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Chloride uptake in freshwater teleosts and its relationship to nitrite uptake and toxicity

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Summary. 1. The relationship between rate of chloride uptake and external chloride concentration was investigated in Rainbow trout, *Salmo gairdneri*, and Perch, *Perca fluviatilis*. The relationship between nitrite uptake and external nitrite and the inhibition of chloride uptake by nitrite was also investigated. Nitrite tolerance tests were performed on a variety of freshwater animals, including Carp, *Cyprinus carpio*, Tench, *Tinca tinca*, Pike, *Esox lucius*, Eel, *Anguilla anguilla*, and tadpoles, *Rana temporaria*.

2. The chloride uptake mechanism is saturable, with maximum uptake rates of 368 $\mu M h^{-1} kg^{-1}$ and 429 $\mu M h^{-1} kg^{-1}$ for the trout and perch, respectively. The half saturation value (K_m, the affinity constant) is 159 $\mu M l^{-1}$ for trout and 333 $\mu M l^{-1}$ for perch.

3. Net nitrite transport was determined in trout, net movement being into the fish against a concentration gradient, with a maximum uptake rate of 281 $\mu M h^{-1} kg^{-1}$; the K_m is 198 $\mu M l^{-1}$. This suggests that nitrite enters the fish via an active uptake process.

4. The data suggest that nitrite is a simple competitive inhibitor of active chloride uptake in both trout and perch. Trout are less tolerant of nitrite than perch (24-h LC_{50} values are 0.7 $mM l^{-1}$ for trout and 1.2 $mM l^{-1}$ for perch) and also have a greater affinity for nitrite.

5. The spectrum of nitrite sensitivity seen in freshwater animals is discussed in relation to chloride uptake kinetics. These data support the hypothesis that nitrite uptake is an active process and furthermore uptake is linked quantitatively with chloride uptake, suggesting that chloride and nitrite enter the fish via the same route.

Introduction

The physiological effects of nitrite toxicity in aquatic organisms have been reported by several authors and reviewed recently (E.I.F.A.C. 1984). At similar environmental nitrite concentrations animals in seawater are more resistant than those in freshwater, this resistance being due mainly to the protective effects of chloride and to a lesser extent those of calcium (Crawford and Allen 1977). Perrone and Meade (1977) showed that addition of chloride to the freshwater environment protected Coho salmon, *Oncorhynchus kisutch*, against nitrite toxicity and this was subsequently confirmed for rainbow trout, *Salmo gairdneri*, by Wedemeger and Yasutake (1978), Bath and Eddy (1980), Russo et al. (1981) and for channel catfish, *Icatalurus punctatus*, by Tomasso et al. (1979). Russo and Thurston (1977) reported for rainbow trout an inverse linear relationship between lethal nitrite concentration and chloride concentration, up to chloride concentrations of 1 $mM l^{-1}$. The protective effect of chloride is species dependent, being most marked in salmonids and least in channel catfish.

Bath and Eddy (1980) reported that nitrite is concentrated to about 3 $mM l^{-1}$ in trout plasma when the fish was exposed to external nitrite concentrations of 0.7 $mM l^{-1}$ while Margiocco et al. (1983) reported that nitrite accumulates in blood and various tissues of the trout to levels far in excess of the environmental nitrite concentration. This evidence supports the hypothesis that the process of nitrite uptake is an active one.

The protective effects of chloride suggest that both chloride and nitrite enter the animal via the same route and compete for uptake into the fish. However, because the uptake mechanism has a greater affinity for chloride, nitrite will tend to be excluded. Anion permeability measurements in cat motoneurons indicated that chloride and nitrite ions behaved in a similar fashion with chloride permeating at a slightly higher rate than nitrite (Araki et al. 1961). The reason for this similar behavior was explained by the similar hydrated size and hydration energy of chloride and nitrite ions.

Gaino et al. (1984) noted an increase in chloride cell turnover and size when trout were exposed to acute nitrite intoxication. Trout can therefore apparently compensate for inhibition of chloride uptake by an increase in chloride cell activity, thus maintaining normal plasma chloride levels. Krous et al. (1982) found that plasma nitrite concentration and the number of lamellar chloride cells were directly correlated in rainbow trout.

The physiological and toxicological effects of nitrite have been studied and the major effects include a relatively rapid conversion of haemoglobin to methaemoglobin in the blood and in the longer term, reactions with certain amines to form potentially carcinogenic N-nitroso compounds (Wolff and Wasserman 1972; Forman et al. 1985). Other physiological effects of nitrite are produced as a consequence of its action as a relaxant of smooth muscle, which results in a general vaso-dilation, with cardiac venous return being increased and sustained by a compensatory reflex tachycardia, which tends to maintain blood pressure. Nitrite induced hypoxia results in tissue damage to the liver and other vital organs resulting in death (Arillo et al. 1984).

In aquatic systems ammonia is produced as a waste product of protein metabolism in animals and larger amounts are produced from certain industrial processes. Nitrate containing agricultural fertilizers are also a potential source of ammonia and nitrite (White 1983). Ammonia is oxidised to nitrate via nitrite by bacteria such as *Nitrosomonas* and *Nitrobacter* and when the second stage of nitrification is inhibited, nitrite levels can build up to toxic levels which can be of importance in aquaculture (Collins et al. 1975).

The present study using whole animals was conducted to explore the relationship between chloride and nitrite uptake and to relate this to the toxicity of nitrite for a variety of freshwater teleosts, using rainbow trout and perch as the principal examples.

Materials and methods

Juvenile rainbow trout *Salmo gairdneri*, 15–50 g in weight, were obtained from a local hatchery (College Mill Trout Farm, Almondbank, Perthshire). Perch, *Perca fluviatilis*, 20–40 g in weight, were taken from Rae Loch and Staredam, Birnam Woods, Perthshire using drift nets. The fish were held in running water containing (in mMl^{-1}), Na^+, 0.22; Ca^{2+}, 0.1; Cl^-, 0.2; pH 6.8 with a 12–12 h photoperiod at a temperature between 10 °C and 14 °C which was also the experimental temperature. Fish were not fed three days prior to or during any experiments.

Sodium, and calcium concentrations, were determined by atomic absorption spectrometry (Pye Unicam SP9) and chloride by coulometric titration (Radiometer CMT10 Chloride Titrator). Nitrite concentration was determined spectrophotometrically using the sulphanilamide-naphthylethylenediamine method of Shechter et al. (1972). ^{36}Cl activity was determined by dissolving water samples in a toluene based scintillant (2:1 Toluene, Triton X100, 4 g $PPOl^{-1}$) and counting in a scintillation counter (Packard 3600).

Twenty four hour LC_{50}'s were used to quantify nitrite toxicity (Eddy et al. 1983); water quality and experimental conditions were the same for all species tested.

Desired nitrite and chloride concentrations were obtained by addition of analytical grade sodium nitrite and sodium chloride to distilled water. Sodium salts were preferred because fish are able to accommodate changes in sodium concentration and also because chloride influx has been reported to be independent of sodium influx (Kerstetter and Kirschner 1972).

At the beginning of each experiment, each fish was placed in a darkened two litre conical flask containing 500 ml of a dilute sodium chloride solution at a predetermined concentration between 0.1 and 4.5 mMl^{-1}. Ca^{2+} concentration ranged from 1 to 14 μMl^{-1} and pH ranged from 6.9 to 7.4 throughout. External chloride concentrations could not be precisely controlled because of movement of chloride ions into and out of the fish during the pre-measurement acclimation period, therefore a range of chloride concentrations around the desired concentration was initially obtained. Dissolved oxygen levels were maintained near to air saturation by constant aeration, which produced continual mixing of the external medium, and also helped to screen the fish from external noises. Water samples of 3 ml for ionic and radioactivity analysis were removed via a tube secured in the flask and thus the sampling procedure produced little or no disturbance to the experimental animal. Prior to the experiment fish were left overnight to acclimatize to their new environment and also to recover from stresses experienced during handling and transfer from stock tanks.

Chloride influx was determined by adding 1 μCi chloride-36 (Amersham International PLC.) to each flask via the sampling tube and then measuring the rate at which radioactivity disappeared from the external medium (Maetz 1956; Wood et al. 1984). This procedure measures whole body chloride fluxes, but in freshwater teleosts the major site of ion exchange is via the gills (Heisler 1984). External specific activity was always more than ten times that of the body fluids, thus no correction for chloride-36 backflux was considered necessary (Kerstetter and Kirschner 1972) and therefore chloride ion influx could be measured unambiguously since any reduction in activity in the external medium was due solely to inward movement of chloride-36. Net nitrite flux was determined by measuring changes in external nitrite concentration. Inhibition of chloride uptake by nitrite was determined by first measuring chloride influx without nitrite for a period of 4–5 h and then measuring chloride influx over a similar period in the presence of nitrite, each fish acting as its own control. This procedure was repeated with various concentrations of chloride and nitrite. The nitrite levels selected were such that the physiological interaction between chloride and nitrite uptake could be fully investigated (Table 2), within the time available before radioisotope equilibrium occurred. Following the acclimation period any fish showing signs of excessive activity were excluded from the study since these fish exhibited erratic changes in chloride influx and it has been noted before that handling stress caused increases in chloride efflux (Eddy and Bath 1979).

Similar methods were used to study other freshwater species (Table 1) but in lesser detail than that for trout and perch.

A problem associated with whole animal experiments is variability between individuals and variability arising through stress, although the experimental design as outlined above was

found to reduce stress effects to very low levels. Whole body studies are almost the only satisfactory preparation available to study ionic exchange in freswhater fish and most likely to reflect the true physiological state of the animal when compared to other preparations such as the perfused head (Perry et al. 1984a; Perry et al. 1984b) which tend to be more amenable to kinetic studies of ion transport.

Results

In both trout and perch the rate of chloride uptake was related to the external chloride concentration with chloride uptake showing saturation kinetics (Fig. 1). The Eadie-Hofstee plot which places less emphasis on low substrate data points than the duble-reciprocal plot (Ainsworth 1977) was used to evaluate the affinity constant K_m (the chloride concentration at 50% maximal uptake) and the maximal rate of uptake. In this paper J_{in} refers to unidirectional ionic influx and $J_{in\ max}$ to maximum rate of ion uptake in $\mu M\,h^{-1}\,kg^{-1}$. Excluding values at the highest external chloride concentration (Fig. 1), $J_{in\ max}$ was 368 $\mu M\,h^{-1}\,kg^{-1}$ and K_m was 159 $\mu M\,Cl^-$ for trout and 429 $\mu M\,h^{-1}\,kg^{-1}$ and 333 $\mu M\,Cl^-$, respectively, for perch (Table 1).

For rainbow trout the net influx of nitrite was

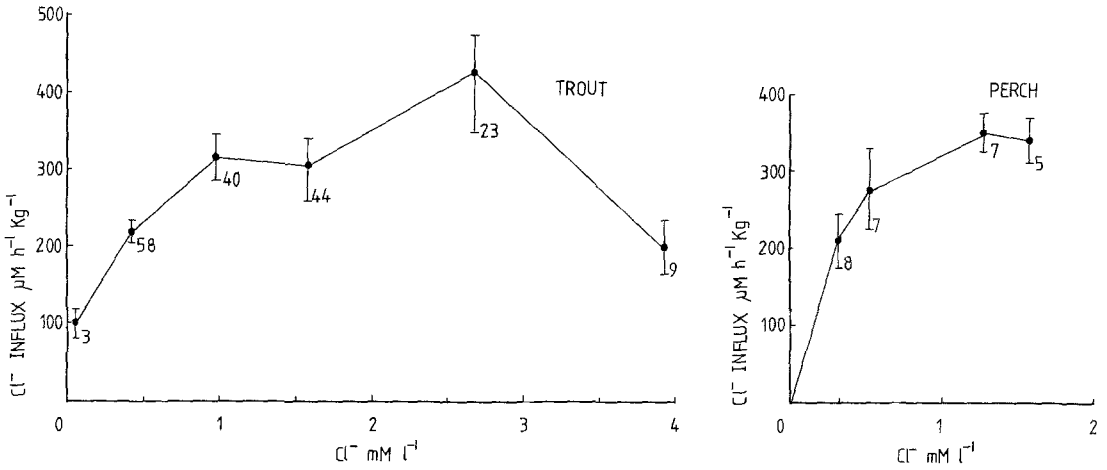

Fig. 1. Relationship between external chloride concentration and chloride uptake in the trout and perch. Each point represents the mean ± SE of the number of fish as indicated

Table 1. Comparative kinetics of chloride uptake and nitrite toxicity for various freshwater animals. Kinetic parameters were determined using the Eadie-Hofstee plot (see text) which can be described by the equation $Y = a - bX$, where a is $J_{in\ max}$, with 95% confidence limits and b is K_m; r is the regression coefficient and the P values indicate significance of regression

Species (weight in g)	(*n*)	Regression analysis of Eadie-Hofstee plots	r	$P<$	LC_{50} (24 h) $NO_2^-\,mM\,l^{-1}$
Trout (15–50) (*Salmo gairdneri*)	(177)	$Y = 368\ (\pm 120) - 159X$	0.8905	0.05	0.7
Perch (20–40) (*Perca fluviatilis*)	(27)	$Y = 429\ (\pm 40) - 333X$	0.9606	0.05	1.2
Carp (2–78) (*Cyprinus carpio*)	(23)	$Y = 25\ (\pm 14) - 169X$	0.8307	0.05	35
Frog (0.07–0.11)[a] (*Rana temporaria*)	(120)	$Y = 162\ (\pm 16) - 93X$	0.9857	0.05	8
		Chloride uptake ($\mu M\,h^{-1}\,kg^{-1}$)			
Yellow eel (59–138) (*Anguilla anguilla*)		(No measurable chloride uptake over 48 h)			80
Silver eel (400–700) (*Anguilla anguilla*)		1.7 (external chloride 0.6 $mM\,l^{-1}$)[b]			
Pike (25–117) (*Esox lucius*)	(12)	258 (external chloride 0.2–0.5 $mM\,l^{-1}$)			
Tench (113–168) (*Tinca tinca*)	(6)	14–41 (external chloride 0.13–2.22 $mM\,l^{-1}$)			50

[a] Premetamorphic tadpoles (3 weeks) [b] Kirsch (1972)

dependent on the external nitrite concentration (Fig. 2), the uptake showing saturation kinetics with $J_{in\ max}$ at 281 $\mu M h^{-1} kg^{-1}$ and K_m at 198 $\mu M l^{-1} NO_2^-$, when the mean external chloride was 0.17 $mM l^{-1}$. Although substrate inhibition is suggested by the data in Fig. 2, the uptake values at nitrite substrate concentrations exceeding 1 $mM l^{-1}$ are not significantly different form preceeding values.

The uptake of chloride was significantly inhibited by the presence of nitrite in the external medium for both trout and perch, and conversely, chloride inhibits nitrite uptake (Tables 2 and 3). The kinetic parameters for chloride inhibition by nitrite were calculated using the Michaelis-Menten equation for a competitive inhibitor according to the methods indicated by Dixon and Webb (1964) from values for $J_{in\ max}$, K_m, chloride concentration and nitrite concentration (Tables 1 and 2).

Trout are more sensitive to nitrite toxicity than perch, the 24 h LC_{50}s being 700 and 1,200 µm $NO_2^-\ l^{-1}$ respectively (Table 1). However, the other species tested (carp, *Cyprinus carpio*, eel, *Anguilla anguilla* and tench, *Tinca tinca*) are much

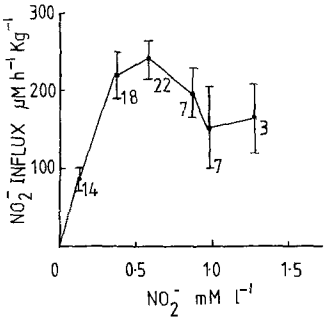

Fig. 2. Nitrite uptake in rainbow trout as a function of external nitrite concentration. Each point represents the mean ± SE of the number of fish as indicated. The kinetic parameters were calculated with external nitrite concentrations from 0–0.9 $mM l^{-1}$ (see Table 1). $J_{in\ max}$ is 281 ± 103 $\mu M h^{-1} kg^{-1}$ and K_m 198 $\mu M l^{-1}$ ($r = 0.909$, $P < 0.05$). Mean external chloride concentration was 0.17 $mM l^{-1}$

Table 3. Variations in nitrite influx as a function of external nitrite concentration in trout at various external chloride concentrations. All values are expressed as means ± SE (N). The difference in mean nitrite uptake with different chloride concentrations was tested using Students *t*-test: * $P < 0.05$, ** $P < 0.01$

External nitrite ($\mu M l^{-1} NO_2^-$)	Nitrite influx[a] ($\mu M h^{-1} kg^{-1}$)	Chloride added ($\mu M l^{-1}$)	Nitrite influx with chloride ($\mu M h^{-1} kg^{-1}$)	
211 ± 6 (10)	158 ± 8 (5)	568 ± 28 (5)	28 ± 14 (5)	**
544 ± 7 (12)	200 ± 36 (6)	585 ± 72 (6)	99 ± 28 (6)	*
208 ± 5 (10)	158 ± 8 (5)	1,884 ± 131 (5)	0 0 (5)	**
522 ± 8 (11)	200 ± 36 (6)	1,920 ± 125 (5)	40 ± 18 (5)	**

[a] Mean external chloride was 0.171 $mM l^{-1}$

Table 2. Variations in chloride influx as a function of external chloride concentration in the absence or presence of external nitrite for trout and perch. All values expressed as means ± SE (N). The difference in mean chloride uptake before and after addition of nitrite was tested on paired values using Students *t*-test: * significantly different at $P < 0.05$, ** $P < 0.01$, N.S., not significantly different at $P < 0.1$. With an external chloride concentration of about 0.556 $mM l^{-1}$ for trout and about 0.45 $mM l^{-1}$ for perch, chloride influx is progressively reduced by increasing nitrite concentration. For trout a plot of chloride influx against the logarithms of external nitrite concentration yields a straight line described by the equation $Y = 194 - 32.4X$, with $r = 0.9468$ and the significance of regression $P < 0.05$. For perch the equation is $Y = 283 - 47.9X$, $r = 0.9997$, and $P < 0.05$, showing that the decrease in chloride uptake is significant in both species. At higher external chloride concentrations (above 1 $mM l^{-1}$) addition of nitrite had no significant effect on chloride uptake at $P < 0.1$ (see text for further details)

External chloride ($\mu M l^{-1}$)	Chloride influx without nitrite ($\mu M h^{-1} kg^{-1}$)	Nitrite added ($\mu M l^{-1}$)	Chloride influx with nitrite ($\mu M h^{-1} kg^{-1}$)	
Trout				
544 ± 38 (9)	192 ± 35 (9)	216 ± 6 (9)	140 ± 31 (9)	*
610 ± 49 (9)	196 ± 38 (9)	535 ± 12 (9)	103 ± 22 (9)	**
514 ± 57 (11)	180 ± 21 (11)	1,197 ± 27 (11)	80 ± 18 (11)	**
1,723 ± 73 (11)	437 ± 75 (11)	236 ± 6 (11)	416 ± 30 (11)	N.S.
1,710 ± 95 (11)	382 ± 71 (11)	533 ± 9 (11)	361 ± 51 (11)	N.S.
1,702 ± 84 (5)	218 ± 50 (5)	1,112 ± 11 (5)	177 ± 14 (5)	N.S.
1,928 ± 154 (10)	418 ± 66 (10)	500 (approx)	416 ± 96 (10)	N.S.
Perch				
412 ± 33 (5)	289 ± 75 (5)	550 ± 19 (5)	150 ± 65 (5)	*
481 ± 33 (8)	278 ± 45 (8)	1,104 ± 4 (8)	139 ± 30 (8)	**
1,179 ± 41 (6)	360 ± 26 (6)	563 ± 14 (6)	300 ± 58 (6)	N.S.
1,559 ± 24 (6)	335 ± 24 (6)	1,109 ± 13 (6)	276 ± 33 (6)	N.S.

more resistant and this is reflected in lower chloride utpake rates while the pike, *Esox lucius*, more closely resembles trout and perch.

Discussion

Chloride uptake kinetics

Absorption of external chloride by trout and perch exhibits saturation kinetics (Fig. 1) and similar observations were reported for the goldfish (de Renzis and Maetz 1973), which has a $J_{in\ max}$ value similar to trout and perch with a lower K_m of 75 μM Cl^- l^{-1}. Using anaesthetized trout of around 250 g Kerstetter and Kirschner (1972) reported that chloride uptake exhibited saturation kinetics, with $J_{in\ max}$ of around 230 μM Cl^- h^{-1} kg^{-1}, and K_m between 200–300 μM Cl l^{-1}. The $J_{in\ max}$ reported by these authors was about 40% lower than the values reported in this study and may be the results of the anaesthetic reducing metabolic activity and therefore influencing $J_{in\ max}$ rather than K_m.

Trout chloride uptake exhibits substrate inhibition at high external chloride levels (Fig. 1) and this had been previously observed in the same species by Kerstetter and Kirschner (1972) who reported that chloride uptake slowed or even stopped when external chloride concentration was above 5.0 mMl^{-1}. Bielawski (1964) studying chloride transport across crayfish (*Astacus pallipes*) gills made similar observations.

Nitrite uptake

There is now evidence to suggest that nitrite transport into the fish is by active transport; nitrite is concentrated in the blood plasma of the rainbow trout against a concentration gradient, e.g. with an external nitrite concentration of 0.7 mMl^{-1}, blood plasma nitrite levels after 24 h exposure were about 7.0 mMl^{-1}, ten times the external nitrite concentration (Eddy et al. 1983), whilst Margiocco et al. (1983) noted a seventy fold concentration in the same species. A second point is that the rate of nitrite uptake by the fish exhibits saturation kinetics (Fig. 2) which suggests that simple diffusive accumulation of nitrite is unlikely while facilitated diffusion seems improbable since nitrite is concentrated against a chemical gradient. Electrical potentials in freshwater rainbow trout are around −10 to +10 mV (Bath and Eddy 1978; Potts 1984) which is insufficient for nitrite to accumulate in the blood via the electrochemical gradient. Nitrous acid, the conjugate acid of nitrite, may be the diffusing species (Colt and Tchobanoglous 1976) which suggests that nitrite concentration in the blood may be pH dependent, but where relevant work has been conducted there is little evidence to support this hypothesis (Bath 1980; Russo et al. 1980; EIFAC 1984).

Inhibition of chloride transport by nitrite

The aleviation of nitrite toxicity by increasing external chloride levels (Perrone and Mead 1977) suggested the possibility that the two ions competed for the same branchial uptake site. Hoffmann (1982) measuring chloride fluxes in Ehrlich cells found that nitrate was a competitive inhibitor of chloride steady-state flux and similar analysis of our data strongly suggests that nitrite behaves in the same way. Inhibition of chloride uptake by nitrite is significant at low external chloride concentrations but at higher chloride levels maximal chloride uptake is not influenced by nitrite, a characteristic of competitive inhibition (Table 2). The converse applies with chloride inhibiting nitrite uptake (Table 3). Estimated values for the inhibitor dissociation constant (K_i) for nitrite show that the chloride uptake mechanism in trout has a greater affinity for nitrite than that of the perch (K_i is 60 and 426 μM NO_2^- for trout and perch, respectively, when the external chloride concentration is 0.61 mMl^{-1}). In perch a concentration of 1,207 μM nitrite l^{-1} is required to inhibit chloride uptake by 50% (external chloride concentration 0.61 m$M\ l^{-1}$), which gives this species increased tolerance to nitrite poisoning (Table 1) compared to the trout where only 292 μM nitrite l^{-1} (external chloride concentration 0.61 mMl^{-1}) produces 50% inhibition of chloride utpake.

Nitrite sensitivity in freshwater animals

Freshwater animals such as carp, eel and tench which have low chloride uptake rates are more resistant to nitrite than animals such as trout, perch and pike which have relatively high rates of chloride influx (Table 1). Furthermore fish such as the trout which has both a high chloride uptake rate and a high affinity for chloride are especially sensitive to nitrite toxicity (Table 1). Thus the rate of chloride uptake may be an indicator of nitrite toxicity for any particular species. The results allow assessment of the likely toxic effects of any environmental nitrite level in terms of external chloride concentration and exposure time (Tables 1 and 3).

Eel, carp and tench can live in water of poor quality and low oxygen levels, conditions favouring nitrite formation, while animals such as trout with relatively high rates of chloride uptake tend to inhabit good quality, well oxygenated water, conditions where nitrite formation is less favoured. Palachek and Tomasso (1984) report that in the

Largemouth bass, *Micropterus salmoides*, plasma nitrite levels did not exceed those present in the environment and the existence of a nitrite exclusion mechanism was suggested, but another possibility is that active chloride uptake is minimal in the Largemouth bass, as in carp, tench and eel (Table 1). The larval amphibian tested, *Rana temporaria*, is unusual in being highly resistant to nitrite while possessing a high affinity for chloride (Table 1). This could be explained if unlike fish gill, the amphibian surface was highly selective towards chloride rather than nitrite and possessed a lower transport rate for chloride.

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