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Freezing and chemical preservatives alter the stable isotope values of carbon and nitrogen of the Asiatic clam (*Corbicula fluminea*)

Jari Syväranta[1, 3, *], Aurélia Martino[1], Dorothée Kopp[1, 2], Régis Céréghino[1] and Frédéric Santoul[1]

[1] EcoLab, UMR 5245 (CNRS-UPS-INPT), Université de Toulouse, bât 4R3, 118, route de Narbonne, 31062 Toulouse Cedex 9, France
[2] IFREMER, Equipe Ecologie et Modèles pour l'Halieutique, Rue de l'Ile d'Yeu, BP 21105, 44311 Nantes Cedex 3, France
[3] Department of Biology and Ecology of Fishes, Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Müggelseedamm 310, 12561 Berlin, Germany

*: Corresponding author : Jari Syväranta, email address : jari.syvaranta@jyu.fi

Abstract:

We tested the impacts of most common sample preservation methods used for aquatic sample materials on the stable isotope ratios of carbon and nitrogen in clams, a typical baseline indicator organism for many aquatic food web studies utilising stable isotope analysis (SIA). In addition to common chemical preservatives ethanol and formalin, we also assessed the potential impacts of freezing on $\delta^{13}C$ and $\delta^{15}N$ values and compared the preserved samples against freshly dried and analysed samples. All preservation methods, including freezing, had significant impacts on $\delta^{13}C$ and $\delta^{15}N$ values and the effects in general were greater on the carbon isotope values (1.3–2.2‰ difference) than on the nitrogen isotope values (0.9–1.0‰ difference). However, the impacts produced by the preservation were rather consistent within each method during the whole 1 year experiment allowing these to be accounted for, if clams are intended for use in retrospective stable isotope studies.

Keywords: Formalin - Freezing - Ethanol - Preservation - Stable isotope analysis

Stable isotope analysis (SIA) from preserved and archived sample materials offers unique possibilities for reconstructing historical food webs and for retrospective ecosystem studies. Many universities, museums and research institutions hold collections of preserved sample materials which potentially could be turned into valuable long-term ecosystem data sets. Analyses of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) stable isotope ratios in sample materials (expressed relative to a standard as $\delta^{13}C$ and $\delta^{15}N$ values) provide information about food web structure, consumer trophic positions as well as energy sources and pathways within ecosystems (Peterson & Fry, 1987; Fry, 2006). However, to date surprisingly few studies have effectively utilised such source of information, perhaps reflecting some prevailing uncertainties about preservation impacts on stable carbon and nitrogen isotope values in sample materials.

In general, however, many reported impacts of preservatives have been relatively small, particularly those on $\delta^{15}N$ values (~1 ‰), suggesting that preserved samples can potentially be utilised in historical food web studies, provided that any impacts can reliably be accounted for. But many contradictions and variability in results and interpretations still exist (see Barrow et al., 2008 and Ventura & Jeppesen, 2009 for recent literature summaries on preservation impacts). For example, Feuchtmayr & Grey (2003) reported elevated $\delta^{13}C$ values in zooplankton after preservation in formalin, whereas many other studies on aquatic animals such as fish, zooplankton and macroinvertebrates (e.g. Mullin et al., 1984; Bosley & Wainright, 1999; Kaehler & Pakhomov, 2001; Edwards et al., 2002; Sarakinos et al., 2002, Syväranta et al., 2008b) have reported opposite impacts. Similarly, some studies on aquatic animals reported strong and significant impacts on $\delta^{15}N$ values (e.g. Sarakinos et al., 2002; Feuchtmayr & Grey, 2003; Kelly et al., 2006), while some only little or no impacts at all (e.g. Mullin et al., 1984; Ogawa et al. 2001; Syväranta et al., 2008b). The magnitude of these reported impacts of preservatives on

58 stable carbon and nitrogen values have been highly variable and range for both $\delta^{13}C$ and $\delta^{15}N$
59 values from no impact to over 2 ‰ difference between control and preserved samples. Similarly
60 contradicting results are reported after ethanol preservation of fish, zooplankton and
61 macroinvertebrate samples (e.g. Kaehler & Pakhomov, 2001; Sarakinos et al., 2002; Feuchtmayr
62 & Grey, 2003; Syväranta et al. 2008b) but fewer studies have considered testing for fixation in
63 formalin and subsequent transfer to ethanol preservation, a technique which is often employed in
64 institutions and museums (Bosley & Wainright, 1999; Carabel et al., 2009), particularly for
65 invertebrate samples. Freezing is likely the most common method to preserve samples for SIA
66 when immediate drying is not possible, but not all studies have considered the potential impacts
67 of freezing of sample on stable isotope ratios. Among the studies that have tested the effects of
68 freezing on aquatic animal tissues and/or whole organisms, some found no impacts (Bosley &
69 Wainright, 1999; Kaehler & Pakhomov, 2001; Sweeting et al. 2004) while others found
70 significant and even strong impacts (Feuchtmayr & Grey, 2003; Dannheim et al., 2007; Barrow
71 et al., 2008; this study) on stable carbon and nitrogen isotope values. In addition, impacts of
72 preservatives seem to be highly taxa-specific and preservation studies are often ran as pilot
73 experiments for other studies and may therefore suffer from extremely low number of replicates
74 or incomplete replication, taxonomic variation within replicates and/or using frozen samples as
75 control.
76 Here we report results from experimental testing of the impacts of chemical preservatives
77 (ethanol and formalin) and freezing on the stable carbon and nitrogen isotope ratios of a
78 freshwater clam (the Asiatic clam *Corbicula fluminea* (O. F. Müller, 1774)). Clams are long-
79 lived primary consumers that are often preferred as baseline indicators in SIA studies of aquatic
80 ecosystems (Post, 2002) and are isotopically shown to closely match the seasonal averages of

zooplankton primary consumers (Syväranta et al., 2008a). *C. fluminea* is a highly invasive species and can form dense clam beds both in European and North-American river and lake bottoms. Albeit invasive, it may serve as a valuable baseline indicator for many present and future SIA studies. Historical samples of clams are likely to be available from many institutional collections which potentially can be used to set "historical stable isotope baselines" for comparisons between and within ecosystems in long-term SIA studies. Despite their apparent importance for stable isotope ecology, only two past studies were identified to specifically test for preservation impacts on clams (Sarakinos et al., 2002; Carabel et al., 2009). Our aim was to provide precise evaluation of the effects of the typical chemical preservatives and freezing methods on $\delta^{13}C$ and $\delta^{15}N$ values of *C. fluminea* on a long-term basis by preserving samples up to one year and comparing the isotope values to freshly dried and analysed samples.

Clam samples were collected with hand nets from the Roques-sur-Garonne area of the river Garonne in Toulouse, southwestern France, in December 2008 and immediately taken to the laboratory for cleaning and processing. Samples were divided into those dissected, dried and analysed immediately (control) and those preserved for 1 week, 1 month, 6 months or 12 months either by freezing (at -20°C) or in ethanol (70 %), formalin (4 %) or by first fixing in formalin for two days and then transferring to ethanol (formalin-ethanol), each group having 5-6 replicate samples. Clams were preserved attached to their shells in all treatments, submerged in the preservative in plastic vials during ethanol/formalin preservation at room temperature and in plastic vials without excess water when frozen. After all preservation treatments, samples were carefully rinsed several times in clean tap water and the foot tissue dissected and cleaned. Only the foot tissue was used for SIA and all samples were oven dried (at 60°C for 48 h) and ground into a fine homogeneous powder using a mixer mill (Retsch MM 200).

Approximately 0.2 mg of sample material was accurately weighed into tin cups and stable isotope ratios of carbon and nitrogen were analysed after combustion in a Carlo Erba NC2500 elemental analyser (Carlo Erba, Milan, Italy) with a Finnigan Mat Delta XP isotope ratio mass spectrometer (Thermo Finnigan, Bremen, Germany). Each analysed sample corresponds to a single individual clam. Stable isotope ratios are expressed as parts per thousand (‰) delta values (δ^{13}C‰ or δ^{15}N‰) referred to the international standards for carbon (PeeDee Belemnite) and nitrogen (atmospheric nitrogen). Data were inspected and corrected using working standards (bass muscle, bovine liver, nicotinamide; SD < 0.2 ‰ for both δ^{13}C and δ^{15}N) that were previously calibrated against International Atomic Energy Agency (IAEA) standards. All stable isotope analyses were performed at the Stable Isotopes in Nature Laboratory, University of New Brunswick, Canada.

Impacts of preservation method and preservation time on stable isotope values were analysed by comparing preserved samples to control samples using analysis of variance (ANOVA) with Tukey's pairwise comparisons tests after testing for data normality and variance homogeneity. All statistical analyses were done using a SPSS 13.0 for Windows software package (SPSS Inc., 2004).

Preservation increased mean (± SD) δ^{13}C and δ^{15}N values by 1.8 ± 0.5 ‰ ($F_{4,108}$ = 163.9, $p < 0.001$) and 1.0 ± 0.3 ‰ ($F_{4,108}$ = 46.4, $p < 0.001$), respectively, compared to the control samples which were dried immediately after collection. Duration of the preservation did not significantly affect the difference in stable isotope values or C:N ratios between control and preserved samples, except for δ^{13}C values in samples preserved with formalin, which after 6 and 12 months of preservation had significantly lower δ^{13}C values compared to one week

126 preservation ($F_{3,23} = 12.3$, $p < 0.001$; Fig. 1). In all other preserved samples the impacts were
127 evident already after one week of preservation.

128 Averaging for the entire preservation period (Fig. 2, Table 1), freezing and ethanol
129 preservation had significantly ($F_{3,92} = 49.8$, $p < 0.001$) more impact on $\delta^{13}C$ values elevating the
130 mean (± SD) by 2.1 ± 0.3 ‰ and 2.2 ± 0.3 ‰, whereas formalin and formalin-ethanol elevated
131 the values by 1.3 ± 0.3 ‰ and 1.6 ± 0.3 ‰, respectively. The impact of preservation on $\delta^{15}N$
132 values was similar for all preservatives as freezing, ethanol, formalin and formalin-ethanol
133 elevated the $\delta^{13}C$ values by 1.0 ± 0.3 ‰, 1.0 ± 0.2 ‰, 0.9 ± 0.2 ‰ and 1.0 ± 0.3 ‰, respectively.
134 Only ethanol preservation significantly affected the C:N ratios in sample materials by lowering
135 the ratios from 3.9 ± 0.4 in control samples to 3.5 ± 0.4. However, the elemental compositions of
136 preserved samples changed during all treatments (Table 1). Carbon content (C%) became
137 significantly lower (by 1.7 to 2.2 units) compared to control samples after the treatments ($F_{4,108} =$
138 6.1, $p < 0.001$), but there were no differences among the treatments or treatment duration.
139 Similarly the N% became lower after treatments by 0.9 to 0.4 units ($F_{4,108} = 36.4$, $p<0.001$),
140 except in ethanol preservation, which significantly increased the N% by 1.0 unit ($p = 0.001$).

141 Our results on the impacts of preservatives on stable isotope ratios showed clear and
142 significant impacts on the $\delta^{13}C$ and $\delta^{15}N$ values of clams, a typical organism used in various
143 ecological SIA studies to set isotope baseline values. Also freezing, perhaps the most common
144 preservation method, significantly elevated both $\delta^{13}C$ and $\delta^{15}N$ values, in contrast to the findings
145 of Bosley & Wainright (1999), Kaehler & Pakhomov (2001) and Sweeting et al. (2004).
146 However, contrary to the impacts of freezing on zooplankton (Feuchtmayr & Grey, 2003) and
147 macrozoobenthos (Dannheim et al., 2007) samples, our clam samples became significantly ^{13}C-
148 enriched (i.e. higher $\delta^{13}C$ values) after freezing at -20°C. Impact of freezing on $\delta^{15}N$ values was
149 similar to previous findings with around 1 ‰ increase (Feuchtmayr & Grey, 2003, Dannheim et

al., 2007). These differences could partly be explained by the freezing methods employed (e.g. -80°C vs. -20°C) and by taxa-specific differences. Clams are protected by their shell but when removed from these they are soft-bodied and easily loose body fluids compared to organisms protected by chitinous exoskeletons. Impacts of the tested preservation methods on isotope ratios of clams were surprisingly similar, particularly with respect to $\delta^{15}N$ where all preservation methods resulted in equally elevated values. Ethanol had the strongest impact on $\delta^{13}C$ values, which in part could relate to the lipid solving properties of ethanol (Syväranta et al., 2008b) as also the C:N ratios of those samples were affected. Similarly strong impact of freezing on the isotope values is more difficult to explain. Most likely this relates to mechanical effects and breakdown of cells allowing leaching of carbon and nitrogen when thawed (Feuchtmayr & Grey, 2003, Dannheim et al., 2007). The duration of the preservation did not affect the $\delta^{15}N$ values, which remained constant throughout the experiment, and only formalin preserved $\delta^{13}C$ values became slightly (but significantly) lower with prolonged preservation time. Preservation did not increase the variation around the mean isotope values either in any case and in general the variation among individual clams was low. Our samples were collected at the same time from a small area and were of equal size so very little among-individual variation was expected. We are therefore confident that all the impacts seen on clam isotope values result from the different preservation methods.

Only two previous preservation studies testing impacts on clam tissues were identified in the literature (Sarakinos et al., 2002; Carabel et al., 2009). The results from those studies agree with our results as Sarakinos et al., (2002) reported 2.18 and 0.67 ‰ increase in $\delta^{13}C$ values after ethanol and formalin preservation, respectively, compared to our 2.2 and 1.3 ‰ increase. Similarly Carabel et al. (2009) reported significant increased in $\delta^{15}N$ values after ethanol-formalin preservation with an increase also around 1 ‰. However, Sarakinos et al. (2002) found

opposing impacts on $\delta^{15}N$ values after ethanol and formalin preservation, and Carabel et al. (2009) found no or only minor impacts on $\delta^{13}C$ values. Both these studies used frozen samples as control treatment and therefore the results may not directly be comparable to ours. If frozen samples would have been used as control in this study, no impacts on $\delta^{15}N$ values and much lower impacts on $\delta^{13}C$ values (both higher and lower values) would have been noticed after preservation in ethanol, formalin or formalin-ethanol. In fact, such result would be in accordance with many previous studies using frozen samples as control.

Since all preservation methods had relatively low and, even more importantly, consistent impact on the $\delta^{15}N$ values (~1 ‰) and as nitrogen isotope values typically fractionate around 3 ‰ per trophic transfer (Peterson & Fry, 1987; Post, 2002), the $\delta^{15}N$ values of preserved clams can offer a suitable and rather reliable baseline indicator for retrospective studies in aquatic ecosystems. Our detailed results (Table 1) provide reliable means to account for preservation effects on sample $\delta^{15}N$ values allowing archived clams to be used for example to estimate historical trophic positions of consumers. The impacts on $\delta^{13}C$ values were greater, and carbon isotope values also fractionate less (<1 ‰) in food webs, making preserved clam samples less desirable for constructing historical food webs based on the $\delta^{13}C$ values. However, even though the effects of preservatives were greater on $\delta^{13}C$ values, the impacts were still rather consistent and provided that these observed impacts on carbon isotope values are accounted for, clams may offer an attractive baseline indicator organism for retrospective studies in aquatic ecosystems.

In conclusion, our results clearly illustrate that common preservation methods, including freezing, significantly affect the stable isotope values in sample materials (here clams) but our well controlled study also illustrates that these impacts are consistent and can be accounted for. Therefore, we conclude that preserved clams can indeed be used for stable isotope analyses but care has to be taken and the values need to be appropriately adjusted for preservation effects, as

shown in this study. However, for studies not restricted to using preserved samples we advise to use only freshly dried samples and to avoid all unnecessary freezing and preserving for SIA. We also recognise a clear need for further taxa-specific, well controlled experiments of preservation impacts, including freezing, on stable isotope sample materials.

Acknowledgments

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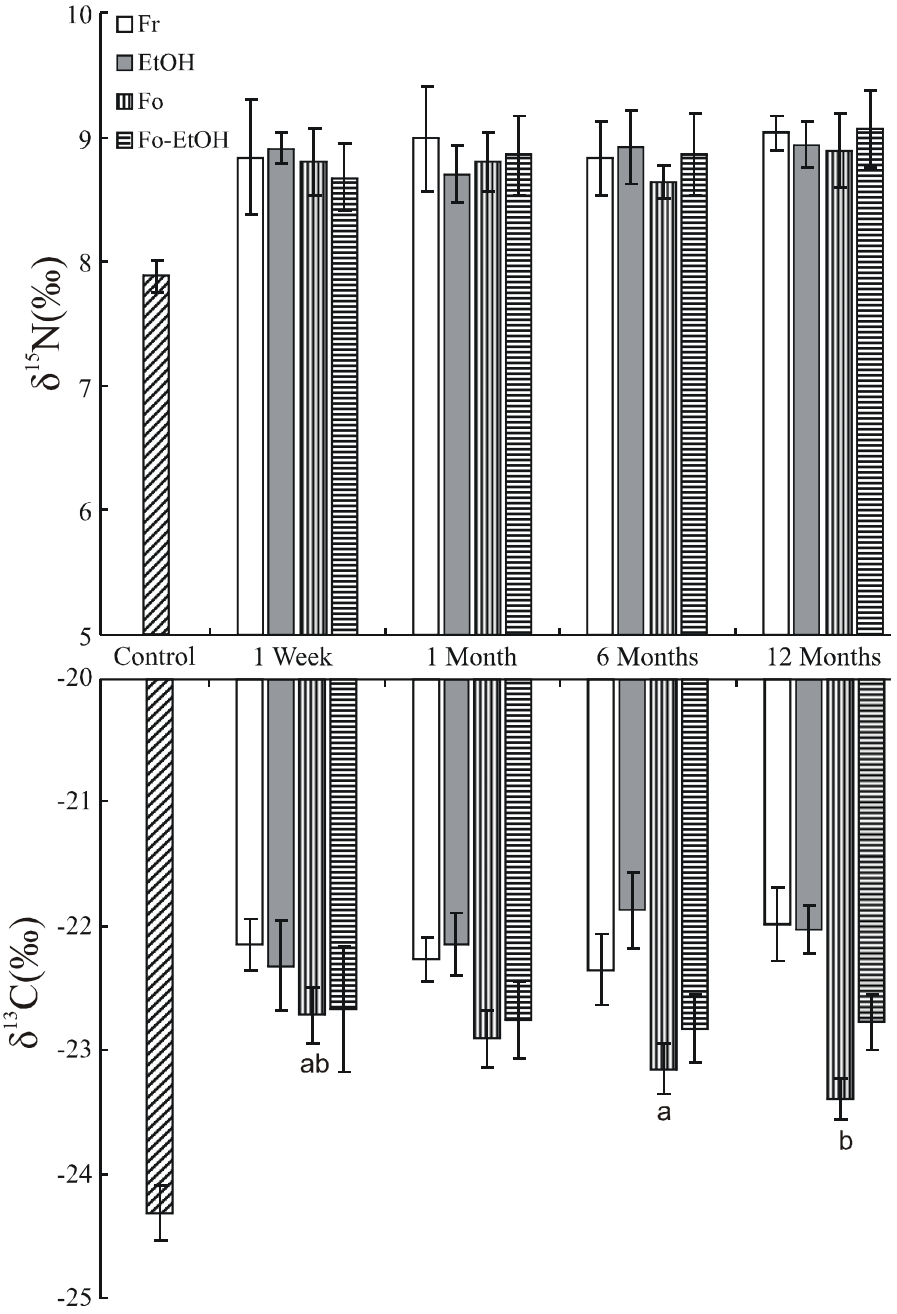
Figure legends

Fig. 1. Changes in $\delta^{15}N$ (upper panel) and $\delta^{13}C$ (lower panel) values after a week, a month, 6 months and 12 months preservation time by freezing (Fr) or in ethanol (EtOH), formalin (Fo) or formalin-ethanol (Fo-EtOH) against the control samples. Stable isotope values significantly differed from control values after all treatments. Bars represent means (± SD) of 5-6 replicate values and letters indicate significant differences ($p < 0.05$) after different preservation times in formalin.

Fig. 2. Stable isotope biplot of mean (± SD) differences in $\delta^{13}C$ and $\delta^{15}N$ values between control and preserved samples (Fr = freezing, EtOH = ethanol, Fo = formalin, Fo-EtOH = formalin-ethanol) for the whole 12-months study period.

268 ***Figures***

269 Fig. 1



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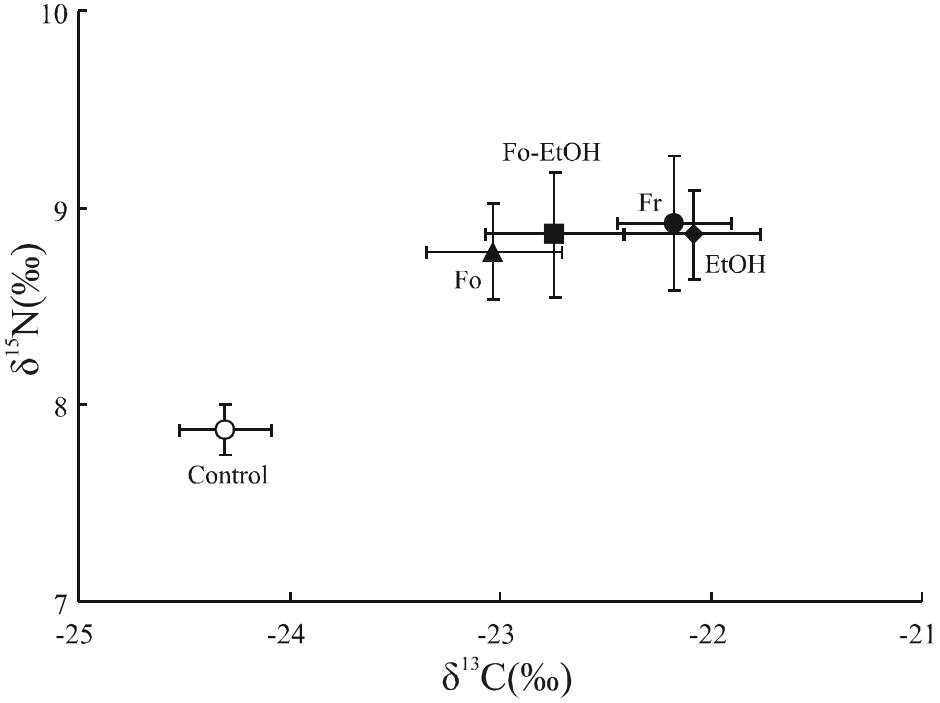
272 Fig. 2



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275 ***Tables***
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Table 1. Mean difference (± SD) of preserved sample $\delta^{13}C$, $\delta^{15}N$, C:N, C% and N% values compared to control samples after each tested preservation period. Sign indicates the direction of the change (+ = higher values, - = lower values) and an overall mean difference to control samples is provided for all tested preservation methods.

	Freezing	Ethanol	Formalin	Fo+EtOH
$\delta^{13}C$				
1 week	+2.2±0.2	+2.0±0.4	+1.6±0.2	+1.6±0.5
1 month	+2.0±0.2	+2.2±0.3	+1.4±0.2	+1.6±0.3
6 months	+2.0±0.3	+2.4±0.3	+1.2±0.2	+1.5±0.3
12 months	+2.3±0.3	+2.3±0.2	+0.9±0.2	+1.5±0.2
mean	*+2.1±0.3*	*+2.2±0.3*	*+1.3±0.3*	*+1.6±0.3*
$\delta^{15}N$				
1 week	+1.0±0.5	+1.0±0.1	+0.9±0.3	+0.8±0.3
1 month	+1.1±0.4	+0.8±0.2	+0.9±0.2	+1.0±0.3
6 months	+1.0±0.3	+1.0±0.3	+0.8±0.1	+1.0±0.3
12 months	+1.2±0.1	+1.1±0.2	+1.0±0.3	+1.2±0.3
mean	*+1.0±0.3*	*+1.0±0.2*	*+0.9±0.2*	*+1.0±0.3*
C:N				
1 week	+0.1±0.1	-0.3±0.1	+0.1±0.1	+0.1±0.2
1 month	0.0±0.2	-0.3±0.3	+0.1±0.1	0.0±0.2
6 months	+0.2±0.1	-0.6±0.0	+0.1±0.1	-0.2±0.1
12 months	+0.2±0.1	-0.5±0.0	+0.2±0.1	-0.1±0.2
mean	*+0.1±0.2*	*-0.4±0.2*	*+0.1±0.1*	*0.0±0.2*
C%				
1 week	-3.1±0.5	-0.4±1.2	-1.6±0.7	-1.5±1.8
1 month	-2.0±1.8	-2.1±1.4	-1.5±1.0	-2.0±0.9
6 months	-2.1±1.2	-2.1±1.1	-1.5±0.5	-2.1±0.2
12 months	-1.5±1.1	-2.6±0.9	-2.1±1.5	-2.8±4.1
mean	*-2.2±1.3*	*-1.7±1.4*	*-1.7±1.0*	*-2.1±2.3*
N%				
1 week	-1.0±0.5	+0.8±0.3	-0.9±0.5	-0.8±0.9
1 month	-0.4±0.7	+0.7±1.1	-0.8±0.4	-0.6±0.6
6 months	-1.1±0.4	+1.7±0.3	-0.6±0.3	+0.1±0.2
12 months	-1.0±0.6	+1.0±0.3	-1.0±0.6	-0.4±1.3
mean	*-0.9±0.6*	*+1.0±0.7*	*-0.8±0.5*	*-0.4±0.9*

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