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PRIMARY RESEARCH PAPER

Evaluating the need for acid treatment prior to $\delta^{13}C$ and $\delta^{15}N$ analysis of freshwater fish scales: effects of varying scale mineral content, lake productivity and CO_2 concentration

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Abstract In order to evaluate the need for using scale acidification to remove carbonates prior to stable isotope analysis, we compared acidified and non-acidified scales of six freshwater fish species (perch, roach, rudd, pike, tench and bream) with contrasting mineral content in their scales. Fish samples were taken from six lakes with variable trophic conditions, ranging from oligotrophic to hypertrophic, and differing in CO_2 concentrations. The scale mineral content of the six species studied ranged between 31.8 and 61.3% dry weight (DW) in tench and perch, respectively. The elemental composition was characterised by high amounts of phosphorus, varying from 4.5 to 9.1% DW. The mineral fraction was dominated by apatite (range 24.4–49.2% DW), carbonates constituted a very small proportion of the total carbon content (average ± SD: 5.5 ± 1.7%). The average effect of acidification was very small for all species (average ± SD: 0.181 ± 0.122 and −0.208 ± 0.243 for carbon and nitrogen, respectively), albeit significant for five out of the six species (excepting tench that had the lowest mineral content). Linear regression slopes between acidified and untreated scales did not differ significantly from one for almost all the species and isotopes. The effects of acidification on the two isotopes were correlated with the relative carbonate content as well as with the CO_2 concentration for carbon and total phosphorus for nitrogen. We conclude that the need for scale acidification depends on the different species and on the system studied, although in most cases the acidification effect will be biologically irrelevant. However, dual analysis of acidified and untreated scales may provide useful information on differences in stable isotope composition of dissolved inorganic carbon and on phytoplankton carbon fractionation generated by varying levels of CO_2 availability.

Keywords Carbon isotope · Nitrogen isotope · Fish scales · Acidification · De-calcification · Stoichiometry

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Introduction

Freshwater ecosystems are suffering from increasing anthropogenic impacts, either directly through introduction of alien species (Garcia-Berthou et al., 2005; Gorokhova et al., 2005) or indirectly via catchment alterations such as increased input of nutrients (Vadeboncoeur et al., 2003; Jeppesen et al., 2007). For some of the impacted freshwater ecosystems, historical monitoring programmes including archived sample material exist. The interest in using such archived material for stable isotope analyses has risen as it allows reconstruction or assessment of the ecological impact of ecosystem changes (Vander Zanden et al., 2003; Chandra et al., 2005; Chasar et al., 2005). Isotopic data can be obtained from any tissue, and samples not collected specifically for dietary studies may therefore be used for food web reconstructions (e.g. Ventura & Jeppesen, 2009). Among these tissues, those allowing non-destructive samplings of the studied individuals are ecologically and ethically most valuable (Estep & Vigg, 1985; Cerling et al., 2006; Ehleringer et al., 2008). Fish scales are of particular interest since they fulfil both characteristics, and therefore their use for ecological reconstructions has become increasingly popular (Estep & Vigg, 1985; Wainright et al., 1993; Satterfield & Finney, 2002; Pruell et al., 2003; Estrada et al., 2005; Kennedy et al., 2005; Gerdeaux & Perga, 2006; Morbey et al., 2007; Grey et al., 2009; Rennie et al., 2009).

Fish muscle is the reference tissue used in most ecological studies and is normally analysed after lipid removal. Basically, fish scales have two components: an organic part formed by two proteins, collagen and ichthylepidin (Seshaiya et al., 1963), in approximately equal proportions, and an inorganic matrix composed mainly of hydroxylapatite (Hutchinson & Trueman, 2006), a calcium phosphate biomineral with different residual mineral contaminations such as carbonates and magnesium (Elliott, 2002). For carbon stable isotope analysis, it is the calcium carbonate fraction that potentially interferes with the stable isotope analysis of proteins. The relative importance of calcium carbonate within the mineral fraction of scales has been only scarcely described. So far, it has been shown that, at least in otoliths, most calcium carbonate comes from dissolved inorganic carbon (DIC; Solomon et al., 2006; Tohse & Mugiya, 2008). Therefore, the use of scales as a substitute or a surrogate of muscle for stable isotope analysis requires either removal of the scale mineral matrix (Estep & Vigg, 1985; Perga & Gerdeaux, 2003; Grey et al., 2009), as commonly done by archaeologists to describe changes in stable isotope composition of bone or other animal tissues (e.g. Chisholm et al., 1983; Lee-thorp et al., 1989), or species-specific calibration of scale and muscle for each habitat (Kelly et al., 2006; Sinnatamby et al., 2008; Syväranta et al., 2008; Blanco et al., 2009; Rennie et al., 2009). Only few studies have specifically tested the effects of acidification on scale carbon and nitrogen stable isotope composition, and opposite conclusions have been reached. In whitefish (*Coregonus lavaretus*), Perga & Gerdeaux (2003) found significant (1.3‰) enrichment of carbon and nitrogen isotopic composition after acidification. Syväranta et al. (2008) found significant effects of acidification on roach $\delta^{13}C$, although they were smaller than the analytical error of the instrument and therefore biologically irrelevant. Sinnatamby et al. (2007) in yellow perch (*Perca flavescens*), walleye (*Sander vitreus*) and Atlantic salmon (*Salmo salar*) and Rennie et al. (2009) in North American whitefish found no significant acidification effects, though the former argued that there might be a species-specific difference in the response to acid treatment.

The different results found in previous studies may be attributed to variations in scale mineral content among species. In addition, more than 75% of scale carbonates come from DIC (Solomon et al., 2006; Tohse & Mugiya, 2008), while all the carbon from scale proteins come from the diet. Therefore, the relative difference between $\delta^{13}C$-DIC and $\delta^{13}C$-diet could be another factor affecting either among or within species effects of scale acidification on $\delta^{13}C$. Since $\delta^{13}C$ changes only negligibly along food webs (Peterson & Fry, 1987), the difference between a $\delta^{13}C$-DIC and a $\delta^{13}C$-diet will most likely depend on the difference in fractionation by algae during photosynthesis, ranging between almost 0 to over 20‰ depending on the extent of carbon limitation (Ventura et al., 2008), as earlier suggested by Perga & Gerdeaux (2004), Gerdeaux & Perga (2006) and Grey et al. (2009) based on their observations of strong correlation between total phosphorus (P_{tot}) and fish scale $\delta^{13}C$.

In order to evaluate further the need for scale acidification prior to stable isotope analysis, we compared acidified and non-acidified scales of six freshwater fish species with contrasting mineral content (from ca. 30 to 60%) sampled along a gradient of lakes varying in nutrient content. Specifically, our objectives were to: (i) investigate the acidification time needed to remove the mineral part of most scales types; (ii) describe the scale elemental (carbon, nitrogen and phosphorus), mineral and protein contents; (iii) determine the effects of acidification on the scale stable isotope composition of carbon and nitrogen, and (iv) investigate the relationships between the possible effects with the scale mineral content, lake productivity and CO_2 concentration.

Materials and methods

Scales were obtained from six different freshwater fish species common in north European temperate lakes: perch (*Perca fluviatilis*), bream (*Abramis brama*), pike (*Esox lucius*), rudd (*Scardinius erythrophtalmus*), roach (*Rutilus rutilus*) and tench (*Tinca tinca*). Fish samples were collected between 2000 and 2006 from six Danish lakes with contrasting nutrient loading from the catchment and, accordingly, with differing gradients of isotopic baselines (Vander Zanden et al., 2005). Therefore, the lakes cover a wide trophic gradient (from oligo- to hypertrophic) and also a strong gradient of CO_2 concentrations, basically driven by the productivity derived pH (Table 1). Most species were found in at least three lakes, with the exception of bream and tench that were present in only one lake each (Table 2). Scales were collected in the field, placed in paper envelopes and stored dry until analysis.

Scales were selected from individuals belonging to five different size classes (1+ to 5+) ranging from ca. 8 to 35 cm. In the case of pike, similar age classes corresponded to larger size (16–90 cm). For tench, we could only find large individuals from the latter age class (35–54 cm). Skin mucus was removed from all scales by soaking in distilled water and gentle scraping with a scalpel under a binocular microscope.

Table 1 Surface area, mean depth, average summer (May–October) chlorophyll *a* (Chl *a*), total nitrogen (N_{tot}), total phosphorus (P_{tot}), the elemental ratio of total nitrogen to total phosphorus, pH, alkalinity and free-CO_2 concentration of the study lakes

Lake	Surface area (km^2)	Depth (m)	Sampling (year)	Trophic status	Chl *a* ($\mu g\ l^{-1}$)	N_{tot} ($mg\ l^{-1}$)	P_{tot} ($mg\ l^{-1}$)	TN:TP (atoms)	pH	Alkalinity ($meq\ l^{-1}$)	CO_2 ($mg\ C\ l^{-1}$)
Slåen	0.182	7.3	2005	Oligotrophic	2.8	0.22	0.01	39.0	7.11	1.39	0.863
Tebstrup	0.360	7.7	2003	Mesotrophic	9.4	1.63	0.04	88.2	8.14	2.83	0.446
Denderup	0.046	1.0	2003	Eutrophic	27.1	0.67	0.06	26.7	7.84	3.01	0.795
Stigsholm	0.209	1.0	2000–2001	Eutrophic	38.3	1.92	0.08	56.2	8.66	1.23	0.083
Væng	0.152	1.2	2006	Eutrophic	85.1	1.07	0.14	17.6	8.01	1.25	0.180
Søbygård	0.381	1.1	2000–2001	Hypertrophic	111.0	1.46	0.29	11.0	8.68	2.34	0.088

	Correlation matrix							
	N_{tot}	P_{tot}	N_{tot}:P_{tot}	CO_2	Alkalinity	pH	Area	Depth
Chl *a*	0.226	0.929	−0.763	−0.733	−0.231	0.637	0.273	−0.681
N_{tot}		0.297	0.390	−0.767	−0.018	0.817	0.596	−0.149
P_{tot}			−0.650	−0.666	0.007	0.716	0.518	−0.533
N_{tot}:P_{tot}				0.200	0.230	−0.092	0.258	0.716
CO_2					0.420	−0.798	−0.472	0.492
Alkalinity						0.204	0.209	0.219
pH							0.587	−0.460
Area								0.329

See Ventura et al. (2008) for a description of analytical methods and CO_2 calculation from pH and alkalinity. Pearson correlation coefficient among the different chemical variables is also shown

Table 2 Number of individuals analysed, lakes where each species was found with number of individuals in each lake (in parenthesis) and mean fork-length (cm) and summary statistics of the different freshwater fish species analysed

Species	No.	Lakes sampled	Fork-length (cm)	SD	Min	Max
Roach	34	Slåen (4), Stigsholm (5), Søbygård (11), Tebstrup (4), Væng (8)	16.9	6.3	7.7	31.0
Perch	31	Denderup (5), Slåen (5), Stigsholm (5), Søbygård (4), Tebstrup (6), Væng (5)	19.4	7.3	8.3	34.5
Rudd	24	Søbygård (10), Tebstrup (2), Væng (11)	16.2	6.7	7.4	36.0
Pike	18	Denderup (3), Stigsholm (2), Søbygård (8), Væng (4)	43.3	23.9	15.5	80.5
Bream	11	Væng (11)	12.6	2.4	9.0	18.7
Tench	7	Denderup (7)	45.4	7.2	35.0	53.5

In order to avoid noise from interscale isotopic differences, scales were spliced into two longitudinal halves, one half being directly dried at ambient temperatures for bulk scale analysis, while the other half was first decalcified, rinsed in distilled water and subsequently dried for analysis. Due to the change in scale weight with fish age, we had to use a different number of scales for each age class and species. The number of scales used ranged from 25 for the +1 fishes to 1 for fishes >27 cm.

Scales of the different fish species exhibited different degrees of mineralisation. Therefore, we investigated the optimal scale decalcification time by soaking scales of roach (moderately mineralised scale) and perch (highly mineralised scale) in 1.2 M HCl. Scales were removed successively at 2 min intervals starting at 2 min, soaked in distilled water, dried at air temperature and weighed. The optimal acidification time was chosen to be that at which scale weight loss stopped. Since carbonates are embedded within apatite, and both are similarly solubilised when soaked in acid, we measured apatite as a measure of the remaining mineral fraction. Initial and remaining apatite contents were estimated from the scale phosphorus content.

For stable isotope analysis we used ca. 1.5 and 2.0 mg of the acidified and untreated scales, respectively. The scales were pre-weighed in tin capsules. From the scales of the larger age classes, we used only a longitudinal piece that was sliced off at the central half of the scale to obtain a representative piece (Hutchinson & Trueman, 2006). Scales were cut into fine slivers using sterile laboratory scissors. Elemental carbon and nitrogen of solid ground material and stable isotope ratios of carbon and nitrogen were analysed at the Stable Isotope Facility, University of Davis, California, USA, using a Europa Hydra 20/20 continuous flow isotope ratio mass spectrometer (CFIRMS; PDZ Europa, Cheshire, UK) coupled with an elemental analyser. Values for ^{13}C, ^{12}C, ^{15}N and ^{14}N were calculated and reported using the standard delta (δ) notation in parts per thousand (‰) as follows:

$$\delta X = \left[\left(R_{\text{sample}}/R_{\text{standard}}\right) - 1\right] \times 1000,$$

where X is $\delta^{13}C$ or $\delta^{15}N$ and R is the corresponding ratio ^{13}C:^{12}C or ^{15}N:^{14}N. Two laboratory standards were included after every 12 samples. We used laboratory standards suitable for the types of samples and their C and N contents, including NIST 1547 peach leaves, NIST 1577b bovine liver, acetanilide, cellulose, glycine, sucrose and ammonium sulphate. Laboratory standards were calibrated against NIST Standard Reference Materials (IAEA-N1, IAEA-N2, IAEA-N3, IAEA-CH7, and NBS-22). Reproducibility was better than 0.1 and 0.3‰ of absolute difference for ^{13}C and ^{15}N, respectively.

Phosphorus in scales was analysed as orthophosphate after 10% HCl digestion (Grasshoff, 1983). Carbon, nitrogen and phosphorus contents were expressed in percentage dry weight. The mineral content of scales was measured as the weight loss after acidification. Within the mineral fraction, we assumed that scales were composed of apatite and carbonates (assumed to be the main compound of the residual fraction). Apatite was assumed to be all hydroxylapatite [$Ca_5(PO_4)_3OH$; molecular weight = 502.32; Elliott, 2002] and was estimated from total phosphorus in the scales by multiplying with the inverse of phosphorus mass fraction in hydroxylapatite (5.41). Carbonates were estimated as the difference between mineral fraction and apatite. Inorganic carbon of

carbonates was calculated assuming that the carbonates were calcium carbonate. Scale protein was estimated from scale nitrogen by multiplying with the inverse of nitrogen mass fraction in fish scale protein (5.31). Scale proteins are composed of collagen and ichthylepidin in approximately equal proportions (Seshaiya et al., 1963), and their average nitrogen content was calculated from the relative amino acid composition and the stoichiometry of the different amino acids in fish scales as described by Ventura (2006).

We calculated the carbon stable isotope composition of the DIC fraction of carbonates from the weighted difference of the carbon stable isotope composition of the untreated scales ($\delta^{13}C_{untreated}$, which have both carbonate and protein carbon) and acidified scales ($\delta^{13}C_{acidified}$, which have only proteins) using the following equations:

$$\delta^{13}C_{untreated} = (1 - C_{ca}) * \delta^{13}C_{acidified} + C_{ca} * \delta^{13}C_{carbonates}, \quad (1)$$

where C_{ca} is the mass fraction of carbonate carbon in the scale. Since only a proportion of the carbon of scale carbonates originates from DIC, we corrected for the proportion of DIC carbon of scales to:

$$\delta^{13}C_{carbonates} = f_{DIC} * \delta^{13}C_{DIC} + (1 - f_{DIC}) * \delta^{13}C_{acidified}, \quad (2)$$

where f_{DIC} is the mass fraction of carbonate carbon originating from DIC. This fraction was assumed to be 0.8 in all the species of this study (Solomon et al., 2006; Tohse & Mugiya, 2008). By inserting Eq. 2 into Eq. 1 and rearranging it, we obtain the equation for the estimation of the carbon stable isotope composition of DIC ($\delta^{13}C_{DIC}$):

$$\delta^{13}C_{DIC} = \frac{\delta^{13}C_{untreated} - (1 - f_{ca} * C_{ca}) * \delta^{13}C_{acidified}}{f_{DIC} * C_{ca}}. \quad (3)$$

A non-parametric Wilcoxon two-sample test was used to test the significance of the mean of the effect of scale decalcification on carbon or nitrogen stable isotope signatures for each of the six fish species studied. Linear regression was used to test for relationships between the acidified and untreated (bulk) samples, and stepwise multiple regression was used to determine relationships with environmental variables (total nitrogen, total phosphorus, chlorophyll *a*, CO_2 concentration, scale mineral content and scale carbonates). Variance partitioning (Legendre & Legendre, 1998) was used to determine the proportion of variance explained exclusively by each significant variable and the proportion of shared variance amongst them. ANCOVA was used to test if slopes differed from 1 and for heterogeneity of slopes (the interaction term of the group variable and the covariate being insignificant). In our case, the slope of the regression resulting from comparing the acidified and non-acidified scales was compared to a slope of 1, resulting from the regression comparing acidified scales in the two axes. All statistical analyses were conducted with SPSS 17.

Results

Optimal scale acidification time and average scale composition

We found that a minimum of 6 min were required to remove almost all the mineral content from the scales (Fig. 1a), the initial apatite content being ca. 35% and afterwards ≤1% of scale dry weight (DW) in the six species studied.

The scale mineral content of the six species ranged between 31.8% in tench to 61.3% DW in perch. The other four species had a similar mineral content (ca. 45% DW; Table 3). The elemental composition was characterised by high amounts of elemental phosphorus due to apatite, ranging from 4.5 to 9.1% DW. The nitrogen content ranged between 6.6% DW in perch to 12.7% DW in tench. Carbon ranged from 17.8 to 31.3% DW (Table 3). The protein content ranged between 35% in perch to 67.6% in tench. Apatite was the most abundant mineral fraction (range 24.4–49.2% DW), the residual fraction varying from 7.4% DW in tench to 13.1% DW in roach (Table 3). We calculated the inorganic carbon in carbonates assuming that carbonates are the only mineral fraction in scales apart from apatite. The average ± SD was 1.3 ± 0.2% DW (range 0.9–1.6), a very small proportion of the total carbon in scales (average ± SD: 5.5 ± 1.7% of total carbon in scales).

Effects of acidification

The average effect of acidification was very small (0.181 ± 0.122 and −0.208 ± 0.243; average ± SD

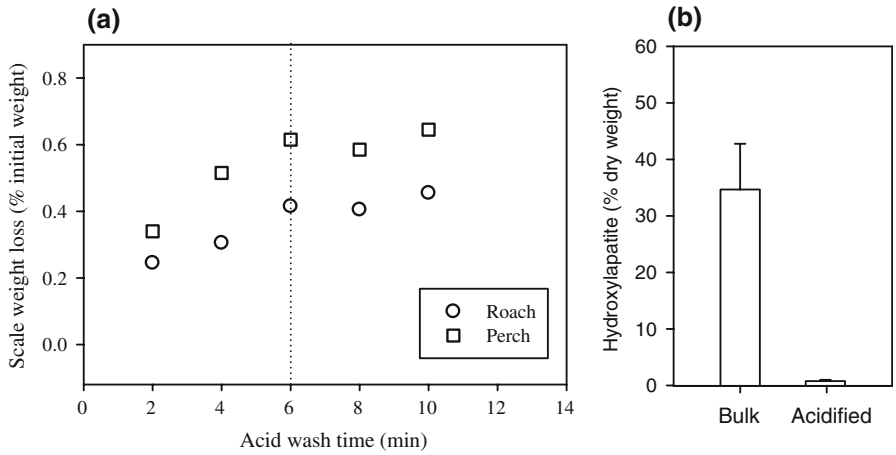

Fig. 1 Scale decalcification (measured as the relative weight loss) with varying acidification time (**a**). The vertical *dotted line* indicates the time at which the scales were most decalcified. The two species tested were representative of a species with highly calcified scales (perch, *Perca fluviatilis*) and a species with moderate scale calcification (roach, *Rutilus rutilus*). (**b**) Average of the six species studied of the apatite content in bulk scales (untreated) and those previously acidified during 6 min

for all species for carbon and nitrogen, respectively) albeit significant for five of our six study species (perch, roach, rudd, pike and bream). It was non-significant for tench, the species with the lowest mineral content. The effect differed between the two isotopes, acidification increased $\delta^{13}C$ (range from 0.02 to 0.398) and decreased $\delta^{15}N$ (range from −0.553 to 0.162; Table 4). Linear regression slopes between acidified and untreated scales were not significantly different from one for all species but one (Figs. 2, 3; Table 5), the only exception being roach that despite a slope of 0.979 differed significantly from 1 for $\delta^{15}N$. Equality of slopes with one suggested that the effect of acidification could be corrected by subtracting the average effect of acidification.

Relationships with mineral content, lake productivity and CO_2 concentration

Since the different species analysed had different scale mineral content and the different lakes had a wide range of nutrient content and CO_2 concentrations, we explored whether the acidification effect on the stable isotope composition of the scales might be related to mineral content, carbonates only, lake surface area, mean depth, nutrients and CO_2 concentrations. We used stepwise multiple regression to select a model with the combination of independent variables that best described the acidification effect on scale $\delta^{13}C$ and $\delta^{15}N$. For the acidification effect on $\delta^{13}C$, the best model had carbonates and CO_2 concentration as significant variables, explaining 58% of the variation (Table 6). CO_2 exclusively explained more variance than carbonates (28.3 and 21.1%, respectively) with 8.4% of shared variance. For the acidification effect on $\delta^{15}N$, the significant variables were total phosphorus and carbonates, explaining 52.2% of the variation, of which 22.1% derived from total phosphorus, 16.1% from carbonates, while 14% of the variance was shared.

A graphical representation of the relationships between the most significant variable in each isotope and acidification effect revealed one outlier. This point belonged to tench, the only species that had no significant acidification effect (Fig. 4a). Therefore, we repeated the stepwise multiple regression without tench. In this case, CO_2 and total phosphorus were the only significant variables for $\delta^{13}C$ and $\delta^{15}N$ (Fig. 5a), respectively, while carbonates were no longer significant (Table 6). A comparison of the acidification effect on carbon isotope composition with the difference between the carbon stable isotope composition of DIC and scale proteins ($\Delta\delta^{13}C_{DIC-acidified}$) revealed a close relationship ($r^2 = 0.923$; $P < 0.001$) with the exception of tench. For this species, no acidification effect was found, but the $\Delta\delta^{13}C_{DIC-acidified}$ was large (Fig. 4b), and therefore tench was not an outlier in the relationship between $\Delta\delta^{13}C_{DIC-acidified}$ and CO_2 (Fig. 4c).

Table 3 Scale elemental composition of phosphorus, nitrogen and carbon, and the relative proportions of apatite and protein content

Species	P (% DW)		N (% DW)		C (% DW)		Apatite (% DW)		Protein (% DW)		Average mineral content (% DW)	Mineral residual. Carbonates (% DW)
	Untreated	Acidified	Untreated	Acidified	Untreated	Acidified	Untreated	Acidified	Untreated	Acidified		
Roach												
Rutilus rutilus L.	6.3 ± 1.0	0.2 ± 0.2	10.1 ± 0.8	16.7 ± 0.8	25.9 ± 1.7	41.0 ± 0.8	33.9 ± 5.5	0.9 ± 1.2	53.4 ± 4.1	88.4 ± 4.1	47.0 ± 3.4	13.1
Perch												
Perca fluviatilis L.	9.1 ± 0.8	0.2 ± 0.1	6.6 ± 0.7	15.8 ± 0.4	17.8 ± 1.7	39.4 ± 0.9	49.2 ± 4.4	1.1 ± 0.6	34.8 ± 3.9	83.9 ± 2.0	61.3 ± 2.6	12.1
Rudd												
Scardinius erythrophtalmus L.	6.2 ± 0.3	0.1 ± 0.1	9.7 ± 0.2	17.5 ± 0.1	25.4 ± 0.3	42.3 ± 0.4	33.5 ± 1.7	0.6 ± 0.5	51.6 ± 1.1	93.1 ± 0.8	45.9 ± 2.8	12.4
Pike												
Esox lucius L.	6.6 ± 0.6	0.1 ± 0.1	9.9 ± 1.7	16.3 ± 0.6	25.1 ± 3.9	40.6 ± 0.6	35.4 ± 3.5	0.6 ± 0.4	52.4 ± 8.8	86.8 ± 3.1	47.1 ± 1.0	11.7
Bream												
Abramis brama L.	5.8 ± 0.8	0.2 ± 0.1	10.5 ± 1.2	16.7 ± 0.3	26.8 ± 3.2	42.6 ± 1.0	31.6 ± 4.4	0.8 ± 0.8	56.0 ± 6.6	88.7 ± 1.4	42.3 ± 5.1	10.7
Tench												
Tinca tinca L.	4.5 ± 0.7	0.1 ± 0.0	12.7 ± 1.1	16.4 ± 0.9	31.3 ± 1.9	41.9 ± 0.6	24.4 ± 4.0	0.5 ± 0.3	67.6 ± 5.6	87.2 ± 4.6	31.8 ± 1.4	7.4

Mineral residual is the proportion of the scale mass not accounted for by apatite and protein. All measures are expressed in mass percentage of dry weight (DW). Scales were analysed before (untreated) or after acidification (acidified) to remove mineral carbonates. Values are averages ± SD of five replicates

Table 4 Effects of acidification on freshwater fish scale $\delta^{13}C$ and $\delta^{15}N$

Species	n	$\Delta\delta^{13}C_{untreated-acidified}$				$\Delta\delta^{15}N_{untreated-acidified}$			
		Average	SD	Z	P_Z	Average	SD	Z	P_Z
Roach	35	0.274	±0.337	−4.259	<0.001	−0.297	±0.255	−4.472	<0.001
Perch	32	0.353	±0.340	−3.782	<0.001	−0.388	±0.385	−4.311	<0.001
Rudd	24	0.074	±0.679	−3.286	<0.001	−0.114	±0.410	−2.543	0.186
Pike	18	0.181	±0.270	−2.769	0.006	−0.094	±0.421	−1.917	0.022
Bream	11	0.179	±0.184	−2.547	0.002	−0.518	±0.230	−2.666	<0.001
Tench	5	0.024	±0.235	−6.74	0.500	0.162	±0.801	−0.405	0.686

Results are the average ± SD difference between a scale before and after acidification. Wilcoxon two-sample tests (Z) were used to test for significantly different means (P_Z)

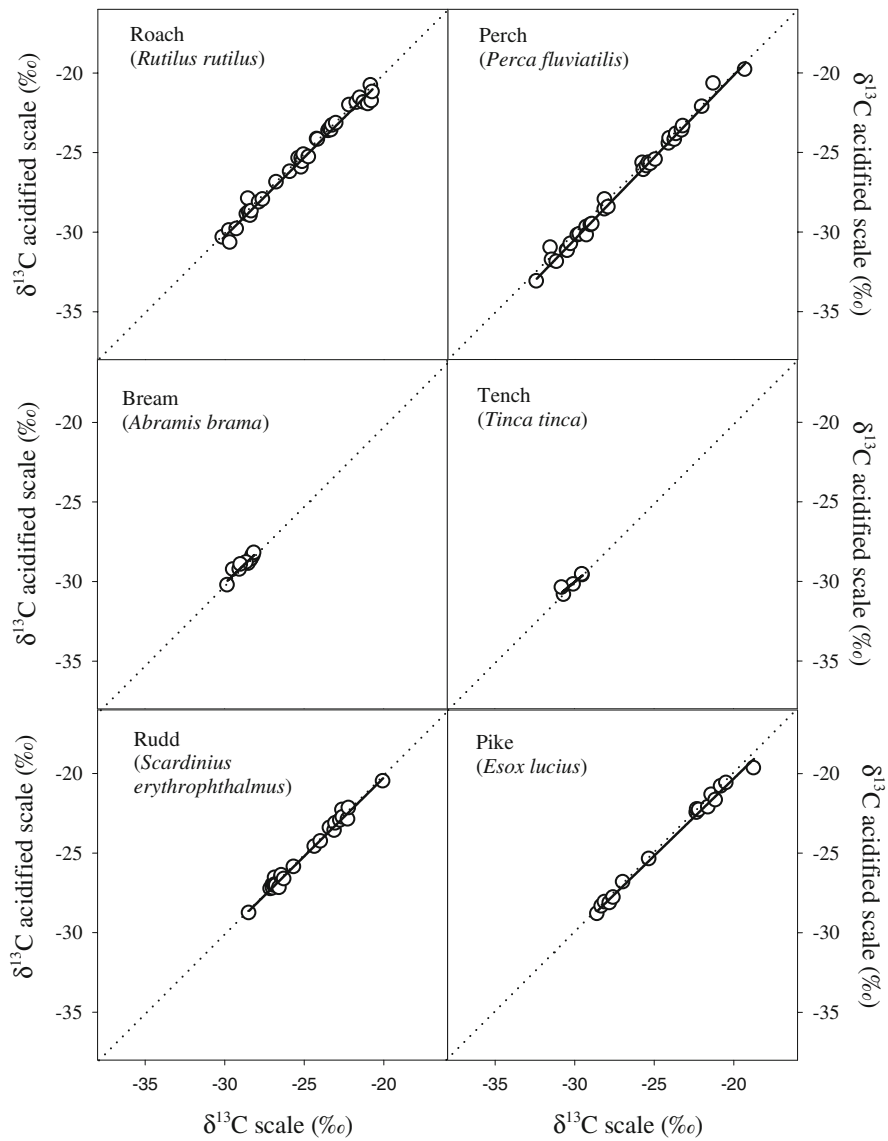

Fig. 2 Non-acidified plotted against acidified scale stable isotope $\delta^{13}C$ signatures for the six freshwater fish species studied. The *solid line* is the fit of a linear regression model and the *dotted line* is the 1:1 relationship. All regressions are significant (see Table 5 for regression parameters and statistical tests)

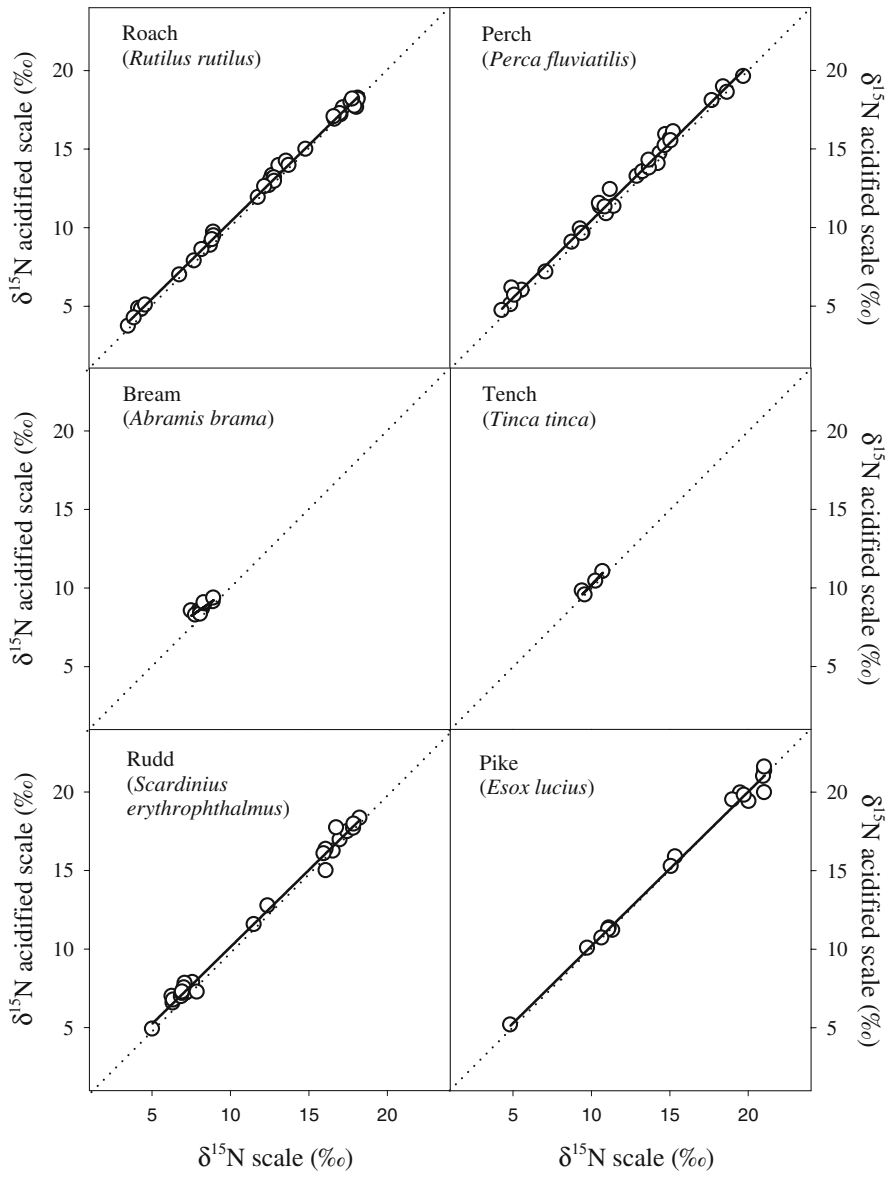

Fig. 3 Non-acidified plotted against acidified scale stable isotope $\delta^{15}N$ signatures for the six freshwater fish species studied. The *solid line* is the fit of a linear regression model and the *dotted line* is the 1:1 relationship. All regressions are significant (see Table 5 for regression parameters and statistical tests)

We also used the stepwise procedure to select the combination of variables (amongst those of Table 1, carbonates and mineral content) that best explained the variability of scale proteins. N_{tot} was the variable that explained most variance for both carbon and nitrogen (73% and 41%, respectively), but, in addition, P_{tot} and CO_2 explained part of the variation (37% and 14%, respectively; Table 6) for nitrogen.

Discussion

In this study, we found small but significant effects of acidification on scale carbon and nitrogen stable isotope composition (average change of +0.18 and −0.21‰ for carbon and nitrogen, respectively) in five of the six species studied (perch, roach, rudd, pike and bream). Previous studies have traced either strong acidification effects (+1.3‰ for both carbon

Table 5 Regression models relating the $\delta^{13}C$ and $\delta^{15}N$ of non-acidified and acidified fish scales and ANCOVA tests (with F and P values) for slopes differing from 1 and intercepts differing from 0

Species	n	$\delta^{13}C$							$\delta^{15}N$						
		Regression statistics				1:1 slope		Non-zero intercept	Regression statistics				1:1 slope		Non-zero intercept
		r^2	P	Slope	Intercept	F	P	F	P	r^2	P	Slope	Intercept	F	P	F	P
Roach	35	0.987	<0.001	0.996	−0.381	0.05	0.831	0.58	0.450	0.997	<0.001	0.979	0.558	6.16	**0.016**	24.59	<0.001
Perch	32	0.992	<0.001	1.032	0.501	3.36	0.072	1.14	0.290	0.992	<0.001	0.985	0.563	0.81	0.372	7.41	0.009
Rudd	24	0.988	<0.001	0.988	−0.509	0.31	0.580	0.85	0.363	0.993	<0.001	0.980	0.338	1.52	0.223	2.9	0.095
Pike	18	0.970	<0.001	0.970	−0.909	2.23	0.147	3.51	0.071	0.993	<0.001	0.982	0.378	0.66	0.422	1.11	0.301
Bream	11	0.896	<0.001	0.956	−1.426	0.35	0.560	0.29	0.598	0.704	0.001	0.697	3.008	4.06	0.059	5.91	**0.026**
Tench	5	0.855	0.024	0.820	−5.444	0.85	0.389	0.85	0.391	0.921	0.04	1.051	−0.321	0.03	0.858	0.04	0.844

and nitrogen) on whitefish (*C. lavaretus*; Perga & Gerdeaux, 2003), small but biologically irrelevant effects on roach (Syväranta et al., 2008) and no effects after scale acidification on yellow perch (*P. flavescens*), walleye (*S. vitreus*), Atlantic salmon (*S. salar*; Sinnatamby et al., 2007) and North American whitefish (*Coregonus clupeaformis*; Rennie et al., 2009). Variability in among species scale characteristics might be one of the explanations for the differences occurring in our study and the other published studies. In this respect, the positive relationship between the acidification effect and the scale mineral content revealed by comparing the results among the six species of this study suggests that an important factor for the among species variability is related to the different mineral content of fish scales. In our study, we found that for fish species with a mineral content lower than 20% (or 7% of carbonates), there are no significant effects of scale acidification, while for the other species, it was close to the analytical error of the instrument and therefore on the limit of being biologically irrelevant.

Other factors may also be responsible for this among species variability, such as incomplete scale decalcification or variability in $\delta^{13}C$-DIC. Perga & Gerdeaux (2003) showed that for whitefish, there was no carbonate left after 2 min acidification. In contrast, Sinnatamby et al. (2007) acidified the scales of three other fish species for 2 min, which might not have been sufficient for decalcifying all species. This could be the case of yellow perch, belonging to the same genus as perch measured in this study and whose scales were heavily calcified.

Our study also revealed that within-species variability in $\delta^{13}C$ was positively correlated with differences in lake CO_2 concentration. The variance of acidification effect explained by CO_2 concentration was 60%, which is high considering that not all carbonates originate from DIC (between 75 and 85%; Solomon et al., 2006; Tohse & Mugiya, 2008). Previous studies have identified a relationship between lake primary productivity and scale $\delta^{13}C$ (Perga & Gerdeaux, 2004; Gerdeaux & Perga, 2006; Grey et al., 2009) and have attributed it mostly to a change in phytoplankton fractionation, but also to some extent to a change in the contribution of respired carbon and to an increase in the DIC pool $\delta^{13}C$ by Rayleigh distillation kinetics and input of atmospheric CO_2, amplified by the alkaline nature of

Table 6 Multiple regression analyses relating the effects of scale acidification on $\delta^{13}C$ and $\delta^{15}N$ ($\Delta\delta^{13}C_{untreated-acidified}$ and $\Delta\delta^{15}N_{untreated-acidified}$, respectively), the difference between the stable isotope composition of carbon of DIC and scale proteins ($\Delta\delta^{13}C_{DIC-acidified}$) and the stable isotope composition of scale proteins ($\delta^{13}C_{acidified}$ and $\delta^{15}N_{acidified}$) to selected environmental variables

	Species included	Coefficient	*P*	R^2	*F*	df	*P*	Variance partitioning (% of total variance)
$\Delta\delta^{13}C_{untreated-acidified}$	All							
Intercept		−0.75	0.02	0.578	11.0	2.16	0.001	
CO_2		0.422	<0.001					28.3
Carbonates		0.071	<0.001					21.1
								Shared: 8.4
$\Delta\delta^{15}N_{untreated-acidified}$	All							
Intercept		0.502	0.169	0.522	8.72	2.16	0.003	
P_{tot}		1.288	0.003					22.1
Carbonates		−0.077	0.017					16.0
								Shared: 14.0
$\Delta\delta^{13}C_{untreated-acidified}$	Excepting tench							
Intercept		0.109	0.013	0.609	24.89	1.16	<0.001	
CO_2		0.480	<0.001					
$\Delta\delta^{13}C_{DIC-acidified}$	All							
Intercept		2.06	0.013	0.587	24.16	1.18	<0.001	
CO_2		9.67	<0.001					
$\Delta\delta^{15}N_{untreated-acidified}$	Excepting tench							
Intercept		−0.472	<0.001	0.546	19.23	1.16	<0.001	
P_{tot}		1.404	<0.001					
$\Delta\delta^{13}C_{acidified}$	All							
Intercept		−33.19	<0.001	0.730	48.66	1.18	<0.001	
N_{tot}		5.66	<0.001					
$\Delta\delta^{15}N_{acidified}$	All							
Intercept		−7.95	<0.001	0.924	64.76	3.16	<0.001	
N_{tot}		9.19	<0.001					40.7
P_{tot}		40.36	<0.001					37.2
CO_2		11.88	<0.001					14.2
								Shared: 0.3

The stepwise procedure was used to select the variables amongst the lake area, mean depth, total nitrogen, total phosphorus (P_{tot}), chlorophyll *a*, CO_2, scale mineral content and scale carbonates. The analyses were run twice for the acidification effects, first with all fish species and second excluding tench, which had no acidification effect. Variance partitioning was used to estimate the amount of variance explained by each variable and the shared variance amongst them when more than one explanatory variable was present in the model

the waters. Perga & Gerdeaux (2004) found that $\delta^{13}C$-DIC was the main factor explaining the change in the scale $\delta^{13}C$, establishing a correlation with P_{tot} only in large lakes. Our results show that the dual analysis of acidified scales and the difference between acidified and untreated scales could be a tool to distinguish between the effects of changing $\delta^{13}C$-DIC and alterations in algal fractionation due to changing CO_2 concentration. Although the procedure used in our study for calculating $\delta^{13}C$-DIC might be less sensitive than capturing CO_2 with cryogenic distillation after acidification for posterior analysis (Lee-thorp et al., 1989), it has the practical advantage of being methodologically simple. We

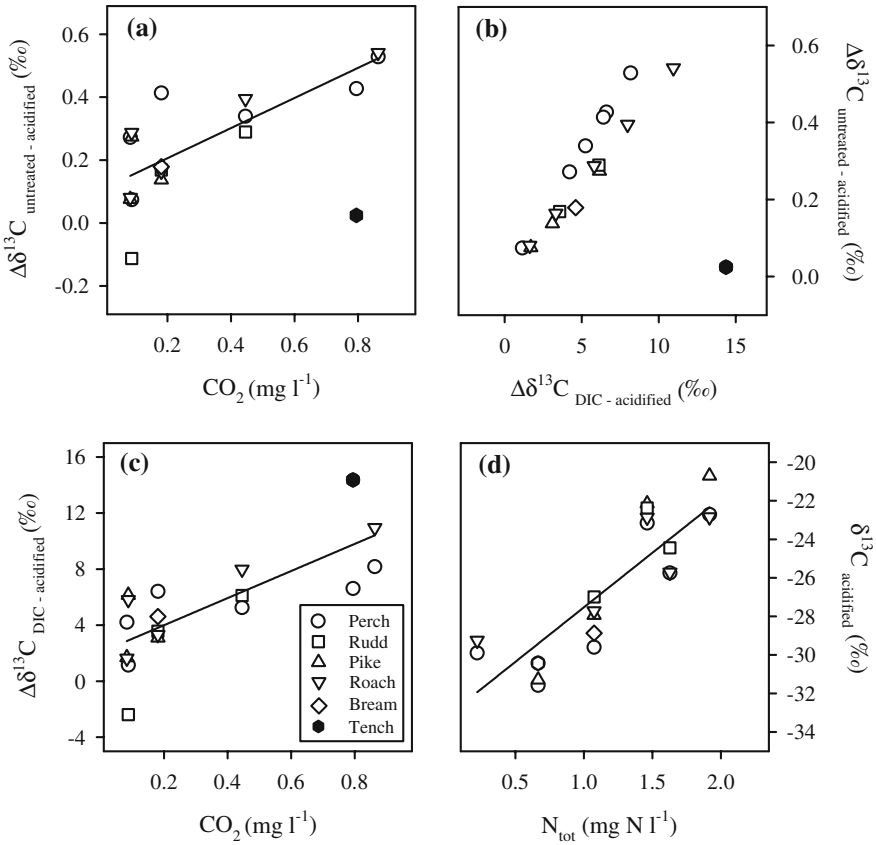

Fig. 4 Relationships between the effect of acidification on scale stable isotope composition of carbon ($\Delta\delta^{13}C_{untreated–acidified}$) with (**a**) average summer free CO_2 concentration, (**b**) with the difference in the stable isotope composition of scale carbonates and protein, in our case acidified scales ($\Delta\delta^{13}C_{carbonates–acidified}$); (**c**) $\Delta\delta^{13}C_{carbonates–acidified}$ with average summer-free CO_2 concentration. See "Materials and methods" section for a description of the procedure for calculating the stable isotope composition of carbonates. (**d**) Relationship between the carbon stable isotope composition of acidified scales ($\delta^{13}C_{acidified}$) and total nitrogen (N_{tot}). Explanatory variables were selected among those of Table 1, scale mineral content and scale carbonates by stepwise linear regression (see Table 6 for regression equations). The regression of (**a**) excludes tench (*in black*), while the other two regressions (**c**, **d**) include tench

found the precision of the method sufficient to capture the fractionation effects created by variable CO_2 concentrations.

Similarly to carbon, we observed significant changes, proportional to the mineral content of scales, in scale $\delta^{15}N$ after acidification. In their study, Perga & Gerdeaux (2003) also found significant and more prominent changes in scale $\delta^{15}N$ after acidification than in our study. Acidification with diluted HCl is unlikely to alter protein nitrogen as proteins are hydrolysed with concentrated HCl at high temperatures (Fountoulakis & Lahm, 1998). In addition, Perga & Gerdeaux (2003) acidified their scales for a shorter time than in our study. The change in $\delta^{15}N$ with the mineral content among the species investigated here indicate that fishes deposit organic nitrogenated compounds together with apatite and carbonates when building scales. This compound likely differs from the other scale proteins (collagen and ichthylepidin) by being more attached to the mineral fraction and is concordingly released when apatite is removed by acidification. Furthermore, the similar correlation between $\Delta\delta^{15}N_{untreated–acidified}$ and $\delta^{15}N_{acidified}$ with nutrients also indicates that the fraction of nitrogen removed by acidification is of the same biogenic origin as

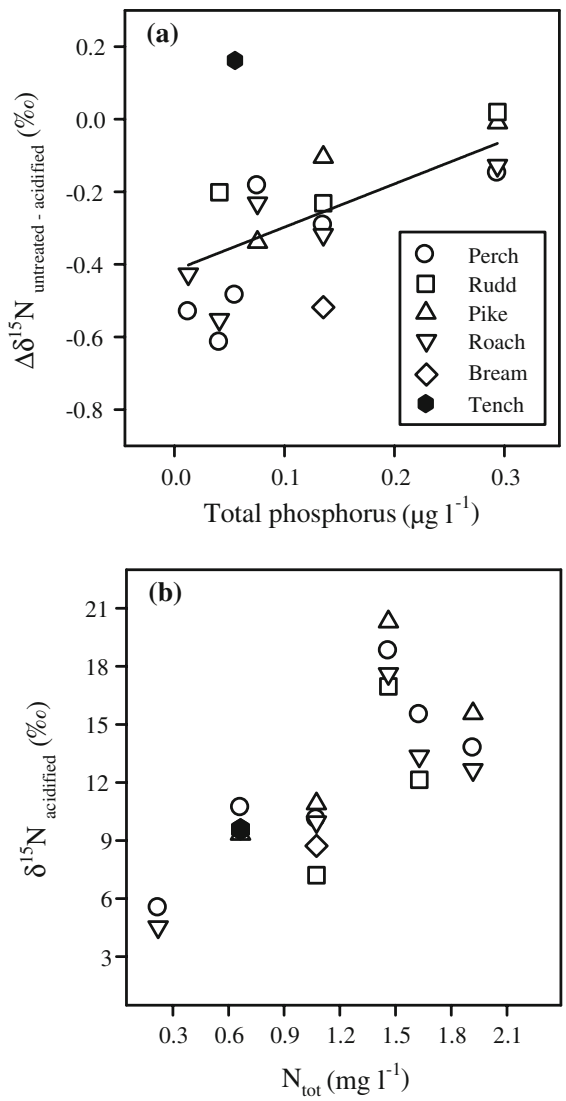

Fig. 5 Relationship between the effect of acidification on scale stable isotope composition of nitrogen ($\Delta\delta^{15}N_{untreated-acidified}$) with total phosphorus (**a**). The regression excludes tench (*in black*), which had no acidification effect due to the low scale mineral content. Relationship between the nitrogen stable isotope compositions of acidified scales ($\delta^{15}N_{acidified}$) with total nitrogen (**b**). Explanatory variables were selected among those of Table 1, scale mineral content and scale carbonates by stepwise linear regression (see Table 6 for regressions equation). In (**b**), total nitrogen was selected together with total phosphorus and CO_2 concentration and was the variable explaining the largest proportion of variance

scale proteins. Therefore, acidification effects on nitrogen provide no additional information than the analysis of scale proteins alone.

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