Ft, ferritin; GPx4, glutathione peroxidase 4; GSH, reduced glutathione; SyOC, pseudo-orthocaspase (SyOC) gene; gengshA, γ-glutamyl-cysteine ligase gene; ggt, γ-glutamyl transpeptidase gene; gpX1, orthologue to human GPx4 gene; gshB, glutathione synthase gene; gpx2, orthologue to human GPx4 gene.

previously reported for other low-level toxicants (Pagano *et al.*, 1986). Nevertheless, this protective effect was not observed in the *M. galloprovincialis* embryos.

The diagram shown in Fig. 1 summarizes the reported data about ferroptosis hallmarks (grouped as morphological, biochemical, protein, and genetic characteristics) previously described in vertebrates which have been seen in aquatic organisms. Once Fe is incorporated into the organisms, through ingestion of food or from the dissolved Fe fraction, oxidative stress and damage can be triggered with an increase in ROS generation and a depletion of the antioxidant system leading to ferroptosis. However, if Fe chelators, certain antioxidants, and specifically regulating genes are activated, ferroptosis can be prevented. Other stressors that might affect Fe homeostasis or trigger signaling factors in common with Fe could also be responsible for the induction of ferroptosis or hormesis, depending on the imbalance in the oxidative condition. Thus, the global scenario is even more complicated than it was previously understood regarding the role of Fe as a toxic component in biological systems. Much more research is needed on this subject to improve the life span and survival of aquatic organisms after exposure to natural and anthropogenic adverse conditions.

Funding Statement: This study was supported by grants from the University of Buenos Aires (UBACyT 20020170100199BA), the National Agency of Research, Technological Development and Innovation (ANPCyT) (PICT-2020-SERIEA-03542), and the National Council for Science and Technology (CONICET) (PIP 11220210100183CO). PMG and SP are career investigators (CIC) from CONICET and JC is a fellow from CONICET.

Author Contributions: The authors confirm contribution to the paper as follows: study conception and design: P.M. González and S. Puntarulo; data collection: P.M. González and J. Cabrera; analysis and interpretation of results: P.M. González and J. Cabrera, S. Puntarulo; draft manuscript preparation: P.M. González and J. Cabrera, S. Puntarulo. All authors reviewed the results and approved the final version of the manuscript.

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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