Improved ultrastructure of marine invertebrates using non-toxic buffers

Jacqueline Montanaro, Daniela Gruber, Nikolaus Leisch

Many marine biology studies depend on field work on ships or remote sampling locations where sophisticated sample preservation techniques (e.g. high-pressure freezing) are often limited or unavailable. Our aim was to optimize the ultrastructural preservation of marine invertebrates, especially when working in the field. To achieve chemically fixed material of the highest quality, we compared the resulting ultrastructure of gill tissue of the mussel Mytilus edulis when fixed with differently buffered EM fixatives for marine specimens (seawater, cacodylate and phosphate buffer) and a new fixative formulation with the non-toxic PHEM buffer (PIPES, HEPES, EGTA and MgCl2). All buffers were adapted for immersion fixation to form an isotonic fixative in combination with 2.5% glutaraldehyde. We showed that PHEM buffer based fixatives resulted in equal or better ultrastructure preservation when directly compared to routine standard fixatives. These results were also reproducible when extending the PHEM buffered fixative to the fixation of additional different marine invertebrate species, which also displayed excellent ultrastructural detail. We highly recommend the usage of PHEM-buffered fixation for the fixation of marine invertebrates.

1   **Improved ultrastructure of marine invertebrates using non-toxic buffers**

3   Jacqueline Montanaro[1], Daniela Gruber[2]and Nikolaus Leisch[3,4*]

4   *[1]Medical University of Vienna, OCUVAC – Center of Ocular Inflammation and Infection, Laura*

5   *Bassi Centers of Expertise, Center for Pathophysiology, Infectiology and Immunology, 1090*

6   *Vienna, Austria*

7   *[2]University of Vienna, Core Facility Cell Imaging and Ultrastructure Research, Althanstrasse*

8   *14, 1090 Vienna, Austria*

9   *[3]Max Planck Institute for Marine Microbiology, Department of Symbiosis, Celsiusstrasse 1,*

10   *28359 Bremen, Germany*

11   *[4]University of Vienna, Department of Ecogenomics and Systems Biology, Archaea Biology and*

12   *Ecogenomics Division, Althanstrasse 14, 1090 Vienna, Austria*

14   *Corresponding author Dr. Nikolaus Leisch

15   Department of Symbiosis

16   Max Planck Institute for Marine Microbiology, Celsiusstrasse 1, 28359 Bremen, Germany

17   Tel.: +49 421 2028 825

18   e-mail: nleisch@mpi-bremen.de

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21 **ABSTRACT**

22 Many marine biology studies depend on field work on ships or remote sampling locations

23 where sophisticated sample preservation techniques (e.g. high-pressure freezing) are often

24 limited or unavailable. Our aim was to optimize the ultrastructural preservation of marine

25 invertebrates, especially when working in the field. To achieve chemically fixed material of the

26 highest quality, we compared the resulting ultrastructure of gill tissue of the mussel *Mytilus*

27 *edulis* when fixed with differently buffered EM fixatives for marine specimens (seawater,

28 cacodylate and phosphate buffer) and a new fixative formulation with the non-toxic PHEM

29 buffer (PIPES, HEPES, EGTA and $MgCl_2$). All buffers were adapted for immersion fixation to form

30 an isotonic fixative in combination with 2.5% glutaraldehyde. We showed that PHEM buffer

31 based fixatives resulted in equal or better ultrastructure preservation when directly compared

32 to routine standard fixatives. These results were also reproducible when extending the PHEM

33 buffered fixative to the fixation of additional different marine invertebrate species, which also

34 displayed excellent ultrastructural detail. We highly recommend the usage of PHEM-buffered

35 fixation for the fixation of marine invertebrates.

36 **INTRODUCTION**

37 Marine research is often dependent upon species sampling from off-shore research stations,

38 marine vessels and submersibles. As highlighted by the Census of Marine Life, many species

39 remain undiscovered, while the complex details about many others remain unknown (Census of

40 Marine Life 2010). One of the routine techniques of marine biology research is to preserve

41 sample specimens for light and electron microscopy, for example for the formal description of a

42 new species. Ultrastructural research is especially valuable for elucidating details on the

43 symbiotic relationships between larger metazoan and prokaryotic organisms, like the mussels

44 of the genus *Bathymodiolus*. These mussels live at hydrothermal vents and cold seeps in the

45 deep sea (reviewed in Dubilier et al. 2008). The mussels harbor chemoautotrophic bacterial

46 symbionts in their gills which exploit the fluid chemistry at these sites to fix carbon and sustain

47 their host (reviewed in Petersen & Dubilier 2009).

48 The first and most crucial step for successful ultrastructure analysis is the fixation of the

49 specimen to preserve the morphology of cells with minimal alteration from the living state

50 (Hayat 2000). There are currently two methods regularly used for sample fixation, high pressure

51 freezing and chemical fixation. High-pressure freezing relies on extremely rapid cooling to vitrify

52 the water in the sample and is usually followed by dehydration at ultra-low temperatures

53 (freeze-substitution) and infiltration with resins (Kuo 2014). Chemical immersion fixation is

54 conventionally based on aldehydes such as glutaraldehyde (GA) or formaldehyde (FA) or a

55 combination of both, which cross-link proteins (Dykstra & Reuss 2003; Hayat 2002). It is

56 followed by stepwise dehydration and infiltration with resins. Due to its convenience, low cost

57 and availability, it remains the most widely used method for preserving biological specimens for

58 electron microscopy. Cellular components and ultrastructural details are adequately preserved,

59 whilst the technique itself is easy to apply and requires minimal equipment and expertise

60 (Hayat 2000). Additionally, when working on research vessels or remote research stations,

61 access to techniques like high-pressure freezing is either extremely limited or nonexistent.

62   Regardless of which fixative is used, any artifact or structural changes introduced during the

63   fixation step (e.g. due to changes of pH or osmolarity), cannot be corrected in later stages and

64   may lead to poor ultrastructure preservation. Therefore, the aldehydes are applied with a

65   buffer, which needs to act as solvent for the fixative, maintain a specific pH and convey tonicity

66   to the final fixative solution. The most commonly used buffers for ultrastructure fixation are

67   cacodylate buffer and phosphate buffer (Dykstra & Reuss 2003). As an adaption for fixation of

68   marine invertebrates, sometimes diluted seawater is used (Dykstra & Reuss 2003; Ettensohn et

69   al. 2004). All of the above buffers come with trade-offs; seawater is, by nature, isotonic to

70   marine samples but has little buffering capacity. Phosphate buffer was reported to cause

71   precipitation artifacts in the tissue (Hayat 2000; Przysiezniak & Spencer 1989) and cacodylate

72   buffer contains arsenic and can have a toxic effect on the sample prior to fixation, which can

73   alter membrane permeability and affect subcellular preservation. Additionally, arsenic gas can

74   be produced in presence of acids, posing a health hazard. According to the Globally Harmonized

75   System of Classification and Labeling of Chemicals, it must be disposed of as hazardous waste

76   (Electron Microscopy Sciences 2015). Some toxic components are essential for electron

77   microscopy (e.g. the fixative for immersion fixation) however, there has been a concerted effort

78   to reduce the toxic materials used (e.g.: replacing uranyl acetate with either gadolinium or

79   samarium (Nakakoshi et al. 2011)).

80   The non-toxic PHEM buffer has a wide pH range, good buffering capacity and causes no

81   precipitations with any reagents used during sample processing. It is a combination of the two

82   zwitterionic chemicals PIPES and HEPES with EGTA and $MgCl_2$ and was proposed by Schliwa and

83   van Blerkom in (1981). HEPES seems to stabilize the lipid components of cell membranes and

84   PIPES causes retention of cellular material, reduces lipid loss in the cells and facilitates

85   extensive cross-linking of cellular material (Baur & Stacey 1977; Hayat 2000). The addition of

86   EGTA, a chelating agent with a high affinity for calcium ions, as well as magnesium chloride

87   enhances the preservation of microtubules and membranes. Therefore, PHEM would seem to

88   be an ideal electron microscopy buffer. However until now, its traditional use has been limited

89   to extraction stabilization of eukaryotic cytoskeleton (Schliwa & van Blerkom 1981),

90   immunofluorescence applications (in e.g. *Dictyostelium discoideum* (Koonce & Gräf 2010),

91   embryos of *Danio rerio* (reviewed in Schieber et al. 2010)) as well as immuno-electron

92   microscopy (in e.g. *Saccharomyces cerevisiae* (Griffith et al. 2008)) of either single cell

93   organisms or cell culture monolayers.

94   The aim of this study was to compare the effect different buffers have on the ultrastructural

95   preservation of marine invertebrates and explore the usage of PHEM buffer in combination

96   with glutaraldehyde. We measured the osmolarity of each of the different buffers and fixatives

97   and adapted the concentration of the PHEM buffer to formulate a new isosmotic buffer-fixative

98   combination. This formulation was compared to established buffer-fixative combinations using

99   the gill tissue of the marine invertebrate *Mytilus edulis*, due to its ready availability. After

100 evaluation of the initial experiment, the PHEM buffered fixative was applied to the fixation of

101 the symbiotic deep-sea mussel *Bathymodiolus childressi*.

102 **METHODS**

103

104   **Buffer and fixative preparation and osmolarity measurements**

105

106   A 10X stock solution of the PHEM buffer was prepared according to (Schliwa & van Blerkom

107   1981) by dissolving 600 mM PIPES, 250 mM HEPES, 100 mM EGTA and 20 mM $MgCl_2$ in 100 ml

108   of ddH_2O. The pH was raised above 7.0 with 10M KOH for all components to fully dissolve. Final

109   pH was adjusted to 7.4.

110   A 10X PBS stock solution (pH 7.4) was prepared by dissolving 137 mM NaCl, 2.7 mM KCl, 10 mM

111   Na_2HPO_4 and 2 mM KH_2PO_4 in 1 liter of ddH_2O. 0.2M Sodium cacodylate buffer (pH 7.4) and

112   25% glutaraldehyde were obtained from Scientific Services, Germany. Unless otherwise

113   indicated, all components were purchased from Carl Roth, Germany.

114   Fixative solutions were prepared according to Table 1. All fixatives were prepared from the

115   same stock of 25% GA and contained a final concentration of 2.5% GA. The osmolarity of the

116   fixative is usually adjusted using non-electrolytes like sucrose, glucose or dextran or electrolytes

117   such as NaCl or $CaCl_2$ (Hayat 2000). To standardize our approach, we supplemented all fixatives,

118   except the seawater, with 9% sucrose, according to the protocol from (Salvenmoser et al.

119   2010).

120   Osmolarity of seawater (salinity 35 PSU), buffer and fixative solutions was measured using

121   either an Osmomat 030 (Gonotec, Germany) or an Advanced Micro Osmometer Model 3MO

122   Plus (Advanced Instruments, USA). All samples were tested in duplicate and measured

123   independently three times. Mean values of sample readings were used for further calculations.

124

125   **Sampling and specimen preparations**

126   *Mytilus edulis* were obtained from a local fish market. They were transferred into an aquarium

127   for 2 days to allow them to recover and to discard dead specimens. Three *M. edulis* were

128   opened by cutting the adductor muscles and the gills were dissected. For each specimen,

129   roughly equal-sized gill pieces were transferred into five different fixatives (Table 1). To avoid

130   bias during the dissection, the tubes containing the fixatives were randomized before starting.

131   Samples were fixed for 12 hours at 4°C and subsequently washed three times in their

132   corresponding buffer solution (1.5X PHEM with 9% sucrose added, 0.1M cacodylate buffer with

133   9% sucrose added, 0.1M PBS with 9% sucrose added or filtered seawater) and post-fixed with

134   1% osmium tetroxide in ddH$_2$O for 1 h. The samples were dehydrated in a graded ethanol series

135   (30%, 50%, 70%, 100% twice), transferred into 100 % dry acetone, and infiltrated using

136   centrifugation (modified from (McDonald 2014)) in 2ml tubes sequentially with 25%, 50%, 75%

137   and 2x 100% Agar Low Viscosity resin (Agar Scientific, United Kingdom). During this process, the

138   samples were placed into the tube and centrifuged for 30 seconds with a bench top centrifuge

139   (Heathrow Scientific, USA) at 2000g for each step. After the second pure resin step, they were

140   transferred into fresh resin in embedding molds and polymerized at 60°C in the oven for 24h.

141   *Bathymodiolus childressi* were collected at 28°07'25.1" N 89°08'23.8" W at a depth of 1071 m

142   using the ROV Hercules in May 2015. Upon recovery, mussels were processed in chilled sea

143   water. Specimen were fixed with PHEM buffered GA and embedded as described for *Mytilus*

144   *edulis*.

145

146 **Light and electron microscopy**

147 Semi-thin (1 μm) and ultra-thin (70 nm) sections were cut with an Ultracut UC7 (Leica

148 Microsystem, Austria). Semi-thin sections were transferred on a glass slide and dried on a

149 heating plate at 60°C. Sections were stained with 1% toluidine-blue solution (Sigma-Aldrich,

150 USA) for 20 seconds, rinsed three times with ddH$_2$O then dried. A drop of LVR resin was placed

151 on the slide, followed by a coverslip, and after polymerization, the sections were viewed using

152 an Olympus BX 53 microscope (Olympus Corporation, Japan) and images were captured using a

153 Canon EOS 700D camera (Canon Inc., Japan).

154 Ultra-thin sections were mounted on formvar coated slot grids (Agar Scientific, United

155 Kingdom) and contrasted with 0.5 % aqueous uranyl acetate (Science Services, Germany) for 20

156 min and with 2 % Reynold's lead citrate for 6 min. Ultrathin sections were imaged at 20kV with

157 a Quanta FEG 250 scanning electron microscope (FEI Company, USA) equipped with a STEM

158 detector using the xT microscope control software ver. 6.2.6.3123. Where needed, brightness

159 and contrast of images was adjusted using Photoshop CS6 and figures were assembled using

160 Adobe Illustrator CS6 (Adobe Systems, Inc., USA).

161

162 **RESULTS AND DISCUSSION**

163 For the comparative part of the study, small pieces of the same gill were fixed in parallel with a

164 set of fixatives to avoid sample bias. Samples from the same animal were always processed

165 simultaneously (e.g. for embedding, polymerization, staining, etc.) to avoid handling bias. This

166 procedure was repeated for 3 different animals to ensure reproducibility. To illustrate the

167 differences between the individual fixative buffers, the result section shows representative

168 images comparing the set of fixatives from the same animal.

Osmolarity measurements and buffer compositions

170 The osmolarity of 2.5 % glutaraldehyde in ddH$_2$O, sterile filtered seawater as well as of the

171 working solutions of buffer and fixatives (Table 1) was measured. As the osmolarity of seawater

172 was 1100 mOsm, all fixative solutions were adjusted to be within a similar osmotic range with

173 either sucrose or additional buffer concentrate. PBS and sodium cacodylate buffer were 300

174 and 339 mOsm respectively at a 0.1M concentration. PHEM buffer in its 1X concentration was

175 only 219 mOsm, therefore, we increased the buffer concentration to 1.5X, resulting in an

176 osmolarity of 323 mOsm, to be comparable with the other two buffers. An increase of the

177 buffer to 3X concentration, to avoid the addition of sucrose in the fixative was measured,

178 resulting in an osmolarity of 1071 mOsm. Comparing the ultrastructure of 3X PHEM fixation and

179 1.5X PHEM + 9% sucrose, little difference was observed (Supplementary Figure 1), however for

180 clarity, we focus only on the sucrose adjusted fixatives.

181 The osmolarity of the fixative solutions ranged from 960 mOsm (Sodium cacodylate buffered

182 GA), 1046 (PBS buffered GA), 1076 (PHEM buffered GA) to 1252 (seawater – GA). For the sake

183 of brevity, we use the following abbreviations throughout the rest of the text: Sodium

184 cacodylate buffered GA (**marCaco**), PBS buffered GA (**marPBS**), PHEM buffered GA (**marPHEM**)

185 and seawater buffered GA (**FSW**).

186

187   **Comparing the effect of different buffers on the fixation of *Mytilus edulis* gill**

188   **filaments**

189   For clarity, images of the same regions of interest were taken from all samples prepared. To

190   facilitate easy comparison, we focused on the typical organelles and structures expected in

191   eukaryotic cells: mitochondria, nucleus, nuclear pores, Golgi apparatus, cilia, microvilli and

192   rough ER. All images show transverse sections through gill filaments showing the ciliated frontal

193   surface.

194

195   **Light microscopy**

196   At the light microscopic level, the variation in staining intensity infers a marked difference in

197   tissue preservation between the different fixations (Figure 1). The sections were stained with

198   toluidine blue, a basic thiazine metachromatic dye which has a high affinity for acidic tissue

199   components, including nucleoids, acidic mucus, RNA, etc. (Sridharan & Shankar 2012). In the

200   FSW fixed gill tissue (Figure 1 A) the outline of the nuclei, the cilia and the overall morphology

201   are visible. By comparison, in the marCaco (Figure 1 B) fixed sample the nuclei are prominent,

202   but the outline of the morphology is hard to detect. In the marPHEM (Figure 1 C) and marPBS

203   (Figure 1 D) fixed samples, the cells are stained more strongly and homogenously with nuclei,

204   cilia and a mucus cell easily discernible.

205   **Electron microscopy**

206   Collectively, the direct comparison of the same gill tissue, fixed at the same time and processed

207   identically, showed a pronounced disparity in terms of membrane contrast and retention of

208   cytosol with different buffers, while larger ultrastructural organelles could be identified in all of

209   them.

210   The FSW fixed samples showed a reasonable detail and preservation in the overview (Figure 2

211   A). The nuclear membranes were smooth but the chromatin was patchily distributed (Figure 2

212   B). The Golgi apparatus looked slightly collapsed and the cytosol was extracted. Due to the

213   extracted cytosol, the rough ER stands out more prominently. Nuclear pores were visible

214   (Figure 2 C). Both the mitochondria and the cilia were well preserved (Figure 2 D).

215   The marCaco fixed sample showed less overall contrast (Figure 3 A). Although nuclear

216   membranes appeared parallel, with nuclear pores visible, the nuclei themselves look extracted

217   and empty (Figure 3 B). The Golgi-Apparatus was well preserved and highly visible, however,

218   the cytosol appeared extracted (Figure 3 C). The cilia were well preserved but the mitochondria

219   appeared grainy and less electron-dense compared to the FSW fixed ones (Figure 3 D).

220   As already suggested by the light micrograph results, the marPHEM fixed sample showed

221   noticeable improvements compared to the two previous samples (Figure 4 A). The nuclei were

222   less extracted in contrast to the FSW and marCaco samples (Figure 4 B, C). The nuclear

223   membranes were well defined and parallel with nuclear pores visible. The individual

224   membranes of the stack of membranes of the Golgi-Apparatus could be easily discerned and

225   the cytosol had overall a much more electron-dense appearance (Figure 4 C). Both microvilli

226   and cilia were well defined and the mitochondria were more electron-dense than in samples

227   fixed with the previous two fixative solutions (Figure 4 D).

228   The marPBS fixed sample showed improved ultrastructural detail when compared to the FSW

229   and marCaco fixed samples, similar to the marPHEM results (Figure 5 A). Membranes were well

230   preserved and parallel, nuclear pores were visible and the cytosol had overall an electron dense

231   appearance (Figure 5 B, C). However, the outline of many of the membrane stacks of the Golgi-

232   apparatus (Figure 5 C) could not be so easily traced compared to the marPHEM sample. As in all

233   other fixations, the cilia were well preserved and the mitochondria of the marPBS (Figure 5 D)

234   appeared similarly well preserved as the marPHEM.

235   This experiment was designed to determine the influence the buffer has on the ultrastructural

236   preservation. Some structures seemed unaffected by the change of buffer, for example cilia. As

237   cilia structures are small and located on the surface of the tissue, they are also the first

238   structures to come in contact with the fresh fixative and hence were well maintained with all

239   methods. On the other hand, preservation of the nucleus and especially the cytosolic

240   components varied strongly. Both marPHEM and marPBS showed generally better resolved

241   ultrastructure than FSW and marCaco fixed tissue. The pronounced improvement in

242   ultrastructural preservation observed in both marPHEM and marPBS suggest that they are a

243   viable alternative for the fixation of marine invertebrates.

244   We were surprised by the comparatively poor performance of marCaco in these experiments,

245   especially regarding the pronounced cytosol extraction we observed, considering it is one of the

246   standard buffers for electron microscopy (Hayat 2000; Kuo 2014). Traditionally, the advantage

247   of using cacodylate-based buffers over phosphate-based buffers was to avoid the formation of

248   precipitates. However, no such precipitates were observed in this study. Looking at the

249   literature, zwitterionic buffers like PIPES and HEPES are often remarked as "a class of buffers

250   little used in electron microscopy" (Dykstra & Reuss 2003) but at the same time it is mentioned

251   that they might yield superior results, as they do not compromise elemental analysis and

252   increase tissue retention (Baur & Stacey 1977; Dykstra & Reuss 2003; Griffith 1993; Hayat 2000;

253   Kuo 2014).

254   When working with buffer systems, one also has to consider the acid dissociation constant

255   (pKa, here given at 20°C). pKa indicates at which pH the buffer system is most effective to resist

256   addition of either acid or base and has the highest buffering capacity. The optimal buffering

257   region is usually considered to be around 1 pH unit on either side of the pKa. PBS (pKa = 7.21),

258   HEPES (pKa = 7.55) and PIPES (pKa = 6.8) are much closer to the pH of the fixative (pH=7.4),

259   compared to sodium cacodylate (pKa = 6.27). This would imply that the buffering capacity of

260   sodium cacodylate at pH 7.4 is reduced, compared to the other buffers, and might explain the

261   difference in preservation.

262

263   **Applying PHEM buffered fixation in the field**

264   The only discernable difference between the marPHEM and marPBS was that the membrane

265   contrast and definition was better in the marPHEM fixative. As our research on *Bathymodiolus*

266   *childressi* requires excellent membrane definition, the marPHEM fixative was used for

267   subsequent work. The gill filament (Figure 6 A) showed excellent preservation, both in the

268   ciliated frontal part as well as in the region bearing the bacteriocytes. The close-up of the

269   bacteriocyte (Figure 6 B) showed the distribution of the methanotrophic bacteria in the cell,

270   with the typical enlarged lysosomes that have been reported in these cells, located basally. The

271   high magnification of the symbiont (Figure 6 C) allowed us to clearly see the host membranes

272   surrounding the symbiont, and the individual membranes of the intracytoplasmic membrane

273   stacks, typical for type I methanotrophs, could clearly be discerned.

274   The current standard for ultrastructural fixation is high pressure freezing (HPF) followed by

275   freeze substitution and resin embedding. However, for samples like the mussels of the genus

276   *Bathymodiolus*, high-pressure freezing is not available. The mussels are retrieved from the

277   seafloor from between 500 to 3000 m water depth and cannot be cultivated in their natural

278   state in the laboratory so far. Any HPF processing would need to happen at sea, on board

279   marine research vessels. This is currently not possible, as HPF equipment is bulky, fragile and

280   requires large volumes of liquid nitrogen. Therefore, optimizing the immersion fixation

281   technique for excellent morphology was the focus of this study.

282   This experiment showed that we could replicate the excellent ultrastructural preservation

283   results we obtained with marPHEM and *Mytilus edulis*, when applying it to the deep-sea mussel

284   *Bathymodiolus childressi*.

285

286   **Application of marPHEM fixation to a wider range of samples and additional**
287   **information**
288   Since the start of this study, marPHEM fixative has been used in our lab to investigate the

289   ultrastructure of multiple marine invertebrates like *Paracatenula galateia* (Supplementary

290   figure 2), multiple nematodes of the sub-family Stilbonematinae (data not shown), the marine

291   acoel *Convolutriloba longifissura* (Supplementary figure 3), and the unicellular ciliate

292   *Kentrophorus* sp. (Supplementary figure 4). A more diluted formulation (2.5% GA in 0.06X

293   PHEM) was used for investigating the terrestrial soil archaea *Nitrososphaera viennensis*

294   (Stieglmeier et al. 2014) as well as *E. coli* (Montanaro et al. 2015) and a 1X PHEM formulation

295   without sucrose was used for the fixation of the limnic flatworm *Stenostomum* cf. *leucops*.

296   (Supplementary figure 5).

297   Experience showed that the 10X PHEM stock solution can be stored frozen at -20°C for at least

298   a year without any obvious detrimental effect. Depending on the concentration used, the liter

299   pricing of PHEM buffer is in the same range as cacodylate buffer, but usage of PHEM buffer

300   results in less hazardous waste being produced.

301

CONCLUSIONS

303   This study compares the influence of different buffers on the resulting ultrastructural

304   morphology preservation and demonstrates the effectiveness of the isosmotic, non-toxic PHEM

305   buffer in combination with aldehydes when applied as an immersion fixative. We have adapted

306   this buffer-fixative combination for ultrastructural fixation of marine invertebrates. The

307   individual components of PHEM buffer seem to enhance ultrastructural detail, reduce

308   extraction and preserve membrane integrity. Our samples showed no evidence of shrinkage,

309   excellent structural preservation and, due to their contrast, easily discernable membranes. As

310   Hayat (2000) already stated "no single buffer can claim universal superiority over the others".

311   We therefore acknowledge that our findings might not be transferrable to every sample, but

312   we would like to encourage other researchers to use marPHEM for two reasons: (a) The above

313 results showcase how changing the buffer in an EM fixative can result in substantial

314 improvements in ultrastructure. Therefore, obtaining satisfying ultrastructural preservation

315 might be as simple as switching to a non-toxic buffer like marPHEM or marPBS. (b) Replacement

316 of toxic solutions with non-toxic alternatives in electron microscopy samples preparation

317 protects both the researchers' health and reduces toxic environmental waste. Even if the

318 results, when switching from sodium cacodylate to e.g. marPHEM, are only on par, the simple

319 fact that the handling and disposal of one more toxic chemical can be eliminated, should

320 provide sufficient motivation.

321 Taken together, our comparative studies showed that isotonic PHEM buffered fixation

322 (marPHEM) gave equal or better fixation and subsequent ultrastructural preservation when

323 directly compared to conventional fixatives. We highly recommend PHEM buffer with

324 glutaraldehyde as an electron microscopy fixative solution for routine use.

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Table detailing the osmolarities

Table 1: Detailed overview of the different fixation and washing buffer formulations and their osmolarity

1   Table 1: Detailed overview of the different fixation and washing buffer formulations and their osmolarity.

Buffer concentration	Buffer type	% Glutaraldehyde [vol/vol]	Addition	Mean osmolarity [mOsm]	s.d.	Comment	Abbreviation
-	-	2.5%	-	287	± 6.9		
-	filtered seawater	-	-	1100	± 8.0		
-	filtered seawater	2.5%	-	1252	± 15.2		**fsw**
1X	PHEM buffer	-	-	219	± 1.2		
1.5X	PHEM buffer	-	-	323	± 1.4		
3X	PHEM buffer	2.5%	-	1071	± 6.9		
1.5X	PHEM buffer	2.5%	9% Sucrose	1076	± 1.6		**marPHEM**
1.5X	PHEM buffer	-	9% Sucrose	714	± 0.5	Washing solution	
0.1M	Phosphate buffer saline	-	-	300	n.d.		
0.1M	Phosphate buffer saline	2.5%	9% Sucrose	1046	± 2.9		**marPBS**
0.1M	Phosphate buffer saline	-	9% Sucrose	645	± 6.0	Washing solution	
0.1M	Sodium-cacodylate buffer	-	-	339	± 4.5		
0.1M	Sodium-cacodylate buffer	2.5%	9% Sucrose	960	± 2.9		**marCaco**
0.1M	Sodium-cacodylate buffer	-	9% Sucrose	632	± 5.8	Washing solution	

1

Light micrograph comparing four differently fixed tissue pieces of *Mytilus edulis*.

Figure 1: Overview of four differently fixed *Mytilus edulis* gills. All images show transverse sections through gill filaments focusing on the ciliated frontal surface. (A) shows a seawater-GA fixed sample, (B) a marCaco fixed sample, (C) a marPHEM fixed sample and (D) a marPBS fixed sample. ci = cilia, mu = mucus granule, nu = nucleus.

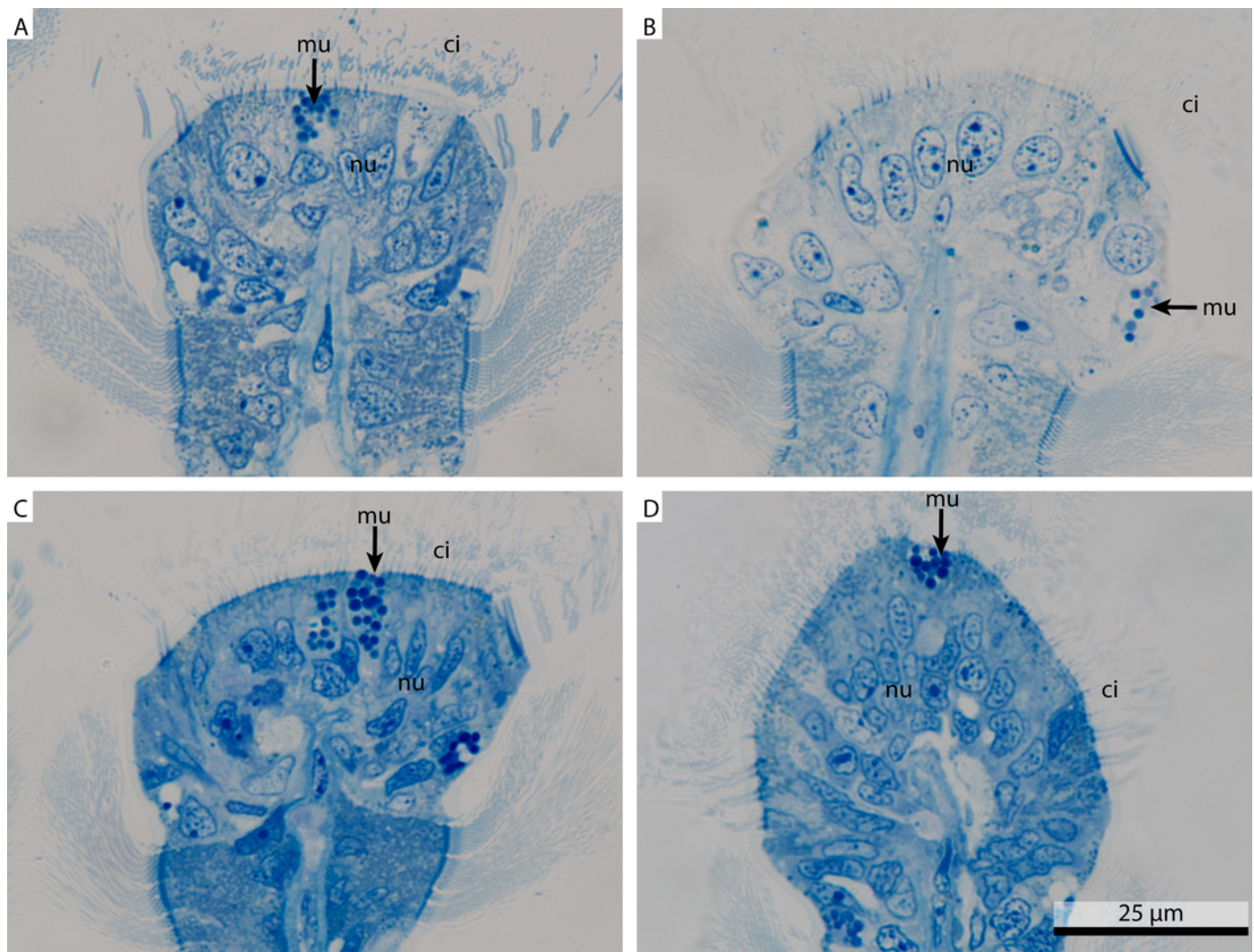
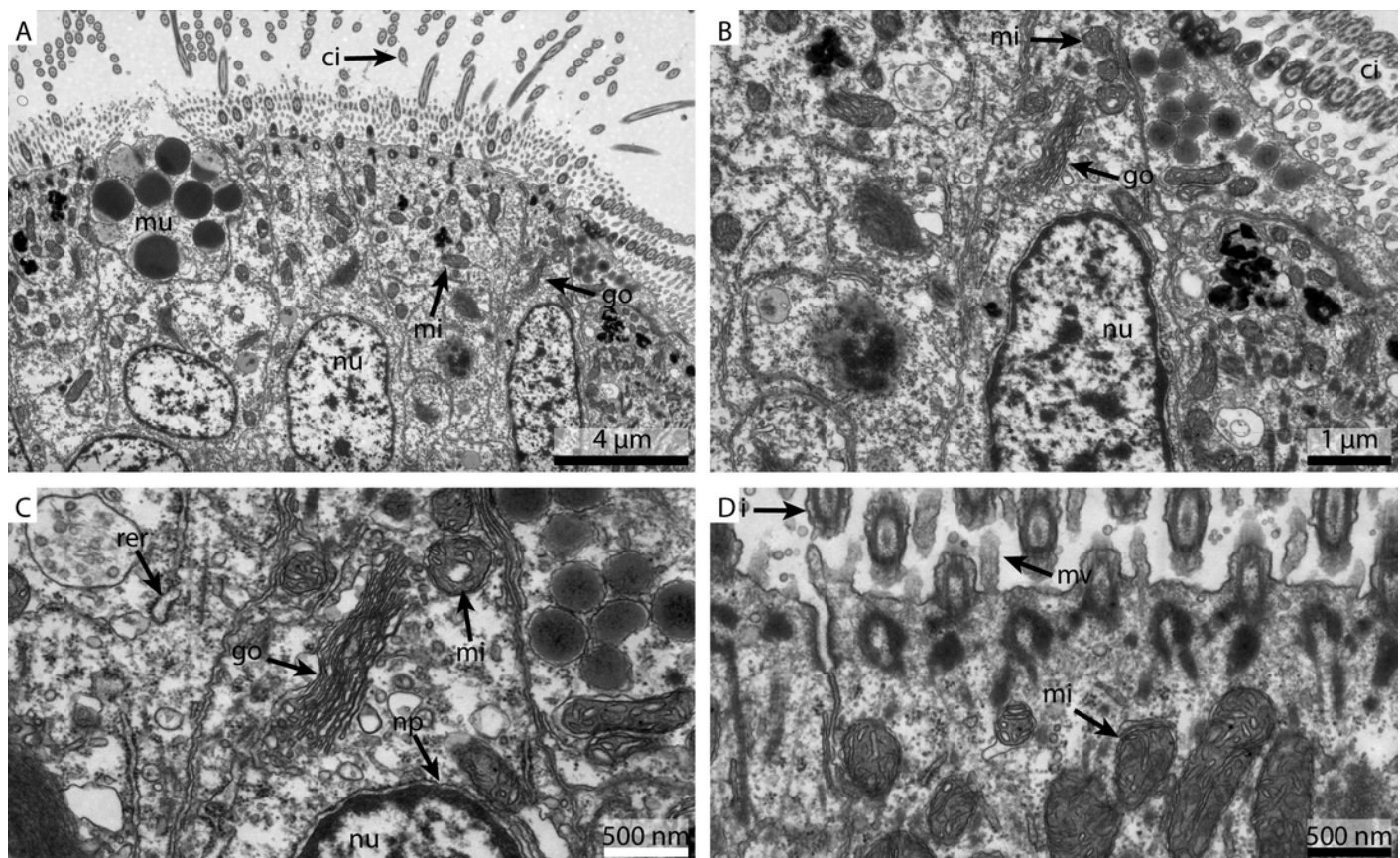
2

Ultrastructural details of FSW fixed gill cell of *Mytilus edulis*

Figure 2: Ultrastructure of FSW fixed gill cell of *Mytilus edulis* (A) shows an overview of the cells of the ciliated frontal surface, (B) is a higher magnification of the same, (C) shows the nucleus in higher magnification and (D) shows details of the cell surface. go = Golgi apparatus, nu = nucleus, ci = cilia, mu = mucus granule, mv = microvilli, np = nuclear pore, rer = rough ER, mi = mitochondria.

3

Ultrastructural details of marCaco fixed gill cell of *Mytilus edulis*

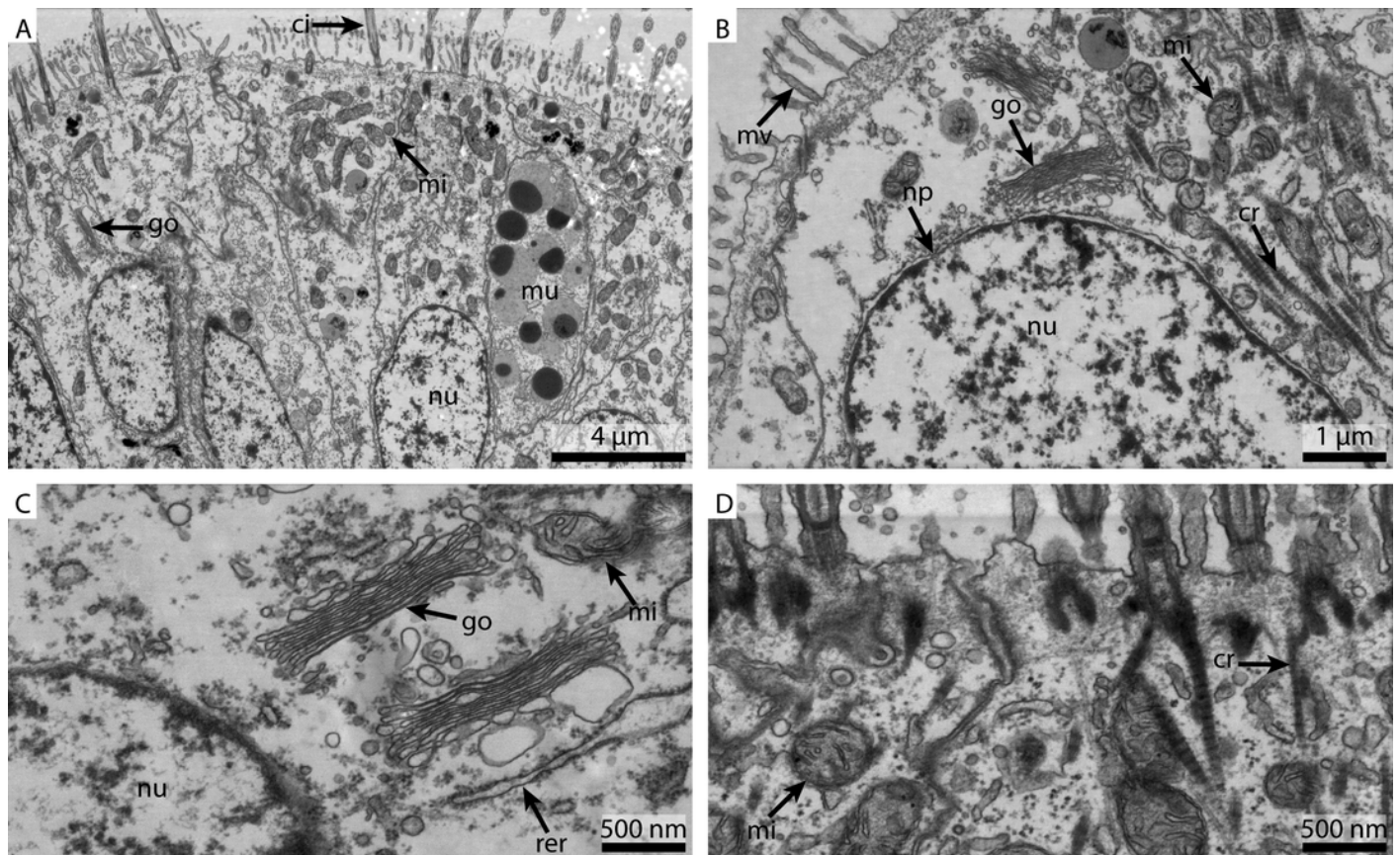
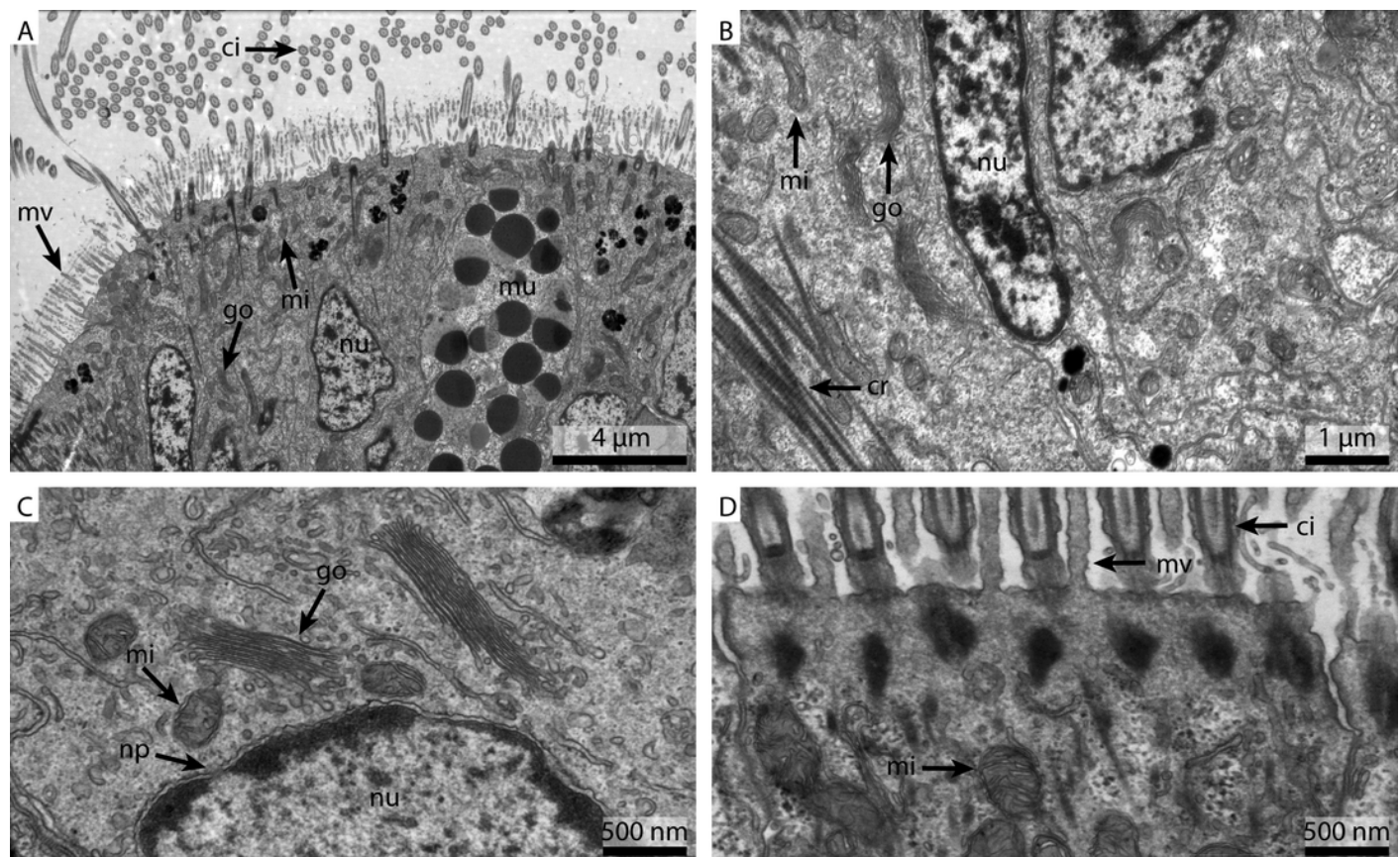
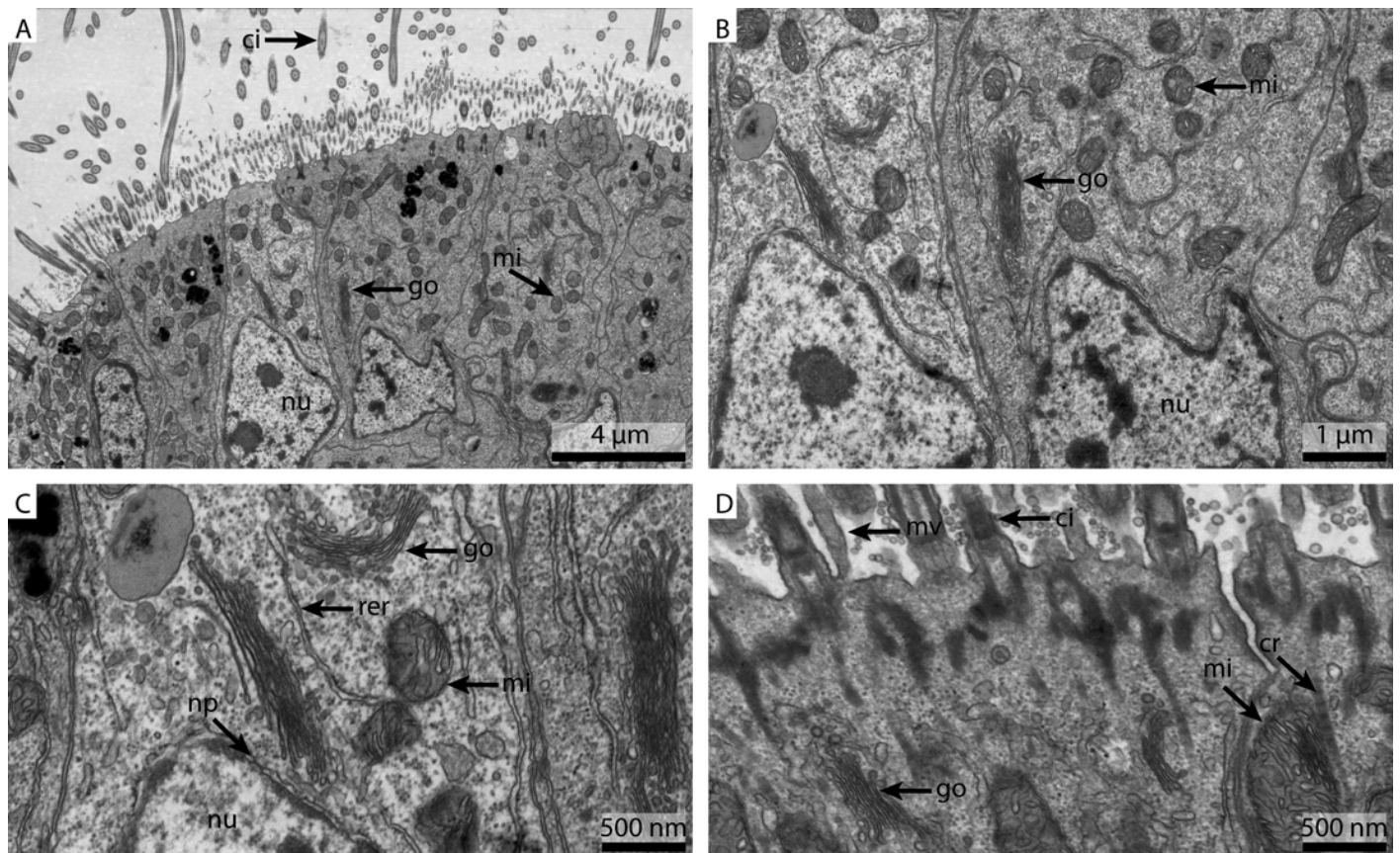
Figure 3: Ultrastructure of marCaco fixed gill cell of *Mytilus edulis* (A) shows an overview of the cells of the ciliated frontal surface, (B) is a higher magnification of the same, (C) shows the nucleus in higher magnification and (D) shows details of the cell surface. go = Golgi apparatus, nu = nucleus, ci = cilia, mu = mucus granule, mv = microvilli, np = nuclear pore, cr = ciliary root, rer = rough ER, mi = mitochondria.

Ultrastructural details of marPHEM fixed gill cell of *Mytilus edulis*

Figure 4: Ultrastructure of marPHEM fixed gill cell of *Mytilus edulis* (A) shows an overview of the cells of the ciliated frontal surface, (B) is a higher magnification of the same, (C) shows the nucleus in higher magnification and (D) shows details of the cell surface. go = Golgi apparatus, nu = nucleus, ci = cilia, mu = mucus granule, mv = microvilli, np = nuclear pore, cr = ciliary root, rer = rough ER, mi = mitochondria.

5

Ultrastructural details of marPBS fixed gill cell of *Mytilus edulis*

Figure 5 Ultrastructure of marPBS fixed gill cell of *Mytilus edulis* (A) shows an overview of the cells of the ciliated frontal surface, (B) is a higher magnification of the same, (C) shows the nucleus in higher magnification and (D) shows details of the cell surface. go = Golgi apparatus, nu = nucleus, ci = cilia, mu = mucus granule, mv = microvilli, np = nuclear pore, cr = ciliary root, rer = rough ER, mi = mitochondria.

6

Ultrastructural detail of marPHEM fixed *Bathymodiolus childressi* gill filament

Figure 6: Ultrastructure of marPHEM fixed *Bathymodiolus childressi* gill filament (A) shows an overview of the ciliated frontal part of the gill filament, with the ciliated cells of the ciliated edge on the right hand side of the image and the symbiont containing bacteriocytes on the left hand side of the image. (B) shows a single bacteriocyte, containing the chemoautotrophic methane-oxidizer symbiont and the characteristic lysosomes. (C) shows a single symbiont surrounded by the hosts membrane, with the bacterial nucleoid, storage vacuoles and the typical methane-oxidizer membrane stacks clearly visible. ba = bacterium, bm = basal membrane, ci = cilium, hm = host membrane, icm = intracytoplasmatic membranes, ly = lysosomes, mu = mucus cell, mv = microvilli, nc = bacterial nucleoid, nu = nucleus, sv = storage vacuoles.