Reviewing the effectiveness of DESS as an alternative preservative for marine zooplankton morphology through image analysis

MSc Thesis
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Abstract

Formalin is the most commonly used fixative for the preservation of zooplankton morphology. The toxic and carcinogenic properties of the substance highlight the need for an alternative solution. DESS solution is an effective alternative to ethanol for the preservation of DNA. In this study, its effectiveness was explored in preserving zooplankton morphology to potentially eliminate the need for two separate preservation methods for DNA and morphology. North Sea mesozooplankton samples were collected and preserved on formalin and DESS. The samples were stored for five months before further processing. Flatbed scanner imagery of the samples was processed in ZooProcess and classified to taxa in EcoTaxa. Additionally, taxa were photographed with an inverted microscope. DESS solution was not adequate in preserving the morphology of the majority of marine zooplanktonic groups. Soft-bodied organisms were typically lost entirely. While soft tissue inside was diminished to varying degrees, exoskeletons of Crustacea remained preserved relatively well, and diagnostic characteristics were largely intact.

Contents

1. Introduction

Plankton are classified as organisms living in the water column that are unable to or have limited ability to move against currents. Considering there is a large diversity among plankton, there are several classifications to distinguish between groups. Firstly, there is a distinction between phyto- and zooplankton. Phytoplankton are photosynthetic microalgae while zooplankton are animals. Zooplankton may also be classified according to life cycle. Holoplankton spend their entire lives as plankton, while meroplankton spend only part of their lives as plankton. Further classification can be through categories such as taxonomy, distribution, habitat and food, and between size classes: megazooplankton (20-200 cm), macrozooplankton (2-20 cm), Mesozooplankton (0,2-20 mm) and microzooplankton (20-200 µm) (Santhanam, Pachiappan, & Begum, 2019; Jak & Slijkerman, 2023). In monitoring practices zooplankton size is important as it dictates which techniques and appliances should be used for optical imaging and processing. The usual practice for the enumeration of sampled microzooplankton is an inverted microscope (Gifford & Caron, 2000). A flatbed scanning system allows for the digital image processing of mesozooplankton >200 µm (Gorsky, et al., 2010).

After collection of zooplankton for the purpose of research, samples must be preserved as soon as possible. There are several ways to preserve zooplankton samples, depending on the desired analysis results. Ethanol is most widely used for the preservation of DNA, while the use of formalin is the most common practice to preserve zooplankton morphology (Steedman, 1985). Formalin is a mixture of gaseous formaldehyde (CH_2O) dissolved into water which works by cross-linking proteins. Ultimately this process makes tissue more rigid and harder to decompose, preserving the physical structure of the organism (Thavarajah, Mudimbaimannar, Elizabeth, Rao, & Ranganathan, 2012). The downside of formalin is that it is a toxic and carcinogenic substance despite that it is used in many industries, highlighting the need for a more harmless alternative (Buesa, 2008). Acid Lugol solution is commonly used as a fixative for phytoplankton, microzooplankton and mesozooplankton. The downside of Lugol is that it causes shrinkage of organisms, distorting their morphology and hindering identification and size estimation (Stoecker, Gifford, & Putt, 1994; Jaspers & Carstensen, 2009).

DESS is an alternative to ethanol for the preservation of DNA that has already been shown to be effective (Beknazarova, et al., 2017; Sharpe, et al., 2020), and while not much researched for other purposes may also have potential in the preservation of morphological features in organisms. Yoder et al. (2006) claimed DESS was suitable for both morphological and molecular analysis of nematodes, eliminating the need for more hazardous chemical counterparts. DESS solution consists of three ingredients: dimethyl sulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA) and sodium chloride (NaCl), first introduced by Seutin, White & Boag in 1991. DMSO enhances the transport of molecules into tissues, membranes and cells, EDTA is a chelator essentially deactivating metal-dependent enzymes diminishing damage to DNA and proteins and NaCl inhibits microbial growth (Naem, Pagan, & Nadler, 2010; Yoder, et al., 2006). Table 1 lists all the mentioned preservatives and components along with their hazard statements and storage precautionary statements (Table 2).

If DESS solution is effective in morphological preservation for zooplankton taxa, it would eliminate the need for two separate preservation methods: one sample preserved with DESS solution could then be used for both morphological and DNA analysis. Thus far, it is not known how effective DESS is in preserving the morphology of most zooplankton taxa.

This study aims to explore the effectiveness of DESS in preserving morphological features of several common zooplankton taxa. The impact of preservatives on morphological features was studied with imaging techniques and microscopy. A flatbed scanning system was applied in combination with web-based classification platform EcoTaxa to semi-automatically classify large quantities of zooplankton

images derived from samples preserved on formalin and DESS through manual classification and trained models. Microscope photography was applied to display morphological features in preservatives. These methods are to shed light on the taxonomic groups that can be identified in DESS, to what taxonomic resolution organisms can be identified and the morphological differences between organisms in DESS and formalin.

Table 1 Common preservatives and their hazard and storage precautionary statements.

Preservative	**Hazard statements**	**Storage precautionary statements**
Ethanol (CH_3CH_2OH)	H225, H319	P403, P235
Formaldehyde (CH_2O)	H301, H311, H331, H314, H317, H341, H350	P403, P233, P405
Acid Lugol solution (I_3K)	H362, H372, H412	
DESS		
DMSO (Dimethyl sulfoxide/ C_2H_6SO)	H315, H319, H335	P405, P403, P233
EDTA (Ethylenediaminetetraacetic acid/ $C_{10}H_{16}N_2O_8$)	H319	
Sodium chloride (NaCl)		

Table 2 Hazard statements (left) and storage precautionary statements (right) related to common preservatives.

Hazard statement	**Hazard**	**Storage precautionary statement**	**Storage**
H225	Highly flammable liquid and vapor	P233	Keep container tightly closed
H301	Toxic if swallowed	P235	Keep cool
H311	Toxic in contact with skin	P403	Store in a well-ventilated place
H314	Causes severe skin burns and eye damage	P405	Store locked up
H315	Causes skin irritation		
H317	May cause an allergic skin reaction		
H319	Causes serious eye irritation		
H331	Toxic if inhaled		
H335	May cause respiratory irritation		
H341	Suspected of causing genetic defects		
H350	May cause cancer		
H362	May cause harm to breast-fed children		
H372	Causes damage to organs through prolonged or repeated exposure		
H412	Harmful to aquatic life with long lasting effects		

2. Methods

2.1 Sample collection

Mesozooplankton samples were collected throughout the Dutch coastal zone from 30 May to 16 June 2023 as part of the MONS monitoring project performed by Wageningen Marine Research (WMR) in commission of Rijkswaterstaat (RWS). The research cruise sailed 980 nautical miles in a crisscross pattern, during which zooplankton samples were collected at 43 stations. A 200 µm WP2-planktonnet was used to collect samples through vertical hauls, which has a 57 cm diameter and a length of 2.6 m (Figure 1). A MiniCTD attached to the WP2-net recorded the depth, allowing for an estimation of water volume sampled (π * net radius^2 * line length). Collected samples from six locations were split on-site using a Motoda box splitter to preserve fractions of a quarter of total volume in formalin and in DESS (van Walraven, Couperus, Jak, & Keur, 2023). Of the six subsamples, four were selected and analyzed due to time constraints of the study. Figure 2 contains a map with highlights indicating sampling stations used in this study. Table 3 lists further information on these samples.

Figure 1 WP2-planktonnet for the vertical collection of zooplankton samples.

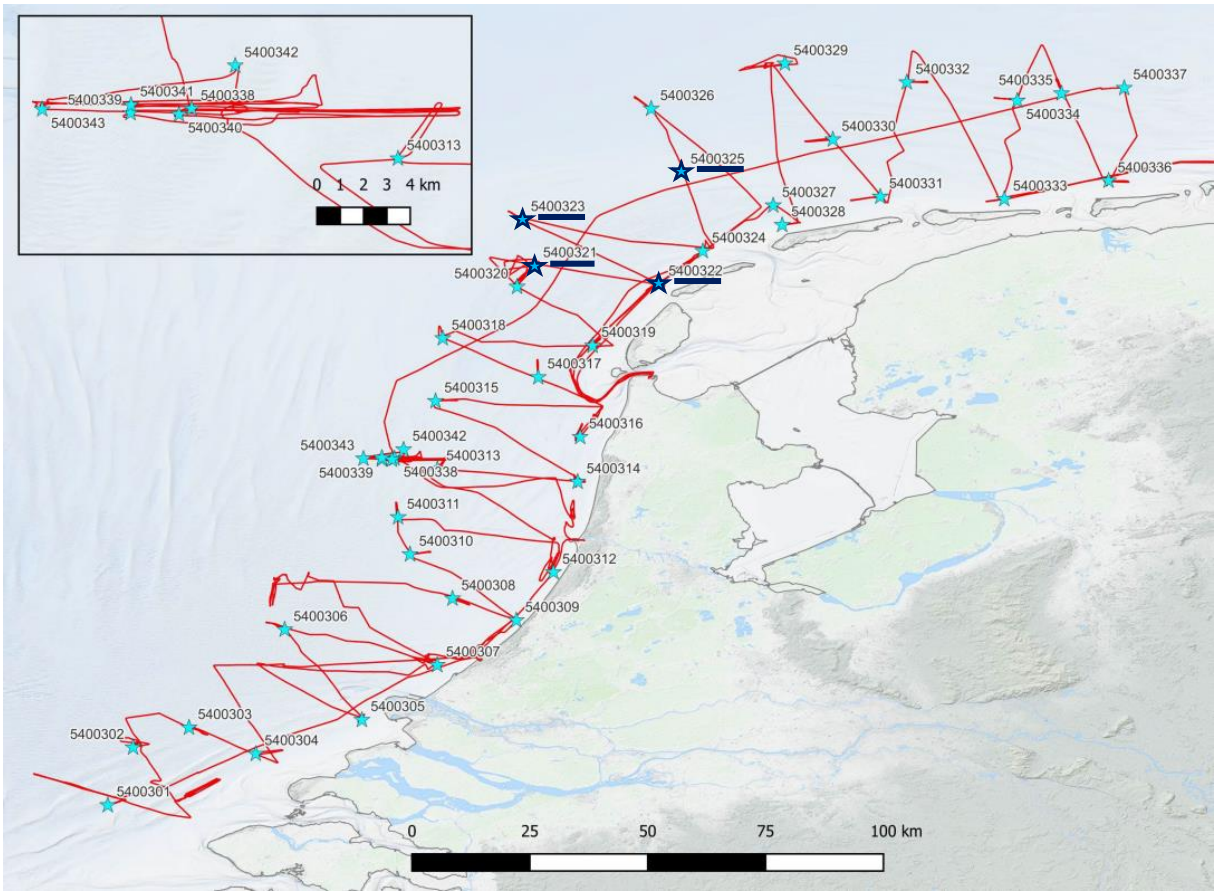

Figure 2 The sailed transect during the research cruise (red line), with sampling stations noted as stars along with stationcodes. Dark blue stars indicate stations where samples used in this study were collected.

Table 3 Overview of samples with sampling dates, stations, depths, volumes and post-split volumes of this study.

Sample	Date (d-m-y)	Station (x,y)	Depth (m)	Sample volume (m^3)	0.25 split volume (m^3)
5400321	08-06-2023	4.43176 53.29927	28	7.145	1.786
5400322	08-06-2023	4.811084 53.24185	17	4.338	1.084
5400323	08-06-2023	4.391903 53.44003	28	7.145	1.786
5400325	09-06-2023	4.881149 53.58337	24	6.124	1.531

2.2 Sample preservation

To test the effectiveness of DESS in the preservation of zooplankton morphology relative to formalin, zooplankton were preserved in formalin and DESS on-site. Formalin samples were preserved in a 4% formalin solution buffered with sodium acetate trihydrate. 1 L of the solution requires 110 mL 37% formalin (37% formaldehyde in water), 42 g sodium acetate trihydrate and 890 mL deionized water. 1 L DESS solution consists of 500 mL 0,5M disodium EDTA, 200 mL DMSO and 300 mL deionized water. 150 to 200 g NaCl is added to saturate the solution. The recipe was taken over from Yoder et al. (2006).

2.3 Imaging

Samples were stored for five months before further processing. In the laboratory, prior to imaging, the samples were stained using rose bengal to increase the contrast in organisms. This allows for small, thin features to be more defined and discernible on the image output of the flatbed scanner and microscope photography as described below.

Flatbed scanner

Prior to scanning, samples were first thoroughly rinsed with seawater over a 180 µm sieve in a fume hood to remove preservatives. Then samples were inspected in a petri dish with sea water to examine the contents. A 500 µm sieve was used to size fraction the sample. High densities within samples were split using a Motoda box splitter to prevent overlap of organisms on the scans and splits were registered in a datasheet. The fractions were then in turns transferred to a glass plate on the flatbed scanner. Overlapping organisms on the glass plate were coarsely separated manually. The samples were then scanned using the flatbed scanner (Epson Perfection V850 Pro). Scans were taken at a 2400 dpi resolution. A list of scans is in Table 4. Here, it states the sample, subsample, scan name, size fraction, laboratory split and finally the derived post-split volume of seawater the scan represents after on-site and laboratory splits.

Table 4 List of scans per sample and preservative subsample, with scan name, size fraction, laboratory split and post-split volumes.

Sample	Subsample	Scan name	Size fraction (µm)	Laboratory split	Post-split volume (m^3)
5400321	Formalin 1	FORM_321_1	<500	0.25	0.447
		FORM_321_2	>500	0.25	0.447
	DESS 1	DESS_321_1	>500	1	1.786
		DESS_321_2	<500	0.125	0.223
		DESS_321_3	<500	0.125	0.223
5400322	Formalin 2	FORM_322_1	>500	0.5	0.542

		FORM_322_2	<500	0.25	0.271
	DESS 2	DESS_322_1	>500	1	1.084
		DESS_322_2	<500	0.5	0.542
		DESS_322_3	<500	0.5	0.542
5400323	Formalin 3	FORM_323_1	>500	0.5	0.893
		FORM_323_2	<500	0.25	0.447
	DESS 3	DESS_323_1	>500	1	1.786
		DESS_323_2	<500	0.25	0.447
5400325	Formalin 5	FORM_325_1	>500	0.5	0.766
		FORM_325_2	<500	0.125	0.191
	DESS 5	DESS_325_1	>500	1	1.531
		DESS_325_2	<500	0.25	0.383
		DESS_325_3	<500	0.25	0.383
		DESS_325_4	<500	0.25	0.383

ZooProcess & Ecotaxa

The sample scans were later processed in ZooProcess, a macro language suite for ImageJ (Gorsky, et al., 2010). This provided regions of interest (ROI) representing particles within the image output. Subsequently, the ROIs were exported into EcoTaxa for taxonomic classification. EcoTaxa is a publicly available web-based application that allows users to upload plankton imagery into an accessible deep learning classification system. The classifiers process imagery into classes based on morphological features provided by a pre-processing software. In the case of this study, ZooProcess. Learning sets were first developed for formalin and DESS samples by classifying thousands of images manually. Then, a portion of the classification was done using the learning sets. However, each image was also manually validated. After classification, EcoTaxa then provides numeric features for each image which can be exported into large datasheets (Picheral, Colin, & Irisson, 2017). In this study, counts of taxa and fitted ellipse data were used. The counts of taxa (Appendix I) were extrapolated to represent individuals per m^3 in the samples (Appendix II). Considering the elongated shape of Copepoda, ellipsoidal area is a more suitable measurement than the equivalent spherical diameter (ESD) (García-Comas, et al., 2016). Ellipse major and minor axes of Copepoda and all taxa were utilized to calculate the ellipse area (π * major axis * minor axis). The resulting data was inserted into count and count density size distribution plots to evaluate size differences between Copepoda and between all taxa combined in formalin and DESS. The EcoTaxa projects can be accessed in the "Explore images" tab on https://ecotaxa.obs-vlfr.fr, using the scan name as search term under "Project".

Microscope photography

Organisms within samples were also manually inspected and photographed with an inverted microscope (ZEISS Axio Observer) to enable comparison of morphological integrity between organisms of the same taxonomic groups conserved with the two different preservatives.

3. Results

Appendix I contains the counts of taxa per sample and scan and size fraction, as well as the derived split volumes of water (m^3) they originate from. In Appendix II these counts are extrapolated to have an estimation of individuals per m^3, or density in the sampling locations in the North Sea.

Combined ellipsoidal area data of Copepoda from all samples showed some minor differences in sizes between formalin and DESS (Figure 3). In formalin and DESS, the largest spike in abundance is just over 0.5 mm^2. In formalin, Copepoda sizes have a secondary spike in abundance around 1 mm^2. Copepoda had an average ellipsoidal area of just under 1 mm^2 in both preservatives. 0.987 mm^2 in formalin and 0.994 mm^2 in DESS. Ellipsoidal area data of all taxa combined showed similar results (Figure 4). Average ellipsoidal area was 1.103 mm^2 in formalin and 0.946 mm^2 in DESS.

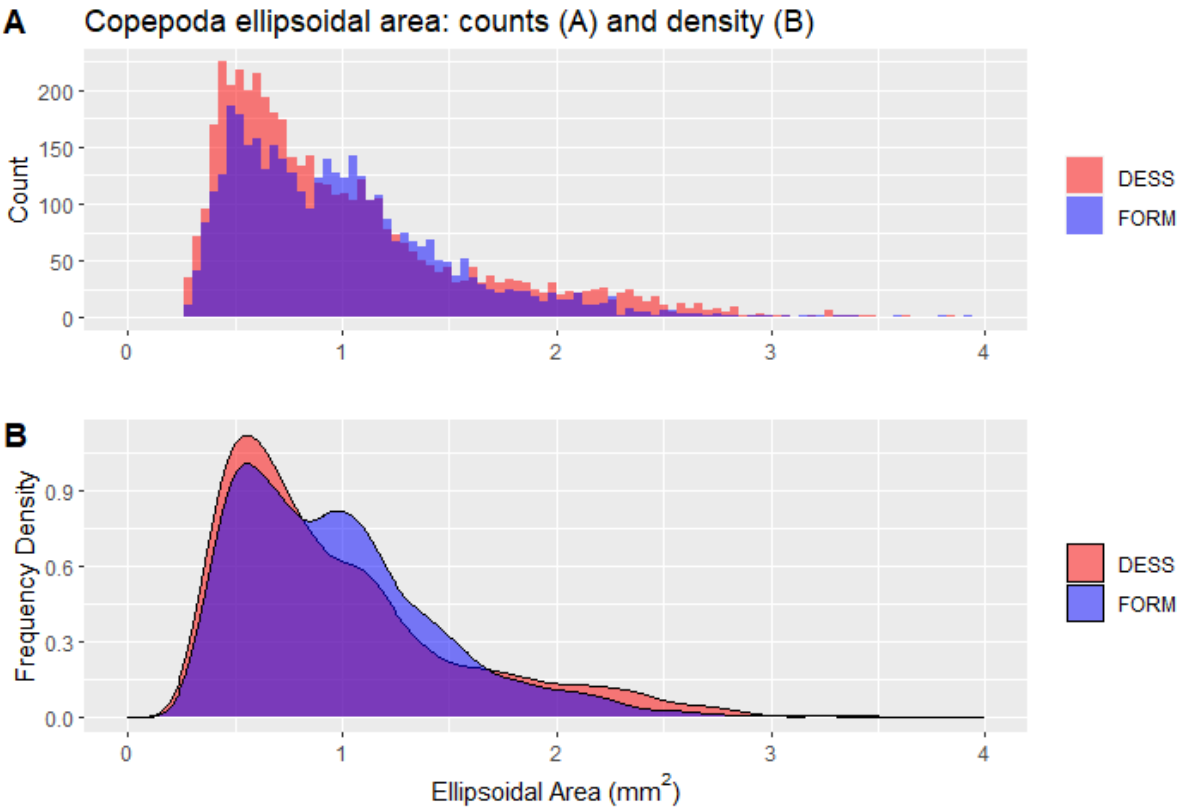

Figure 3 Ellipsoidal area distribution of Copepoda on formalin and DESS. A. ellipsoidal area frequency distribution counts. B: ellipsoidal area frequency distribution density.

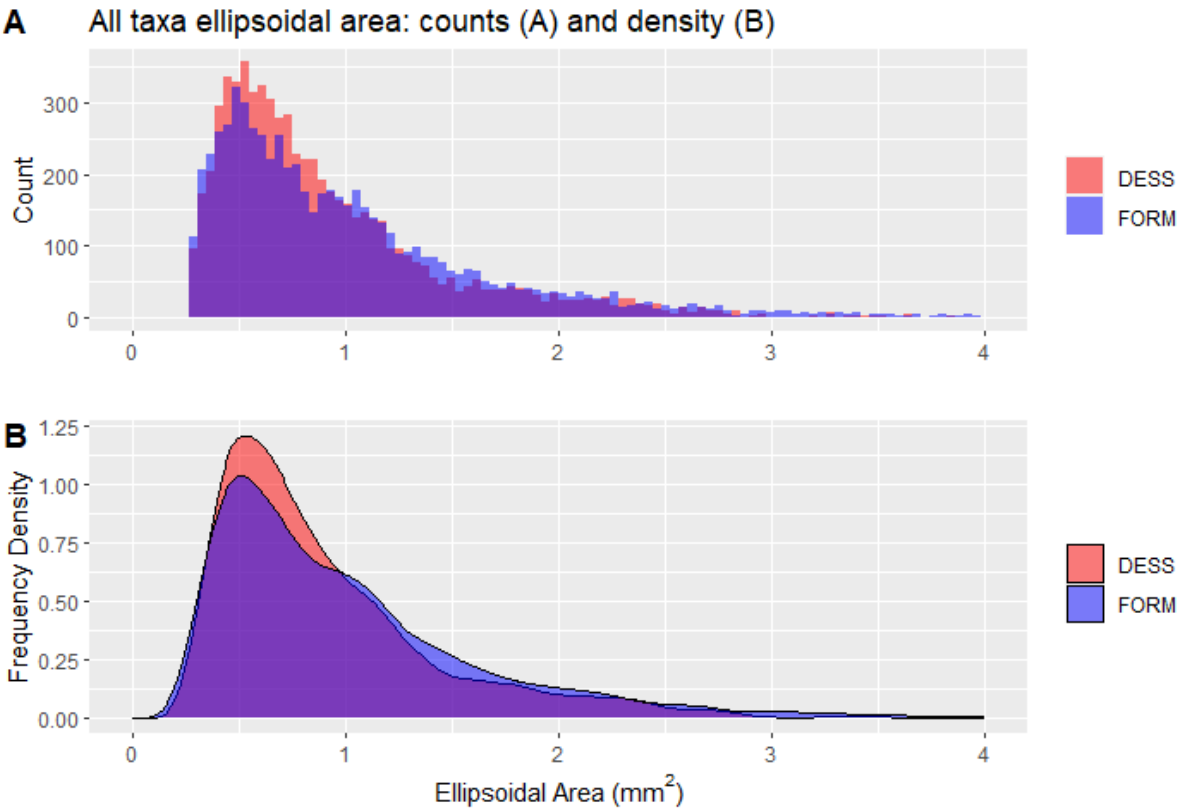

Figure 4 Ellipsoidal area distribution of all taxa on formalin and DESS. A. ellipsoidal area frequency distribution counts. B: ellipsoidal area frequency distribution density.

As expected, little to no morphological disintegration was seen in formalin samples five months after collection. Significant morphological deterioration of organisms could be seen in DESS samples, the level of which depended highly on the taxonomic group the organism belongs to (Figure 5). In the figure, values left of the dotted red lines indicate higher densities of individuals in formalin, and vice versa. Note that the axes are on a logarithmic scale. The best results in favour of DESS appear to be in Crustacean taxa, and a worse performance in soft-bodied organisms.

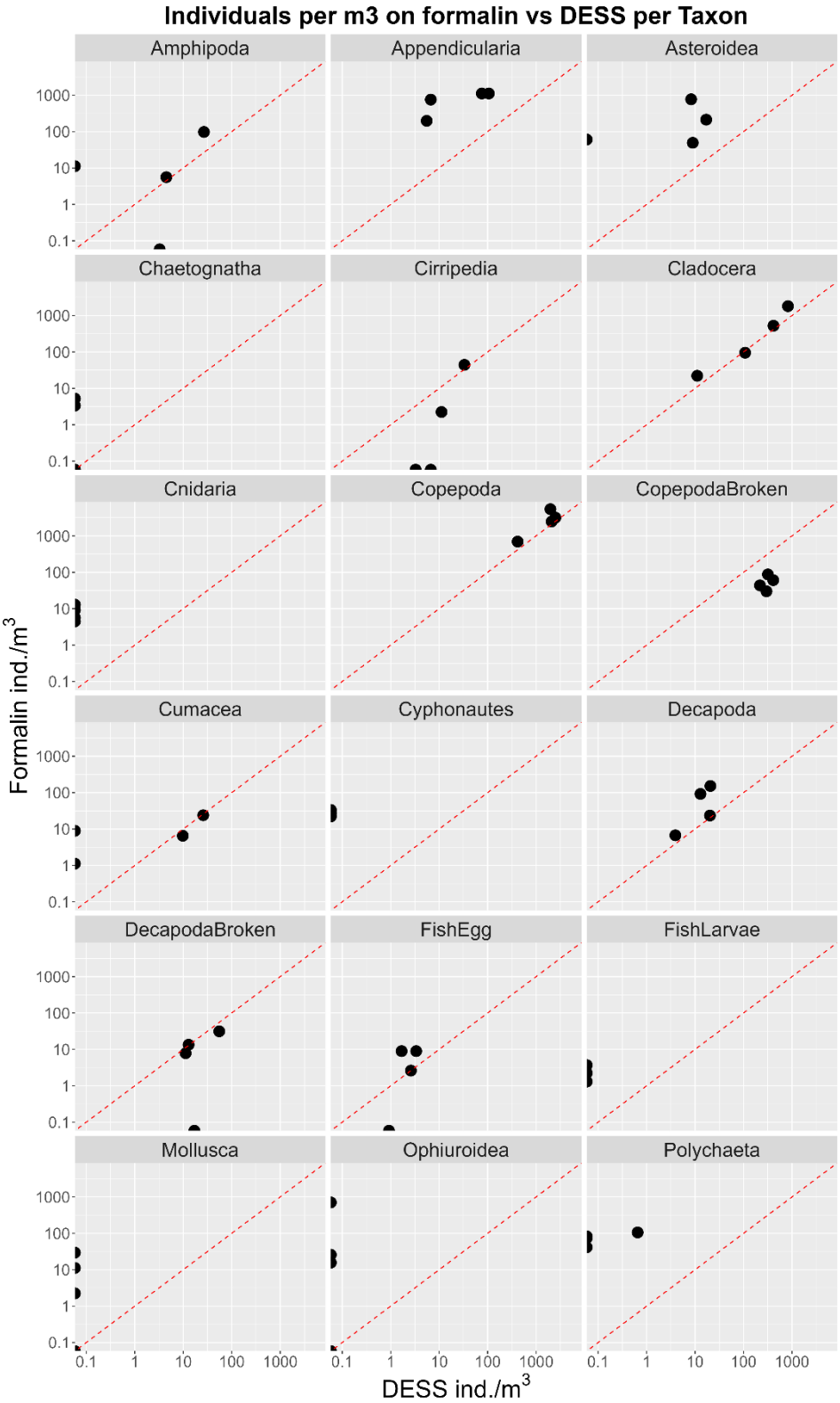

Figure 5 Individuals per m³ on formalin vs DESS per taxon. Black dots indicate the different samples. The dotted red lines are the equality lines, on which taxa would have equal numbers of individuals per preservative.

Table 5 gives the ratio of individuals in DESS relative to formalin, the estimated taxonomic resolution observable organisms can be identified to in DESS and a description of the morphological integrity of observable organisms in DESS. Note that Cirripedia performed better in DESS than in formalin here by chance, due to the low abundance of Cirripedia in samples.

Table 5 An overview of the effectiveness of DESS at preserving morphology of zooplankton taxa. The ratio of individuals in DESS to formalin, the taxonomic resolution identifiable in DESS and a description of morphology in DESS.

Taxon	**Proportion DESS/FORM**	**Taxonomic resolution identifiable in DESS**	**Morphology in DESS**
Appendicularia	0.061	Family or genus	Minor decay or decapitated
Bryozoa (cyphonautes)	0	-	Absent
Chaetognatha	0	-	Absent
Cnidaria	0	-	Absent
Crustacea			
Amphipoda	0.301	Species	Minor to moderate shrinkage of soft tissue, exoskeleton intact
Cirripedia	1.169	Species	Minor shrinkage of soft tissue, exoskeleton intact
Cladocera	0.552	Species	Minor to significant shrinkage of soft tissue, exoskeleton intact
Copepoda	0.592	Species	Minor to significant shrinkage of soft tissue, exoskeleton occasionally in pieces
Cumacea	0.878	Species	Minor to significant shrinkage of soft tissue, exoskeleton intact
Decapoda	0.210	Family, genus or species	Minor to significant shrinkage of soft tissue, exoskeleton often in pieces
Echinodermata			
Asteroidea	0.031	Class	Highly degenerated blobs
Ophiuroidea	0	-	Absent
Ichthyoplankton			
Fish eggs	0.418	Class	Largely dissolved embryos, chorion intact
Fish larvae	0	-	Absent
Mollusca	0	-	Absent
Polychaeta	0.002	Class	Highly degenerated blobs, mucus tubes intact

3.1 Amphipoda

Amphipoda were not abundant in the samples, leading to very inconsistent distributions (Figure 6). The Amphipoda observed in both preservatives were in relatively good shape (Figure 7A-E), with only slightly shrunken soft tissue inside the exoskeletons in DESS (Figure 7C). This is unusual for observed Crustacea as described for other taxa in this study. Considering the well-preserved exoskeleton, including the body segments, limbs and setae, Amphipoda should be identifiable to species level on either preservative.

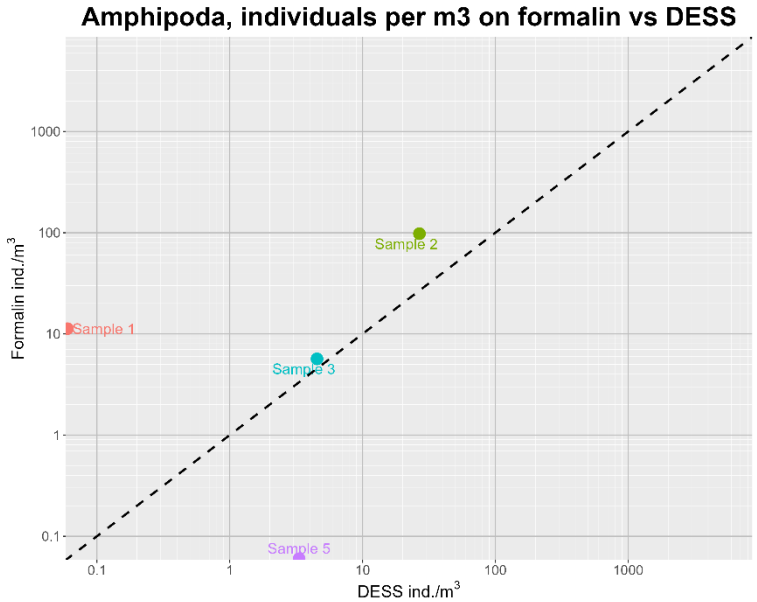

Figure 6 Amphipoda individuals per m³ in each preservative per sample.

Figure 7 Inverted microscope photography and scan images of Amphipoda in the samples. A-B: Amphipoda on formalin; C-E: Amphipoda on DESS.

3.2 Appendicularia

Appendicularia were abundantly found in formalin samples, but certainly not entirely absent in DESS samples (Figure 8). In terms of morphological integrity, the counts of Appendicularia consist of only complete organisms, yet on both DESS and formalin decapitated tails were present, raw counts of which are in Appendix I. Some scan images of complete Appendicularia (Figure 9B,E) and tails (Figure 9C,F) were clearer than others, with unclear images stemming mostly from DESS samples. Although not counted, loose trunks were infrequently observed in formalin and even less in DESS. There was a large variability in the level of decay. Regardless, specimens of a similar high quality were observed in both preservatives (Figure 9A,D). There is, however, some decay visible in the trunk of the specimen on DESS (Figure 9D), possibly complicating accurate identification to species level. Such well-preserved Appendicularia should be identifiable at least to family level.

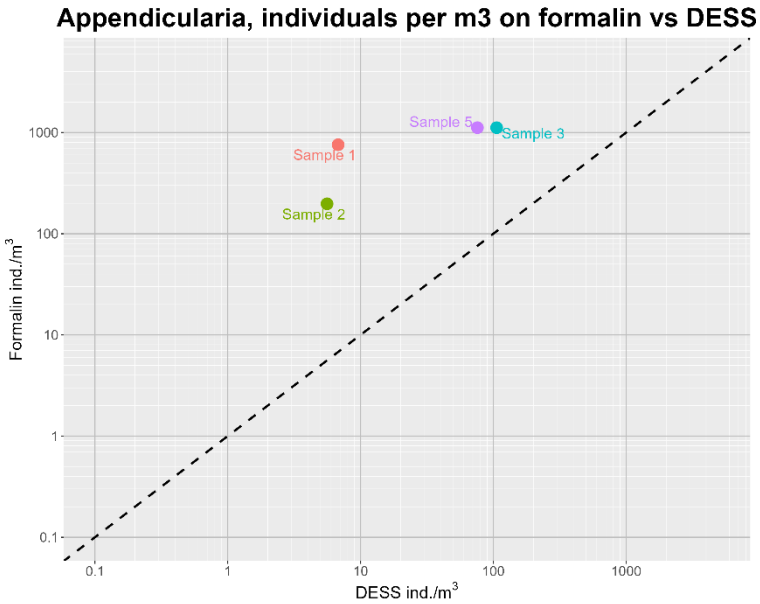

Figure 8 Appendicularia individuals per m^3 in each preservative per sample.

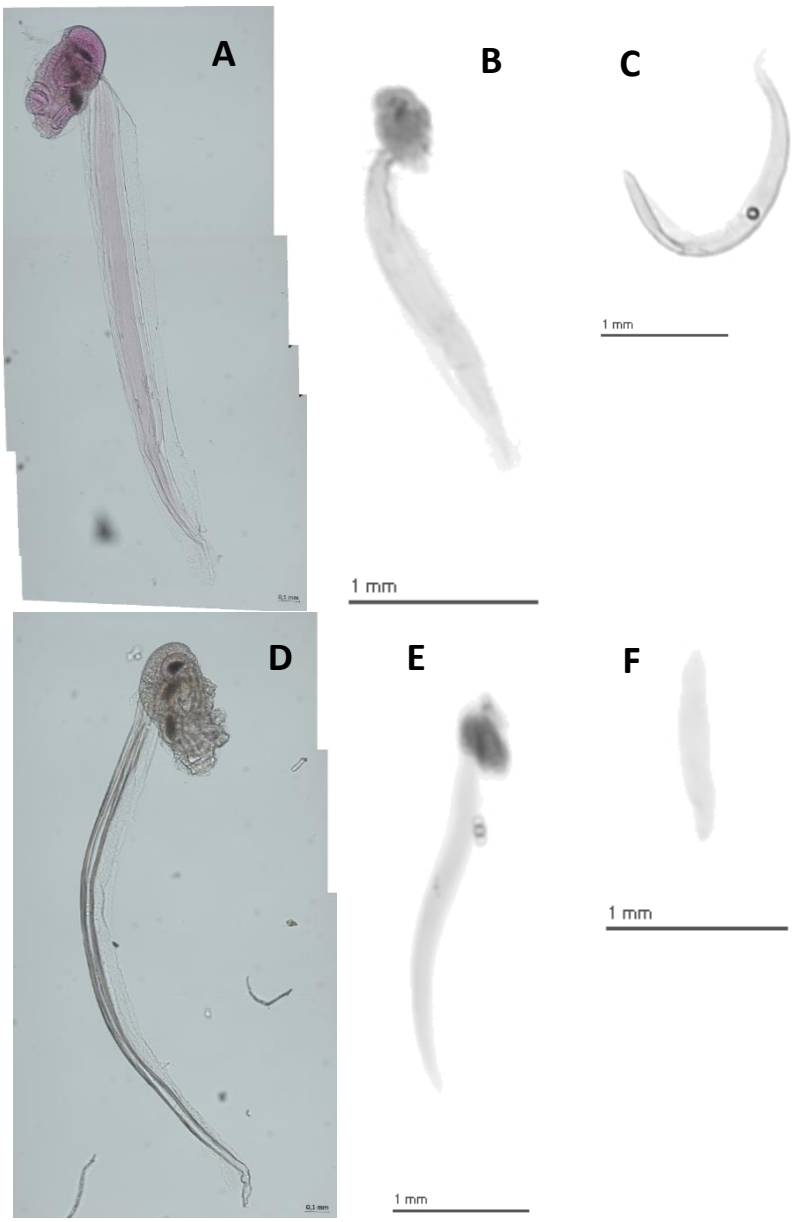

Figure 9 Inverted microscope photography and scan images of Appendicularia in the samples. A-B: Oikopleura sp. on formalin; C: Appendicularia tail on formalin D-E: Oikopleura sp. on DESS; F Appendicularia tail on DESS.

3.3 Asteroidea

Several formalin samples had high abundances of Asteroidea, yet only few could be found in the samples' DESS counterparts (Figure 10). Remains were typically found to be in the middle of disintegration or nearly unrecognizable blobs (Figure 11D-G). In formalin, most numerously, juveniles were seen still carrying their larval brachiolaria bodies (Figure 11A-C). In DESS, brachiolaria were nearly entirely absent, and juvenile bodies were observed to be mostly absent or in the process of breaking down.

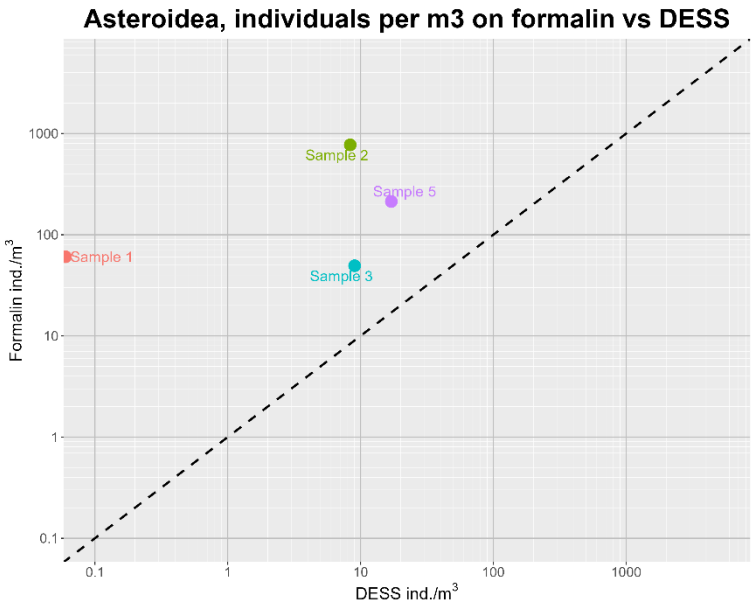

Figure 10 Asteroidea individuals per m^3 in each preservative per sample.

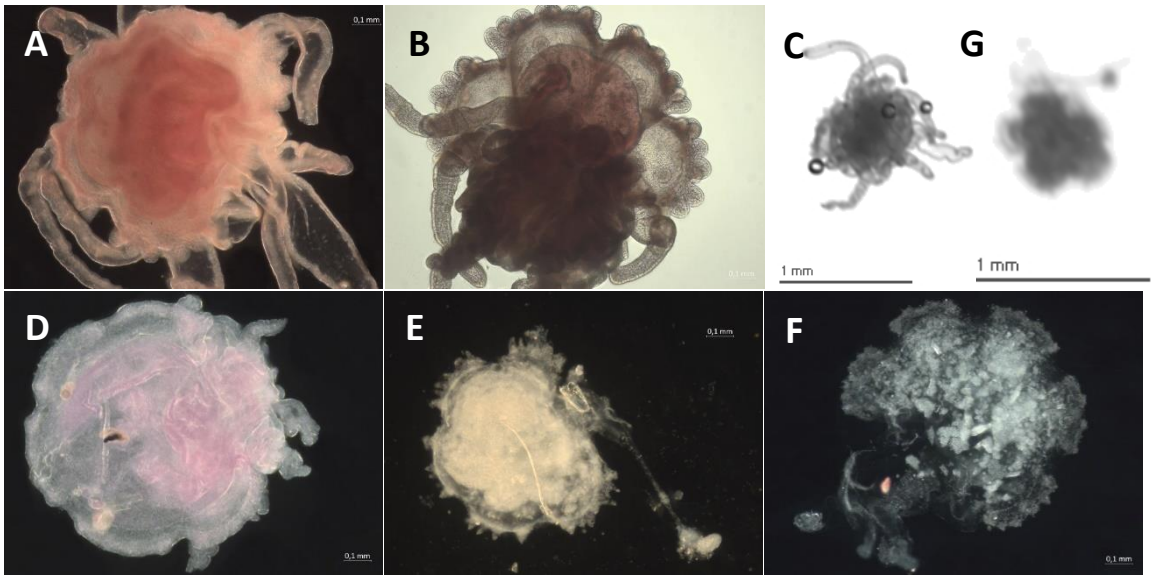

Figure 11 Inverted microscope photography and scan images of Asteroidea in the samples. A-C: Asteroidea on formalin, with developing juvenile body and the remaining Brachiolaria attached intact; D-F: gradations of deterioration seen in bodies of Asteroidea on DESS; G: scan image of Asteroidea on DESS.

3.4 Chaetognatha

In contrast with their relative abundance in formalin samples, Chaetognatha were entirely absent in DESS samples (Figure 13). Their soft bodies were well preserved in formalin, with defining features still discernable (Figure 12A-B). Given their large size, they also appeared clearly on the scans (Figure 12C).

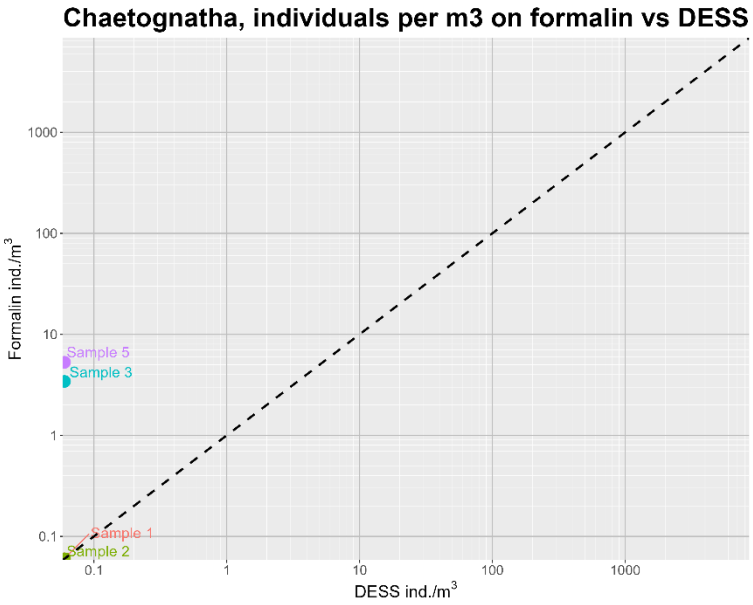

Figure 13 Chaetognatha individuals per m^3 in each preservative per sample.

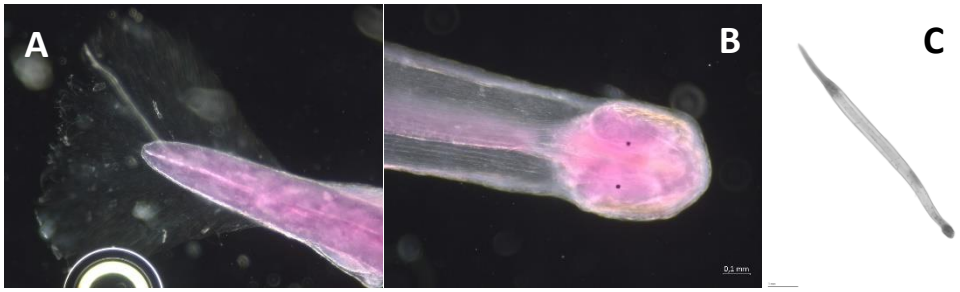

Figure 12 Inverted microscope photography and scan images of Chaetognatha in the formalin samples. A: tail fin; B: head; C: scan image.

3.5 Cirripedia

Observations of Cirripedia were limited in the samples, providing unbalanced results (Figure 14). This class includes nauplii and cyprids, both of which were seen in mostly good condition in both formalin and DESS (Figure 15). Shrinkage of soft tissue in DESS was minimal and exoskeletons surrounding it were typically unscathed. This holds true for both the nauplii and cyprid stages. With exoskeletons remaining of high quality, nauplii can still be distinguished from other taxa by observing their fronto-lateral horns. Naupliar stages and species can also be identified through the observation of shapes and lengths of parts and counts of setae, all features visible in the exoskeleton.

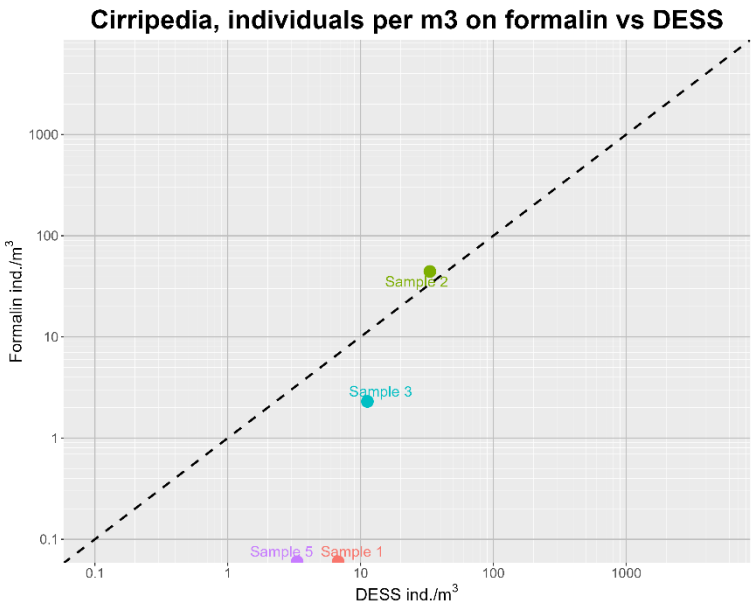

Figure 14 Cirripedia individuals per m^3 in each preservative per sample.

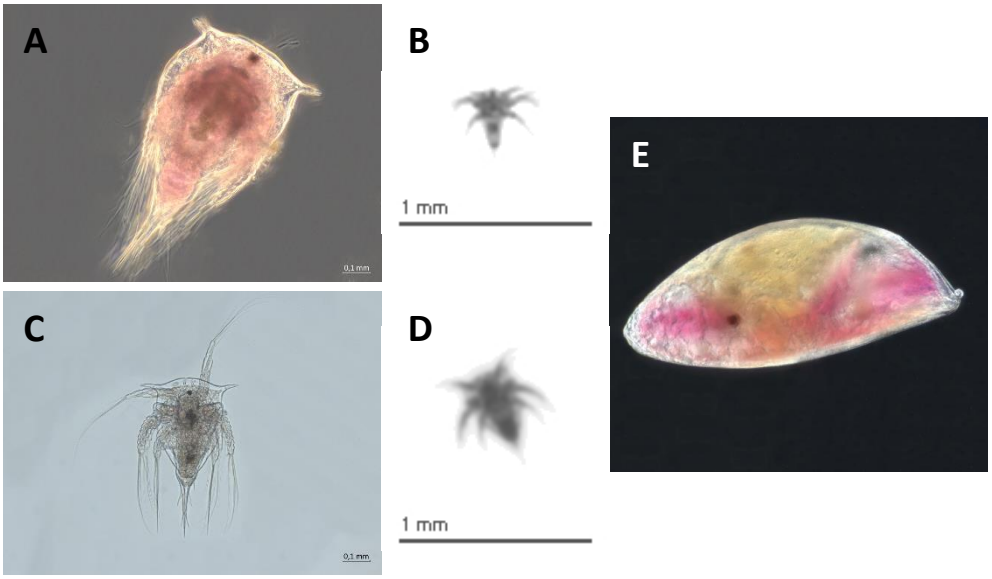

Figure 15 Inverted microscope photography and scan images of Cirripedia. A-B: Cirripedia nauplii on formalin; C-D: Cirripedia nauplii on DESS; E: Cirripedia cyprid on DESS.

3.6 Cladocera

Cladocera performed very well on DESS relative to formalin. Similar numbers of individuals were identifiable in each sample (Figure 16). While there was still significant shrinking of soft tissue within exoskeletons in DESS (Figure 17D-F), specimens were still easily identifiable as Cladocera and to family level, probably just as well as specimens in formalin (Figure 17A-C). Positive identification to species level requires counts of setae on thoracopods which should also be possible considering the good preservation of exoskeletons (Figure 17G). Even embryo baring females were observed in DESS samples (Figure 17H).

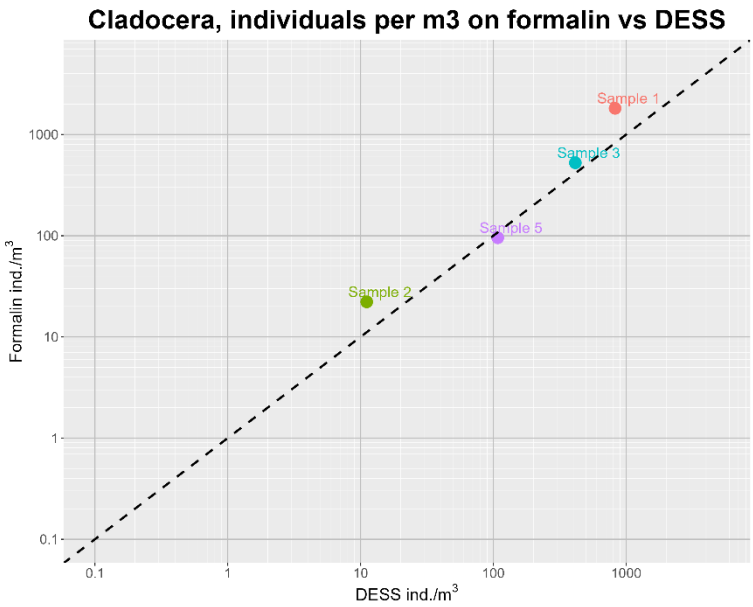

Figure 16 Cladocera individuals per m³ in each preservative per sample.

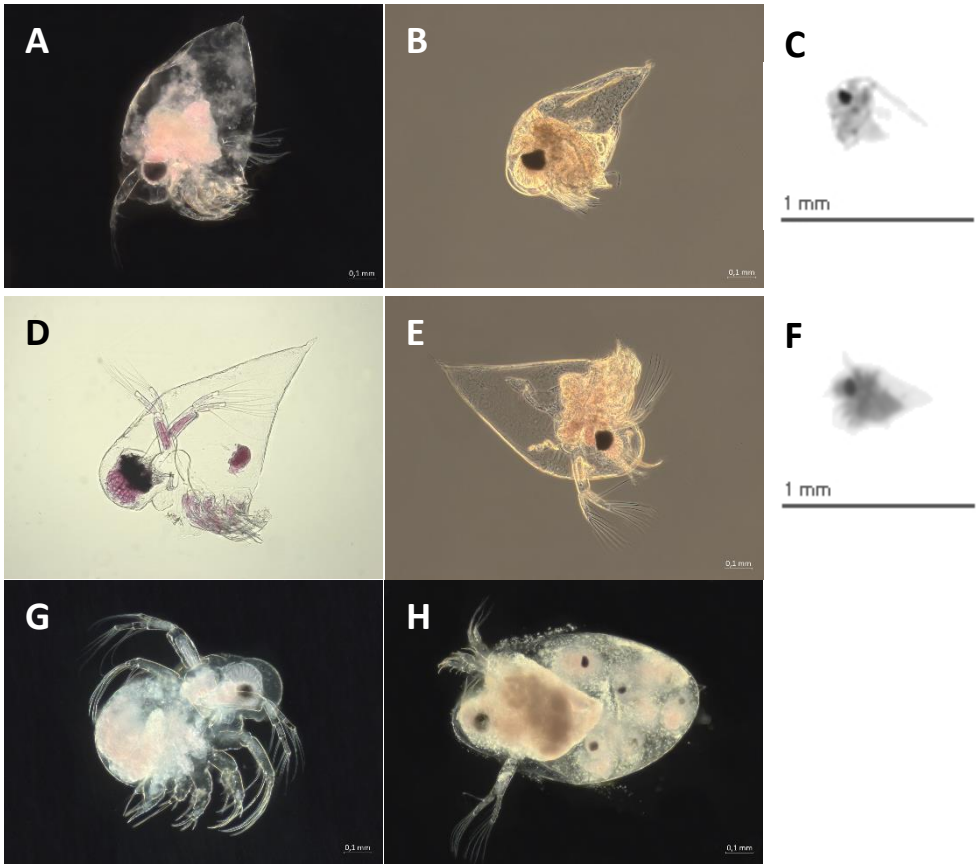

Figure 17 Inverted microscope photography and scan images of Cladocera. A-C: Evadne on formalin; D-F: Evadne on DESS; G: Podon on DESS; H: Cladocera baring embryos on DESS.

3.7 Cnidaria

Cnidaria were scarcely found in formalin and absent in DESS (Figure 18). Regardless of their soft gelatinous bodies, most were observed to be in good condition in formalin (Figure 19A-E).

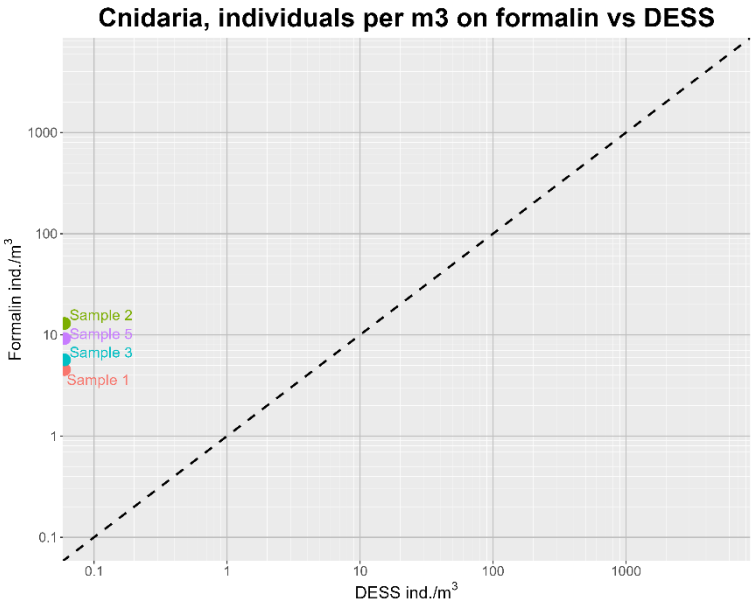

Figure 18 Cnidaria individuals per m³ in each preservative per sample.

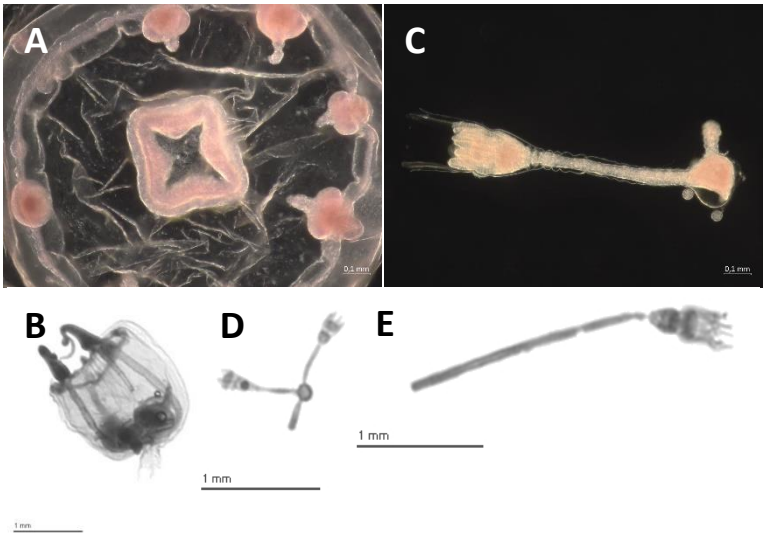

Figure 19 Inverted microscope photography and scan images of Cnidaria on formalin. A-B: Cnidaria spp.; C-E: Leptothecata.

3.8 Copepoda

Copepoda were preserved quite well in DESS according to Figure 20, although there is no distinction between levels of deterioration, of which there was great variation (Figure 21A-D). While some specimens had nearly the quality of preservation that formalin specimens had, the soft tissue inside copepods was most often shrunken greatly in DESS. Many broken parts of copepods were also found in DESS samples (Figure 21E-H). A commonly observed breaking point was between the cephalosome and the metasome. Regardless of shrinking and apart from the broken parts, complete exoskeletons were preserved well. Without the soft tissue inside, it should still remain possible to identify copepods to species level. A very small and often essential part for identification is the fifth pair of legs (P5), which, like other parts of the exoskeleton, remained intact and held its diagnostic characteristics in both preservatives regardless of shrinkage in DESS. This is exemplified by the comparison of *Temora longicornis* on formalin and DESS in Figure 22. Lastly, smaller limbs appeared to be less susceptible to diminishing soft tissue than the larger bodies.

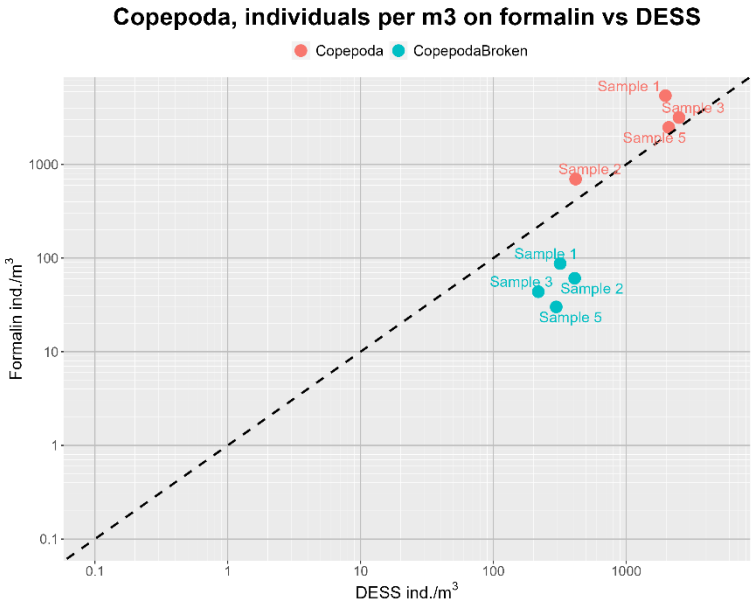

Figure 20 Copepoda individuals and broken parts per m^3 in each preservative per sample.

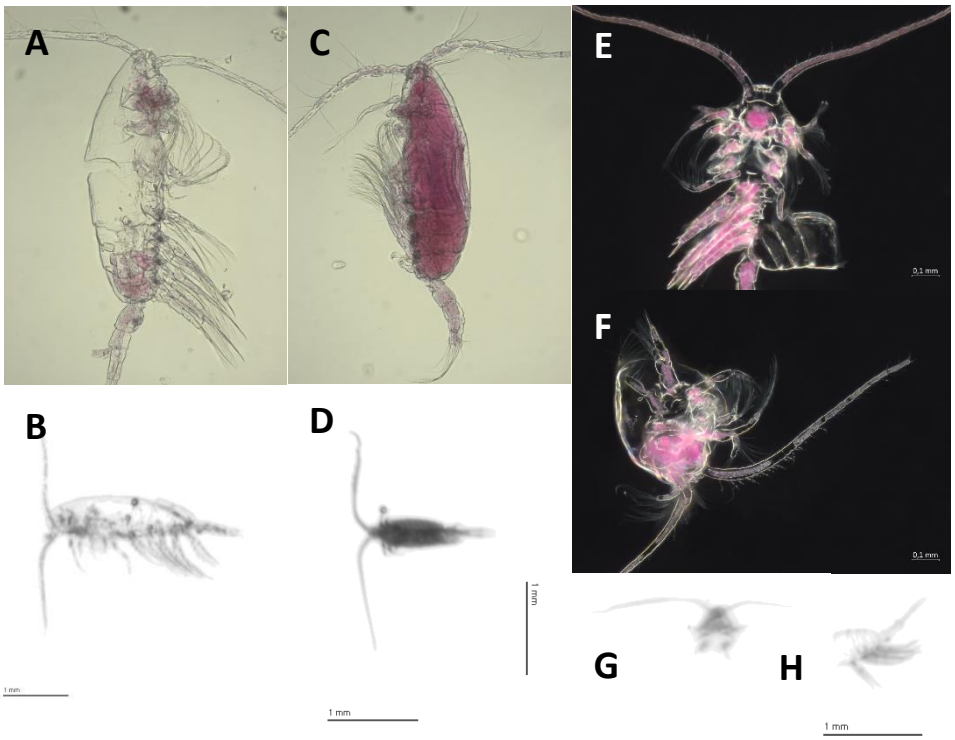

Figure 21 Inverted microscope photography and scan images of Copepoda on DESS. A-B: soft tissue largely reduced; C-D: soft tissue mostly preserved; E-H: broken parts.

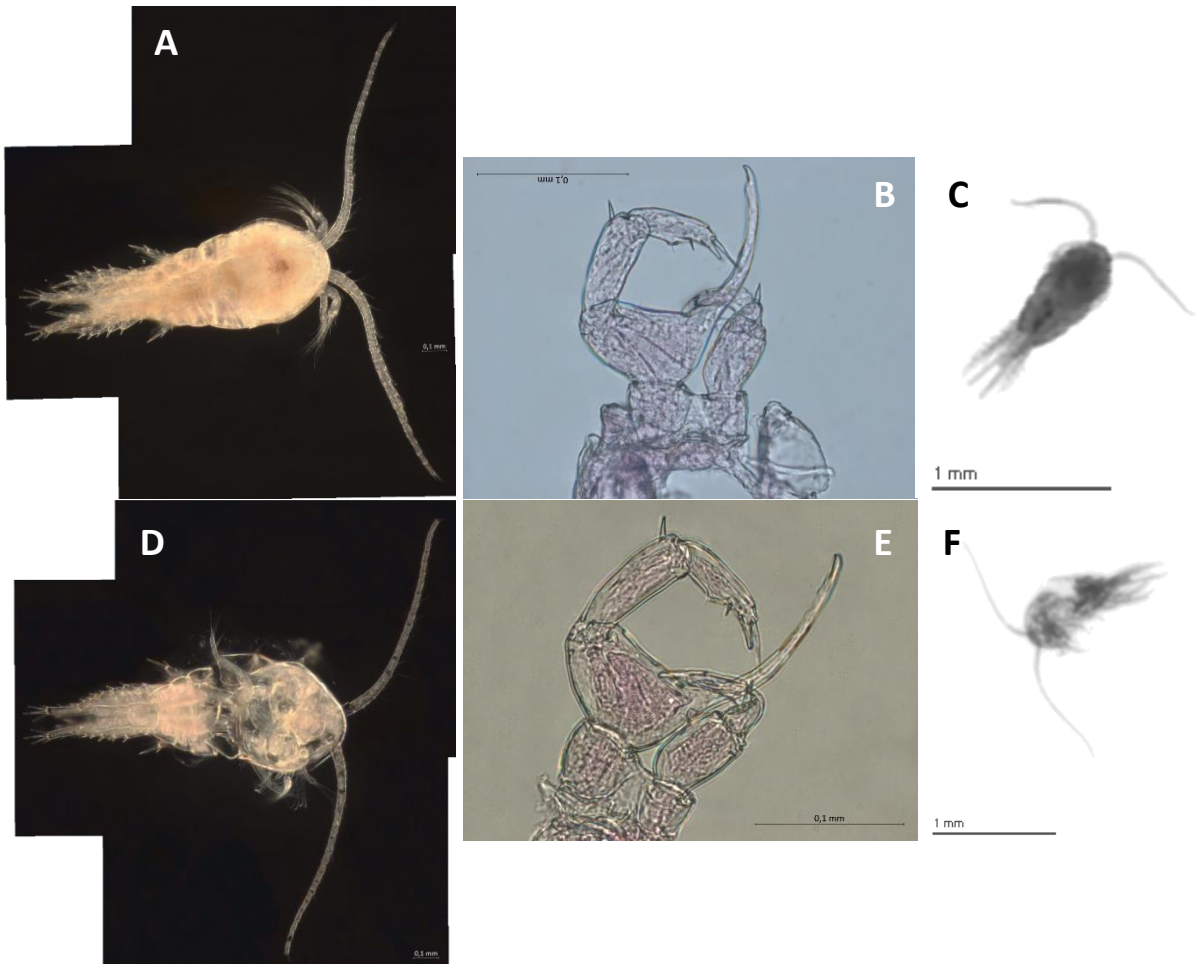

Figure 22 Inverted microscope photography and scan images of Temora longicornis in the samples. A-C: T. longicornis photo, P5 and scan image on formalin, respectively; D-F: T. longicornis photo, P5 and scan image on DESS, respectively.

3.9 Cumacea

Cumacea were preserved relatively well in DESS, although observations were limited in both preservatives (Figure 23). Cumacea in formalin were mostly unscathed (Figure 24A-B). Like in other *Crustacea*, shrinking of soft tissue was common in Cumacea in DESS, although the level of which varied greatly among individuals. Reduction ranged from hardly visible to nearly entirely void of soft tissue (Figure 24C-E). Again, however, the exoskeletons of Cumacea were typically free of decay and fragmentation. With exoskeletons remaining preserved, it should be possible to identify Cumacea to species level in DESS.

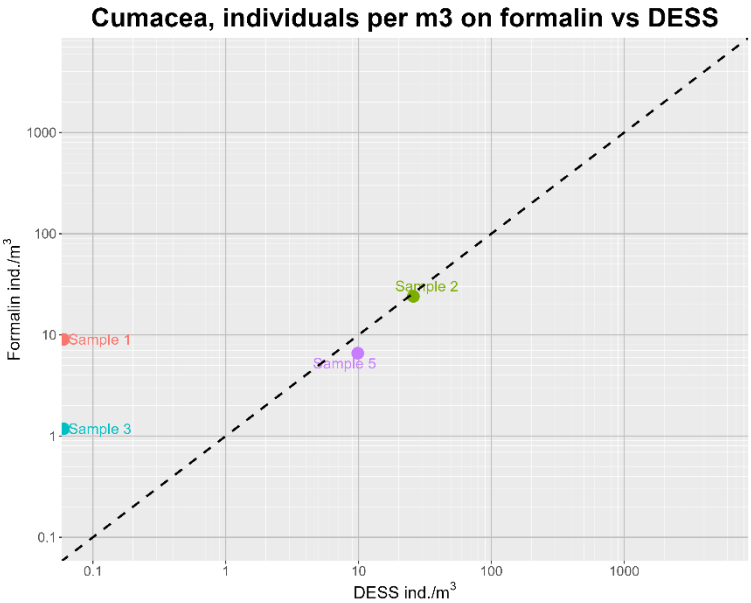

Figure 23 Cumacea individuals per m³ in each preservative per sample.

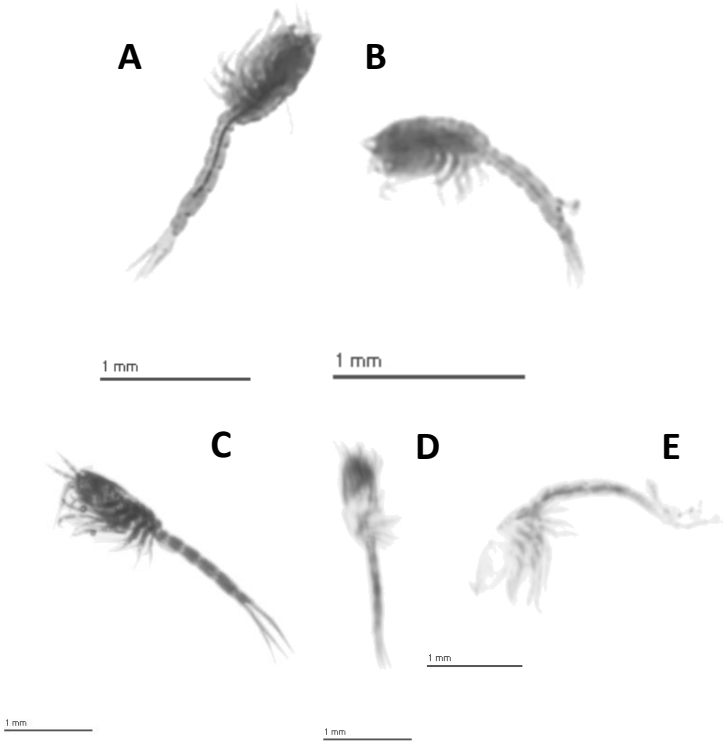

Figure 24 Scan images of Cumacea. A-B: Cumacea on formalin; C-E: gradations of tissue reduction in Cumacea on DESS.

3.10 Cyphonautes

Cyphonautes, bryozoan larvae, were present and well preserved in all formalin samples while entirely absent in DESS samples (Figure 25). In both photography and scans cyphonautes were well recognizable in formalin (Figure 26).

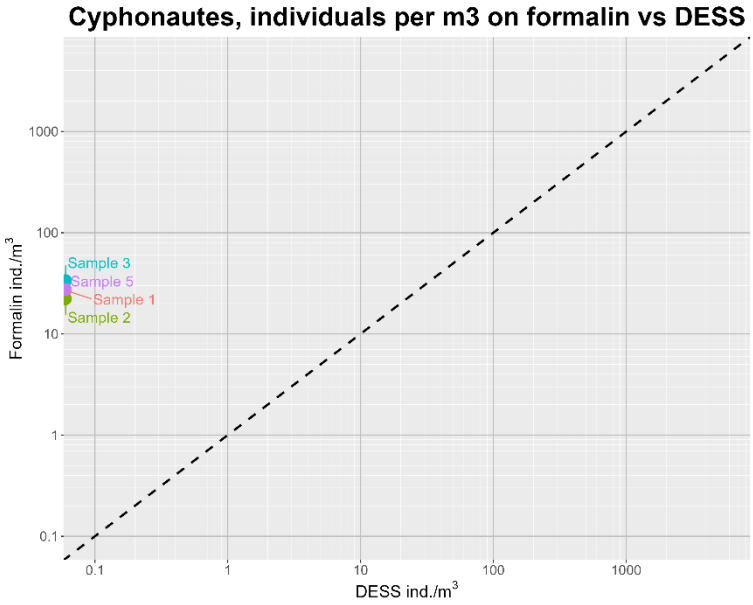

Figure 25 Cyphonautes individuals per m^3 in each preservative per sample.

Figure 26 Inverted microscope photography and scan image of cyphonautes in the samples.

3.11 Decapoda

DESS effectively preserved the exoskeletons of Decapoda, yet formalin performed better (Figure 27). Decapoda in formalin exhibited little to no shrinkage of soft tissue (Figure 28A,C,E), while tissue shrank and dissolved most obviously in Decapoda in DESS (Figure 28B,D,F). Bodies were largely deprived of soft tissue, while appendages displayed the ongoing shrinkage and loss of soft tissue (Figure 29A-B). There was an abundance of broken off pereopods and eyes of Decapoda (Figure 29C-F), which was also seen in formalin samples to a lesser extent. Brachyura zoea and megalopa were typically very fragile, nearly always missing appendages and the majority of soft tissue. Identification to species level can be difficult due to commonly missing body parts.

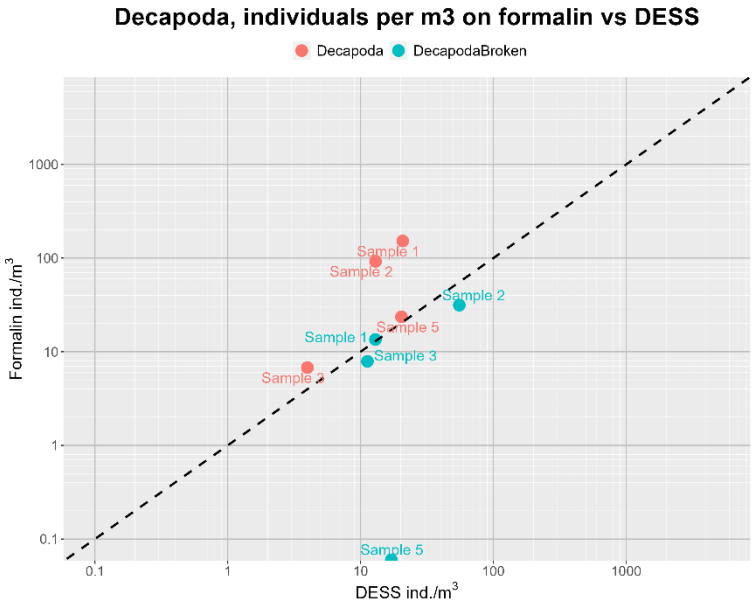

Figure 27 Decapoda individuals and broken parts per m^3 in each preservative per sample.

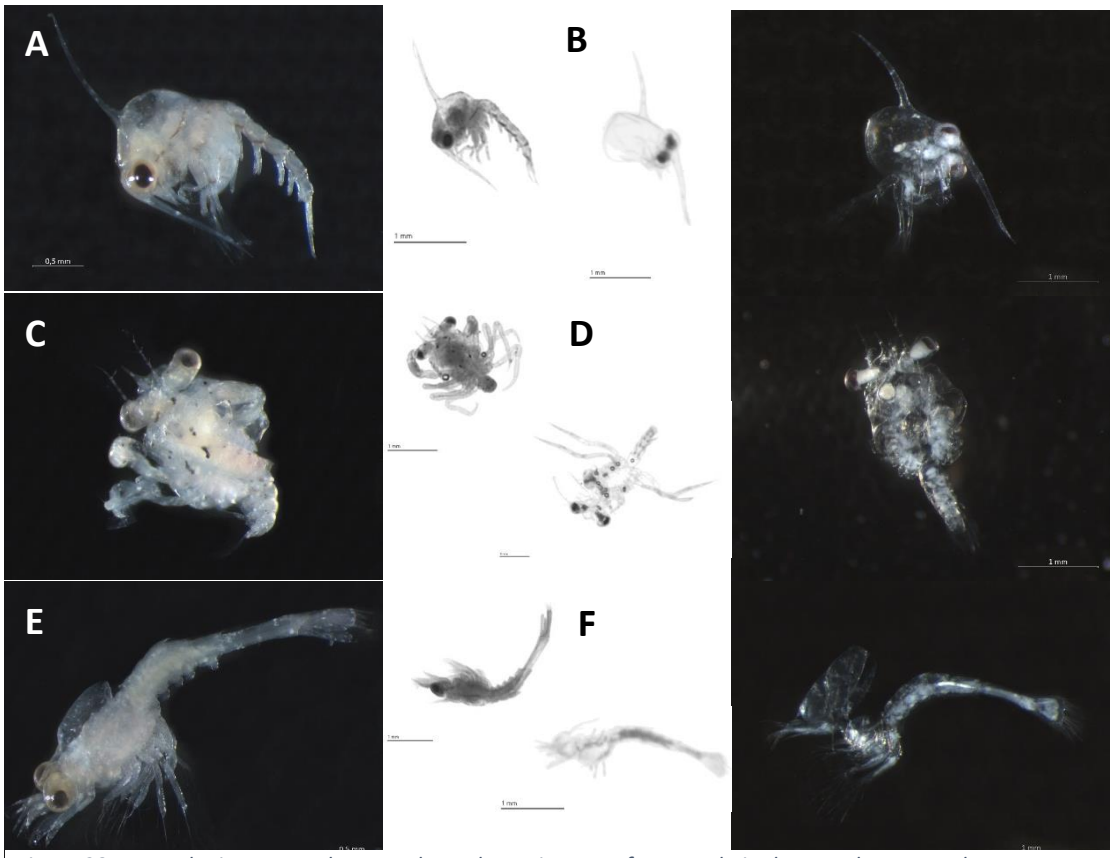

Figure 28 Inverted microscope photography and scan images of Decapoda in the samples. A: Brachyura zoea on formalin; B: Brachyura zoea on DESS; C: Brachyura megalopa on formalin; D: Brachyura megalopa on DESS; E: Caridea zoea on formalin; F: Caridea zoea on DESS.

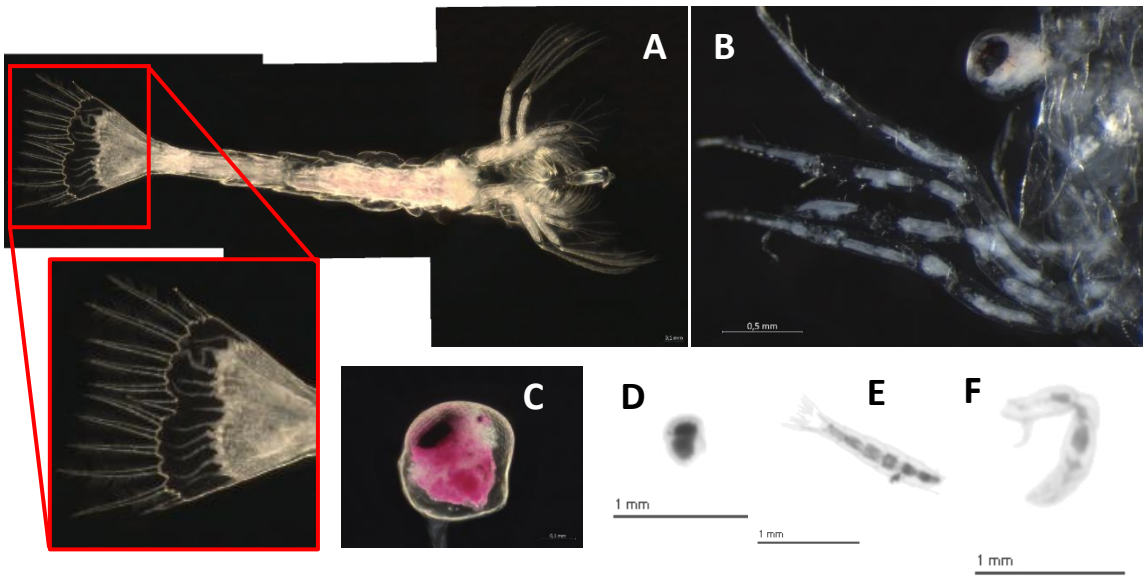

Figure 29 Inverted microscope photography and scan images of Decapoda soft tissue and broken parts in DESS. A: Caridea with shrunken soft tissue out of telson spines; B: shrunken soft tissue in pereopods of Brachyura megalopa; C-F: broken parts of Decapoda.

3.12 Ichthyoplankton

Ichthyoplankton in samples were most commonly fish eggs. Some fish larvae were observed, but only in the formalin samples. Fish eggs were observed in formalin and DESS (Figure 30). Although the insides were always heavily decayed in DESS. The fish embryos inside seemingly dissolved into the entirety of the eggs (Figure 31E-F), deeming them unrecognizable. In formalin samples, fish larvae in and out of eggs kept their shape (Figure 31A-D), potentially remaining identifiable to a certain taxonomic level, although shrinkage and loss of pigmentation may complicate this. In DESS, fish eggs shells seemingly did not decompose, possibly allowing for accurate counts of eggs.

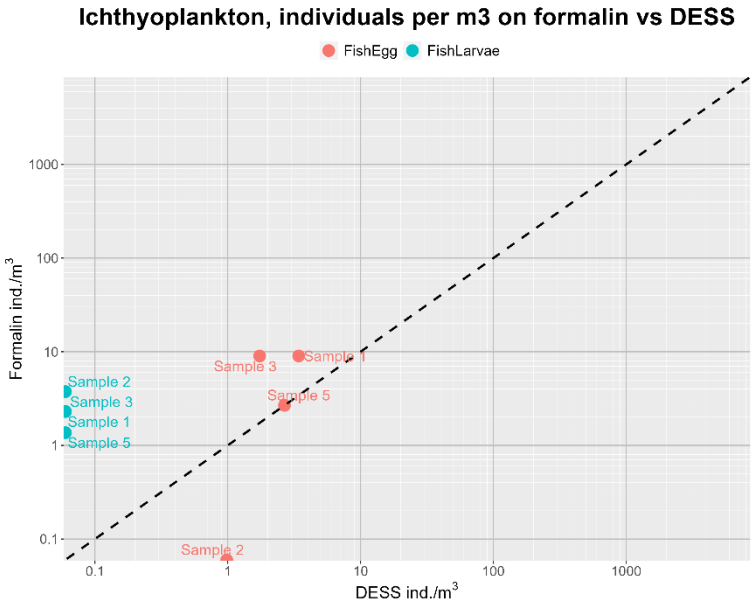

Figure 30 Ichthyoplankton egg and larvae individuals per m³ in each preservative per sample.

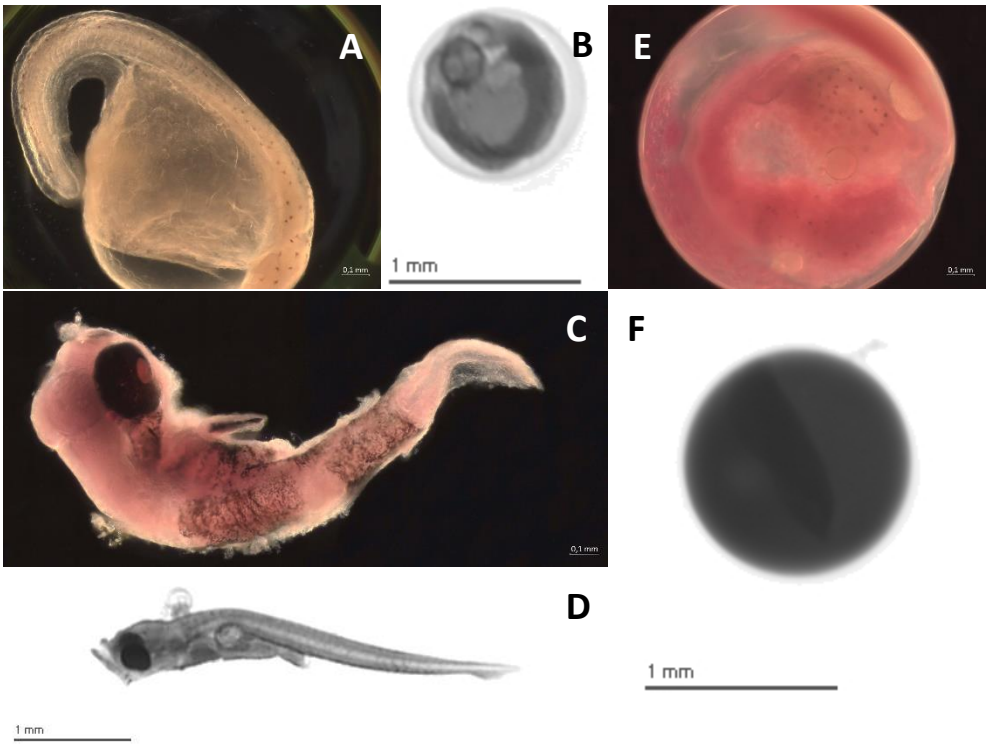

Figure 31 Inverted microscope photography and scan images of Ichthyoplankton in the samples. A-B: fish eggs on formalin; C-D: fish larvae on formalin; E-F: fish eggs on DESS.

3.13 Mollusca

Mollusca were merely observed in formalin samples (Figure 32). With few exceptions, all were bivalve larvae. Mollusc taxa were discernible in photography and scan imagery alike (Figure 33).

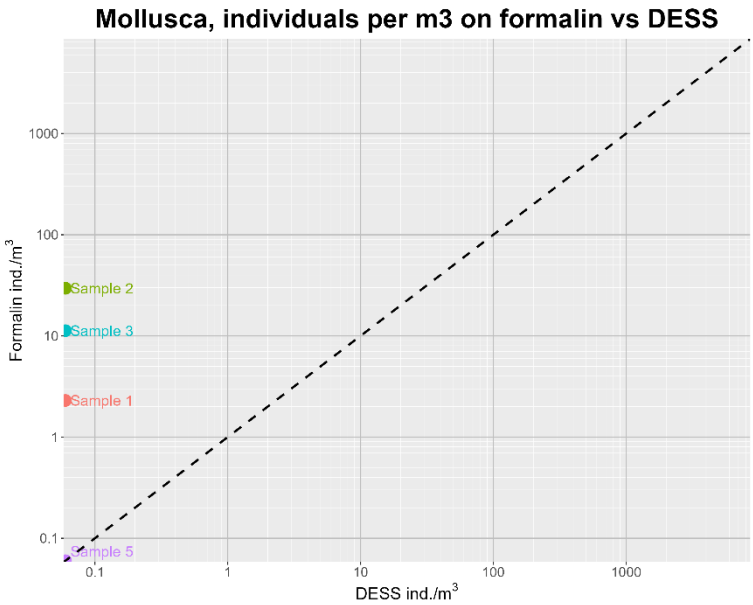

Figure 32 Mollusca individuals per m^3 in each preservative per sample.

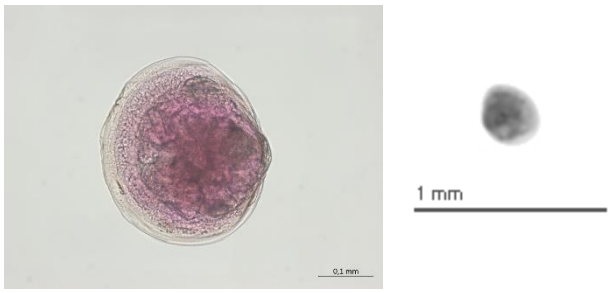

Figure 33 Inverted microscope photography and scan image of Bivalvia in the samples.

3.14 Ophiuroidea

There were no observations of Ophiuroidea in DESS, while they were abundantly present in FORM, namely in sample 3 (Figure 34). The majority of observations were pluteus larvae that remained largely intact (Figure 35A). Although small and with thin features, they were still visible on the scans (Figure 35D). Formalin also contained some well-preserved post-metamorphosis Ophiuroidea (Figure 35B-C).

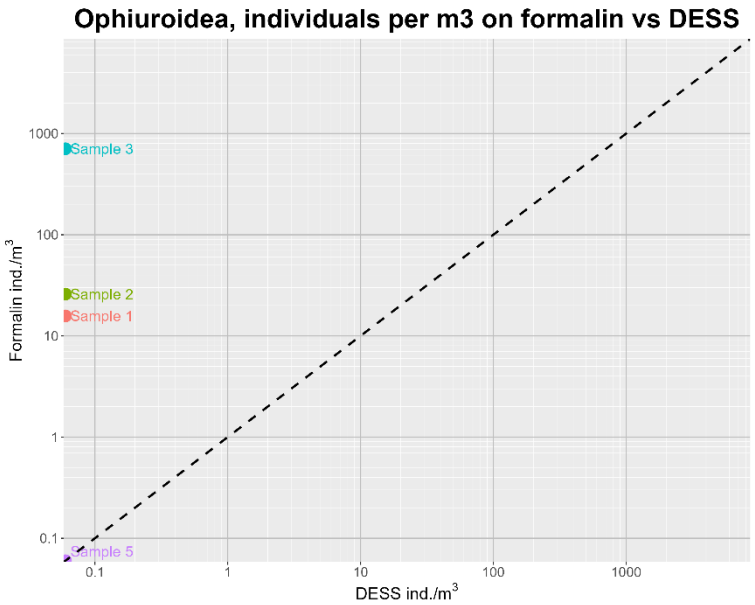

Figure 34 Ophiuroidea individuals per m^3 in each preservative per sample.

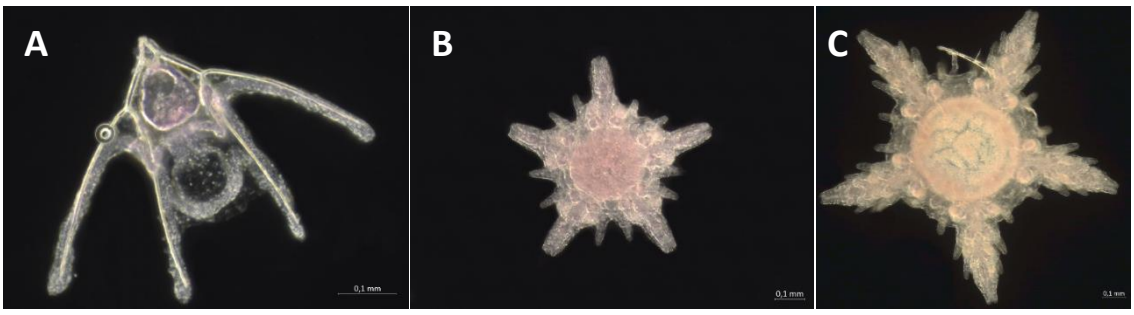

Figure 35 Inverted microscope photography and scan images of Ophiuroidea in the formalin samples. A: mostly undamaged Ophiopluteus; B-C: post-metamorphosis Ophiuroidea; D: scan image of Ophiuroidea.

3.15 Polychaeta

Polychaeta were seen in multitudes in formalin samples, with their entire body morphology seemingly unharmed. In DESS samples Polychaeta were a rare find (Figure 36). Remains found were only recognizable as Polychaeta due to the mucus tube surrounding them (Figure 37A-B), meaning non tube-dwelling Polychaeta were either entirely dissolved or simply no longer recognizable as such (Figure 37C-D). In formalin, several taxa within polychaeta could be recognized (Figure 38).

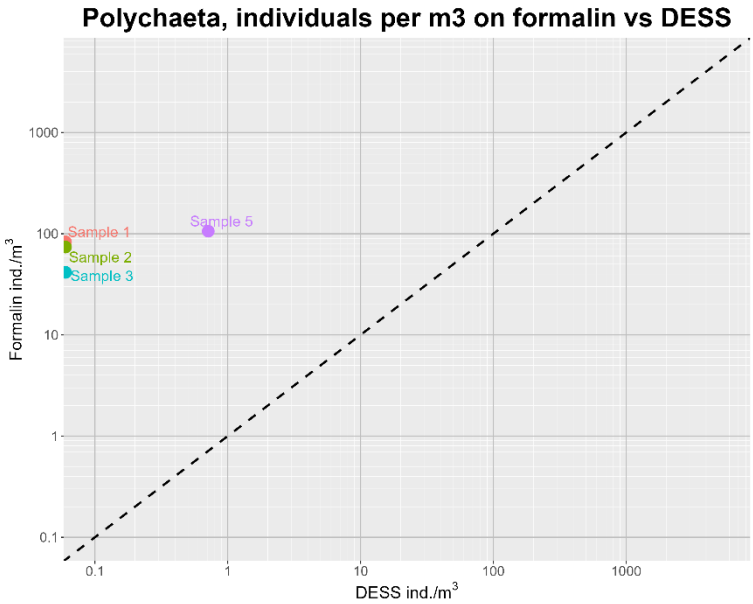

Figure 36 Polychaeta individuals per m^3 in each preservative per sample.

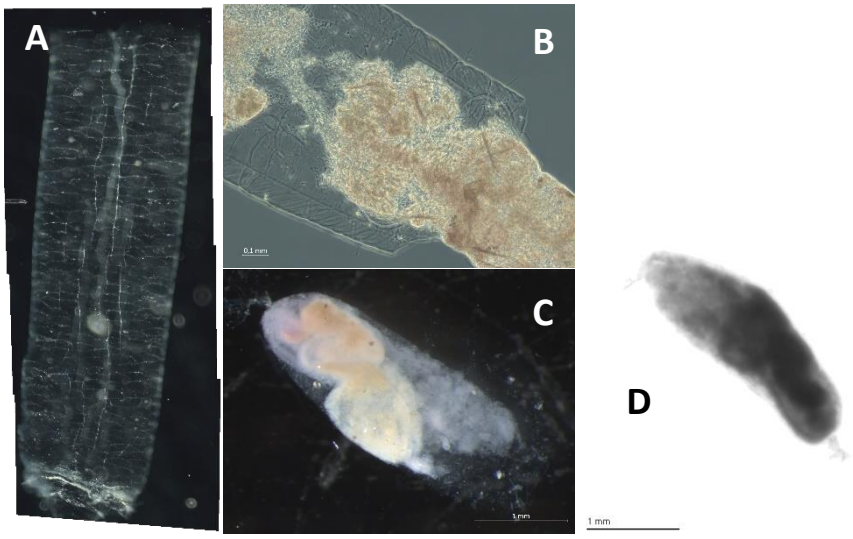

Figure 37 Inverted microscope photography and scan images of Polychaeta on DESS. A-B: Polychaete tubes, with remains in B; C-D: Unknown remains of a Polychaeta.

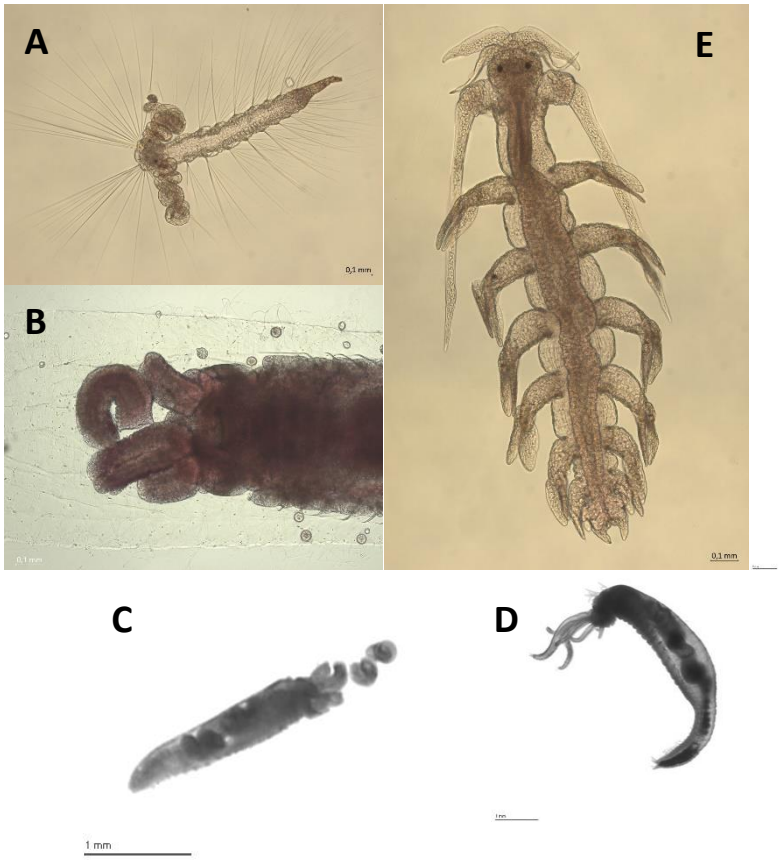

Figure 38 Inverted microscope photography and scan images of Polychaeta on formalin. A: Magelona sp.; B-D: Polychaete tube worm aulophora; E-F: Tomopteris sp.

4. Discussion

The image analysis showed varying levels of damage to the preserved organisms in both formalin and DESS. The little deterioration that was seen in formalin samples as compared to DESS can likely be attributed to the sieving process at sampling stations and in the laboratory, during which samples are rinsed and poured into a scanning plate. Preserved organisms may experience some level of force that causes them to collapse and break.

The minor differences found in ellipsoidal area of Copepoda data between formalin and DESS may be explained by scan image quality. With soft tissue decay and shrinkage inside Copepoda exoskeletons, the quality of scan imagery worsens as well. Less soft tissue results in a more transparent exoskeleton. This transparency can cause issues in the flatbed scanners performance and output such as the occurrence of noise, blur, distortion and other image artifacts. Ultimately, this may lead to wrong estimations of size. In the comparison of all taxa combined, considering soft-bodied taxa typically dissolved entirely in DESS solution, shrinkage of these organisms did not result in large differences of ellipsoidal areas between preservatives as they could not be measured. As samples are dominated by Copepoda and other Crustacea, the combined taxa largely follow the same trends as the isolated data of Copepoda.

Relative to other Crustacea, Amphipoda, Cumacea and Cirripedia had low abundance in the samples giving way to pure chance in the comparison of formalin and DESS. Had there been more in the samples, a trend may have been observable as it was for Copepoda and Decapoda. However, it appeared that soft tissue inside Amphipoda remained preserved better than that of other Crustacea in DESS. Planktonic crustaceans typically have thin exoskeletons with prolonged limbs, deeming them vulnerable in samples (Omori & Fleminger, 1985). In all Crustacea, chitin skeletons did not seem to decompose at all, although soft tissue inside Copepoda and Decapoda exoskeletons was typically shrunken greatly. With soft tissue diminishing the stability of the exoskeleton decreases, likely causing them to break into pieces during sieving. A common breaking point in Copepoda was between the cephalosome and the metasome. In Decapod megalopa and zoea limbs separated from the body, resulting in large amounts of loose pereopods and eyes in DESS samples. Loose parts were also seen in formalin samples presumably due to the toll sieving takes on delicate bodily connections. Crustacea shed their skin as they grow or as they advance to a subsequent stage in their life cycle and there was also some dubious scan imagery of DESS samples due to the transparency of crustacea caused by the lack of soft tissue. Due to this, counts of crustacea in this DESS samples may contain some discrepancies. The bodies of Amphipoda are highly segmented, offering some flexibility perhaps contributing to their robustness post-preservation. According to Baas, et al. (1995), chitin is highly resistant to decay, suggesting why crustacean exoskeletons are so effectively preserved relative to other taxa. Regardless of the observed shrinking or dissolving soft tissue, chitin exoskeletons usually remain with defining features intact, such as setae on appendages, body shape, segmentation, and shapes and sizes of appendages like the maxilla, maxilliped and fifth pair of swimming legs (Castellani & Edwards, 2017). If a research discipline does not require more pristine specimens, DESS could be a viable option for preservation of most crustaceans. In some cases, it will be necessary to account for the common separation of appendages from bodies of Copepoda and Decapoda. Exoskeletons of Cladocera and Cirripedia larvae are not as segmented as those of Copepoda, which may be part of the reason for their complete exoskeletons in DESS. With their small sizes and appendages, they may not be as susceptible to breaking points as some other Crustacea.

Considering the soft gelatinous structure of Appendicularia (Jaspers, et al., 2023), the trunk and tail of the animal must easily separate during the sieving process, especially when a preservative such as DESS

poorly protects an organism from decay. In preserved Appendicularia specimens, the tails of are often in better conditions than the trunks (Fenaux, 1985), which was valid here for specimens in formalin and DESS. Laakmann & Holst (2014) described the morphology of hydromedusae in DESS to lose structure completely and nearly dissolve, in contrast with specimens in 4% formaldehyde, which maintained body structures and morphological characteristics. Hydromedusae or any other Cnidaria species were also entirely absent in the samples described in this study. Cnidaria largely consist of mesoglea, a highly water-based substance giving them their soft, fragile, gelatinous structures (Sarras, et al., 1991). Mesoglea has long been a challenging obstacle to biologists in the fixation of gelatinous zooplankton (Haddock, 2004). Gelatinous zooplankton can be challenging to preserve morphologically (Mitchell, Edgar, & Martindale, 2021), so it is not unexpected that taxa such as Cnidaria, Chaetognatha and Bryozoa break down and essentially disappear from samples. It is surprising that relatively large numbers of Appendicularia were still found.

Fish larvae were not preserved in DESS, yet fish eggs had relatively good results. After fertilization of eggs, the soft chorion, essentially a protective membrane, hardens. Chorion hardening transforms the fish egg into a sturdy, chemically resistant structure (Ohtsuka, 1960). This may be reason for the preservation of fish eggs in DESS samples.

Given the similarities that polychaeta have with nematodes, one might expect similar results when preserved with DESS. In this study, polychaeta were poorly preserved on DESS, with only minor traces of them found, while Yoder, et al. (2006) reported DESS to be an effective preservative of nematode morphology. Hence, one should consider how components differ among the groups. Nematodes have a cuticle that functions as a protective layer, which consists of extensively crosslinked collagen-like proteins (Johnstone, 1994), perhaps the crosslinked proteins offer nematodes some additional protection from degradation in preservation. Many polychaeta, however, also have heavily collagen-based cuticles (Hausen, 2005). Perhaps the cuticle is not well-developed enough for robust protection in larval stages.

Unlike the larval stages of Ophiuroidea and Echinoidea, Asteroidea larvae are soft bodied, lacking calcareous skeletons (McEdward & Janies, 1993). They typically do not form an endoskeleton until reaching the point of metamorphosis. Perhaps early juvenile Asteroidea were still present (although rarely) due to a level of robustness owing to the starting development of a calcium carbonate skeleton. The calcium carbonate shells of gastropods were not present in DESS, however, and expected would be for at least some pluteus larvae to have remained observable in DESS sample 3, considering the copious amounts of pluteus larvae seen in its corresponding formalin sample. Pluteus larvae are smaller and thinner, which is reason to believe they may be subject to more rapid deterioration. Later stages of Ophiuroidea were not commonly found in formalin samples (table x) and therefore expectedly entirely absent in DESS samples.

EDTA is a chelating agent meaning it forms bonds with metal ions. It binds calcium most effectively. This can influence the speciation of metals in solutions. Perhaps EDTA chelation destabilizes select skeletons such as the calcium carbonate skeleton in Echinodermata formed by calcium and magnesium, both key components in the taxa (Dubois, 2014). However, Motekaitis & Martell (1987) found in experiments that EDTA has little effect on speciation of calcium and magnesium among other metal ions, and solid calcium carbonate is not dissolved by conversion to EDTA complexes. It is noteworthy that in their study, concentrations of EDTA used were orders of magnitude much lower. DMSO is a penetrative substance potentially too severe in the preservation of not only Echinodermata but also other zooplankton due to their small size and fragility. It is commonly used as a solvent for chemical reactions, potentially even promoting the natural degradation of organisms. High NaCl concentrations may be problematic by forming salt crystals in the samples and within tissues of

organisms. Salt crystals were in fact observed in abundance in some of the samples. Even in DNA preservation not all DESS ingredients may be necessary. Sharpe et al. (2020) tested variations of DESS for their effectiveness preserving high molecular weight DNA and concluded DMSO and NaCl may be mostly redundant components, stating that EDTA was the only contributor to DNA preservation of three species representing molluscs, arthropods and annelids.

Considering DMSO is used as a penetrative substance to aid in rapid transport of molecules into tissue it may also not be necessary in small animals such as the majority of zooplankton. NaCl may be in the solution because it has the ability to inhibit microbial growth (Li, et al., 2021), yet precipitation caused by the salt may be problematic for the recovery of small organisms in samples (Sharpe, et al., 2020). EDTA may inhibit damage to DNA and proteins through chelation (Naem, Pagan, & Nadler, 2010), raising interest in its effectiveness by itself to preserve zooplankton morphology.

Acidity is an important variable in preservatives as a poorly configured substance can cause damage to calcareous organisms or soft tissue. Especially planktonic Mollusca are sensitive to acids due to their highly soluble aragonite shells (Heyman, 1981). Yet, according to Omori & Fleminger (1985), driving the pH above 8 can cause damage to the internal tissue of *Crustacean* zooplankton. Depending on the nature of a project, such as an interest in specific taxa, a preservative should be configured accordingly.

This study provides a good indication of the effectiveness of DESS in preserving zooplankton morphology. It also raises more questions. In further research, the comparison should be made not only with formalin, but also with other preservatives and the lack of a preservative. Experiments with different configurations of DESS solution would clarify which of the components DMSO, EDTA and NaCl are in fact beneficial, necessary or the contrary in the preservation of zooplankton.

5. Conclusion

This study explored the effectiveness of alternative preservative DESS to preserve the morphology of zooplankton as substitute for formalin. A common problem with organisms in DESS samples was the shrinkage and decay of soft tissues. The only group with reasonable results was Crustacea. While the soft tissue was subjugated to decay, chitin exoskeletons can be effectively preserved. Delicate parts of crustacea are susceptible to detachment and fragmentation, however. Cladocera seemed to have the best results. Regardless of some soft tissue degeneration, there were no observations of broken exoskeletons.

Concluding, DESS solution does not seem to be adequate in preserving the majority of marine zooplanktonic taxonomic groups for morphological identification. Soft-bodied organisms typically dissolve entirely, while the exoskeletons of Crustacea remain preserved to varying degrees. While soft tissue inside exoskeletons experiences strong shrinkage, microscope imagery and observations suggest that DESS keeps Crustacean diagnostic characteristics largely intact. Depending on the aim and requirements of a study, DESS may be a preservative to be considered when combining morphological and DNA analysis of Crustacea. For a more definitive verdict, subsequent research should focus on the effectiveness of different configurations of DESS solution and its components on the morphology of zooplankton.

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Appendix I

EcoTaxa counts per sample, scan and size fraction. Including sampling information such as depth, splits and derived volumes.

Sample	ScanFile	Depth (m)	Volume (m3)	Split	SubSplit	VolumePostSplit (m3)	SizeFraction	*Amphipoda*	*Appendicularia*	*AppendicolariaTail*	*Asteroidea*	*Bivalvia*	*Brachiolaria*	*CarideaZoea*	*Chaetognatha*	*Cladocera*	*Cnidaria*	*Cumacea*	*CirripediaNauplii*
DESS1	DESS_321_1	28	7.14492417	0.25	1	1.786231043	>500	0	0	0	0	0	0	0	0	5	0	0	0
	DESS_321_2	28	7.14492417	0.25	0.125	0.22327888	<500	0	1	0	0	0	0	0	0	203	0	0	2
	DESS_321_3	28	7.14492417	0.25	0.125	0.22327888	<500	0	2	3	0	0	0	0	0	164	0	0	1
DESS2	DESS_322_1	17	4.33798967	0.25	1	1.08449742	>500	1	0	0	0	0	0	0	0	0	0	4	0
	DESS_322_2	17	4.33798967	0.25	0.5	0.54224871	<500	15	4	6	4	0	0	0	0	6	0	10	13
	DESS_322_3	17	4.33798967	0.25	0.5	0.54224871	<500	13	2	9	5	0	0	0	0	6	0	14	19
DESS3	DESS_323_1	28	7.14492417	0.25	1	1.786231043	>500	0	1	0	0	0	0	0	0	0	0	0	0
	DESS_323_2	28	7.14492417	0.25	0.25	0.44655776	<500	2	47	30	4	0	0	0	0	185	0	0	2
DESS5	DESS_325_1	24	6.12422072	0.25	1	1.53105518	>500	1	2	5	0	0	0	0	0	3	0	0	0
	DESS_325_2	24	6.12422072	0.25	0.25	0.38276379	<500	1	32	69	10	0	0	0	0	49	0	1	0
	DESS_325_3	24	6.12422072	0.25	0.25	0.38276379	<500	1	25	80	3	0	0	0	0	32	0	5	0
	DESS_325_4	24	6.12422072	0.25	0.25	0.38276379	<500	0	20	76	10	0	0	0	0	42	0	0	0
FORM1	FORM_321_1	28	7.14492417	0.25	0.25	0.44655776	<500	5	278	62	4	0	0	0	0	763	1	4	0
	FORM_321_2	28	7.14492417	0.25	0.25	0.44655776	>500	0	60	29	23	0	0	0	0	47	1	0	0
FORM2	FORM_322_1	17	4.33798967	0.25	0.5	0.54224871	>500	5	3	3	324	1	0	0	0	0	0	3	2
	FORM_322_2	17	4.33798967	0.25	0.25	0.27112435	<500	24	52	5	35	12	8	0	0	6	0	5	11
FORM3	FORM_323_1	28	7.14492417	0.25	0.5	0.89311532	>500	1	75	6	27	1	0	3	3	27	4	1	0
	FORM_323_2	28	7.14492417	0.25	0.25	0.44655776	<500	2	461	21	6	2	4	0	0	221	0	0	0
FORM5	FORM_325_1	24	6.12422072	0.25	0.5	0.76552759	>500	0	79	6	87	0	0	0	4	9	2	1	0
	FORM_325_2	24	6.12422072	0.25	0.125	0.19138189	<500	0	194	7	16	0	3	0	0	16	1	1	0

Sample	ScanFile	*CirripediaCypris*	*FishLarvae*	*FishEgg*	*Ophiuroidea*	*Pluteus*	*Polychaeta*	*Copepoda*	*CopepodaBroken*	*CopepodaNaupii*	*Cyphonaute*	*BrachyuraMegalopa*	*BrachyuraZoea*	*CrustaceaBroken*	*Tomopteris*	*Leptotheca*	*Gastropoda*
DESS1	DESS_321_1	0	0	2	0	0	0	61	0	0	0	1	2	3	0	0	0
	DESS_321_2	0	0	0	0	0	0	459	67	1	0	0	0	2	0	0	0
	DESS_321_3	0	0	1	0	0	0	407	75	0	0	0	4	3	0	0	0
DESS2	DESS_322_1	1	0	1	0	0	0	6	0	0	0	0	0	0	0	0	0
	DESS_322_2	1	0	0	0	0	0	208	205	0	0	0	5	32	0	0	0
	DESS_322_3	2	0	0	0	0	0	237	239	0	0	0	2	28	0	0	0
DESS3	DESS_323_1	0	0	3	0	0	0	21	1	0	0	3	2	0	0	0	0
	DESS_323_2	3	0	0	0	0	1	1110	97	2	0	0	0	5	0	0	0
DESS5	DESS_325_1	1	0	4	0	0	0	238	11	0	0	1	4	6	0	0	0
	DESS_325_2	2	0	0	0	0	0	770	115	9	0	0	1	3	0	0	0
	DESS_325_3	0	0	0	0	0	0	712	107	0	0	0	2	7	0	0	0
	DESS_325_4	0	0	0	0	0	0	737	70	2	0	0	1	4	0	0	0
FORM1	FORM_321_1	0	0	0	1	6	18	1725	32	3	12	0	2	0	0	0	0
	FORM_321_2	0	1	4	0	0	19	698	7	0	0	4	25	6	0	0	0
FORM2	FORM_322_1	0	0	0	0	0	12	84	3	0	0	2	16	1	0	0	0
	FORM_322_2	0	1	0	4	3	14	147	15	0	6	0	1	8	0	0	0
FORM3	FORM_323_1	0	2	8	1	10	24	500	7	0	0	0	1	3	1	1	0
	FORM_323_2	1	0	0	0	310	6	1165	16	0	15	0	0	2	0	0	1
FORM5	FORM_325_1	0	1	2	0	0	37	484	3	0	1	0	3	0	0	1	0
	FORM_325_2	0	0	0	0	0	11	355	5	0	5	0	0	0	0	0	0

Appendix II

Counts converted to estimated individuals per m^3, with some taxa grouped to overarching taxa.

Preservati	Sample	*Amphipoda*	*Appendicularia*	*Asteroidea*	*Chaetognatha*	*Cladocera*	*Cnidaria*	*Cumacea*	*Cirripedia*	*FishLarvae*
DESS	1	0	6.718055902	0	0	824.6413619	0	0	6.718055902	0
DESS	2	26.740497	5.532516625	8.2987749	0	11.0650325	0	25.81841	33.1950975	0
DESS	3	4.47870393	105.8093804	8.9574079	0	414.2801139	0	0	11.19675984	0
DESS	5	3.26572162	75.76474156	16.981752	0	107.7688134	0	9.797165	3.265721619	0
FORM	1	11.1967598	756.9009649	60.462503	0	1813.875093	4.478703934	8.957408	0	2.239352
FORM	2	97.741127	197.3264263	772.70816	0	22.1300665	12.90920546	23.97424	44.260133	3.688344
FORM	3	5.59837992	1116.316956	49.265743	3.359027951	525.1280363	5.598379918	1.119676	2.239351967	2.239352
FORM	5	0	1116.876794	212.92505	5.22515459	95.35907127	9.144020533	6.531443	0	1.306289

Preservati	Sample	*FishEgg*	*Ophiuroidea*	*Polychaeta*	*Copepoda*	*CopepodaBroken*	*Cyphonautes*	*Decapoda*	*DecapodaBroken*	*Mollusca*
DESS	1	3.35902795	0	0	1973.428921	317.9879793	0	20.71401	12.87627381	0
DESS	2	0.9220861	0	0	415.860833	409.4062302	0	12.90921	55.32516625	0
DESS	3	1.67951398	0	0	2497.437281	217.7769788	0	3.918866	11.19675984	0
DESS	5	2.6125773	0	0.6531443	2091.368125	297.1806673	0	20.24747	16.98175242	0
FORM	1	8.95740787	15.67546377	82.856023	5425.949816	87.33472672	26.87222361	152.2759	13.4361118	2.239352
FORM	2	0	25.81841092	73.766888	697.0970947	60.85768287	22.1300665	92.20861	31.35092754	29.50676
FORM	3	8.95740787	706.5155456	41.428011	3168.683034	43.66736336	33.59027951	6.718056	7.837731885	11.19676
FORM	5	2.6125773	0	105.80938	2487.173585	30.04463889	27.4320616	23.5132	0	0