REVIEWS IN Aquaculture

Reviews in Aquaculture (2018) **10**, 525–542 doi: 10.1111/raq.12184

Toxic effects of nitrite on freshwater organisms: a review

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Received 1 July 2016; accepted 26 September 2016.

Abstract

High nitrite concentrations may occur mainly in recirculating aquaculture systems, but can also be found under certain conditions in natural waters. Among studied freshwater organisms, molluscs and worms followed by fish are the most resistant to nitrite. On the other hand, crustaceans and aquatic insects followed by amphibians are the most sensitive. Wide interspecific differences in nitrite susceptibility can be found within freshwater insects, crustaceans and amphibians. Chloride concentration in water is supposed to be the most important factor influencing nitrite toxicity. Generally, a positive chloride effect on nitrite toxicity reduction can be expected in all aquatic animals (or their early stages) employing gills for breathing and ion exchange. This phenomenon has already been observed in an amphipod (*Eulimnogammarus toletanus*, Pinkster & Stock), a planarian (*Polycelis felina*, Dalyell), two species of crayfish, several fish species and in amphibians in early development stages. A relatively huge amount of data on nitrite effects is available for fish, but other freshwater organisms were less frequently studied. Information on chronic effect of nitrite is nearly completely missing.

Key words: amphibians, aquaculture, crustaceans, fish, insect, toxicity.

Introduction

In natural waters, nitrites typically accompany nitrates and forms of ammoniacal nitrogen. Owing to their chemical and particularly biochemical lability, nitrites typically occur at low concentrations and never constitute the dominant form of inorganic nitrogen. The reason is that in oxic environments, they are subject to nitrification and quickly transformed into nitrates, whereas in anoxic environments, they undergo mainly denitrification to gaseous end products, elemental nitrogen (N_2) or nitrous oxide (N_2O), or other biochemical reductions (Philips *et al.* 2002; Pitter 2009). In the summer, however, nitrite concentrations can increase to levels ranging from tenths up to 1 mg L^{-1} NO_2^- in surface waters mainly in those across agricultural watersheds (Smith *et al.* 1995; von der Wiesche & Wetzel 1998; Corriveau *et al.* 2010). In wastewater, nitrite concentrations may exceed the level of 1 mg L^{-1}. Wastewater produced by some sections of engineering plants may contain up to hundreds of mg L^{-1} of nitrite nitrogen, while the levels in composite wastewater of the whole plant may be in tens of mg L^{-1} (Pitter 2009).

Increased nitrite concentrations (ranging from tenths to tens of mg L^{-1} NO_2^-) may be detected in waters used for intensive farming of commercial and aquarium fish or other aquatic organisms, particularly in recirculating aquaculture systems (RAS), at the very beginning of operation or as a result of insufficiently effective biological filtering (Avnimelech *et al.* 1986; Kamstra *et al.* 1996; Burford *et al.* 2003; Svobodová *et al.* 2005a; Buřič *et al.* 2016). Nitrite was found, for example, in commercial eel (*Anguilla anguilla*, Linnaeus) farms in concentrations up to 49 mg L^{-1} NO_2^- (Kamstra *et al.* 1996) or in RAS stocked with Blue tilapia (*Oreochromis aureus*, Steindachner) at even higher concentrations reaching 99 mg L^{-1} NO_2^- (Avnimelech *et al.* 1986). In biological filters, the process of nitrification takes place, reducing levels of ammonia, the principal waste product of nitrogen metabolism in fish (Wood 1993). In the course of nitrification, ammoniacal nitrogen is first biochemically oxidised to nitrites and then to nitrates, which are less toxic than ammonia and nitrites to aquatic organisms. If the second nitrification phase is partly or completely inhibited, nitrite accumulation occurs. Ultra violet irradiation which is used for water disinfection in recirculating fish culture systems (Sharrer *et al.* 2005) may be another source of nitrite if the treated water contains high nitrate levels, as nitrate can be converted to nitrite by UV irradiation (Lu *et al.* 2009). Nitrite accumulation in

aquaculture systems often results in deterioration of health in stocked specimens and sometimes in mass dying (Tucker & Schwedler 1983a; Svobodová *et al.* 2005a; Jørgensen *et al.* 2009; Kouba *et al.* 2012).

Nitrite uptake

Exposed to nitrites, most freshwater fish actively accumulate this ion in their plasma (Jensen 2003). Nitrite concentrations in fish blood plasma may reach levels up to 60 times higher than those in the surrounding water (Fontenot *et al.* 1999). To a lesser extent, nitrites are also accumulated in some fish tissues, namely in the gills, liver, brain and in muscles (Margiocco *et al.* 1983). They penetrate into fish body through the eosinophilic chloride-secreting gill cells, which, among other functions, perform the exchange of Cl^-/HCO_3^- ions (uptake/excretion) between the organism and the environment. Nitrites act as a competitive inhibitor of chloride uptake, and vice versa (Crawford & Allen 1977; Williams & Eddy 1986; Harris & Coley 1991). If water contains nitrites, fish take them in at the expense of chlorides.

Fish sensitivity to nitrite is therefore closely related to the rate of their chloride uptake. Fish manifesting a high rate of chloride uptake through their gills, for example rainbow trout (*Oncorhynchus mykiss*, Walbaum), Eurasian perch (*Perca fluviatilis*, Linnaeus), channel catfish (*Ictalurus punctatus*, Rafinesque) and northern pike (*Esox lucius*, Linnaeus), have been found to be very sensitive to nitrite. Conversely, fish characterised by a very low or zero rate of chloride uptake, for example European eel (*A. Anguilla*, Linnaeus), common carp (*Cyprinus carpio*, Linnaeus), tench (*Tinca tinca*, Linnaeus) and bluegill (*Lepomis macrochirus*, Rafinesque), are resistant to nitrite (Williams & Eddy 1986, 1988; Tomasso & Grosell 2005). The mechanism of nitrite uptake also explains why increased Cl^- levels in water protect fish from nitrite uptake and its toxic effects (Jensen 2003).

Freshwater crayfish also actively take in nitrites through their gills, just as they absorb chlorides, and accumulate them at high concentrations in their hemolymph (Harris & Coley 1991; Jensen 1996b). Exposed to the same nitrite concentrations under the same environmental conditions, European crayfish (*Astacus astacus*, Linnaeus) will manifest a nitrite level in their hemolymph up to four times higher than trout (Jeberg & Jensen 1994; Stormer *et al.* 1996; Jensen 2003). The reason for higher nitrite levels in crayfish hemolymph remains unknown as yet, however, crayfish are assumed to manifest a higher rate of chloride uptake compared to fish, and/or nitrites show a higher affinity to ion exchange occurring in crayfish gills compared to fish (Jensen 1996a).

Some authors state that diffusion of uncharged nitrous acid (HNO_2) across gill membranes is an alternative route of nitrite uptake by fish or crustaceans as nitrite occurs in water not only in the form of nitrite ions (NO_2^-) but also HNO_2. However, HNO_2 is a medium strong acid with $pK_a = 3.35$ ($t = 25°C$) and thus NO_2^- highly prevails over HNO_2 at pH range of 6–9 (>99.8% vs. <0.2%, resp.) (Pitter 1999), that is at pH acceptable for most fish and crustacean species. Thus contribution of HNO_2 to nitrite toxicity is negligible. HNO_2 cannot be compared with, for example HCN which is a weaker acid prevailing in dissociated form (CN^-) at much higher pH (>10). Therefore, HCN contributes significantly to cyanide toxicity even within natural pH range (e.g. at pH 7 HCN represents nearly 100% of both cyanide species).

Toxicity mechanisms

In fish, nitrites make their way from blood plasma into erythrocytes, where they oxidise bivalent iron in haemoglobin to trivalent iron, producing methaemoglobin, which is incapable of carrying oxygen, and so reducing the capacity of blood to transport oxygen (Bodansky 1951). As methaemoglobin is brown, its increased concentration in blood results in a brown colouring of blood and gills. The proportion of methaemoglobin in fish blood and nitrite concentration in blood plasma have a linear relationship (Fig. 1). It is to be noted here that fish blood commonly contains a certain proportion of methaemoglobin even in the absence of nitrites, and concentrations of up to 10% are by no mean uncommon (Cameron 1971; Soldatov 2002). The maximal level of methaemoglobin, 27%, without visible symptoms of pathology was reported for Atlantic cod (*Gadus morhua*, Linnaeus) (Graham & Fletcher 1986). This is because haemoglobin in fish is more prone to auto-oxidation by oxygen than in mammals (Beutler 1968; Kiese 1974). When fish that have suffered nitrite poisoning are moved to clean water, methaemoglobin levels in the blood usually drop to normal physiological values within 24–72 h (Huey *et al.* 1980; Knudsen & Jensen 1997), unless nitrite poisoning has progressed too far. Recovery from methaemoglobinemia in rainbow trout was observed also after chloride supplementation into the water even in the presence of nitrite (Zusková *et al.* 2013). At least two mechanisms are supposed to play an important role in the process of recovery, that is NADH-methaemoglobin reductase system which carries out reduction in methaemoglobin (Fe^{3+}) back to haemoglobin (Fe^{2+}) (Huey & Beitinger 1982) and nitrite oxidation (detoxification) to almost nontoxic nitrate presumably carried out by catalase and cytochrome oxidase (cytochrome aa_3) (Doblander & Lackner 1996, 1997). Fish also respond to oxidative stress caused by nitrite exposure by upregulating mRNA expression of genes encoding important antioxidant enzymes (Sun *et al.* 2014).

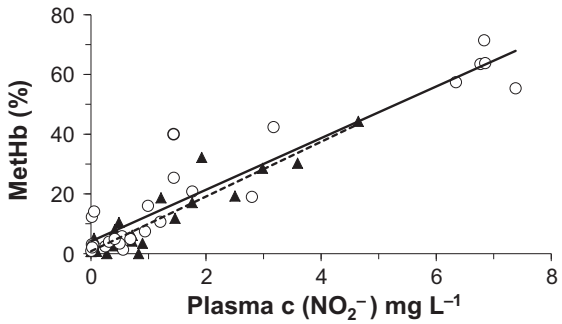

Figure 1 Linear relationship between the proportion of blood methaemoglobin (MetHb) and nitrite concentration in blood plasma in Eurasian perch (*Perca fluviatilis*) and largemouth bass (*Micropterus salmoides*). Adapted from Kroupova *et al.* (2013). (▲) Eurasian perch $y = 9.2x + 0.8$; $R^2 = 0.87$ and (○) largemouth bass $y = 8.7x + 4.1$; $R^2 = 0.90$.

Methaemoglobinemia is not the only negative effect of nitrites in fish. Nitrites can also affect, for example the process of ion level regulation in the body (Jensen *et al.* 1987; Knudsen & Jensen 1997; Gisbert *et al.* 2004), respiratory processes (Cameron 1971; Aggergaard & Jensen 2001), cardiovascular functions (Aggergaard & Jensen 2001), and endocrine and excretory processes (Jensen 2003). Details can be found in comprehensive review by Jensen (2003) which is still up to date. Moreover, fish exposed to momentary or chronic sublethal nitrite concentrations are more prone to infectious diseases, which suggests a negative impact of nitrites on the fish immune system (Hanson & Grizzle 1985; Carballo & Muñoz 1991; Carballo *et al.* 1995).

Methaemoglobinemia was described in frogs after exposure to nitrites (Huey & Beitinger 1980a). However, in frogs and their early development stages, nitrite uptake and the mechanisms of its toxic effects are less thoroughly described than in fish. Some aquatic animals, for example some molluscs and arthropods, use haemocyanin as their respiration protein to bind oxygen molecules instead of haemoglobin. Unlike haemoglobin, haemocyanin does not contain bivalent iron, but monovalent copper. It has been found that the copper in crayfish haemocyanin may be oxidised by nitrites to a higher oxidation state (Tahon *et al.* 1988). The result of this process is methaemocyanin, which, just like methaemoglobin, cannot carry oxygen. However, methaemocyanin is usually produced at low pH and in the presence of surplus nitrites (Tahon *et al.* 1988; Jensen 1996a), while its production is insignificant at physiological pH. Consequently, nitrites affect haemocyanin capacity to transport oxygen less significantly than in haemoglobin, which explains why nitrite disturbs oxygen transport less strongly in crustaceans than in fish (Jensen 1996a). In crayfish, nitrite exposure is also associated with disruption of osmoregulation manifested by lower hemolymph Cl^-, Na^+ and osmolality (Jensen 1990, 1996b; Harris & Coley 1991). Similar to fish, it has been shown that nitrite exposure depresses immunological function and thus increases pathogenic susceptibility of crustaceans (Cheng *et al.* 2002; Romano & Zeng 2013).

At low concentrations, nitrites can be used by aquatic algae and cyanobacteria as a source of nitrogen when other substrates (ammonia or nitrates) are missing (Yang *et al.* 2004; Chen *et al.* 2009). High nitrite concentrations, however, may be harmful to both algae and cyanobacteria (Abe *et al.* 2002; Yang *et al.* 2004; Chen *et al.* 2011). Chen *et al.* (2011), for example, recorded a drop in growth and symptoms of oxidative stress in the cyanobacteria *Microcystis aeruginosa* ((Kutzing) Lemmermann) exposed to nitrite concentration of 33 mg L^{-1} NO_2^- (at 18 mg L^{-1} Cl^-). Higher concentrations of nitrites are also known to negatively affect photosynthetic processes (Sahay *et al.* 2006). However, we have not observed any growth inhibition in the green freshwater algae *Desmodesmus subspicatus* ((Chodat) E. Hegewald & A. Schmidt) when exposed up to 1314 mg L^{-1} NO_2^-, and 72hIC50 value was as high as 3552 mg L^{-1} NO_2^- (12.7 mg L^{-1} Cl^-, Material & Methods in Supporting Information).

Nitrite is also known to negatively influence microorganisms and their processes as it is a bacteriostatic molecule due to its affinity for the metal ions in the centre of enzymes. Nitrites inhibit, for example, ammonia oxidation activity, nitrite oxidation, denitrification, both anoxic and aerobic phosphate removals, methanogenesis and cell growth (reviewed, e.g. by Philips *et al.* 2002). However, these effects can be observed first when nitrite levels reach tens or hundreds of mg L^{-1} (Philips *et al.* 2002).

Toxicity

Different fish vary substantially in their sensitivity to nitrites. Acute lethal concentration levels (LC50s) may range from units to hundreds of mg L^{-1} NO_2^- (Table 1). The most sensitive fish species include salmonids and perseids. Conversely, a high degree of nitrite resistance is manifested, for example by largemouth bass (*Micropterus salmoides*, Lacepède) (Palachek & Tomasso 1984b) and green sunfish (*Lepomis cyanellus*, Rafinesque) both members of family *Centrarchidae* (Tomasso 1986).

Comparing nitrite toxicity to aquatic insects, crustaceans and molluscs, Soucek and Dickinson (2012) concluded that aquatic insects were the most sensitive, followed by crustaceans, while molluscs proved to be most resistant (Table 2). It is to be emphasised that lethal concentrations for the respective aquatic species can only be compared if they were generated from tests employing dilution water of comparable chloride concentrations. The validity of studies

Table 1 Acute toxicity of nitrite (NO_2^-) to freshwater vertebrates

Group	Order	Organism	Stage	Temperature (°C)	Cl^- (mg L^{-1})	Time	LC50 (mg L^{-1})	Reference
Amphibians	Anura	Oregon Spotted Frog (*Rana pretiosa*)	Larva	15	NR (tap water)	4 d	22.4	Marco *et al.* (1999)
						7 d	4.3	
						15 d	1.87	
		Red-legged Frog (*Rana aurora*)	Larva			4 d	18.37	
						7 d	13.14	
						15 d	3.91	
		Western Toad (*Bufo boreas*)	Larva			4 d	>23.0	
						7 d	17.68	
						15 d	5.75	
		Northern Pacific Treefrog (*Hyla regilla*)	Larva			4 d	18.07	
						7 d	11.82	
						15 d	4.04	
	Caudata	North-western Salamander (*Ambystoma gracile*)	Larva			4 d	6.24	
						7 d	5.06	
						15 d	3.32	
		Small-mouth salamander (*Ambystoma texanum*)	Larva	25	5	96 h	1.09	Huey and Beitinger (1980b)
Fish	Cypriniformes	Grass carp (*Ctenopharyngodon idella*)	Larva	22.0	9–10	24 h	143.0	Korwin-Kossakowski *et al.* (1996)
						48 h	61.4	
		Zebra fish (*Danio rerio*)	Juvenile	23.0	19	96 h	242.4	Pištěková *et al.* (2005)
					50		318.0	
					100		387.4	
			Larva	23.0	19	96 h	386.0	Voslářová *et al.* (2006)
					50		458.7	
					100		672.4	
		Common carp (*Cyprinus carpio*)	Juvenile	28	1.0	48 h	21.23	Hasan and Macintosh (1986)
					5.0		23.53	
					10.5		68.32	
					27.5		139.20	
					45.0		305	
					1.0	96 h	8.38	
					5.0		18.90	
					10.5		47.35	
					27.5		89.58	
					45.0		160	
					1.0	120 h	7.43	
					5.0		18.9	
					10.5		43.9	
					27.5		86.7	
					45.0		148.5	
					1.0	168 h	7.13	

Table 1 (continued)

Group	Order	Organism	Stage	Temperature (°C)	Cl^- (mg L^{-1})	Time	LC50 (mg L^{-1})	Reference
					5.0		18.73	
					10.5		39.56	
					27.5		80.64	
					45.0		144.2	
			Juvenile	20.0	11	96 h	68.0	Machova and Svobodova (2001)
					40		199.3	
		Roho labeo (*Labeo rohita*)	Juvenile	26.4–27.3	10.3	24 h	97.9	Ciji *et al.* (2012)
						48 h	47.98	
						72 h	38.22	
						96 h	37.07	
		Tiger barb (*Puntius tetrazona*)	Juvenile	22.2	11	96 h	32	†
		Harlequin rasbora (*Trigonostigma heteromorpha*)	Juvenile	21	11	96 h	83	†
		Vimba bream (*Vimba vimba*)	Larva	21.5	11	96 h	35	†
			Juvenile	21.5	11	96 h	25	†
		Mrigal carp (*Cirrhinus mrigala*)	Juvenile	30	9.9	24 h	28.9	Das *et al.* (2004)
						48 h	16.3	
						72 h	10.7	
						96 h	10.4	
		Catla[a] (*Catla catla*)	Juvenile	28	14	24 h	397 (in static system) 386 (in flow-through system)	Tilak *et al.* (2002)
		Fathead minnow (*Pimephales promelas*)	Juvenile	23.0	22	24 h	700	Palachek and Tomasso (1984a)
						48 h	280	
						72 h	260	
						96 h	230	
			NR			24 h	185	
						48 h	155	
						72 h	150	
						96 h	150	
		Topeka shiner (*Notropis topeka*)	Juvenile	24.7	0.64–1.04	96 h	27.3	Adelman *et al.* (2009)
			Adult	25.0	0.64–1.04		20.0	
		Tench (*Tinca tinca*)	Larva	23.0	9–10	24 h	135	Korwin-Kossakowski *et al.* (1995)
						48 h	85.7	
						96 h	64.4	
		Tench – diploid (*Tinca tinca*)	Juvenile	18.5	11	96 h	32	†
		Tench – triploid (*Tinca tinca*)	Juvenile	18.5	11	96 h	20	†
	Perciformes	Eurasian perch (*Perca fluviatilis*)	Juvenile	22.5	10.5	48 h	11.0	Kroupova *et al.* (2013)
		Largemouth bass (*Micropterus salmoides*)	Juvenile	22.5	10.5	48 h	882.0	

Table 1 (continued)

Group	Order	Organism	Stage	Temperature (°C)	Cl^- (mg L^{-1})	Time	LC50 (mg L^{-1})	Reference
		Largemouth bass (*Micropterus salmoides*)	Juvenile	23	22	96 h	460.7	Palachek and Tomasso (1984b)
		Green Sunfish (*Lepomis cyanellus*)	Juvenile	23.0	22	96 h	526.8	Tomasso (1986)
		Guadalupe bass (*Micropterus treculi*)	NR	23	22	96 h	616	Tomasso and Carmichael (1986)
		Pike-perch (*Sander lucioperca*)	Juvenile	22	40	24 h	32.2	Wuertz *et al.* (2013)
						48 h	32.2	
						72 h	32.2	
						96 h	24.6	
						120 h	20.0	
		Nile tilapia (*Oreochromis niloticus*)	Juvenile (4.4 g)	25.8	6	96 h	266	Atwood *et al.* (2001)
					319		1110	
			NR (90.7 g)		6		26.3	
			Juvenile	25 ± 1	35		28.18	Wang *et al.* (2006)
					70		44.67	
		Blue tilapia (*Oreochromis aureus*)	Juvenile	23	22	96 h	53.2	Palachek and Tomasso (1984b)
	Salmoniformes	Rainbow trout (*Oncorhynchus mykiss*)	Juvenile	14.8	10	24 h	31.9	Kroupova *et al.* (2008)
						48 h	25.1	
						72 h	11.9	
						96 h	11.2	
		Cutthroat trout (*Salmo clarki*)	Juvenile	12	0.44	96 h	1.71	Thurston *et al.* (1978)
		Chinook salmon (*Oncorhynchus tshawytscha*)	Juvenile	9.1	NR (tap water)	48 h	19	Crawford and Allen (1977)
	Siluriformes	Channel catfish (*Ictalurus punctatus*)	Juvenile	23.0	22	96 h	23.4	Tomasso (1986)
		Wels catfish (*Silurus glanis*)	Juvenile	19.5	11	96 h	23	†
		Striped catfish (*Pangasianodon hypophthalmus*)	Juvenile	28	10.6	96 h	75.9	Lefevre *et al.* (2011)
		South American catfish[b] (*Rhamdia quelen*)	Juvenile	25	3.94	96 h	20.46	de Lima *et al.* (2011)
		North African catfish (*Clarias gariepinus*)[c]	Juvenile	NR	NR (tap water)	96 h	92	Hilmy *et al.* (1987)
			Juvenile				105	
			Juvenile	23	22	96 h	23.0	Ekwe *et al.* (2012)
		Yellow catfish (*Tachysurus fulvidraco*)[d]	Juvenile	22.5	52.4	96 h	45.85	Sohn *et al.* (2015)
	Anguilliformes	European Eel (*Anguilla anguilla*)	Juvenile	21.2	117	96 h	472.0	Kamstra *et al.* (1996)
	Cyprinodontiformes	Guppy (*Poecilia reticulata*)	Juvenile	20.0	10	96 h	25.6	Kroupová *et al.* (2004)
					145		259.6	
			Juvenile	24 ± 1	18.5–19.1	96 h	30.2	Doleželová *et al.* (2011)

Table 1 (continued)

Group	Order	Organism	Stage	Temperature (°C)	Cl^- (mg L^{-1})	Time	LC50 (mg L^{-1})	Reference
	Acipenseriformes	Siberian sturgeon (*Acipenser baeri*)	Juvenile	18.0	130.5	72 h	427.0	Huertas *et al.* (2002)
		Shortnose sturgeon (*Acipenser brevirostrum*)	Juvenile	17.9 ± 0.31	<1	96 h	37.1	Fontenot *et al.* (1998)
	Characiformes	Cachama[e] (*Colossoma macropomum*)	Juvenile	25	<0.5	96 h	1.82	da Costa *et al.* (2004)
		Matrinxã (*Brycon cephalus*)	Juvenile	24 ± 1	0.35	96 h	2.82	Avilez *et al.* (2004b)
		African River pike (*Hepsetus odoe*)	Juvenile	23	22	96 h	46.0	Ekwe *et al.* (2012)
	Gonorynchiformes	Milkfish (*Chanos chanos*)	Juvenile	27.4–27.7	26.79	48 h	39.4	Almendras (1987)

†Unpublished data by authors of this review (Material & Methods described in Supporting Information).
d, days; h, hours; NR, not reported.
(i) English or Latin names of species were taken from www.fishbase.org following recommendation of FAO (Food and Agriculture Organization of the United Nations). Upper indexes indicate the name of the species as it is stated in the original study, [a]Indian major carp, [b]silver catfish, [c]*Clarias lazera*, [d]*Psuedobagurus fulvidraco*, [e]the Amazonian fish. (ii) Studies not taking into account chloride concentration in water were excluded with an exception of those which were carried out in tap water. Chloride concentrations in tap water can be expected to have been low (in units or tens of mg L^{-1}).

not taking into account chloride concentrations in water is very low because this parameter affects nitrite toxicity most significantly of all the factors explored. Bearing this fact in mind, we also made an attempt to compare nitrite toxicity among different classes of freshwater organisms for which acute toxicity data and chloride concentration of dilution water used for toxicity tests are available. The ratio between chloride concentration in water and 48- to 96-h median lethal concentration of nitrite nitrogen ($N–NO_2^-$) for different species of respective animal classes was used as a measure of nitrite toxicity (Fig. 2). Based on these results, it can be concluded that freshwater molluscs and worms followed by fish are the most resistant among studied phyla/classes. On the other hand, crustaceans and aquatic insects followed by amphibians are the most sensitive. Interestingly, there are wide interspecific differences in nitrite susceptibility within classes of freshwater insects, crustaceans and amphibians.

Nitrite toxicity to fish is substantially affected by a number of internal and external factors. The most important external factors include water quality, namely water temperature, oxygen saturation, cation and anion concentrations, in particular chloride concentrations, and, obviously, the length of exposure (Crawford & Allen 1977; Palachek & Tomasso 1984a; Lewis & Morris 1986; Svobodová *et al.* 2005b; Kroupová *et al.* 2006a). Important internal factors include the fish species, age, size and individual fish sensitivity (Eddy *et al.* 1983; Palachek & Tomasso 1984a; Williams & Eddy 1988; Bartlett & Neumann 1998; Aggergaard & Jensen 2001). The importance of the separate factors is subject to continuous verification and correction. Studies of other aquatic animals primarily focus on the effects of chlorides in the water (Kozák *et al.* 2005; Alonso & Camargo 2008; Shinn *et al.* 2013), while the other factors are not addressed in the available literature.

The most prominent factor affecting nitrite toxicity to fish is chloride concentration in water. This is so because nitrites and chlorides enter organisms in the same way, as described in more detail above in the section on toxicity mechanism. Test conducted with several fish species has confirmed a tight linear relationship between nitrite toxicity and chloride concentration in the water (Table 3, Fig. 3), with addition of chlorides into water producing a more prominent increase in nitrite resistance in fish less sensitive to nitrites (Table 3), that is those manifesting a low chloride uptake rate (Jensen 2003; Tomasso & Grosell 2005). The European Inland Fisheries Advisory Commission recommends keeping the $Cl^-/N–NO_2^-$ weight ratio above 17 in salmonid aquaculture, and above 8 in the aquaculture of rough species (FAO, 1984). Our experience shows that death or damage may occur in fish even at higher $Cl^-/N–NO_2^-$ weight ratios. For instance, cases of wels catfish (*Silurus glanis*, Linnaeus) and tench dying in

Table 2 Acute toxicity of nitrite (NO_2^-) to freshwater invertebrates

Group	Order	Organism	Stage	Temperature (°C)	Cl^- (mg L^{-1})	Time (h)	LC50 (mg L^{-1})	Reference
Decapod crustacean	Decapoda	Narrow-clawed crayfish (*Astacus leptodactylus*)	Adult	13.0	35	48	29.4	Yildiz and Benli (2004)
					61		49.2	
		Spiny-cheek crayfish (*Orconectes limosus*)	Juvenile	18.0	11	96	15.9	Kozák *et al.* (2005)
					400	96	317.4	
		Southern plains crayfish (*Procambarus simulans*)	NR	25	≤5	24	31.5	Beitinger and Huey (1981)
						48	13.8	
						72	8.6	
						96	6.1	
		Redclaw crayfish (*Cherax quadricarinatus*)	Juvenile	24	79	24	28.3	Liu *et al.* (1995)
						48	23.7	
						72	19.4	
						96	15.4	
			Juvenile	25	450	24	140	Meade and Watts (1995)
						48	122	
						96	85	
		Signal crayfish (*Pacifastacus leniusculus*)	Adult	NR	17.7	24	113	Harris and Coley (1991)
						48	31	
		Red swamp crayfish (*Procambarus clarkii)*	NR	23	22	24	46	Gutzmer and Tomasso (1985)
						48	37	
						72	32	
						96	28	
			NR		100	24	~115	
						48	~95	
						72	~87	
						96	~80	
		Giant river prawn (*Macrobrachium rosenbergii*)	Juvenile	27	15	96	27.9	Chen and Lee (1997)
					24		36.8	
					34		42.3	
						96	10.3	
		Chinese mitten crab (*Eriocheir sinensis*)	Juvenile	20	122–155	24	29.25	Hong *et al.* (2009)
						48	26.83	
						72	25.91	
						96	25.91	
Small crustaceans	Amphipoda	Amphipod (*Echinogammarus echinosetosus*)	Adult	15.5	56	24	25.9	Alonso and Camargo (2006)
						48	12.4	
						72	9.6	
						96	8.5	

Table 2 (continued)

Group	Order	Organism	Stage	Temperature (°C)	Cl^- (mg L^{-1})	Time (h)	LC50 (mg L^{-1})	Reference
		Amphipod (*Eulimnogammarus toletanus*)	Adult	15.5	56	24	60.8	
						48	14.2	
						72	8.8	
						96	6.9	
		Sideswimmer (*Gammarus fasciatus*)	Juvenile	20	26	96	21.3	Ewell *et al.* (1986)
		Amphipod (*Hyalella azteca*)	Juvenile	22.7	72.9	96	41.1	Soucek and Dickinson (2012)
		Freshwater shrimp (*Gammarus*)	NR	18	30	96	40.4	Kelso *et al.* (1999)
	Cladocera	Water flea (*Daphnia*)					~62	
		Water flea (*Daphnia magna*)	Juvenile	20	26		32	Ewell *et al.* (1986)
	Isopoda	Pillburg (*Asellus intermedius*)					>66.7	
Aquatic insects	Ephemeroptera	American mayfly (*Hexagenia*)	NR	18	30		4.6	Kelso *et al.* (1999)
		Mayfly (*Ephemerella*)					8.2	
	Plecoptera	Stonefly (*Allocapnia vivipara*)	Larva	11.5	1.9	96	4.9	Soucek and Dickinson (2012)
		Stonefly (*Amphinemura delosa*)		12.4			3.3	
Molluscs	Basommatophora	Snail (*Helisoma trivolvis*)	Juvenile	20	26	96	39.3	Ewell *et al.* (1986)
		Pond snail (*Lymnaea stagnalis*)	Juvenile	20.3	3.8	96	183.0	Soucek and Dickinson (2012)
	Unionoida	Freshwater mussel (*Lampsilis siliquoidea*)	Juvenile	20.0	1.9	96	580.0	
	Veneroida	Fingernail clam (*Sphaerium simile*)	Juvenile	22.7	1.9	96	183.0	
	Littorinimorpha	Aquatic snail (*Potamopyrgus antipodarum*)	NR	20.4	NR (bottled drinking water)	24	7012	Alonso and Camargo (2003)
						48	2786	
						72	2050	
						96	1758	
Aquatic worms	Tricladida	Platyhelminth (*Polycelis*)	NR	18	30	96	202	Kelso *et al.* (1999)
		Flatworm (*Dugesia tigrina*)	Juvenile	20	26	96	>66.7	Ewell *et al.* (1986)
		Planarian (*Polycelis felina*)	Adult	15.5	56	24	2582	Alonso and Camargo (2006)
						48	463	
						72	262	
						96	197	
	Lumbriculidae	Segmented worm (*Lumbriculus variegatus*)	Juvenile	20	26	96	>66.7	Ewell *et al.* (1986)

h, hours; NR, not reported.

Studies not taking into account chloride concentration in water were excluded.

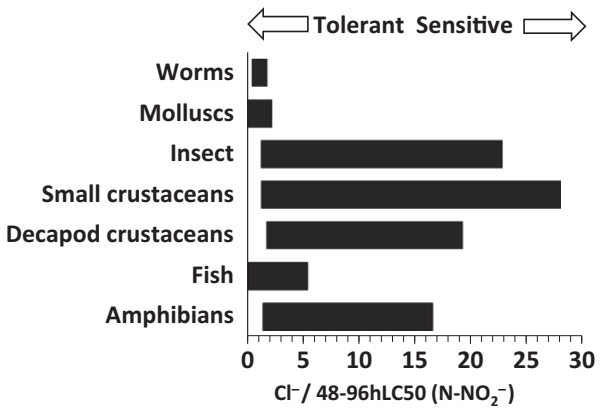

Figure 2 Comparison of nitrite toxicity among different groups of freshwater organisms using ratio between chloride concentration in water and 48–96-h median lethal concentration (LC50) of nitrite nitrogen (N–NO_2^-) for different species of the respective animal groups as a measure.

Table 3 Linear dependence of lethal nitrite concentrations on chloride concentrations in water for five fish species

Fish species	Cl^- to 96hLC50 (NO_2^-) dependence	
Rainbow trout[1]	$y = \mathbf{0.96}\,x + 1.7$, $R^2 = 1.0$	Strength of effect of Cl^-
Eurasian perch[2]	$y = \mathbf{1.14}\,x - 2.8$, $R^2 = 1.0$	
Guppy[3]	$y = \mathbf{1.40}\,x + 43.6$, $R^2 = 0.93$	
Zebra fish juveniles[4]	$y = \mathbf{1.75}\,x + 217.2$, $R^2 = 0.97$	
Common carp[5]	$y = \mathbf{3.37}\,x + 4.9$, $R^2 = 0.99$	
Zebra fish larvae[6]	$y = \mathbf{3.61}\,x + 302.6$, $R^2 = 0.98$	

y = 96hLC50 NO_2^- (mg L^{-1}), x = Cl^- concentration (mg L^{-1}), R^2 = coefficient of determination.

Source of data for relationship calculation: [1]Russo and Thurston (1977); [2]unpublished data by authors of this review (Material & Methods described in Supporting information); [3]Kroupová *et al.* (2004); [4]Pištěková *et al.* (2005); [5]Hasan and Macintosh (1986); [6]Voslářová *et al.* (2006).

Slopes of the regression lines are in bold.

intensive recirculation farming units have been recorded at Cl^-/N–NO_2^- weight ratios ranging between 13 and 28, and 11 and 19, respectively (Svobodová *et al.* 2005a). According to Buřič *et al.* (2016), it is safe to rear rainbow trout, brook trout (*Salvelinus fontinalis*, Mitchill), brown trout (*Salmo trutta m. fario*, Linnaeus), Siberian sturgeon (*Acipenser baeri*, Brandt) and Russian sturgeon (*Acipenser gueldenstaedti*, Brandt & Ratzeburg) in an open recirculating system at the Cl^-/N–NO_2^- weight ratio ≥100 (calculated based on given chloride and maximum nitrite concentrations measured over the three-year rearing period including winter seasons with slow nitrification rate in biofilter).

Generally, a positive chloride effect on nitrite toxicity reduction can be expected in all aquatic animals (or their early stages) employing gills for breathing and ion exchange (i.e. also for chloride uptake from the environment). This phenomenon has already been observed in an amphipod (*Eulimnogammarus toletanus*; Alonso & Camargo 2008), a planarian (*Polycelis felina*; Alonso & Camargo 2008), two species of crayfish (Fig. 3; Yildiz & Benli 2004; Kozák *et al.* 2005) and in amphibians in early development stages (Huey & Beitinger 1980a; Shinn *et al.* 2013). However, it is not known whether there is any effect of chloride on nitrite toxicity to freshwater algae or cyanobacteria.

Among other water quality parameters, the presence of bromides has been confirmed to have a positive effect on reduction in nitrite toxicity to fish (Eddy *et al.* 1983). The reason is that their chemical properties are similar to those of chlorides. Similarly, increased calcium concentrations have been found to have a positive effect on reduction in nitrite toxicity to fish by some researchers, albeit less prominent than that of chlorides (Crawford & Allen 1977; Mazik *et al.* 1991; Fontenot *et al.* 1999). Other anions and cations are of marginal importance with respect to nitrite toxicity reduction. Similarly, negligible is the effect of pH on nitrite toxicity, particularly at levels commonly occurring in natural waters (Lewis & Morris 1986). On the other hand, factors potentially increasing fish sensitivity to nitrites include low oxygen concentrations in water (Bowser *et al.* 1983) and higher water temperature (Huey *et al.* 1984; Kroupová *et al.* 2006a). However, the latter factor may accelerate the process of fish recovery after nitrite poisoning (Huey *et al.* 1984).

Fish sensitivity to nitrites has also been found to vary with age and size. It has been repeatedly confirmed that older fish are more sensitive than early development stages (Russo *et al.* 1974; Perrone & Meade 1977; Bartlett & Neumann 1998; Kroupova *et al.* 2010). The lower sensitivity of early development stages of fish may be due to the higher activity of methaemoglobin reductase, an enzyme reducing methaemoglobin back to haemoglobin, in younger fish compared to older ones. Another possible explanation is that, unlike juveniles or adults, the early development stages do not possess fully developed gills, and their respiration therefore occurs mostly through their skin, lowering the importance of oxygen transport through blood (Bartlett & Neumann 1998). Atwood *et al.* (2001) and Hvas *et al.* (2016) reported that smaller fish are less sensitive to nitrite than larger individuals. Hvas *et al.* (2016) showed that smaller fish had a higher capacity for detoxifying nitrite to nitrate than larger fish. However, in any case, it was not clear whether both studied groups were juveniles or whether the larger fish were already adult, that is whether it is size or both size and stage dependent effect.

Compared to relatively huge amount of data on nitrite acute toxicity to freshwater organisms, there are only few

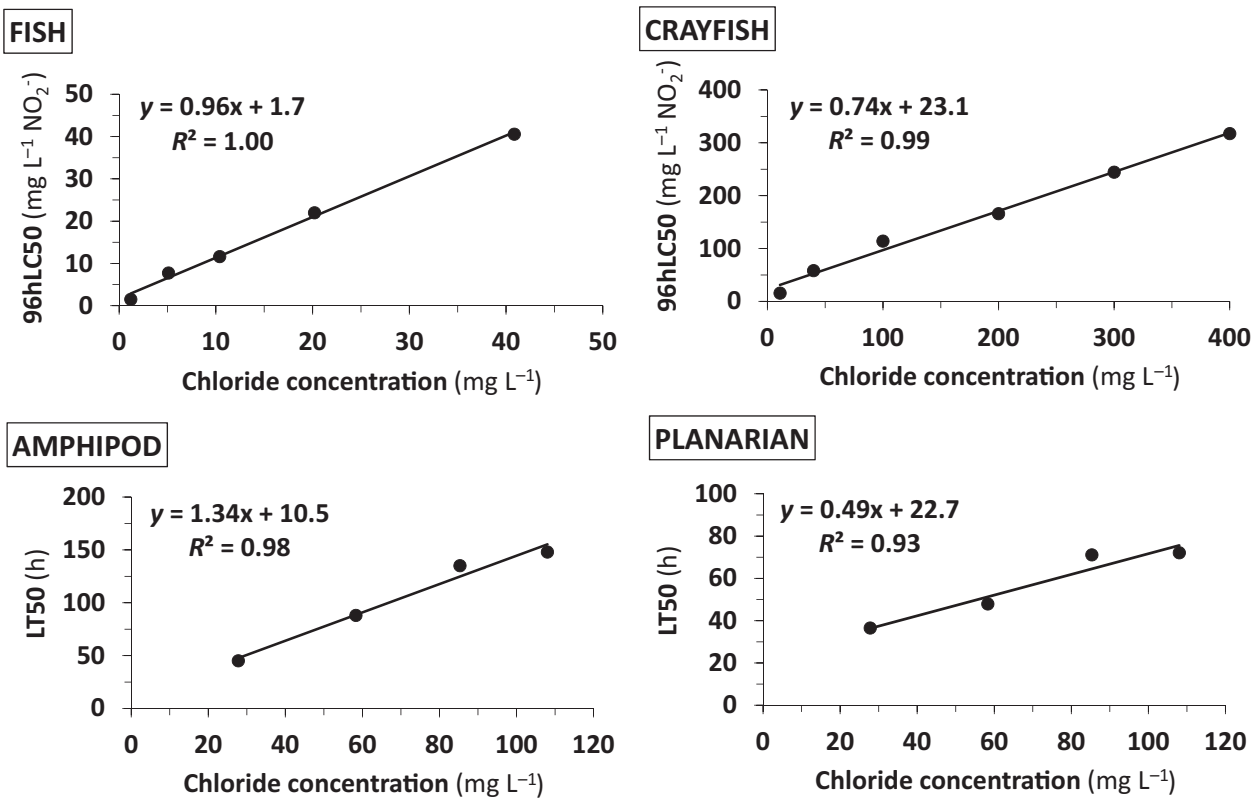

Figure 3 Linear dependence of median lethal nitrite concentrations (LC50) or median lethal nitrite times (LT50) on chloride concentrations in water in a fish species (rainbow trout, *Oncorhynchus mykiss*), a crayfish (eastern crayfish, *Orconectes limosus*), an amphipod (*Eulimnogammarus toletanus*) and a planarian (*Polycelis feline*). Adapted from Russo and Thurston (1977), Kozák *et al.* (2005) and Alonso and Camargo (2008).

studies dealing with nitrite subchronic effects and those studies are exclusively focused on fish (Table 4; studies not taking into account chloride concentration in water were excluded). To the best of our knowledge, only one study has been dealing with chronic toxicity of nitrite to freshwater fish so far (Wedemeyer & Yasutake 1978); no chronic studies on other freshwater organisms are available. Prolonged exposure to sublethal nitrite concentrations was reported to result mainly in decrease in specific growth rate (Table 4) and/or slight changes in biochemical profile of the blood plasma, haematological parameters and histology of gills of exposed fish (Kroupova *et al.* 2008; Wuertz *et al.* 2013; Zusková *et al.* 2013). Interestingly, methaemoglobin levels together with plasma nitrite concentrations were shown to decrease with prolonged exposure to nitrite indicating ability of adaptation in fish (Doblander & Lackner 1997; Zusková *et al.* 2013). Doblander and Lackner (1997) reported the adaptation to be the result of increased oxidation of nitrite to nitrate, which is then possibly excreted via urine and bile. NADH-methaemoglobin reductase may be also involved in the process of adaptation. However, whether the enzyme system can be accelerated remains uncertain. Woo and Chiu (1997) found the system unresponsive in sea bass (*Lates calcarifer*, Bloch). However, study by Avilez *et al.* (2004a) indicated that the enzyme system may be activated by nitrite concentration and/or duration of exposure. Another strong evidence of ability to acclimate to nitrite was presented by Tucker and Schwedler (1983b) who observed higher methaemoglobin levels in fish that had not previously been exposed to nitrite than in fish with an immediate past history of exposure (for min. 2 weeks) to relatively high NO_2^-/Cl^- molar ratios.

Clinical symptoms and pathological–morphological signs of acute poisoning

The fish are lethargic, their escape reflex is weakened, and tonic–clonic muscle contractions can be observed. The following stage is agony and death in lateral or dorsal position.

The amphibians affected are generally lethargic (Vršková & Knotek 2008), the larvae of tailless amphibians manifest a drop in activity and loss of balance bordering on paralysis (Marco & Blaustein 1999).

Table 4 Subchronic and chronic nitrite (NO_2^-) toxicity to freshwater fish

Order	Organism	Stage	Temperature (°C)	Cl^- (mg L^{-1})	Time	NO_2^- (mg L^{-1})	Effect on target organism	Reference
Cypriniformes	Zebra fish (*Danio rerio*)	Juvenile	23	18.5–19.1	28 d	73	LOEC for SGR	Voslářová *et al.* (2008)
	Topeka shiner (*Notropis topeka*)	Juvenile	25.1	0.64–1.04	30 d	18.2	LOEC for survival	Adelman *et al.* (2009)
	Fathead minnow (*Pimephales promelas*)	Juvenile	24.7			13.3		
		Embryo – larvae	25		32 d	13.8	LOEC for survival and growth	
	Common carp (*Cyprinus carpio)*	Embryo – larvae	20–22.5	10	29 d	0.7	Delay in development; ↓ FCF	Kroupova *et al.* (2010)
						28	LOEC for mortality	
	Roho labeo (*Labeo rohita*)	Juvenile	26.1–27.4	10.2	45 d	6.6	↓ SGR, WBC, Hb ↑ NO_2^- in gills, liver and muscle	Ciji *et al.* (2013)
Anguilliformes	European Eel (*Anguilla anguilla*)	Juvenile	24.9	117	77 d	65.7	NOEC for mortality and SGR	Kamstra *et al.* (1996)
Perciformes	Pike-perch (*Sander lucioperca*)	Juvenile	22	40	32 d	1.4	↑ NO_2^- in plasma and muscle	Wuertz *et al.* (2013)
						11.5	↑ NO_2^- in plasma and muscle ↑ MetHb (71%) NOEC for mortality	
Salmoniformes	Steelhead trout (*Salmo gairdneri*)	Juvenile	10	2.3	6 mo	0.05	↑ MetHb (only 2.7%); NOEC for mortality, SGR, histopathology	Wedemeyer and Yasutake (1978)
	Rainbow trout (*Oncorhynchus mykiss*)	Juvenile	14–15.5	10	28 d	0.2	LOEC for SGR	Kroupova *et al.* (2008)
						1.0	↑ NO_2^- in plasma	
		Juvenile	14.3–15.2	11	14 d	1.0	↑ MetHb (13%) ↑ NO_2^- in plasma, ↓ Hc	Zusková *et al.* (2013)
					22 d		↔ MetHb (9%) ↑ NO_2^- in plasma, ↓ Hc	
			7.9	0.25	14 d	0.32	↑ NO_2^- in plasma, ↑ MetHb (25.6%)	Doblander and Lackner (1997)
					48 d		↔ NO_2^- in plasma; ↔ MetHb (3.6%)	
Siluriformes	Silver catfish (*Rhamdia quelen*)	Juvenile	25	3.9	40 d	1.52	100% mortality	de Lima *et al.* (2011)
						1.19	NOEC for mortality and SGR	
	African catfish (*Clarias gariepinus)*	Juvenile	25.7	0.02	28 d	2.0	EC10 for SGR	Roques *et al.* (2015)
						3.9	EC10 for feed intake	

d, days; EC, effect concentration; FCF, Fulton's condition factor; Hb, haemoglobin; Hc, haematocrit; LOEC, lowest observed effect concentration; MetHb, methaemoglobin; mo, months; NOEC, no observed effect concentration; SGR, specific growth rate; WBC, white blood cell count.
Studies not taking into account chloride concentration in water were excluded.

The macroscopic sign of nitrite poisoning is chocolate brown colour of blood and gills. The brown colour in the gills is already visible when methaemoglobin content reaches ca 25% (Svobodová *et al.* 2005a). Microscopically, oedema and hyperplasia of the gill epithelium can be observed or an increase in eosinophilic chloride cells (Svobodová *et al.* 2005b; Kroupova *et al.* 2008).

The principal diagnostic sign of nitrite poisoning in amphibians is chocolate brown colour of blood and brown colouring of gill filaments in the larvae of tailed amphibians (Vršková & Knotek 2008).

Diagnosis and therapy

The initial step in diagnosing nitrite poisoning in all aquatic animals is water analysis and identification of nitrite and chloride concentrations. The diagnosis can subsequently be confirmed if laboratory tests reveal high levels of blood methaemoglobin. Methaemoglobin concentration in nitrite-poisoned fish is ≥20–25% of total haemoglobin, and death occurs at methaemoglobin concentrations of over 70% (Svobodová *et al.* 2008). The fish and crayfish affected are also characterised by high nitrite concentrations in blood plasma and in tissues sometimes exceeding nitrite levels in the water.

Nitrite-poisoned fish must be moved to clean water or the water should be supplemented with chlorides as soon as possible (Kroupova *et al.* 2006b; Zusková *et al.* 2013). Treatment of fish with 1 mg L^{-1} methylene blue (an agent reducing trivalent iron in blood pigment back to bivalent iron) reduces dramatically the methemoglobin levels within a few hours which might be an advantage in severely poisoned fish (Wedemeyer & Yasutake 1978). Amphibians may be treated in a moderately aerated bath containing methylene blue at concentrations of 2 mg L^{-1} for larvae and 4 mg L^{-1} for adult amphibians (Vršková & Knotek 2008).

Prevention

The essential preventive measures in recirculating fish-rearing facilities include the following (may apply also for intensive rearing of other freshwater organisms, e.g. crayfish and prawns):

1 When operation is started, fish should be stocked gradually and feed portions should be optimised in accordance with the current efficiency of biological filters, to preserve water quality required for fish farming. Full stocking is only possible after the biofilters become fully functional. Water pH and concentrations of oxygen, ammonia, nitrites, nitrates and chlorides, and possibly also the weight chloride/N-nitrite ratio, must be tested continuously (Svobodová *et al.* 2005a).
2 Treatment of fish in recirculating systems should not employ antibiotics or most types of disinfectants in bath form. Treatment must take place outside the recirculating system to prevent biofilter damage (Svobodová *et al.* 2008).
3 Where nitrite accumulation may occur in fish-rearing facilities, $Cl^-/N\text{–}NO_2^-$ weight ratio in water is recommended to be kept at a minimum of 100 (Svobodová *et al.* 2005a; Buřič *et al.* 2016). Increased chloride concentrations are achieved by adding common salt (sodium chloride). A dose of 165 mg of salt per litre of water (or 165 g m^{-3} water) raises chloride concentration by 100 mg L^{-1}.
4 Another purifying unit may be included to improve water quality in addition to nitrifying biofilters which are incorporated in most recirculating systems. For instance, constructed wetland was proved to decrease and stabilize nitrite levels in recirculating hatchery (Burič *et al.* 2015). Denitrifying reactor may be also included in the recirculating system as it is supposed not only to remove nitrite and nitrate but also to raise alkalinity which is being lost during intensive nitrification resulting in pH decline in culture water (van Rijn *et al.* 2006).
5 Fish feed is recommended to have a high ascorbic acid content (ca 8000 mg kg^{-1}) to prevent nitrite-induced methaemoglobinemia (Wise *et al.* 1988). Moreover, dietary supplementation with higher amounts of vitamin E (300 mg kg^{-1}) was found to be beneficial for nitrite-exposed roho labeo (*Labeo rohita*, Hamilton) (Ciji *et al.* 2013).

Conclusions

Nitrites continue to cause serious problems in aquaculture facilities frequently leading to health impairment of cultured species or even to their mass mortalities. Adverse effects of nitrite on fish and other freshwater organisms can be reduced by increasing chloride concentration in the water because it was unequivocally demonstrated that chlorides have a positive effect on the resistance of the most aquatic organisms towards nitrites. It is also known that nitrite concentrations can raise up to 1 mg l^{-1} NO_2^- under certain conditions in rivers. However, there is no information available on the consequences of such nitrite exposure for freshwater ecosystem. Therefore, nitrite effects on aquatic organisms are a topic which still deserves our attention.

A relatively huge amount of data on nitrite effects is available for fish, but other freshwater organisms have been much less frequently studied. Information on chronic effects of nitrite exposure is nearly completely missing for all freshwater organisms. Further research should be focused on these very subjects.

Acknowledgements

The study was supported by the Ministry of Education, Youth and Sports of the Czech Republic – Projects 'CENAKVA' (No. CZ.1.05/2.1.00/01.0024) and 'CENAKVA II' (No. LO1205 under the NPU I program). P.Š. was supported by the project of the Grant Agency of University of South Bohemia in Ceske Budejovice (No. 012/2016/Z).

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Appendix S1. Acute toxicity tests on selected fish species and green algae *Desmodesmus subspicatus*.