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ORIGINAL ARTICLE

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Preservation effects on wet weight, dry weight, and ash-free dry weight biomass estimates of four common estuarine macro-invertebrates: no difference between ethanol and formalin

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Abstract Two preservative treatments traditionally used in aquatic sciences, formalin (4%) and ethanol (70%), were compared for their effects on biomass estimations. The effects of both preservatives on wet weight, dry weight, and ash-free dry weight were determined for samples preserved for 10, 21, and 90 days. The effects were studied in four different macrofauna species commonly found in German estuaries: *Heteromastus filiformis* (Capitellidae, Polychaeta), *Hediste diversicolor* (Nereididae, Polychaeta), *Corophium* sp. (Amphipoda, Crustaceae), and *Gammarus* spp. (Amphipoda, Crustacea). The biomass estimates of preserved samples were compared with those of unpreserved samples. In all four species the loss in wet weight, dry weight, and ash-free dry weight was most pronounced within the first 10 days, and an additional weight loss was recorded between days 10 and 21. However, there was no further loss in weight for samples kept for as long as 90 days in the preservatives. In general, crustaceans exhibited higher weight loss than polychaetes, and smaller species (*H. filiformis* and *Corophium* sp.) showed higher weight loss and a higher variability than larger species. As our main result, significant differences between the two preservative treatments did never occur. Our results contradict some earlier investigations on this matter where formalin has been reported to be superior to alcoholic preservatives because weight loss was less pronounced than in ethanol. Factors affecting biomass estimates are discussed and we conclude that, for the macrofauna groups tested, the use of the toxic formalin solution is not justified when the major intent is biomass estimation.

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Introduction

In aquatic studies biomass estimates are commonly performed on preserved material because extensive sampling often requires preservation in the field before determination and analysis can be carried out. In marine studies the most common preservative for macrofauna is formalin (4% final concentration), while in limnology the use of ethanol (70–80% final concentration) prevails. Formaldehyde is a colorless gas highly soluble in water. It is commercially sold as saturated aqueous solution with concentration of about 37%, conventionally called 100% formalin. Working solutions are normally buffered with sodium tetraborate (Borax), hexamethylene tetramine (e.g. Ruhmor 1990), or phosphate in order to prevent the formation of formic acid, which can substantially degrade the tissue quality.

The health hazard involved with the use of formalin has been covered extensively (e.g. Black and Dodson 2003) and has been recognized by most scientists today. In brief, as a rule of thumb, the slightest odor of formalin in the air indicates concentrations as small as 0.6 ppm (AIHA 1989), which can already cause acute exposure effects such as irritation of eyes, nose, and throat in some individuals. Higher concentrations, in the range of 3–5 ppm, will cause eyes to water (an experience most biologists have made at least once during their career), while concentrations above approximately 25 ppm can cause severe injuries of the respiratory tract. Moreover, short-term contact with formalin solutions can result in severe skin irritation and the individual can be sensitized to formalin resulting in a allergic reaction during future contacts. In addition, the carcinogenic nature of formaldehyde has to be taken into account. It is a risk, which might

even exist when individuals are exposed to formaldehyde concentrations below the 0.6 ppm olfactorial threshold. As a consequence, the use of formalin should be reduced as much as possible.

Despite the considerable human health concerns involved with its use, many marine biologists continue to use formalin, because it has become a standard procedure for preserving fauna samples (e.g. Ruhmor 1990) and is recommended by many official sources such as the Baltic Marine Environment Protection Commission (Helsinki Commission, HELCOM) (e.g. HELCOM 1988), the International Council for the Exploration of the Sea (ICES 1994; ICES 1996), and the Convention for the Protection of the Marine Environment of the North-East Atlantic (OSPAR Convention) (OSPARCOM 1997). In some cases formalin is a reasonable fixative especially when animals are collected for museum collections (one aspect of fixation with formalin is that it stiffens the body while ethanol often results in rather squashy specimens). The price difference between the two preservatives also plays a considerable role. In Germany, for example, some laboratory retailers charge almost twice as much for ethanol (96%, industrial grade) as for the same amount of formaldehyde solution (>35%). In countries where alcohol is even more prohibitively priced this difference can be even higher. However, a different perspective might be achieved, if the additional costs for the increasing safety requirements (protective close, respirators, fume hoods, etc.) and if differences in hazardous waste fees are taken into account.

In most studies samples are collected in order to gain information about species compositions and abundance, a task similarly achievable when formalin is replaced by ethanol. However, some previous studies have reported that for the assessment of biomass, an important parameter describing standing crop in benthic communities, ethanol is not recommended because weight loss is higher than in formalin preserved specimens (Howmiller 1972; Dermott and Paterson 1973; Landahl and Nagell 1978; Leuwen et al. 1985), while other studies could not confirm this observation (Mason et al. 1983; Gaston et al. 1996). Moreover, in some cases ethanol-fixed specimens continued to lose weight over time (e.g. Dermott and Paterson 1973) – a fact which would make biomass estimates unreliable, and most likely resulted in the recommendation of formalin as the fixative of choice for marine benthos.

In this study, we (1) established conversion factors for wet weight (WW), dry weight (DW), and ash-free dry weight (AFDW) for four common macrofauna species (*Heteromastus filiformis*, *Hediste diversicolor*, *Corophium* sp., and *Gammarus* spp.), and (2) tested if there are significant differences between biomass estimates when samples are preserved in either 4% formalin or 70% ethanol. In addition the weight loss is described over a time period of 90 days.

Material and methods

Measurements were made on material collected in the Weser estuary, Germany. This estuary is located at the southern coast of the North Sea and discharges the river Weser (watershed area 46,306 km^2) into the Wadden Sea. The estuary mouth is located close to the city of Bremerhaven and is characterized by diurnal tides (mean tidal range at the Bremerhaven tidal gage is about 3.8 m) and mean water temperatures range from approximately 5°C in the winter time to 20°C in summer, while salinity can fluctuate between about 8 to 15‰ depending on season, river run-off, and tidal cycle.

For the present investigation four species from the two most abundant macrofauna taxa, polychaetes, and crustaceans were chosen. This species selection was guided by their relative abundances in the estuary and the desire to compare larger and smaller species from the same taxa in order to cover possible differences in conservation effects on biomass estimates between larger and smaller animals. *Heteromastus filiformis* (Claparede 1864) was chosen as a smaller polychaete (length up to 100 mm), while *Hediste diversicolor* (O.F. Müller 1776) was selected as a representative of a larger polychaete (length up to 200 mm). In crustaceans we chose *Corophium* sp. Latreille, 1806 as a small (length up to 10 mm) and *Gammarus* spp. Fabricius, 1775 (*Gammarus salinus* Spooner, 1947 and *G. zaddachi* Sexton, 1912) as a larger species (length up to 20 mm). Most of these species are inhabitants of eulitoral muddy sediments and were collected at low tide on a tidal flat close to the city limit of Bremerhaven (53°33.63′N, 8°31.34′E, WGS 84, Mercator projection, ellipsoid). Only the gammarids, were collected from artificial hard substrate periphyton (surface marker buoy No. 33 Fedderwarder Ost, 53°41.72′N, 8°20.06′E, WGS 84, Mercator projection, ellipsoid). Samples were collected and sorted directly at the sites, then flushed with water to remove any adhering sediment or detritus. Living animals were brought to the laboratory, were they were divided into replicate subsamples prior to fresh weight (WW of unpreserved samples, FW) estimation (see below). Due to the large differences in weights between the different taxa and sizes, cohorts of several individuals in each replicate where used, each made up of 10 (*H. filiformis*), 5 (*H. diversicolor*), 100 (*Corophium* sp.), and 10 individuals (*Gammarus* spp.). This procedure helped to increase precision of weight estimates. Altogether, 84 subsamples (21 per macrofauna species) were prepared and randomly assigned to one of the treatments (7 per species, see below). However, 10 days treatments in *H. diversicolor* were excluded from the analysis due to extreme variabilities in weight estimates.

In one treatment of each group, weight estimates (FW, DW, and AFDW) were measured using unpreserved samples. For the remaining six treatments the FW was estimated by blotting the live animals on tissue

Table 1 DW and AFDW in percent of the initial FW of unpreserved samples for four different macrofauna groups (means ± SD)

	DW (% of FW)		AFDW (% of FW)		AFDW (% of DW)	
H. filiformis	17.3	±0.5	9.2	±0.2	53.4	±0.7
H. diversicolor	13.8	±2.9	10.6	±0.5	78.0	±11.5
Corophium sp.	14.3	±0.5	9.8	±0.5	68.6	±2.2
Gammarus spp.	26.1	±0.2	18.0	±0.3	69.1	±0.7

paper for 1 min (cf. Dermott and Paterson 1973) prior to weighing to the nearest ±0.1 mg. Then the samples were preserved either in borax buffered formalin (4%) or ethanol (70%), were they were kept for 10, 21, or 90 days at about 15°C in the dark. After that, samples were blotted for 1 min on tissue paper and the WW was estimated to the nearest ±0.1 mg. Next the DW of the samples was measured. Samples were placed for 24 h in an oven at 100°C, cooled down to room temperature in a desiccator (3–4 h), and then weighed. To determine the ash content of the samples, these were placed into a muffle furnace at 550°C for 1 h, cooled down to room temperature (6 h) in a desiccator, and then weighed. The AFDW was calculated by subtracting the ash-weight from the DW.

The WW was calculated as percentages of the initial FW, while DW and AFDW are given as percentages of the respective weights of unpreserved samples. In order to identify significant differences between treatments we calculated the 95% confidence intervals (CI) using Tukey's honestly significant difference method (Tukey's HSD or T-method, see Sokal and Rohlf 1998). Non-overlap in 95% CI corresponds to significant differences at $\alpha = 0.05$ between samples. Statistical power (type II error probability, 1-β) was calculated using algorithms for balanced analysis of variance tests (see Crawley 2002). High power values (i.e. >80%, also commonly expressed as 0.8) indicate high probabilities to find significant differences when they exist. All calculations and figure compilations were performed using the freely available R computer language, version 1.7 (Ihaka and Gentleman 1996, for information and downloads see also http://www.r-project.org).

Results

The DW (in percent of the initial FW, Table 1) of unpreserved samples were species-specific and ranged from 13.8% in the large polycheate, *H. diversicolor*, to

Table 2 Means of WW, DW, and AFDW (in percent of the corresponding weight estimates from unpreserved samples ±95% CI; DW and AFDW are also given in percent of WW ±95% CI) in four macrofauna groups preserved in formalin (4%) or ethanol (70%) for 10, 21, and 90 days, respectively

	Ethanol (70 %)			Formalin (4 %)			95% CI	
	10 days	21 days	90 days	10 days	21 days	90 days		
WW (% of WW from unpreserved samples = FW)								
H. filiformis	81.7	67.5	70.7	75.0	62.2	63.3	±	10.0
H. diversicolor	—	87.2	86.1	—	95.0	86.9	±	6.6
Corophium sp.	69.7	66.7	63.2	71.0	65.5	64.4	±	6.2
Gammarus spp.	79.0	76.3	73.3	75.6	69.4	71.9	±	4.7
DW (% of DW from unpreserved samples)								
H. filiformis	76.6	56.3	63.8	69.2	55.1	53.2	±	17.5
H. diversicolor	—	81.3	82.3	—	88.7	83.8	±	14.3
Corophium sp.	69.2	64.6	62.1	70.5	60.0	60.3	±	7.5
Gammarus spp.	78.9	76.6	72.7	74.8	68.8	67.9	±	5.4
DW (% of WW)								
H. filiformis	13.2	9.7	11.0	12.0	9.5	9.2	±	3.0
H. diversicolor	—	11.2	11.4	—	12.3	11.6	±	2.1
Corophium sp.	9.9	9.2	8.9	10.1	8.5	8.6	±	1.1
Gammarus spp.	20.6	20.0	19.9	19.5	17.9	17.7	±	1.4
AFDW (% of AFDW from unpreserved samples)								
H. filiformis	72.7	61.8	57.3	74.9	64.8	56.7	±	14.8
H. diversicolor	—	70.4	65.1	—	79.7	67.1	±	7.6
Corophium sp.	61.9	54.6	52.6	71.5	64.3	65.0	±	7.6
Gammarus spp.	73.4	70.8	66.6	76.2	71.3	71.1	±	5.7
AFDW (% of WW)								
H. filiformis	6.7	5.7	5.3	6.9	6.0	5.2	±	1.4
H. diversicolor	—	7.4	6.9	—	8.4	7.1	±	0.8
Corophium sp.	6.1	5.4	5.2	7.0	6.3	6.4	±	0.8
Gammarus spp.	13.2	12.7	12.0	13.7	12.8	12.8	±	1.0

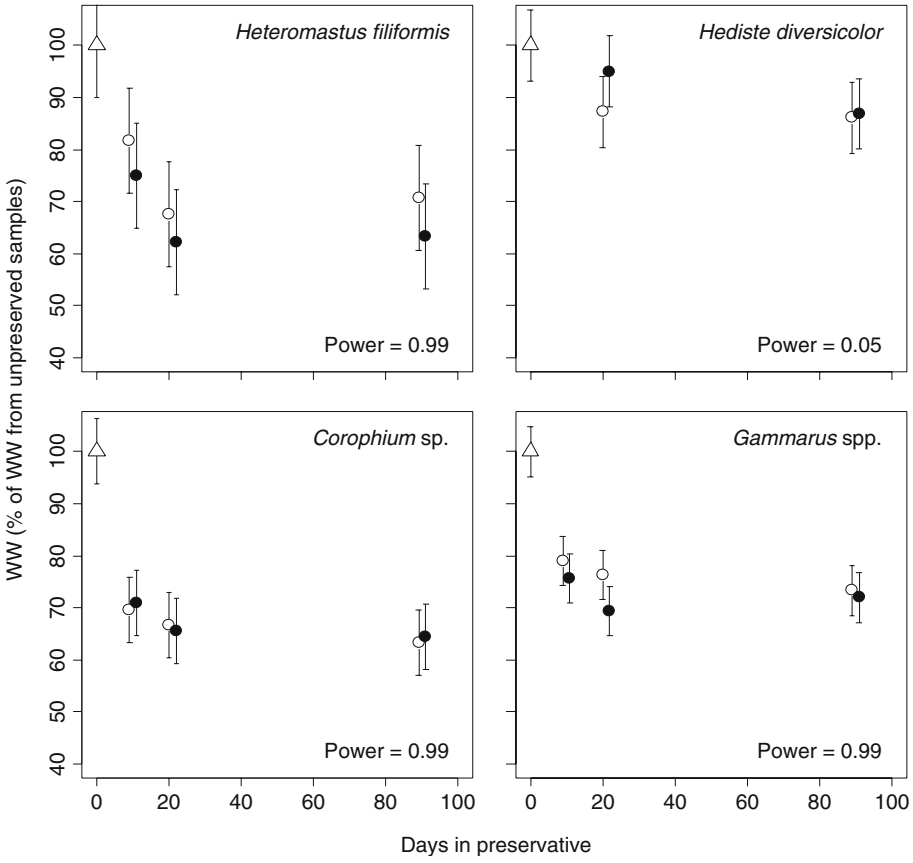

Fig. 1 The WW of samples preserved in 70% ethanol (*open circles*) and 4% formalin (*filled circles*) for 10, 21, or 90 days in percent of WW of unpreserved samples in four macrofauna species. WW (FW) of unpreserved samples (day 0, *open triangles*). *Error bars* represent 95% CI. A small offset (±1 day) has been applied between treatments with ethanol and formalin to avoid graphic overlap

26.1% in the larger crustacean, *Gammarus* spp. The variability (standard deviation, SD) was low (ranging from 0.2 to 0.5) except for *H. diversicolor* (SD ±2.9). The AFDW for unpreserved samples (Table 1) is given, for better comparison, both as percentages of FW and of DW values (both types of information can be found in the literature). The AFDW values of unpreserved specimens ranged from 9.2% (*H. filiformis*) to 18.0 in persent of FW (*Gammarus* spp.) and SD ranged from 0.2 to 0.5%.

Preservation with formalin or ethanol resulted in a pronounced loss of WW (Table 2, Fig. 1). Weight loss was largely restricted to the first 21 days of preservation. Thereafter, however, no significant weight loss could be observed. Weight loss ranged from 13.1% (*H. diversicolor*) to 36.7% (*H. filiformis*) in samples kept for 90 days in formalin. In general, crustaceans exhibited higher weight loss than polychaetes, and small specimens showed higher weight loss and a higher variability than large ones. Only small and non-significant differences could be observed between the two preservatives, and power analysis (values in Fig. 1) revealed sufficiently high power (>0.8) values for all four macrofauna groups tested except for *H. diversicolor*, where power (0.05) was low.

The results for DW in percentages of the DW from unpreserved samples is shown in Fig. 2 and Table 2. In general, DW values corresponded well to WW results. Similar to DW estimates from unpreserved samples, a pronounced drop in DW was detectable within the first 21 days in all four macrofauna species, while no significant changes in DW occurred thereafter. Weight loss ranged from 16.2% (*H. diversicolor*) to 46.8% (*H. filiformis*) in samples kept for 90 days in formalin. Again, crustaceans exhibited higher weight loss than polychaetes, and smaller species showed higher weight loss and a higher variability than larger ones. Only small and non-significant differences could be observed between the two preservatives. Again, power analysis revealed sufficient power (>0.8) for all tests except for *H. diversicolor* (power = 0.60).

The AFDW estimates (Fig. 3, Table 2) basically reflected the results found for WW and DW (higher weight loss in crustaceans and in small species, largest weight loss within the first 21 days, thereafter no further significant weight loss, no significant difference between preservatives). Power analysis indicated sufficient (>0.8) power for all groups except for the smaller crustacean *Corophium* sp. where power was low (0.06). However, weigh loss was highest in the smaller crustacean *Corophium* sp. (47.4%) kept in ethanol, while the lowest

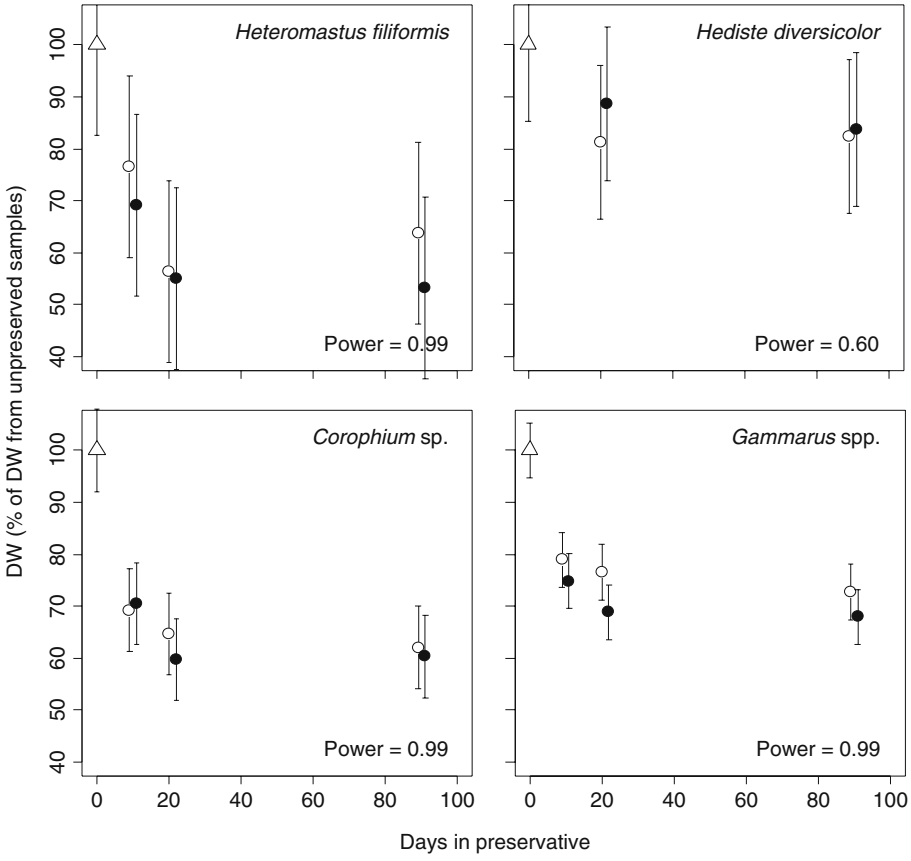

Fig. 2 The DW of samples preserved in 70% ethanol (*open circles*) and 4% formalin (*filled circles*) for 10, 21, or 90 days in percent of DW of unpreserved samples in four macrofauna species. The DW of unpreserved samples (day 0, *open triangles*). *Error bars* represent 95% CI. A small offset (±1 day) has been applied between treatments with ethanol and formalin to avoid graphic overlap

weight loss was again recorded in the larger polychaete *H. diversicolor* (32.9%) preserved with formalin.

Discussion

In this study, we established conversion factors for biomass estimates for four common estuarine macrobenthos species preserved either in formalin (4% final concentration) or ethanol (70% final concentration) and demonstrated that the choice of the preservative is irrelevant for biomass estimation. In other words, ethanol preserved specimen did not lose more weight than specimens preserved with formalin. Nevertheless, both preservation methods resulted in a weight loss, which was most pronounced within the first 21 days. After 21 days, however, weight had stabilized and only small differences have been observed between samples kept for 21 and 90 days in fixative solutions. With our species selection we covered two common macrobenthos taxa, polychaetes, and crustaceans, both with a small and a large species. Smaller species showed greater weight loss (compared to unpreserved specimens) and exhibited higher variabilities than large ones. Though we cannot be sure that our conversion factors are entirely applicable to other related species within the same size category, they probably will not deviate too much.

In general, our data of biomass estimates of unpreserved samples are well in range compared with available data from the literature. A comprehensive compilation of some biomass conversion factors including those from unpublished sources and gray zone literature can be found for example in Rumohr et al. (1987). Therein, DW values (in persent of WW values) are given ranging from 12.9 to 29.9% (for *Corophium*) and from 15.4 to 26.4% (for *Gammarus*). Our results (14.3% for *Corophium* and 26.1% for *Gammarus*) are well placed within these ranges.

Previous studies on organisms collected from fresh water habitats (mainly chironomids) have reported higher weight losses of WW, DW or AFDW (e.g. Howmiller 1972; Dermott and Paterson 1973; Landahl and Nagell 1978; Leuwen et al. 1985) as well as higher losses of carbon content (Salonen and Sarvala 1985) in samples fixed with ethanol in comparison to formalin fixed specimens. Nevertheless, in some cases mass gain has been reported for ethanol fixed samples (e.g. Wiederholm and Eriksson 1977). However, our results for common marine/brackish macrofauna species do not support these findings. On the contrary, in most cases we found lower masses, though not significant, in samples preserved with formalin. Similar findings have been

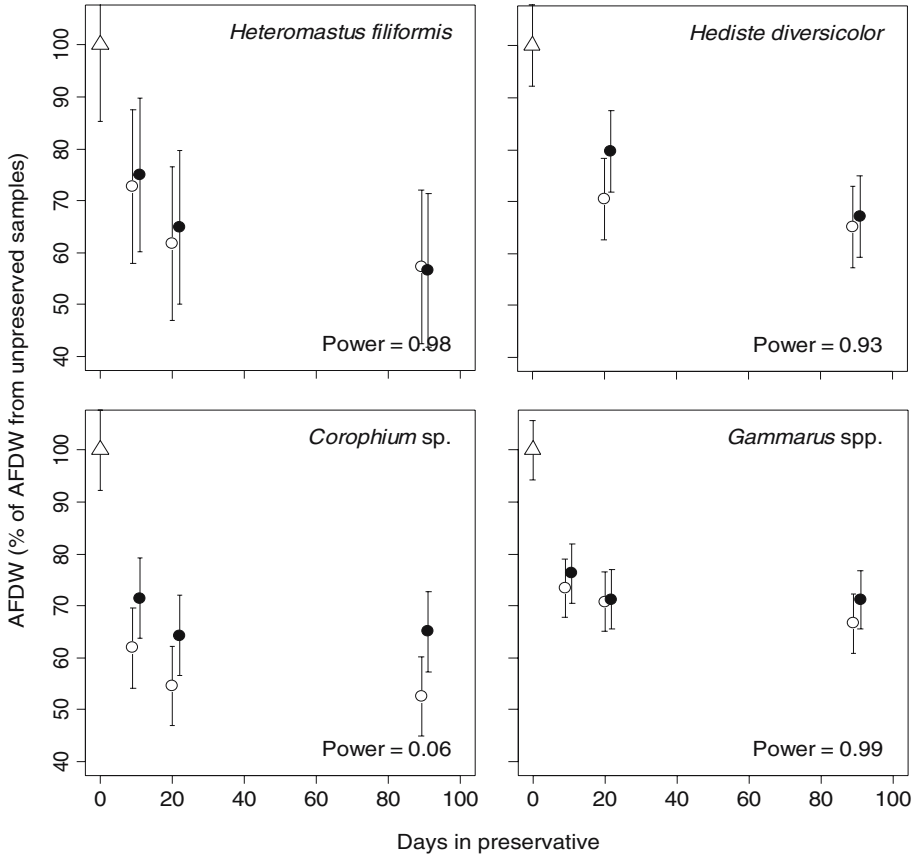

Fig. 3 The AFDW of samples preserved in 70% ethanol (*open circles*) and 4% formalin (*filled circles*) for 10, 21, or 90 days in percent of AFDW of unpreserved samples in four macrofauna species. The AFDW of unpreserved samples (day 0, *open triangles*). *Error bars* represent 95% CI. A small offset (±1 day) has been applied between treatments with ethanol and formalin to avoid graphic overlap

reported by Mason et al. (1983) and Gaston et al. (1996) for marine macroinvertebrates. One reason for these differing findings might be that insect larvae contain higher amounts of lipids in preparation for metamorphosis (e.g. Cavaletto et al. 2003), a substance easily soluble in alcoholic solvents. Marine invertebrates, in contrast, accumulate much less proteins and rarely have vast stores of lipids, except when gravid (Gaston et al. 1996). Moreover, our own observations on fixation practice with ethanol in freshwater species showed that fixation with highly concentrated ethanol preservatives (70–90%) can often result in the fast increases in body volume of chironomides causing cracks at the abdomen or head capsule with the consequence of losses in body fluids. It is evident that this will distort biomass estimates.

Sample handling is a great source of error for biomass estimation. For example: the removal of externally adhering fluids prior to weight estimation is a necessary step in WW estimation and different methods have been applied with different levels of success. Centrifugation of specimens (e.g. Howmiller 1972), for example, is less likely to produce reliable results because this most likely results in the loss of fluid from body tissues of preserved specimens (Wiederholm and Eriksson 1977) which, especially in cases of repetitive WW estimation, easily results in assumed weigh losses; intensity and duration of centrifugation most likely influence the result further (Mason et al. 1983). Alternatively, blotting samples with filter paper for defined time periods has been shown to be the most appropriate procedure although, the time period samples are blotted can significantly influence weight estimates (Dermott and Paterson 1973): one min of blotting has been shown to be most efficient and least error-prone. However, blotting followed by "air drying" for undefined time periods (Howmiller 1972; Hamilton and Kingston 1985) are not recommended, especially when comparisons of preservatives with different volatilities are intended (e.g. Hamilton and Kingston 1985, alcohol versus formalin). About 5–10 min of air exposure can induce WW losses of up to 25% (Jonasson 1972). Likewise, drying temperature is known to influence biomass estimation considerably. Higher weight losses have been reported when drying temperature in preparation of DW estimation exceeded 160°C, while temperatures below 500°C have proven to result in inhomogeneous AFDW estimates (Mason et al. 1983). Similarly important seem to be storage conditions. Leuwen et al. (1985) reported significant lower DW and AFDW looses for the gastropod *Radix peregra* when samples were stored in the dark.

Specimen condition, which can be substantially impaired by sample treatment (excessive sieving), also influences biomass estimates. Damaged specimens with punctured cuticles, or even lacking appendages and other body parts can lose indefinable amounts of body fluids during preservation, which introduces an immeasurable error into biomass estimation (Gaston et al. 1996). Especially, fragile taxa without protective exoskeletons, such as annelids, are more likely to suffer from such treatment than others. Loss of body fluid can only be partially compensated for when the entire preservative is included in DW and AFDW estimation. Dermott and Paterson (1973) found significantly higher DW estimates when the entire preservative solution was allowed to dry with the samples. Likewise, filling state of the animals alimentary tract can account for some extra weight. In chironomids, for examples, detritus and sand grains can amount for 4% of the measured DW (Landahl and Nagell 1978), a factor relevant especially when deposit feeding specimens are investigated. Moreover, gut content material such as sand grains affect estimates of DW and AFDW.

Considering all potential error sources mentioned, the effect of different preservatives seems to be negligible. In our opinion, biomass estimation is mainly affected by the sampling procedure itself, and by the sample handling rather than by the choice of the preservative. If sample treatment and biomass estimation are carried out with reasonable care, convenient conversion factors for preserved samples may be established for probably any species. When the same methods of preservation and sample handling are adopted as in the present paper, the conversion factors reported herein may be used. However, caution is advised for different species. Although our factors may be applicable for related species as well (same taxa and same size class), this has not been tested yet.

The use of ethanol as a preservative has, in addition to the lower health risk for operator/laboratory personal, also some additional advantages, e.g. if molecular determination of specimens using DNA samples is desired. Though, DNA amplification by polymerase chain reaction techniques (PCR) can be carried out using formalin fixed samples (e.g. Greer et al. 1991), this is difficult and error-prone (Quach et al. 2004). Besides, spills, which can occur in the heat of sampling, can be taken with much more calmness because the fixative is less hazardous to man and nature. However, we do not want to leave unmentioned that ethanol use is not without inconvenience. First, for the same amount of samples much more fixative solution is required using ethanol (96%) to obtain the final (70%) concentration, especially when the samples contain high portions of water. Using a formalin stock solution (e.g. 36%) only small amounts need to be added to samples to achieve the desired 4% final concentration. Second, the high vapor pressure of ethanol can make regular refilling of sample vials necessary (Black and Dodson 2003); and third, and probably most important, ethanol preserved specimen tend to be rather soft and mushy compared to formalin-fixed samples so that they are not usable for museum collections and care has to be taken not to harm fragile body parts or to puncture the cuticle during post fixative sample handling.

Conclusions

In this study, we have presented compelling evidence that the choice of the preservative has little effect on biomass loss. Both preservatives result in pronounced losses of biomass estimates, which however, is largely restricted to the first 3 weeks. Several other factors (e.g. sampling and sample handling) affect the precision of biomass estimation probably much more than the choice of the preservative. We conclude that the use of the toxic formalin solution should be abandoned, or at least limited to the few useful applications (e.g. museum collections and meiofauna fixation). The considerable advantage is clear: lower health risks for scientists and laboratory personnel. Furthermore, our biomass conversion factors provide other researchers with a technique to calculate the pre-fixative biomass in four estuarine macroinvertebrates from preserved specimens. These factors may even be applied to other related species within the same size category, but further research is welcome to test this hypothesis and to obtain values for other benthic species. Especially values from other common marine and estuarine invertebrates would be an excellent completion.

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