The use of stable isotope analyses has been increasing in studies of aquatic food webs (Post 2002). Samples for isotope analyses are usually preserved by freezing because fixation can affect isotope signatures. Hence, sample identification must be made for live or frozen samples. However, identifying live samples is often difficult for small aquatic invertebrates. This difficulty also applies to frozen samples without fixation. For example, thawed polychaetes without fixation become slimy and difficult to identify. However, if the effect of fixation is known, fixed organisms can also become useful samples for isotope analyses (e.g. Vanderzanden et al. 2003, Gorokhova 2005).

The main preservatives for aquatic invertebrates are formalin and ethanol. Several studies have investigated the change in isotope signatures by formalin fixation in invertebrate species (Mullin et al. 1984, reviewed in Sarakinos et al. 2002, Kelly et al. 2006). In most cases, formalin fixation depletes carbon signatures, but weakly enriches nitrogen signatures. For ethanol fixation, a few studies have investigated the change effect on isotopic signatures in aquatic invertebrates (e.g., Mullin et al. 1984, reviewed in Sarakinos et al. 2002, Kelly et al. 2006). In these studies, ethanol fixation enriches carbon signatures, though there is no trend in the shifts of nitrogen signatures. In marine benthos, however, we are aware of only one study, on Octopus vulgaris (Kaehler & Pakhomov 2001) and therefore, more information is needed for marine benthos. Addressing this issue, we report the effect of ethanol fixation on the isotope signatures in several marine macrobenthic organisms, and discuss the efficacy of ethanol-fixed samples for stable isotope analyses featuring shift of signatures.

We collected benthic organisms from tidal flats and subtidal zones in the inner part of Ariake Bay (Table 1) on December 13th, 2008, using a Smith McIntyre grab (sampling size: 22.5×22.5 cm²) from a boat. The samples were brought to the laboratory and identified under a stereoscopic microscope during the day. Of the collected benthic organisms, we chose the following six species that were present in numbers sufficient for isotope analysis: the arcid bivalve Scapharca kagoshimensis (Tokunaga) from stations C to F, Tegillarca granosa (Linnaeus) from B and C, the pinnid bivalve Atrina lischkeana (Clessin) from G to I, mytilid bivalve Modiolus metcalfei (Hanley) from D and F, the nereidid polychaete Nectoneanthes sp. from G to I, and the spionid Prionospio membranacea from B and C. Half of these organisms were preserved in 80% solution of 99% synthetic ethanol (Seiko Co., Ltd.), and the rest were frozen at −30°C until analysis as controls. As most...
animals were collected from several stations, the sample sizes from each station were equalized between fixed and control treatments for each species.

Sample preparation was performed similarly for fixed and raw samples. For *Prionospio membranacea* samples, about 20 individuals were combined into one sample depending on size. For the *Nectoneanthes* sp., individuals were cut into two, and one was used as a fresh sample and the other was used as a sample for fixing. For the arcid bivalves (i.e., *S. kagoshimensis* and *T. granossa*), the pedal muscle was used for measurement. For *A. lischkeana*, the adductor muscle was used for measurement. Samples were dried at 60°C in an oven for 24 h and then powdered. The powder was immersed in 1.2 N HCl solution for 10 min. This duration was long enough to acidify the carbonate because no CO₂ gas was produced following a second immersion in HCl. After rinsing with distilled water, we dried the samples again. The benthos samples were then immersed in a mixture of methanol and chloroform (1:2) to remove the lipid components. All samples were oven-dried and placed in tin capsules, and the carbon and nitrogen contents and isotope ratios of the samples were analyzed using a mass spectrometer (ANCA-GSL, Sercon Inc.). Stable isotope values were denoted as δ, a measure of the amount of the heavier isotope in a sample relative to known standards, which is calculated as

\[ \delta^{13}C = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1000 \%, \]

\[ \delta^{15}N = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1000 \%, \]

where R indicates \(^{13}C/^{12}C\) or \(^{15}N/^{14}N\), respectively. The standards for C and N are Pee Dee Belemnite and atmospheric nitrogen, respectively.

The effects of fixation varied depending on the species (Table 2). No significant effect of fixation was found in both the carbon and nitrogen signatures for the arcid bivalves *S. kagoshimensis* (ANOVA, both \(F_{1,22}<0.85\), both \(p>0.37\)) and *T. granossa* (both \(F_{1,5}<1.63\), both \(p>0.25\)). In *S. kagoshimensis*, only the carbon content of the fixed samples was significantly lower than the frozen samples (\(F_{1,22}=8.91, p=0.007\)), though no difference was found in either nitrogen or carbon contents in *T. granossa* (both \(F_{1,5}<1.01\), both \(p>0.35\)). For the nereid polychaete *Nectoneanthes* sp., fixation showed a significant enrichment of carbon signatures by 1.3‰ (\(F_{1,6}=6.37, p=0.045\)) and almost significant enrichment of nitrogen sig-

### Table 1. Sampling stations.

| Area          | Stn. | Latitude  | Longitude |
|---------------|------|-----------|-----------|
| Tidalflat A   | 33°08’01” | 130°10’40” |
| Tidalflat B   | 33°07’53” | 130°10’45” |
| Tidalflat C   | 33°07’37” | 130°10’53” |
| Subtidal D    | 33°07’21” | 130°11’01” |
| Subtidal E    | 33°07’02” | 130°11’14” |
| Subtidal F    | 33°06’48” | 130°11’27” |
| Subtidal G    | 33°06’24” | 130°11’39” |
| Subtidal H    | 33°05’06” | 130°11’55” |
| Subtidal I    | 33°05’32” | 130°12’10” |

### Table 2. Isotope signatures of ethanol fixed (EtOH) and frozen samples. Carbon and nitrogen contents are also presented. Δ indicates the difference between ethanol-fixed and frozen samples. n = sample size, S.D. = standard deviations.

| Species          | Treat       | \(\delta^{13}C\) mean ± S.D. (%) | \(\delta^{15}N\) mean ± S.D. (%) | Carbon content mean ± S.D. (mg g⁻¹) | Nitrogen content mean ± S.D. (mg g⁻¹) | n |
|------------------|-------------|---------------------------------|---------------------------------|-------------------------------------|--------------------------------------|---|
| *Atrina lischkeana* | EtOH        | -21.1 ± 0.4                     | 11.9 ± 0.2                      | 410.7 ± 6.2                         | 117.7 ± 5.8                          | 4 |
|                  | frozen      | -20.8 ± 0.3                     | 12.5 ± 0.1                      | 451.8 ± 24.2                        | 133.3 ± 7.2                          | 4 |
|                  | Δ(EtOH-frozen) | -0.3                             | -0.6 *                        | -41.1 *                             | -15.5 *                              |   |
| *Modiolus metcalffi* | EtOH        | -20.9 ± 0.4                     | 10.6 ± 0.6                      | 432.2 ± 14.8                        | 124.9 ± 7.0                          | 6 |
|                  | frozen      | -21.4 ± 0.5                     | 11.2 ± 0.5                      | 438.5 ± 9.7                         | 123.0 ± 6.3                          | 6 |
|                  | Δ(EtOH-frozen) | 0.5 †                  | -0.6 †                        | -6.3                                | 1.9                                  |   |
| *Nectoneanthes sp.* | EtOH        | -17.0 ± 0.5                     | 13.1 ± 0.1                      | 408.8 ± 74.2                        | 124.9 ± 25.9                         | 4 |
|                  | frozen      | -18.3 ± 1.0                     | 12.3 ± 0.7                      | 336.2 ± 97.2                        | 84.9 ± 36.1                          | 4 |
|                  | Δ(EtOH-frozen) | 1.3 *                             | 0.8 †                        | 72.6                                | 40.0                                 |   |
| *Prionospio membranacea* | EtOH        | -19.2 ± 0.2                     | 9.2 ± 0.2                       | 405.2 ± 20.9                        | 112.9 ± 7.4                          | 4 |
|                  | frozen      | -20.1 ± 0.2                     | 9.5 ± 0.4                       | 332.3 ± 37.8                        | 77.7 ± 10.9                          | 4 |
|                  | Δ(EtOH-frozen) | 0.9 *                             | -0.3                        | 73.0 *                             | 35.2 *                              |   |
| *Scapharca kagosimensis* | EtOH        | -20.1 ± 0.8                     | 11.8 ± 0.5                      | 452.1 ± 8.1                         | 129.5 ± 6.8                          | 12 |
|                  | frozen      | -19.8 ± 0.5                     | 11.8 ± 0.4                      | 463.8 ± 11.0                        | 127.5 ± 3.6                          | 12 |
|                  | Δ(EtOH-frozen) | -0.2                             | 0.0                          | -11.8 *                             | 2.1                                  |   |
| *Tegilarca granossa* | EtOH        | -18.4 ± 0.6                     | 10.0 ± 0.3                      | 451.4 ± 9.4                         | 133.6 ± 7.2                          | 4 |
|                  | frozen      | -18.9 ± 0.5                     | 10.0 ± 0.4                      | 453.7 ± 7.9                         | 129.8 ± 2.2                          | 4 |
|                  | Δ(EtOH-frozen) | 0.5                             | 0.0                           | -2.3                                | 3.8                                  |   |

* Δ(EtOH-frozen) is statistically significant in ANOVA.

† Overall Δ(EtOH-frozen) is statistically significant in MANOVA.

‡ Δ(EtOH-frozen) is marginally significant in ANOVA.
The effect of ethanol fixation on stable isotope signatures in benthic organisms

... by 0.8‰ (F_{1,6}=1.16, p=0.32), but the nitrogen signatures were significantly depleted by fixation (−0.3‰; F_{1,6}=2.2, p=0.18). Moreover, both the carbon and nitrogen contents of the fixed samples were significantly higher than those of the frozen samples (both F_{1,6}=11.42, both p<0.015). In the mytilid bivalve *M. metcalfei*, ANOVA showed no significant changes in either carbon (F_{1,10}=3.45, p=0.09) or nitrogen signatures (F_{1,10}=3.23, p=0.10), but MANOVA showed that the overall shift was significant (Pillai’s trace=0.76, p=0.0078). In the pinnid bivalve *Atrina lischkeana*, the carbon signatures did not change (−0.3‰; F_{1,6}=1.16, p=0.32), but the nitrogen signatures were significantly depleted by fixation (−0.3‰; F_{1,6}=18.9, p=0.005). Both carbon and nitrogen contents of the fixed samples were significantly lower than those of the frozen samples (F_{1,6}=10.78 and p<0.017 for both types of samples).

Ethanol fixation changed both carbon and nitrogen signatures variously, which can affect estimation of food web structures. In nitrogen, the isotope signatures shifted from −0.6 to +0.8‰ due to fixation. According to a review by Kelly et al. (2006), shifts in nitrogen signatures of ethanol-fixed aquatic invertebrates range from −0.4 to +0.8‰, which is consistent with the findings of the present study. These changes by fixation may be relatively small for the general fractionation of the nitrogen isotope in food webs (i.e., enrichment by 3–4‰ with trophic level; Post 2002), but would not be negligible. Shifts in carbon signatures by fixation were more serious than for nitrogen. Carbon signatures were enriched by 0.5–1.3‰ due to fixation. These changes are relatively large for general carbon fractionation (i.e., enrichment by 0–1‰ with trophic level; Post 2002), and can have a greater bias in estimating the quantitative contribution of energy sources in food webs. For example, suppose we want to estimate the relative contribution of phytoplankton and benthic diatoms to target species, and the carbon signatures of phytoplankton and benthic diatoms are −19‰ and −16‰ (e.g., Fry & Sherr 1986), respectively. If the actual contribution of phytoplankton to the target consumer is 30%, the contribution in fixed samples will be overestimated to be from 40% to 60% depending on the degree of enrichment (i.e., 0.3–0.9‰) in the simple source-mixing model of single isotope (e.g. Phillips & Gregg 2001, Schindler & Lubekin 2004). In dual isotope incorporating nitrogen signatures, the error might be larger.

Although it is not the main focus of this study to elucidate the cause of the shift attributed to fixation, most of the cases can be explained either by selective loss or uptake of materials during preservation. As ethanol does not contain nitrogen, the changes in nitrogen signatures would be due to leaching by tissue hydrolysis during preservation. However, the direction of the shift in nitrogen signatures and nitrogen content was not consistent. For example, the significant depletion in the nitrogen signature of *A. lischkeana* may be due to hydrolysis of an isotopically heavier protein because the nitrogen content of the fixed samples was significantly lower than that of the frozen samples. In contrast, the nitrogen signature of *P. membranacea* decreased, although the nitrogen content of the fixed samples was significantly higher than that of the frozen samples.

The enrichment in carbon signatures of ethanol-fixed samples can be explained by the leaching of lipid contents during preservation (e.g., Kaehler & Pakhovom 2001, Arrington & Winemiller 2002, Sweeting et al. 2004). In fact, a few of the fresh specimens were viscous and sticky even after oven drying (48 h), while there were no such specimens in the fixed samples (Yoshino, personal observation). As lipids are isotopically lighter than proteins (DeNiro & Epstein 1977), lipid extraction enriches carbon signature. In the Arctic char *Salvelinus alpinus*, ethanol fixation enriched the carbon signature by 0.78‰ when no lipid extraction processing was applied, but by 0.2% in lipid extracted samples. In this study, however, we extracted lipid contents in both fixed and raw samples. Nevertheless, some organisms showed much higher enrichment than 0.2%. In such cases, the uptake of ethanol-derived carbon may also occur. According to Kelly et al. (2005), the ethanol carbon signature was −10.8‰. If this is the average carbon signature of ethanol, the uptake will make sample carbon signatures more positive. The two polychaete species may be manifesting this case, since carbon contents are higher in the fixed samples than in the frozen samples.

However, we should note that both polychaete species show considerably lower carbon contents than is general in marine invertebrates (i.e. 40 to 50%). Moreover, the content of fixed samples is higher than that in frozen samples by more than 20% in carbon, and in nitrogen 50% of their own contents. For this improbable situation, we speculate the effect of organically low materials in the gut (i.e. feces) not removed in this study. Dilution of sample organic contents by feces can explain well the low organic contents in frozen polychaetes. Furthermore, if fixation shock induced animals to excrete feces, organic contents will increase in fixed samples. The present changes of isotope signatures by fixation in polychaetes may also be overestimated as a result.

It also should be noted that the carbon signatures of the fixed samples of *A. lischkeana* and *S. kagoshimensis* decreased, although the depletion of their signatures was not significant. However, especially in *A. lischkeana*, this nonsignificance could be due to the small sample size. The carbon contents of fixed samples of both species were significantly lower compared with those of the frozen samples. These results are counterintuitive because both lipid extraction and uptake of ethanol-derived carbon should enrich carbon signatures as described above. Although the mechanism for the depletion is unclear at present, a similar case is found in *Drosophila melanogaster*, in which ethanol preservation significantly depleted carbon signatures by more than 1% (Ponsard & Amlou 1999). Quail muscle was also depleted by 0.44%, although this was not statistically significant (Hobson et al. 1997). However, we are not aware of any findings concerning the depletion of carbon signatures due to ethanol fixation in aquatic animals.
More investigation is needed to precisely evaluate the depletion in carbon signatures.

In conclusion, ethanol fixation changes both carbon and nitrogen signatures variously depending on the species in marine benthos, and the direction of shifts in the signatures may also be inconsistent. Ethanol fixation therefore may not necessarily be a smart choice for stable isotope analyses because of the inconsistency of shifts in isotope signatures. However, if samples were processed appropriately (e.g. removing gut contents) and signature shifts were precisely evaluated for each species, ethanol-fixed samples can be practically useful for stable isotope analyses by correcting the shifts for each species. Such a retrospective approach has been adopted for estimating ecosystem changes using archived samples fixed by other preservatives (e.g. Vanderzanden et al. 2003, Gorokhova 2005). Ethanol-fixed samples should be useful for the same purpose.

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