Appendix A

The use of trichloroacetic acid fixation and propylene phenoxetol conservation in quantitative sampling of ctenophores

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Introduction

Lobate ctenophore species are notoriously difficult to sample due to their fragility and preservation in traditional media such as buffered formaldehyde solution or ethanol is difficult. A method which has largely been unnoticed or possibly considered as too labor intensive (Purcell, 1988) is a method developed for preservation of individual specimens from samples by Adams et al. (1976). This method consists of fixation using a trichloroacetic acid (TCA) solution and subsequent preservation in a seawater solution containing propylene phenoxytol, propylene glycol and formaldehyde. In 2009, we tested this method for preservation and fixation of field samples and subsequently applied it with some modifications successfully for a year-round quantitative sampling programme of the ctenophores Mnemiopsis leidyi, Pleurobrachia pileus and Beroe gracilis in the western Wadden Sea (this study). This appendix describes the use of this modified fixation method and its advantages and disadvantages.

Materials and methods

Original fixation and preservation method for single specimens

The fixation and preservation method for individual ctenophores was the result of an investigation of some eighty reagents by the SCOR Working Group 23 project (Adams et al., 1976). For fixation a solution of 1 g tri-chloracetic acid or 1 g p-toluenesulphonic acid in 99 ml sea water is used. For preservation, a solution of 5 ml propylene phenoxytol, 45 ml propylene glycol and 50 ml 40% formaldehyde is prepared. The original protocol includes the following steps:

1. separate the ctenophores from other plankters and place them in a beaker with sea water;
2. drain the sea water away from the ctenophores by using bolting silk;
3. pour the ctenophores gently into the fixative;
4. fix for 30 minutes during which the specimens change slightly from transparency to translucency;
5. remove the fixative and replace it with a 1% preservation/sea water solution;
6. preserve for 5–7 days;
7. transfer from a 1% to a 5% preservation/sea water solution and store between 5 and 20 °C.
Modified protocol for fixation and preservation of quantitative samples

In applying the method of Adams et al. (1976) for quantitative samples, the following criteria were used when testing different modifications:

1. fixation and preservation of all species occurred;

2. after shrinkage fresh length and mass could be reconstructed;

3. stomach analysis could be performed on preserved individuals.

These criteria were applied in a three-step procedure: first different concentrations were tested for optimal fixation and preservation. Next, shrinkage and reconstruction of fresh mass was determined and finally, regurgitation was measured.

Ctenophores were caught using the method and gear as described in Van der Veer and Sadée (1984) in tidal gullies in the western Wadden Sea, the Netherlands. The whole sample or a subsample was examined on a 1mm sieve and if possible macroalgae such as Ulva lactuca were removed. The sample or a subsample of maximum 500 ml was put in a 800 ml glass jar, which was then filled up with the fixation solution. Solutions were prepared on board by adding different quantities of 100 g/l TCA-seawater solution to ambient seawater. In summer, the fixated samples were stored in a styrofoam box with ice for cooling.

In the lab the TCA-fixed samples were transferred on the day they were caught to sea water containing 1% preservation stock solution by volume. For this, samples were put on and drained in a sieve of ca. 0.5 mm mesh size, returned in the jar and subsequently the preservation solution was added. After 5-8 days the samples were transferred to sea water with 5% preservation stock solution. Samples were stored at a constant temperature of 4 °C.

Ctenophores of 10 mm or smaller in length were measured submerged in a petri dish using a stereo microscope with a measuring eyepiece. Larger ctenophore lengths were measured using a Vernier caliper. For Pleurobrachia pileus and Beroe gracilis the length measurement taken was the polar length, for Mnemiopsis leidyi the distance from the mouth to the statocyst.

Live ctenophores were fixed and preserved individually in glass jars to study shrinkage. Length and weight was measured prior to fixation, when transferred from the 1% to the 5% preservation solution. After preservation length and weight were measured twice with intervals of multiple months. Measurements were taken as described above.

Wet mass was determined by putting individual ctenophores on a drain for 20 sec to remove adhesive water. Ash Free Dry Mass (AFDM) was determined by first drying for 2 to 3 d at 60 °C in a ventilated stove, weighing and incinerating for 2 h at 520 °C. The weight loss after incineration was considered to represent AFDM.
Appendix A

Data analysis

All data were stored in a Microsoft Access database. Data analysis was carried out using R (R Core Team, 2014) and SigmaPlot 12.0. The allometric relationship

\[ V = a \times l^b \]  

(A.1)

between the fresh volume \( V \) (ml) and oral–statocyst length before and after fixation \( l \) (mm), was estimated using the non-linear least squares function of R.

Results and discussion

When the TCA solution was added the effect could be observed almost instantly. Structures such as the comb rows, meridional canals, oral lobes and walls of the stomodaeum became increasingly opaque white (Fig. A.3). This property greatly increased the visibility, especially when sorting in black sorting trays.

The fixation and preservation method worked in preserving ctenophores individually as well as together with hydromedusae, crustacean- or ichthyoplankton in a single sample. When samples contained large quantities of plant-peat- or macroalgae fragments or high quantities of crustaceans, often only the mouth, stomodaeum and statocyst complex of the \( M. leidyi \) ctenophores were left after preservation. Overall ca. 95% of ctenophores could be measured in preserved samples. Large samples of ctenophores smaller than 5 mm length could be subsampled using a Folsom plankton splitter without major damage to the specimens. Of the first 30 \( M. leidyi \) that were individually fixed, only one could not be measured on the last measuring moment 475 days after fixation in total 7 out of 128 (5.5%) \( M. leidyi \) disintegrated or were too damaged to measure. One Pleurobrachia pileus had disintegrated and all Beroe gracilis could be measured. Relationships between lengths and weights of fresh and fixed \( M. leidyi \) are shown in Fig. A.1.

A significant allometric relationship between fixed and fresh lengths and fresh weights of individually fixed ctenophores could be estimated for \( M. leidyi \) (Fig. A.1; Table A.1) and \( P. pileus \) (Table A.1). Mean shrinkage by volume as measured when transferring the samples from the 1% to the 5% stock solution was 55% for large \( M. leidyi \) \((n=8, sd=4.9)\) and 54% for small \( M. leiydi \) \((n=80, sd=13.1)\). Percentage of shrinkage was highly variable in small ctenophores.

Mean shrinkage by length was 16% \((n=88, sd=13.0)\) post-fixation and 19% \((n=53, sd=19.8)\) measured on the second measuring moment. Mean shrinkage by length of fixed \( P. pileus \) measured on the first measuring moment was 19% \((n=12, st.dev=7.8)\) and also 19% \((n=12, sd=9.7)\) measured on the second measuring moment. Mean shrinkage by length of fixed \( B. gracilis \) after the first and second measuring moment was respectively 26% \((n=20, sdv=7.0)\) and 28% \((n=18, sd=9.1)\).

There was a positive relationship between ash free dry weight and fresh ctenophore volume (Fig. A.2) for ctenophores weighing less than 20 g \((afdm = 0.0088 \times freshweight)\). For the ctenophores weighing more than 20 g the sample size was too low to see a clear relationship.
Table A.1: Parameter estimates and standard errors and p-values of the estimated length (fixed or fresh) - fresh volume relationship $V = a \cdot l^b$.

<table>
<thead>
<tr>
<th>Species</th>
<th>n</th>
<th>estimate</th>
<th>SE</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>$M. leidy_i^{fresh}$</td>
<td>128</td>
<td>a 0.0145</td>
<td>0.0030</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>$M. leidy_i^{fresh}$</td>
<td>128</td>
<td>b 2.0000</td>
<td>0.0580</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>$M. leidy_i^{fixed}$</td>
<td>128</td>
<td>a 0.0079</td>
<td>0.0013</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>$M. leidy_i^{fixed}$</td>
<td>128</td>
<td>b 2.3202</td>
<td>0.0481</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>$P. pileus^{fixed}$</td>
<td>89</td>
<td>a 0.0011</td>
<td>0.0003</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>$P. pileus^{fixed}$</td>
<td>89</td>
<td>b 2.6809</td>
<td>0.1075</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>$B. gracilis^{fixed}$</td>
<td>20</td>
<td>a 0.0022</td>
<td>0.0019</td>
<td>0.273</td>
</tr>
<tr>
<td>$B. gracilis^{fixed}$</td>
<td>20</td>
<td>b 2.2282</td>
<td>0.3281</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

No regurgitation was observed in 10 individuals of $M. leidy_i$ after 10 minutes following addition of the TCA solution to a Petri dish containing the specimen. Food was observed moving towards the mouth opening, but not expelled. After the final preservation step however the stomodaeum wall had turned from translucent to opaque and was often disintegrated into small particles making stomach content analysis difficult or impossible. Only larger crustacean zooplankton prey items such as cyprid larvae and copepods could be distinguished, and in one instance a 6mm long goby larva.

These results show that the method of Adams et al. (1976) for the preservation of single species also worked well for the preservation of field samples of ctenophores, allowing detailed quantitative measurements in the lab. Heavily damaged specimens could often still be measured because the mouth-stomodaeum-statocyst complex remained intact (Fig. A.3). This method, while being slightly more labour intensive than the method using Lugol’s solution proposed by Engell-Sorensen et al. (2009), could potentially be more effective as it does not have the disadvantages of the high variation in shrinkage and discoloration. The percentages of ctenophores that are still measurable are also higher using this method. The Lugol’s solution method has been shown to work also for larval ctenophores (Sullivan and Gifford, 2009), whether that is also the case for this method is still unknown. The fixed oral-statocyst length – fresh mass relationship for $M. leidy_i$ and the other species can be used to accurately estimate the wet mass or bio-volume of live ctenophores. Overall, this method will be very useful for sampling ctenophore populations more efficiently and accurately. A further major improvement to the method would be finding a suitable replacement for the toxic formaldehyde used in preservation.
Figure A.1: Relationships between fresh length and fresh weight (a), fixed length and fresh weight (b), fresh- and fixed weight (c) and fresh and fixed length (d) of individual *Mnemiopsis leidyi*. 
Figure A.2: Relationship between ash free dry weight (afdw, g) and fresh weight of *Mnemiopsis leidyi* ctenophores with fitted regression lines.

Figure A.3: Photographs of preserved ctenophores: *Mnemiopsis leidyi*: (a) specimen of 8 mm oral–aboral length after 231 days in the preservation solution, (b) specimen of 16 mm oral–aboral length after 475 days in the preservation solution, (c) specimen of 34 mm oral–aboral length after 397 days in the preservation solution, (d) damaged preserved specimen of 32 mm oral-aboral length after 397 days in the preservation solution. Note that the statocyst-stomodaeum-mouth complex is still intact allowing measurement. (e) specimen of *Pleurobrachia pileus* 14 mm length after 397 days in the preservation solution. (f) specimen of *Beroe gracilis* of 16 mm length after 358 days in the preservation solution.