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Ethanol: A Better Preservation Technique For Daphnia

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Ethanol: a better preservation technique for *Daphnia*

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Abstract

A 4% formalin-40% sucrose solution has been used by limnologists for three decades as the preferred freshwater zooplankton preservative because it kills and fixes cladocera (Branchiopoda) with relatively little distortion. Because of the increasing evidence of health hazards related to formalin, we sought an alternative, safer preservative that satisfies the need for low distortion. Our results suggest the ethanol preservative methods (70% and 95% treatments) are as good or better as using 4% sugar formalin to fix and store samples. Our results indicate the best method is to fix samples in 95% EtOH followed by storage in 70% EtOH. This technique gave us the least frequent distortion, the highest average number of eggs per female, and the fewest embryos lost from the brood chamber. None of the techniques appeared to have positive or negative effects on body length. Using hot water to fix animals before storage is not recommended.

Plankton ecologists often kill and fix zooplankton specimens for future study. The standard protocol used by limnologists is a sucrose-formalin solution first recommended by Haney and Hall (1973). This solution is an aqueous solution of 4% formalin (final concentration) and 40 g of sucrose per liter. Commercially available formalin is a 37% by weight, or a 40% by volume, aqueous solution. Thus, a 4% formalin solution is a 1.5% formaldehyde solution.

The 4% formalin solution was recommended because it reduced morphological distortion, even of soft animals like *Daphnia*, while taking advantage of fixation and preservation properties of formalin. Minimum distortion is particularly desirable in studies of size-selective predation or reproductive rates, in which eggs need to stay in the brood chamber of branchiopods. Whereas there are advantages of formalin fixation, there also exists significant human health concerns for formaldehyde exposure.

Acute effects of exposure—The effects of greatest interest to limnologists are caused by inhalation and contact. The greatest acute exposure occurs during analysis of samples when the limnologist looks at plankton samples through a microscope, often for hours at a time. Labs are often poorly ventilated, leading to increased exposure through inhalation of formaldehyde vapors. This inhalation is highly irritating to the upper respiratory tract and eyes.

According to the United States Occupational Safety and Health Administration (OSHA 2001),

> concentrations of 0.5 to 2.0 ppm (in air) may irritate the eyes, nose, and throat of some individuals. Concentrations of 3 to 5 ppm also cause tearing of the eyes and are intolerable to some persons. Concentrations of 10 to 20 ppm cause difficulty in breathing, burning of the nose and throat, cough, and heavy tearing of the eyes, and 25 to 30 ppm causes severe respiratory tract injury leading to pulmonary edema and pneumonitis. A concentration of 100 ppm is immediately dangerous to life and health. Deaths from accidental exposure to high concentrations of formaldehyde have been reported.

The OSHA statement concerning short-term contact is that "formalin is a severe skin irritant and a sensitizer. Contact with formalin causes white discoloration, smarting, drying, cracking, and scaling. Prolonged and repeated contact can cause numbness and a hardening or tanning of the skin. Previously exposed persons may react to future exposure with an allergic eczematous dermatitis or hives."

Acute exposure and hypersensitivity—Some individuals have an allergic-like response to formaldehyde. In our experience, a few students have developed a red rash or difficulty breathing immediately after exposure to low levels of formaldehyde. In one case, a student developed hives <1 min after a dropper bottle of formalin solution was opened in a large and well-ventilated room. Another student experienced extreme tachycardia when exposed to imperceptible fumes. A colleague that

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is allergic to formalin experiences bronchial constriction, is often incapacitated (on the floor barely able to crawl), and has even passed out.

Chronic effects of exposure—Long-term effects of formaldehyde exposure include cancer and mutation (OSHA 2001). Chronic exposure is experienced when formalin-preserved samples are stored in offices or laboratories. The fumes are often undetectable, but the samples are observed to lose formalin over a period of years. Screw-top jars often unscrew themselves. Wooden cabinets once used to store formalin samples retain the odor for decades after the samples are removed.

Protective measures—United States OSHA policy requires that employees handling formaldehyde solutions of 1% or more must wear "material impervious to formaldehyde" (OSHA 2001). Such material includes gloves, aprons, full facepiece negative pressure respirators equipped with replaceable filters, and goggles. If exposure levels are more than the 0.5 ppm time-weighted 8-h action level in the air, OSHA (2001) policy requires that interior space be ventilated to reduce the formaldehyde concentration.

Exposure indicators—OSHA policy requires that an employee exposed to more than the 0.5 ppm action level or the 2 ppm Short-Term Exposure Limit (averaged over 15 min) must be monitored for exposure by the employer. Anyone working with formaldehyde in an enclosed space is encouraged to contact the appropriate safety officer or OSHA representative to have the working space monitored. This is especially true if any odor or irritation is experienced. In general, it is not reliable to use odor to assess whether there is a dangerous level of exposure. This is because the perception of formaldehyde by odor or irritation is reduced by chronic exposure. For this reason, safety officers recommend monitoring whenever formaldehyde is being used. Nevertheless, a rule of thumb is that the slightest odor of formaldehyde is probably in the range of the 0.5 ppm action level. According to the American Industrial Hygiene Association (AIHA), the odor of formaldehyde is detectable at concentrations as low as 0.6 ppm (AIHA 1989), which is slightly above the 0.5 ppm action level.

The 4% formalin formula has been adopted by limnologists throughout the world. Although it is unpleasant to use because of its noxious odor and stickiness, it does an acceptable job of fixing and preserving zooplankton. The problem is that formaldehyde presents a number of human health issues. These issues were not appreciated prior to 1973. However, research over the last several decades has revealed a plethora of acute and chronic effects of formaldehyde exposure. Despite the generally recognized health hazards of formaldehyde, limnologists and freshwater fisheries biologists continue to use it as the preferred preservative because it is has become a standard method. Our goal was to find an alternative preservative with a lower health hazard and equal or better preservation characteristics.

Materials and procedures

A plankton sample was removed from the sampling vessel (plankton net or plankton trap) and collected on a small sieve with a mesh size adequate to retain the smallest of the zooplankton. We tapped the bottom of the sieve to remove as much water as possible. The sieve was then set into a beaker containing 95% ethanol for 15 s. The depth of the 95% ethanol should not exceed half of the height of the sieve. Following the 15-s fixing of the sample, the sieve was removed from the 95% ethanol, and the contents of the sieve were washed into a sample bottle with 70% ethanol for preservation. The volume of the animals in the sample should not exceed 30% of the volume (animals plus preservative) in the sample bottle.

Assessment

We compared morphology of groups of *Daphnia* killed with formaldehyde, heat, and two levels of ethyl alcohol (EtOH): 70% and 95%. We chose not to include a sugar-alcohol treatment because our personal experiences over the last 20 y have indicated excellent results with ethanol alone. Further, lack of sucrose in one's storage solution eliminates sticky field and laboratory equipment.

Procedures used to test the effects of the different fixation and storage techniques included collecting one large sample of zooplankton, dividing the sample into 40 portions, then fixing and preserving each portion using one of four preservation treatments. Samples were allowed to sit 60 to 90 d, and then they were processed to collect measures, which would be similar to those used by the typical limnologist and reflect the preservation technique effects. Samples were processed again after 18 months of storage to determine the long-term effects of the preservation techniques.

Zooplankton were collected 16 Nov. 2001 from the Turnbull Laboratory Pond (47°27′17″ N, 117°34′20″ W), a small eutrophic (surface area 1.7 ha, maximum depth 2.0 m) and fishless water body located on the Turnbull National Wildlife Refuge, Spokane County, WA, USA, by multiple vertical tows using a 20-cm diameter, 250-μm mesh conical plankton net. The plankton community included *Daphnia pulex* (abundance > 20 individuals L^{-1}), *Diaptomus leptopus*, and *Chaoborus americanus* (abundance of both < 1 individual L^{-1}). The sample was divided into 40 portions of approximately 500 *Daphnia* per portion. Individual portions were then treated as separate samples and randomly assigned to one of four fixation and preservation treatments (ten replicates per treatment). Treatments included (1) putting live animals directly into sample bottles of 70% EtOH (the "70%" treatment); (2) collecting live animals on a 153-μm mesh sieve, immersing the sieve into 50°C tap water, then transferring the animals to samples bottles containing 70% EtOH (the "heat" treatment); (3) collecting live animals on 153-μm mesh sieve, immersing the sieve in 95% EtOH (similar to the technique described in de Bernardi [1984]) for 15 s, then transferring the sample to 70% EtOH (the "95%"

treatment); and (4) collecting live animals on 153-μm mesh sieve, then transferring live animals directly into sample bottles containing 4% sugar-formalin prepared as per Haney and Hall's (1973) technique (the "SF" treatment).

Following 60 to 90 d of storage, samples were selected at random and the first 70 *Daphnia* (including a mixture of juveniles and adults) in each sample were examined to determine individual body length, number of eggs in the brood chamber, and distortion. Body lengths were recorded as the distance from the anterior margin of the helmet to the base of the tail spine. Distorted animals were considered those with a ballooned carapace, the abdomen extended ventrally at least 45° toward the carapace margin, or both. Percent distorted was calculated for each sample. Measures were not collected from males or ephippial females. Sample averages of the eggs per female and body length measures were averaged to determine treatment averages for each measure. One way ANOVA and Tukey's HSD were used to detect significant treatment effects and identify significant differences among treatments (SYSTAT version 8.0).

After 18 months of storage, the samples were processed again using the methods described above. Additionally, the proportion of embryos that had become dislodged from the brood chamber was calculated for each sample by dividing the number of loose embryos by the total number of embryos retained in brood chambers and loose in the sample bottle.

Body length results are presented in Fig. 1. There were no significant differences in body length among *Daphnia* in the four treatments after 60 to 90 d of storage ($F_{3,36}$, $P = 0.473$), nor were there significant differences in body length among treatments after 18 months of storage ($F_{3,36}$, $P = 0.926$). However, in all of the treatments, average body length did decrease over the 15 months that separated the sample examination periods. Average body length decreased approximately 2% in the heat treatment. In the 95% treatment, body length decreased on average by approximately 4%. In the 70% and SF treatments, body lengths decreased by 5% and 6%, respectively. Thus, there is some shrinkage that occurs when animals are stored in either 70% ethanol or the 4% formalin solution, but there is little difference among the preservatives, and after 18 months of storage, shrinkage amounted to 6% or less.

ANOVA results indicate that there were significant treatment differences in the percent of individuals with a ballooned carapace or an extended abdomen for each storage duration ($F_{3,36}$, $P < 0.001$ for each) (Fig. 2). At 34% and 38% for the 60- to 90-d storage duration and the 18-month storage duration, respectively, percent distortion was significantly higher in the heat treatment than in the other treatments ($P < 0.001$). Percent distortion in the 70% and SF treatments ranged from 16% to 20%, and there were no significant differences between these two treatments on either of the exam-

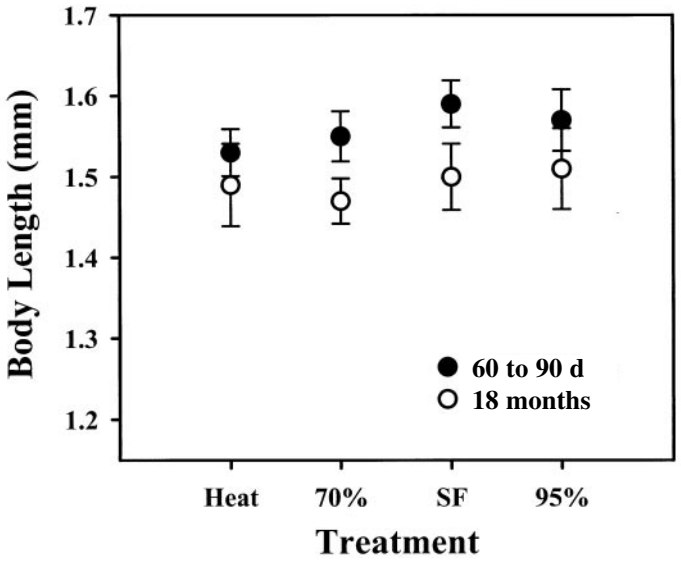

Fig. 1. Mean (± 1 SE) body length calculated from the *Daphnia pulex* in samples preserved by each of four fixation and preservation treatments (10 samples per treatment) after 60 to 90 d and 18 months of storage. Treatments include: 70%, live animals were immediately put in sample bottles containing 70% EtOH; Heat, animals were collected on a sieve, immersed in 50°C water, and then put in sample bottles of 70% EtOH; 95%, animals were collected on a sieve, immersed in 95% EtOH for 15 s, and then transferred to 70% EtOH for storage; and SF, animals were immediately put in sample bottles containing a 4% sugar formalin solution (as per Haney and Hall 1973).

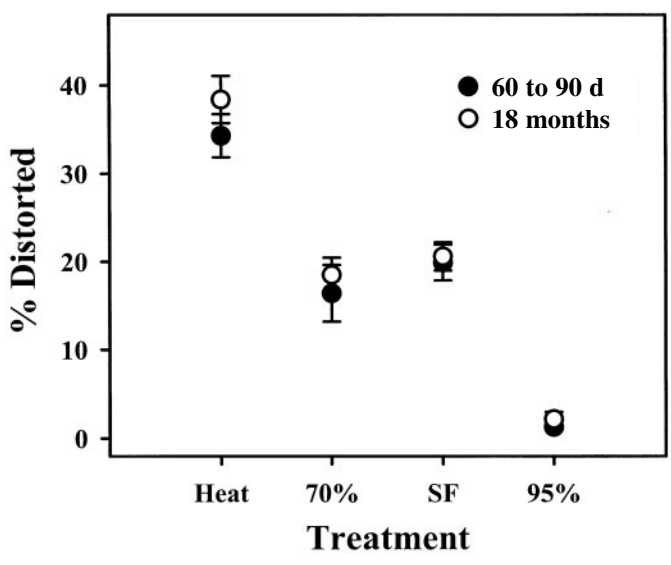

Fig. 2. Mean percent distortion (± 1 SE) calculated from the *Daphnia pulex* in samples preserved by each of four fixation and preservation treatments (10 samples per treatment) after 60 to 90 d and 18 months of storage. Treatments include: 70%, live animals were immediately put in sample bottles containing 70% EtOH; Heat, animals were collected on a sieve, immersed in 50°C water, and then put in sample bottles of 70% EtOH; 95%, animals were collected on a sieve, immersed in 95% EtOH for 15 s, and then transferred to 70% EtOH for storage; and SF, animals were immediately put in sample bottles containing a 4% sugar formalin solution (as per Haney and Hall 1973).

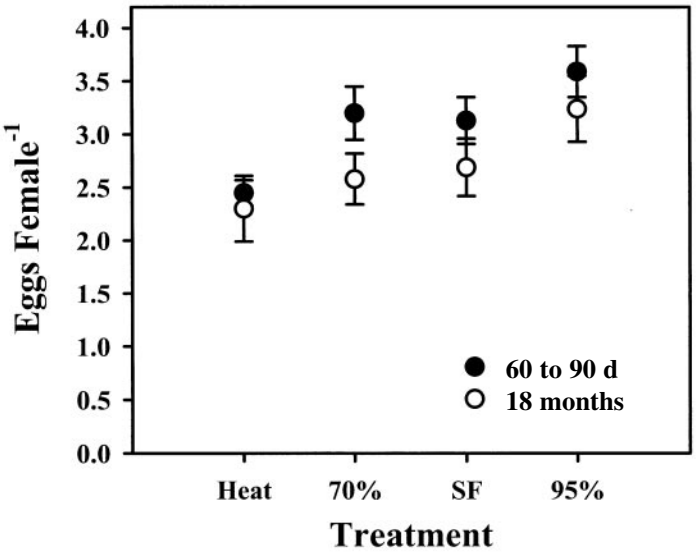

Fig. 3. Mean (± 1 SE) eggs per female calculated from the *Daphnia pulex* in samples preserved by each of 4 fixation and preservation treatments (10 samples per treatment) following 60 to 90 d and 18 months of storage. Treatments include: 70%, live animals were immediately put in sample bottles containing 70% EtOH; Heat, animals were collected on a sieve, immersed in 50°C water, and then put in sample bottles of 70% EtOH; 95%, animals were collected on a sieve, immersed in 95% EtOH for 15 s, and then transferred to 70% EtOH for storage; and SF, animals were immediately put in sample bottles containing a 4% sugar formalin solution (as per Haney and Hall 1973).

ination dates (60 to 90 d, $P = 0.71$; 18 months, $P = 0.859$). The percent distorted in the 95% treatment was significantly lower than in the other treatments during each examination period ($P < 0.001$ for each: Fig. 2).

The average number of eggs per female for each of the treatments is presented in Fig. 3. The lowest values consistently occurred in the heat treatment where distortion was more common, and the highest values occurred in the 95% treatment where distortion was less frequent. *Daphnia* in the 70% and SF treatments tended to possess an intermediate number of eggs per individual. ANOVA results indicate that there was significant difference among treatment variation in the number of eggs per female *Daphnia* after the 60- to 90-d storage period ($F_{3,36}$, $P = 0.006$). Multiple comparisons indicate eggs per female in the heat treatment (average = 2.45) were significantly less ($P = 0.003$) than in the 95% treatment (average = 3.59). No other differences among the treatments were detected. Following 18 months of storage, no among treatment variation was detected ($F_{3.36}$, $P = 0.138$) although the same among treatment pattern is evident (Fig. 3).

Fig. 4 illustrates the percent of embryos encountered that had been released from the brood chamber during fixation and after 18 months of storage. ANOVA results indicate significant among treatment variation in the percent of loose embryos ($F_{3,36}$, $P < 0.001$). The least number of loose embryos was observed in the 95% treatment (average = 2.6%) and was significantly lower when compared to all of the other treatments ($P < 0.001$). In the 70% and SF treatments, loose embryos averaged 10% and 14% and were not significantly different from one another ($P = 0.202$). The highest observed value was seen in the heat treatment that averaged 19% and was significantly higher than in the 70% percent treatment ($P = 0.002$) and the 95% treatment ($P = 0.001$).

As one would expect, there exists a negative correlation between the percent distorted and the average number of eggs per female in the sample. Among the 40 samples examined after 60 to 90 d of storage, this correlation was –0.448 (df = 38, $P < 0.01$), and after the 18-month storage period the correlation was –0.299 (df = 38, $0.05 < P < 0.06$). Furthermore, after 18 months of storage, there was a significant positive correlation between the percent-distorted individuals and the occurrence of loose embryos in the sample (correlation coefficient = 0.639, df = 38, $P < 0.01$) and a significant negative correlation between the number of eggs per female and the occurrence of loose embryos (correlation coefficient = –0.493, df = 38, $P < 0.01$). All of the correlations above suggest distortion results in decreased egg counts and an increase of embryos lost from the brood chamber.

Of additional note was subtle variation in tissue quality of the animals fixed and stored by the various techniques. *Daph-*

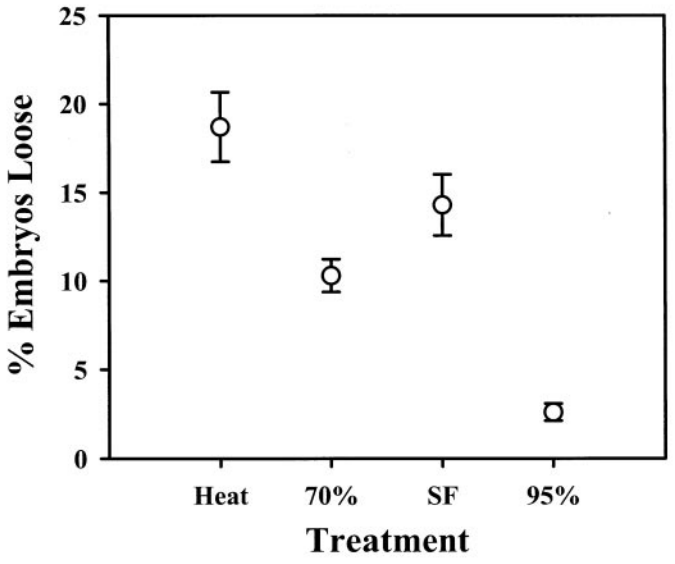

Fig. 4. Mean (± 1 SE) percent loose embryos calculated from the *Daphnia pulex* in samples preserved by each of 4 fixation and preservation treatments (10 samples per treatment) following 18 months of storage. Treatments include: 70%, live animals were immediately put in sample bottles containing 70% EtOH; Heat, animals were collected on a sieve, immersed in 50°C water, and then put in sample bottles of 70% EtOH; 95%, animals were collected on a sieve, immersed in 95% EtOH for 15 s, and then transferred to 70% EtOH for storage; and SF, animals were immediately put in sample bottles containing a 4% sugar formalin solution (as per Haney and Hall 1973).

nia stored in the SF treatment were qualitatively different in three ways. First, tissues remained more transparent, easing, for example, egg counts. In contrast, after 18 months of storage, *Daphnia* stored in ethanol possessed tissues with a yellow tint. However, although tinted, *Daphnia* did remain transparent and anatomical features and embryos were readily viewed and could be easily examined, measured, etc., when observed under a dissecting microscope. However, embryos in the brood chamber, especially those early in development, lose their transparency. This was not a problem when conducting eggs counts, but if one was attempting to discern the egg stage (as per Threlkeld 1979), one might encounter difficulty. Second, SF *Daphnia* were more fragile and could be easily damaged (dented or smashed) during sample handling and processing. Although Steedman (1976) suggests crustacean zooplankton stored in ethanol became brittle, we detected no evidence that *Daphnia* were brittle or fragile even after 18 months of storage in our 70% and 95% treatments. Instead, they were resilient to deformation, or being dented or crushed, as they were manipulated in the sorting tray and positioned for egg and body length measures. Third, during both sample-processing events, it was observed that in the SF treatment the eggs of ovigerous individuals were often dislodged from the brood chamber and were loose within the carapace. Combined with the fragile nature of the fixed adults, this meant great care was required to prevent spilling embryos from the adult. *Daphnia* fixed and stored by the 70% and 95% techniques were far more durable and the embryos within the carapace of ovigerous individuals tended to remain as a cohesive mass and intact within the brood chamber. Although not statistically significant, our results do indicate the highest egg counts were observed in the 95% treatment and those in the SF treatment averaged 13% fewer eggs per female after 2 to 3 months of storage, and 17% fewer eggs per female after 18 months of storage. This suggestion is further supported by our observing that the proportion of loose eggs in the 95% treatment averaged only 20% of the proportion observed in the SF treatment.

Discussion

Zooplankton fixation in 95% ethanol and storage in 70% ethanol is a low toxicity and, for *Daphnia*, an equally effective alternative to the sugar formalin solution many limnologists currently use. Ethanol has neither the harmful health effects, nor the storage and disposal complications and costs that come with the use of formalin. Low cost solvent grade ethanol is readily available in large volumes at reasonable costs. And, although many institutions require adherence to flammable storage regulations and documenting use of ethanol as a volatile organic, it has been our experience that compliance with environmental health regulations associated with ethanol use is far easier than for situations where formalin is used. Ethanol-stored tissues have the added advantage that they may be used for genetic analysis, whereas formalin stored tissues may not (Sambrook et al. 1989). Finally, when using ethanol as a preservative, one need not be concerned with contaminating laboratory equipment nor does one have to be concerned with keeping the fixative and contaminated equipment away from live animals.

We recognize that 95% ethanol fixation, followed by storage in 70% ethanol, is likely not suitable for all taxa nor for all collecting purposes. For many of the common productivity and demographic studies conducted by limnologists, our 95% technique should work well for most branchipods. Our results provide no information concerning the value of ethanol used for long-term museum storage where fragile anatomical parts may be examined after several years of storage or for studies where other parameters might be collected from the branchiopoda. Additionally we have no evidence to suggest whether our 95% technique will be an effective fixation and preservation method for other taxa.

We should also emphasize that we did not test the effects of first anesthetizing animals in soda water prior to fixation and storage. It is our suspicion that the soda water technique could only improve the results of each fixation and preservation method, and our not using soda water is perhaps responsible for the high proportion of distorted individuals observed in the 70% and SF treatments. However, our experience using the 95% method and the results for that technique presented in this manuscript indicate soda water is not a necessary step. Only 2% of the animals fixed and preserved with the 95% technique were distorted, and only 2.6% of the embryos in the sample were observed to have been lost from the brood chamber.

Ethanol use as a preservative is not without some inconvenience. Ethanol can evaporate from screw cap sample bottles and snap cap vials and thus require topping-off with 70% ethanol approximately once every 1 to 2 y. We have had the best luck with screw cap polypropylene sample bottles. Additionally, Steedman (1976) reports crustacean zooplankton can become brittle when stored in ethanol. Thus, ethanol may not be the best choice for long-term museum storage or situations where fragile anatomical parts may be examined several years after collection. However, this method works as well as sugar-formalin for much of the monitoring, productivity, and demographic work conducted in the plankton of freshwaters and when samples are processed within a few months of collection.

References

AIHA [American Industrial Hygiene Association]. 1989. Odor thresholds for chemicals with established occupational health standards. American Industrial Hygiene Association. Table 5.3.

de Bernardi, R. 1984. Methods for the estimation of zooplankton abundance, p 59-86. *In* J. A. Downing and F. H. Rigler [eds.] A manual on methods for the assessment of secondary productivity in fresh waters. IBP Handbook 17. Blackwell.

Haney, J. F. and D. J. Hall. 1973. Sugar-coated *Daphnia*: a preservation technique for cladocera. Limnol. Oceanogr. 18:331-333.

OSHA [Occupational Safety and Health Administration]. 2001. Regulations (Standards-29 CFR) Substance technical guidelines for formalin-1910.1048. <http://www.osha-slc.gov/SLTC/formaldehyde/>

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory.

Steedman, H. F. 1976. Zooplankton fixation and preservation. Volume 4: Monographs on oceanographic methodology series. UNESCO. 350 pp.

Threlkeld, S. T. 1979. Estimating cladoceran birth rates: the importance of egg mortality and the egg age distribution. Limnol. Oceanogr. 34:390-396.

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