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RESEARCH REPORT

A cold bath for a formalin-free laboratory: alternative fixative methods in early developmental stages of the sea urchin *Paracentrotus lividus* (Lamarck, 1816)

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Abstract

Fixatives are widespread in biological and medical research because they allow preserving specimens for a long time. Historically, formaldehyde has been the most used fixative so far, but new solutions are needed because of its carcinogenicity. In this study, we tested alternative fixative methods to find a harmless, economic, and simple-to-use methodology to fix samples for larval morphological analysis in *Paracentrotus lividus*. In two separate experiments, *P. lividus* embryos were fixed after 48 h post-fertilization by adding Formalin Free Tissue Accustain™, NaOH-buffered Formalin Free Tissue Accustain™, glacial ethanol and denatured ethanol at different concentrations (from 10 % to 70 %) and by submerging the vials containing the larvae in seawater at 0 °C and maintained at 4 °C for 144 h. Our results suggested that all the alternative fixatives tested do not guarantee a good quality of larvae for morphological purposes, while larvae that faced the thermal shock and were kept at 4 °C did not show any evidence of damage throughout time. The results of this study candidate this method as a good and safe substitute of formalin in studies that require morphological and taxonomic recognition and shed light on its use in other kinds of studies as well.

Key Words: formalin; ethanol; alternative fixatives; larval development; sample preservation; sea urchin

Introduction

Fixation is a crucial step in life science and medical research since it allows to preserve specimens from decay and to analyse samples after a long time from the collection. Since its invention, in 1859, formaldehyde has become very popular in research laboratories because of its low cost and high efficacy in fixation (Chesnick *et al.*, 2010).

It is a gas which condenses forming a liquid known as 'formalin' usually available at the standard concentration of 37 % with a pH range from 2.8 to 4.0 (Schander and Halanych, 2003), however it can be buffered for specific purposes such as the preservation of calcareous organisms (Prado *et al.*, 2012; Munari *et al.*, 2016). In the liquid phase (from 4 % dilution) formalin is present as methyleneglycol, which can react with the $-NH_2$ groups of proteins, forming methylene protein bridges during a process called 'cross-linking' (Benerini Gatta *et al.*, 2012).

Historically, formaldehyde has been widely used for different categories of marine species: for example, in invertebrates research, it is used for the fixation of benthic species with pelagic early life stages (Munari *et al.*, 2016; Oliva *et al.*, 2016; Foo *et al.*, 2020), meiofauna (Pusceddu *et al.*, 2016; Bertocci *et al.*, 2019; Rizzo *et al*., 2020), corals (Calcinai *et al*., 2015), annelids (Gravina *et al*., 2018), hydroids (Fraschetti *et al.*, 2002), colonizers on artificial panels used for the study of invertebrates (Martell *et al.*, 2018) and vertebrates like fishes (Vacchi *et al*., 2007; Meneghesso *et al*., 2013). Formalin is also used to fix vegetal tissues of seaweeds (Falace *et al.*, 2005; Pinna *et al.*, 2020) and seagrasses (Vasapollo and Gambi, 2012).

Over the years the 'formalin dogma', considered an irreplaceable fixative, has started to be questioned (Zanini *et al.*, 2012) because of its carcinogenicity (European Parliament, 2008), the shrinkage effect on some fish larvae (Fowler and Smith, 1983; Morkert and Bergstedt, 1990), and several difficulties for DNA extraction protocols (Schander and Halanych, 2003). In recent years a lot of patented fixatives, that do not contain formalin, have been commercialized to replace formaldehyde (*i.e*., CellBlock, CyMol, FineFix, Greenfix, Holland, Lugol, NotoXhisto, Paga, Rcl2, Upm and zinc-based fixatives) in biological, clinical and pathology studies (Acton *et al*., 2005; Benerini Gatta *et al.*, 2012;

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Oselladore *et al.*, 2012; Zanini *et al.*, 2012; Yang *et al.*, 2017). Among them, to be mentioned is Formalin Free Tissue Accustain™ whose use has started for the preservation of marine invertebrates (Regoli *et al*., 2019). A more classical method is represented by ethanol that has been historically used for the preservation of tissues and entire organisms such as fishes, crustaceans, nematodes, echinoderms (Black and Dodson, 2003; Uthicke *et al.*, 2004; Fonseca and Fehlauer-Ale, 2012; La Mesa *et al.*, 2017).

In studies concerning sea urchin species, glutaraldehyde is often used instead of formaldehyde, alone (Ruocco *et al.*, 2020) or mixed with acrolein (Jubinville *et al.*, 1967).

However, formalin-free fixatives could cause collateral effects that can compromise several protocols from molecular to morphological ones (i.e. shrinking of fishes larvae) more than formalin (Fowler and Smith, 1983).

Moreover, depending on the number and the kind of species present in the sample, the chemical composition of the fixative may act differently (Fiocca *et al.*, 2014). For all of these reasons it is then difficult to find the 'perfect fixative' since each compound can be used for species-specific (Fonseca and Fehlauer-Ale, 2012; Yang *et al.*, 2017) or aim-specific (Acton *et al.*, 2005; Zanini *et al.*, 2012) purposes. Also, the possibility to observe small organisms under the microscope without a prior fixation of any kind is not always suitable since their movement in the media makes it difficult to conduct morphological and taxonomic observations.

In this study we tested alternative compounds on the larval stages of *Paracentrotus lividus* with the aim to find a fast fixative method that can be economic and harmless to the operator for morphological analyses, to be carried out in a short time. Formalin Free Tissue Accustain™ (FFFA), glacial ethanol (GE) and denatured ethanol (DE) at different concentrations were tested as a possible substitute to formaldehyde. Furthermore, larvae were also maintained at a 4 °C degree in seawater as an alternative method to stop larvae swimming and preserve them over time.

Materials and methods

Animal collection and adult treatments

Paracentrotus lividus adults were collected in the marine protected area (MPA) 'Regno di Nettuno' along the coast of Ischia island (40°44'47.9"N 13°56'39.3"E) by scuba divers at a depth of 2-5 m and immediately transported in the laboratory of the Ischia Marine Centre (IMC) of the Stazione Zoologica Anton Dohrn in cool boxes to avoid further stress. In the laboratory, adults were induced to spawn by injecting 1 mL of 0.5 M KCl solution into the coelom (Byrne *et al.*, 2008) and shaking the specimens to allow a homogeneous distribution of KCl solution. Sperms were collected dry to avoid instantaneous activation and kept on ice until use. Females were let to spawn upside down in beakers filled with 0.22 µm filtered seawater (FSW). Before the fertilization, both sperms and eggs were checked for anomalies with an optic microscope (Chiarore *et al.*, 2020). Fertilization was performed using a constant sperm:egg ratio (1250:1) (Moschino and Marin, 2002) and observed after 30 minutes to check the elevation of the jelly coat. Embryos were successively maintained in 25 mL vials, for 48 h post-fertilization (hpf) at 22 °C, at a concentration of 50 larvae/mL. All experimental procedures on animals were done according to the guidelines of the European Union (Directive 609/86).

Larval fixation procedures

After 48 hpf, in a set of vials, calculated volumes of FSW were removed with a modified plastic Pasteur pipette equipped with 60 µm mesh to be sure to not lose larvae from the vial. Successively, the volume was restored, and larval development was stopped by adding the chosen fixatives. In another set of vials, development was stopped by thermal shock, submerging the vials into a tank filled with seawater at 0 °C for 30 minutes at least. This method numbs larvae making them fall on the bottom, facilitating their collection for morphological analyses.

Two separate experiments were conducted to test the fixatives at different concentrations.

In the first experiment, three non-formalin fixatives (GE, DE and FFFA) were tested.

Based on the results of the first experiment, in the second one, only two non-formalin fixatives (DE, buffered FFFA named BFFFA) were used. BFFFA was prepared by manually buffering the 4.76 pH of FFFA adding 1 M NaOH until a value of 7.22 pH units was reached.

All of these fixatives were tested following concentrations in Table 1. After fixation, samples

Table 1 Fixatives and relative concentrations were calculated in % as volume of fixative per total volume of solution (V/V). GE= glacial ethanol; DE= Denaturated ethanol; FFFA= Ready to use Formalin Free Fixative, Accustain™; BFFFA= NaOH buffered Formalin Free Fixative, Accustain™. 1stE = Concentrations used in the first experiment. 2ndE = Concentrations used in the second experiment

	Fixative concentration (% V/V)				
Fixatives	**10**	**20**	**30**	**50**	**70**
GE			1stE	1stE	1stE
DE	2ndE	2ndE	1stE	1stE	1stE
FFFA			1stE	1stE	1stE
BFFFA	2ndE	2ndE	2ndE		

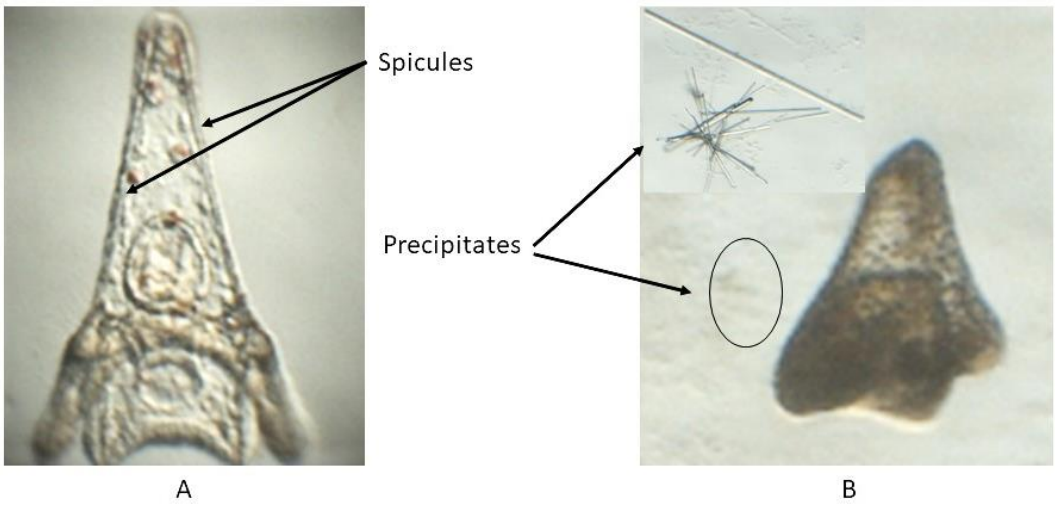

Fig. 1 Examples of sea urchin larvae considered in the analysis. A= Tissue state: Not Damaged; Transparency: Transparent; Spicules State: Visible; Precipitates: Absent. B= Tissue state: Damaged; Transparency: Non transparent; Spicules State: Not visible; Precipitates: Present

were preserved at 4 °C until microscope observation. Each method was tested in three replicates and three times after fixation (named 24 h-afx, 72 h-afx and 144 h-afx) were considered to evaluate the efficiency of each method.

Larval developmental observation and classification

A minimum of 100 larvae per replicate were observed and photographed in Petri dishes under an optical macroscope (Leica Z16 APO), equipped with a Leica DFC 300FX camera connected to a computer with the Leica LAS program (Leica Application Suite, Version 4.5) for the three times after fixation.

Each vial was removed from the fridge just before larvae had to be photographed to avoid any degradation in particular for the FSW method.

The different fixatives efficiency was evaluated through the analyses of four variables, necessary for the morphological analyses, as shown in Figure 1 and Table 2.

Statistical Analysis

The statistical analyses were performed with a non-parametric PERmutational Multivariate Analysis Of Variance, PERMANOVA (Anderson, 2001) applied on the Euclidean distance matrix of raw data. Two different PERMANOVA designs were used to test differences between larvae treated with different fixatives.

To test the effect of different fixatives on larval tissue morphology at three times after fixation, a two-factors design, time (3 levels) and fixatives (5 levels), was chosen. Only 30 % concentration was taken into account since it was the only shared concentration among the different substances.

To test the effect of different fixatives at low concentrations (10 %, 20 %, 30 %) on larval tissue morphology at three times after fixation, a two-factor design, considering the combined factor Concentration x Fixatives (named as Condition, 7 levels in total) was performed evaluating effects of DE, BFFFA and FSW. The effects of high concentrations (50 % and 70 %) of fixatives were

Table 2 Morphological variables analysed

Tissue state	Damaged: the tissue appeared to be degraded or the different anatomical structures were not clearly distinguishable
	Not damaged: the tissue was intact, and the anatomical structures were clearly distinguishable
Transparency	Transparent: larvae were clear
	Non transparent: larvae were opaque
Spicules state	Visible: it was not possible to distinguish the spicules because of degradation
	Not visible: spicules were clearly visible
Precipitates	Absent: no precipitation of fixatives occurred
	Present: salt, clumps, or a cloudy mixture was present at the microscopic observation

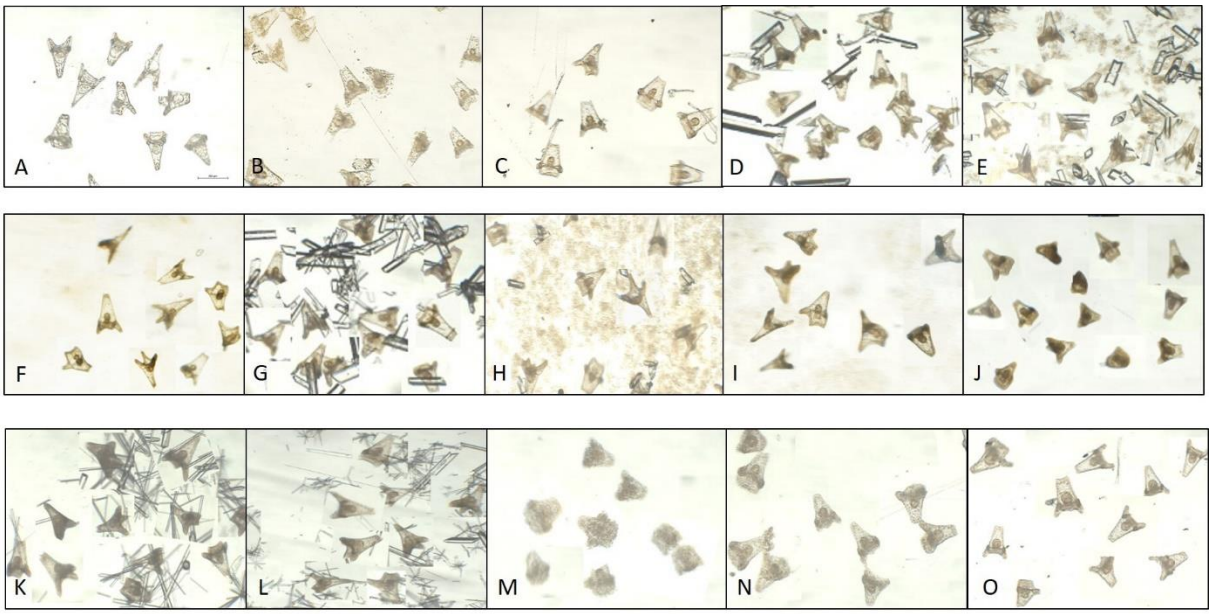

Fig. 2 Morphology of larvae 144 h after fixation. White (A), Den 10 % (B), Den 20 % (C), Den 30 % (D), Den 50 % (E) Den 70% (F), Eth 30% (G), Eth 50 % (H), Eth 70 % (I), FFFA 30 %(J), FFFA 50 %(K), FFFA 70 % (L), FFFB 10 % (M), FFFB 20% (N), FFFB 30% (O)

excluded from the statistical analysis. The tissue and spicule states were analysed as a percentage, while transparency and precipitates as presence/absence.

A Non-metric multidimensional scaling (nMDS) was also conducted to highlight the pattern of aggregation among the different methods of fixation.

The software package PRIMER 6 PERMANOVA Plus (PRIMER-E Ltd, Plymouth, UK) was used for all statistical analyses and the nMDS.

Results

The morphology of *P. lividus* larvae kept at the different fixation methods after 144 h is shown in Figure 2. The results of the different fixatives efficiency at the three times after fixation on the morphological parameters are shown in Table 3.

First Experiment

PERMANOVA results of the effect of the interaction between the two factors are reported in Table 4.

Table 3 Results of considered variables for all the methods at the three times after fixation. Tissue State: D= Damaged, ND= Not Damaged; Transparency: T= Transparent, NT= Non transparent; Spicules State: V=Visible, NV= Not visible; Precipitates: P= Present, A= Absent

Time	**24 h-afx**														
Method	**FSW**	**GE**			**DE**					**FFFA**			**BFFFA**		
% V/V	**//**	**30%**	**50%**	**70%**	**10%**	**20%**	**30%**	**50%**	**70%**	**30%**	**50%**	**70%**	**10%**	**20%**	**30%**
Tissue State	ND	D	D	D	D	D	D	D	D	D	D	D	D	D	D
Transparency	T	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Spicule State	V	NV	NV	NV	V	V	NV	NV	V	NV	NV	NV	NV	V	V
Precipitate	A	P	P	P	A	A	A	P	P	P	P	P	A	A	A
Time	**72 h-afx**														
Method	**FSW**	**GE**			**DE**					**FFFA**			**BFFFA**		
% V/V	**//**	**30%**	**50%**	**70%**	**10%**	**20%**	**30%**	**50%**	**70%**	**30%**	**50%**	**70%**	**10%**	**20%**	**30%**
Tissue State	ND	D	D	D	D	D	D	D	D	D	D	D	D	D	D
Transparency	T	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Spicule State	V	NV	NV	NV	V	V	NV	NV	NV	NV	NV	NV	NV	NV	V
Precipitate	A	P	P	P	A	A	P	P	P	P	P	P	A	A	A
Time	**144 h-afx**														
Method	**FSW**	**GE**			**DE**					**FFFA**			**BFFFA**		
% V/V	**//**	**30%**	**50%**	**70%**	**10%**	**20%**	**30%**	**50%**	**70%**	**30%**	**50%**	**70%**	**10%**	**20%**	**30%**
Tissue State	ND	D	D	D	D	D	D	D	D	D	D	D	D	D	D
Transparency	T	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Spicule State	V	NV	NV	NV	V	V	NV	NV	NV	NV	NV	NV	NV	NV	V
Precipitate	A	P	P	P	A	A	P	P	P	P	P	P	A	A	A

Table 4 PERMANOVA results. Pseudo-F values and permutational p-values for all morphological variables analysed in *P. lividus* larvae throughout the maintenance of samples at five fixation methods (FSW; Glacial ethanol 30 %; Denatured ethanol 30 %; Ready to use Formalin Free Fixative, Accustain™ 30 %; NaOH-buffered Formalin Free Fixative, Accustain™ 30 %), at different times (24 h, 72 h and 144 h) after fixation are listed. Significant results are in bold

Source	df	SS	MS	Pseudo-F	p-value
Fixatives (Fi)	4	120940.0	30235.0	24.396	**<0.001**
Time (Ti)	2	282.2	141.1	0.114	0.892
Fi X Ti	8	1822.6	227.8	0.184	0.993

PERMANOVA highlighted a significant effect of the fixation method used on larvae preservation, while the time of maintenance and its interaction with the fixation method did not influence larval preservation (Table 4). Furthermore, the nMDS evidenced a pattern of distribution with a high Euclidean distance between FSW and all the other fixatives which instead showed a pattern of aggregation (Figure 3).

Second Experiment

Significant differences among different combinations of concentrations and fixatives were highlighted by PERMANOVA (Table 5). The time of maintenance and its interaction with the different methods, did not show to influence larval preservation.

Results from the nMDS analysis, calculated as the distance among centroids (Figure 4), showed that there was a pattern of distribution with a high Euclidean distance between the FSW and all the fixatives which instead showed a pattern of aggregation.

Results showed that spicules in fixed larvae were well visible and measurable only in the FSW at all times of maintenance. The same result has been obtained using DE, but only after 24 h after fixation while after 144 h larvae were not so well maintained as shown in Figure 2.

Furthermore, results showed that larval tissues appeared to be intact only in FSW at all times of maintenance, while all the remaining fixating methods did not succeed in preserving the integrity of the tissues (Figure 2).

Discussions and conclusions

The present study aimed to test the fixation efficacy of four no-formalin, alcohol-based substances for morphological purposes on sea urchin larvae. Different concentrations of glacial ethanol, denatured ethanol, ready to use Formalin Free Fixative Accustain™, Buffered Formalin Free Fixative Accustain™ were tested during 144 h after fixation. Moreover, an alternative non-chemical method, based on numbing the larval with a cold temperature shock, was tested. Some of the fixatives used in this study (GE, DE and FFFA) formed salt precipitates, as observed by Neuhaus *et al.* (2017) for ethanol, and flocculates when used at concentrations of 50 % and 70 %.

In the first experiment, the effects of the

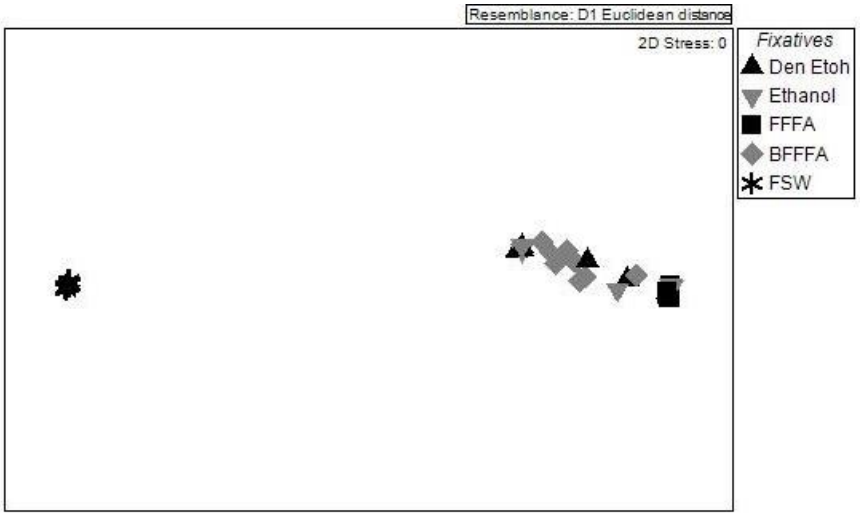

Fig. 3 n-MDS ordination plot of Euclidean distances for all the data at the 30 % concentration

Table 5 PERMANOVA results. Pseudo-F values and permutational p-values for all morphological variables analysed in *P. lividus* larvae throughout the maintenance of samples at seven different conditions of fixation (FSW; Denatured ethanol at 10, 20 and 30 %; NaOH-buffered Formalin Free Fixative, Accustain™ at 10, 20 and 30 %), at different times (24 h, 72 h and 144 h) after fixation are listed. Significant results are in bold

Source	df	SS	MS	Pseudo-F	p-value
Condition (Co=Concentration x Fixative)	6	150000	24982.0	38.758	**< 0.001**
Time (Ti)	2	1134.2	567.1	0.880	0.428
Co x Ti	12	8812.0	734.3	1.139	0.363

common 30 % concentration of the four formalin-free substances and FSW on the larval development parameters were evaluated. PERMANOVA highlighted a significant difference among methods, in particular there was a pattern between FSW, and all the fixatives used as demonstrated by the nMDS. This common pattern among fixatives could be explained considering that all of them are alcohol-based. In the same experiment, the PERMANOVA showed for each method of fixation that the time of maintenance was not a significant factor, at least for 144 h, being no differences among 48 h post-fertilization larvae observed at different times from fixation. However, the formalin-free fixatives used in the first experiment did not demonstrate to be good substitutes since already after the first observation time (24 h) deleterious morphological effects were evident.

In order to reduce precipitates and flocculates, in the second experiment, larvae of *P. lividus* were fixated and maintained in DE, BFFFA at lower concentrations (10 %, 20 %, 30 %; kept at 4 °C) as well as in FSW (cold bath at 0 °C; kept at 4 °C) for 144 h. PERMANOVA highlighted a difference among the different conditions of maintenance with an evident pattern between FSW and compared to the others, similarly to the first experiment.

In general, the FSW method showed to be the best in both experiments. In terms of larval calcareous structures, only in the FSW method, there was a 100 % of larvae with visible spicules during the 144 h of maintenance. A similar result was obtained at the lowest concentration (10 %) of the DE. However, spicules visibility was the only variable in common with the FSW methods since larval tissues were as damaged as for the other conditions of preservation. Indeed, results showed that only applying the FSW method, and at all the time considered, there were 100 % of not-damaged larvae, while fixatives at all concentrations considered had severe effects on larval tissues even after the first 24 h of fixation. It is important to mention that also BFFFA induced a dramatic effect on larval quality, leading to the hypothesis that the low pH of FFFA was not the main driver of the scarce success of the fixation observed in the first experiment, but probably the effect must be sought in the composition of this product.

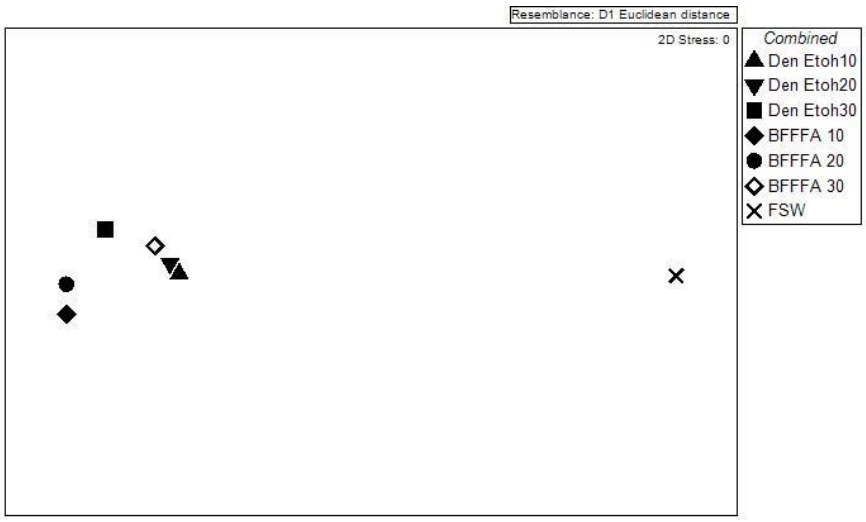

Fig. 4 n-MDS ordination plot of Euclidean distances among centroids for FSW, DE and BFFFA at low concentrations (10 % - 20 % - 30 %)

In conclusion, our result showed that all the compounds tested are not suitable for all research based on sea urchin larval morphology, such as ecotoxicological investigations (Chiarore *et al.*, 2020; Foo *et al.*, 2020). On the contrary, applying a thermal shock close to 0 °C on larvae, and their maintenance at 4 °C, did not negatively influence larval morphological parameters for 144 h. This method could be considered very suitable for all laboratories, considering its cheapness and simplicity. An aspect that could be further investigated as development of this experiment could be assessing the mid and long-term fixation efficacy of the thermal-shock procedure. However, according to Fonseca and Fehlauer-Ale (2012) Fiocca *et al.* (2014) and Yang *et al.* (2017) it is likely that every fixation method could be considered efficient for a specific application, a specific organism and even a specific life stage. Future investigations are needed to verify this inexpensive method also for non-morphological applications and the feasibility with other groups of organisms at their different life stages with different geographical origins, from poles to the tropical areas.

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