Improved ultrastructure of marine invertebrates using non-toxic buffers

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

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ABSTRACT

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 marine EM fixatives (seawater, cacodylate and phosphate buffer) and a new fixative formulation with the non-toxic PHEM buffer (PIPES, HEPES, EGTA and $MgCl_2$). 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.

INTRODUCTION

Marine research is often dependent upon species sampling from off-shore research stations, marine vessels and submersibles. As highlighted by the Census of Marine Life, many species remain undiscovered, while the complex details about many others remain unknown (Census of Marine Life 2010). One of the routine techniques of marine biology research is to preserve 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; Przsiezniak & 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 *M. edulis*, due to its ready availability. After evaluation

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

101 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 137mM NaCl, 2.7mM KCl, 10mM

111 Na_2HPO_4 and 2mM KH_2PO_4 in 1 liter of ddH_2O. 0.2M Sodium cacodylate buffer (pH 7.4) and 25%

112 glutaraldehyde were obtained from Scientific Services, Germany. Unless otherwise indicated, all

113 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 specimen. Three *M. edulis* were opened

128 by cutting the adductor muscles and the gills were dissected. For each specimen, roughly equal-

129 sized gill pieces were transferred into five different fixatives (see Table 1). To avoid bias during

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

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

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

133 added, 0.1M PBS with 9% sucrose added or filtered seawater) and post-fixed with 1% osmium

134 tetroxide in ddH$_2$O for 1 h. The samples were dehydrated in a graded ethanol series (30%, 50%,

135 70%, 100% twice), transferred into 100 % dry acetone, and infiltrated using centrifugation

136 (modified from (McDonald 2014)) in 2ml tubes sequentially with 25%, 50%, 75% and 2x 100%

137 LVR resin. During this process, the samples were placed into the tube and centrifuged for 30

138 seconds with a bench top centrifuge (Heathrow Scientific, USA) at 2000g for each step. After

139 the second pure resin step, they were transferred into fresh resin in embedding molds and

140 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 1071m

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

Light and electron microscopy

146
147 Semi-thin (1µm) 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. The glass slide was contrasted with 1% toluidine-blue solution (Sigma-

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

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

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

153 using a 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. Images were processed

159 using Photoshop CS6 and Illustrator CS6 (Adobe Systems, Inc., USA).

160

161 **RESULTS AND DISCUSSION**

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

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

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

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

166   differences between differed fixative buffers, the result section shows representative images

167   comparing the set of fixatives from the same animal.

168   **Osmolarity measurements and buffer compositions**

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

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

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

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

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

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

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

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

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

178   1.5X PHEM + 9% sucrose, little difference was observed (Supplementary Figure 1), however for

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

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

181   GA), 1046 (PBS buffered GA), 1071 (PHEM buffered GA) to 1246 (seawater – GA). For the sake

182   of brevity, we'll use the following abbreviations throughout the rest of the text: Sodium

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

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

185

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

187   **filaments**

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

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

190   eukaryotic cells: Mitochondria, nucleus, nuclear pores, Golgi-Apparatus, cilia, microvilli and

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

192   surface.

193

194   **Light microscopy**

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

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

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

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

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

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

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

202   D) fixed samples, the cells are stained more strongly and homogenously with nuclei, cilia and a

203   mucus cell easily discernible.

204   **Electron microscopy**

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

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

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

208   them.

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

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

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

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

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

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

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

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

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

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

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

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

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

222   membranes are well defined and parallel with nuclear pores being visible. The individual

223   membranes of the stack of membranes of the Golgi-Apparatus can be easily discerned and the

224   cytosol has overall a much more electron-dense appearance (Figure 4 C). Both microvilli and

225   cilia are well defined and the mitochondria are more electron-dense than in samples fixed with

226   the previous two fixative solutions (Figure 4 D).

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

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

229   preserved and parallel, nuclear pores are visible and the cytosol has overall an electron dense

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

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

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

233   appear similarly well preserved as the marPHEM.

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

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

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

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

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

239   components varied strongly. Both FSW and marCaco showed generally less well resolved

240   ultrastructure than marPHEM and marPBS fixed tissue. The pronounced improvement in

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

242   viable alternative for the fixation of marine invertebrates.

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

244   especially the pronounced cytosol extraction we observed, considering its one of the standard

245   buffer for electron microscopy (Hayat 2000; Kuo 2014). Traditionally, the advantage of using

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

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

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

249   little used in electron microscopy" (Dykstra & Reuss 2003) but at the same time its commented

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

251   increase tissue retention (Baur & Stacey 1977; Dykstra & Reuss 2003; Hayat 2000; Kuo 2014).

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

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

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

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

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

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

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

259   difference in preservation.

260

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

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

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

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

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

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

267   bacteriocyte (Figure 6 B) shows the distribution of the methanotrophic bacteria in the cell, with

268   the typical big lysosomes that have been reported in these cells, located basally. The high

269   magnification of the symbiont (Figure 6 C) allows us to clearly see the host membranes

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

271   stacks, - typical for type I methanotrophs - can clearly be discerned.

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

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

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

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

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

277   marine research vessels. This is currently not possible, as the HPF equipment is bulky, fragile

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

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

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

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

282   *Bathymodiolus childressi*.

283

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

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

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

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

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

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

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

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

294   (Supplementary figure 5).

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

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

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

298   results in less hazardous waste being produced.

299

300   **CONCLUSION**

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

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

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

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

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

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

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

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

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

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

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

312 improvements in ultrastructure. Therefore, getting satisfying ultrastructural preservation might

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

314 toxic solutions with non-toxic alternatives in electron microscopy samples preparation protects

315 both the researchers' health and reduces toxic environmental waste. Even if the results, when

316 switching from sodium cacodylate to e.g. marPHEM, are only on par, the simple fact that the

317 handling and disposal of one more toxic chemical can be eliminated, should provide sufficient

318 motivation.

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

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

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

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

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336 REFERENCES

337 Baur PS, and Stacey TR. 1977. The use of PIPES buffer in the fixation of mammalian and marine tissues
338   for electron microscopy. *Journal of Microscopy* **109**:315-327. 10.1111/j.1365-
339   2818.1977.tb01145.x

340 Census of Marine Life. 2010. The First Census of Marine Life: Highlights of a Decade of Discovery.
341   *Available at http://www.coml.org/comlfiles/partner2010/Final%20Report%209-14%20small.pdf*
342   (accessed 12 2015).

343 Dubilier N, Bergin C, and Lott C. 2008. Symbiotic diversity in marine animals: the art of harnessing
344   chemosynthesis. *Nat Rev Micro* **6**:725-740. 10.1038/nrmicro1992

345 Dykstra MJ, and Reuss LE. 2003. *Biological electron microscopy: theory, techniques and troubleshooting*.
346   New York: Springer.

347 Electron Microscopy Sciences. 2015. Sodium Cacodylate buffer Material Data Safety Sheet. *Available at*
348   *https://www.emsdiasum.com/microscopy/technical/msds/11654.pdf* (accessed 12 2015).

349 Ettensohn CA, Wray GA, and Wessel GM. 2004. *Development of Sea Urchins, Ascidians, and Other*
350   *Invertebrate Deuterostomes: Experimental Approaches*. San Diego: Elsevier Academic Press.

351 Griffith J, Mari M, De Mazière A, and Reggiori F. 2008. A cryosectioning procedure for the ultrastructural
352   analysis and the immunogold labelling of yeast *Saccharomyces cerevisiae*. *Traffic* **9**:1060-1072.
353   10.1111/j.1600-0854.2008.00753.x

354 Hayat M. 2000. *Principles and techniques of electron microscopy: biological applications*. Cambridge:
355   Cambridge University Press.

356 Hayat M. 2002. Fixation and Embedding. *Microscopy, Immunohistochemistry, and Antigen Retrieval*
357   *Methods*. New York: Springer US, 53-69.

358 Koonce MP, and Gräf R. 2010. *Dictyostelium discoideum*: A Model System for Ultrastructural Analyses of
359   Cell Motility and Development. In: Müller-Reichert T, ed. *Methods in Cell Biology - Electron*
360   *Microscopy of Model Systems*. San Diego: Academic Press, 197-216.

361 Kuo J. 2014. *Electron microscopy: methods and protocols*. New Jersey: Humana Press.

362 McDonald KL. 2014. Rapid Embedding Methods into Epoxy and LR White Resins for Morphological and
363   Immunological Analysis of Cryofixed Biological Specimens. *Microscopy and Microanalysis*
364   **20**:152-163. 10.1017/S1431927613013846

365 Montanaro J, Inic-Kanada A, Ladurner A, Stein E, Belij S, Bintner N, Schlacher S, Schuerer N, Mayr UB,
366   and Lubitz W. 2015. *Escherichia coli* Nissle 1917 bacterial ghosts retain crucial surface properties
367   and express chlamydial antigen: an imaging study of a delivery system for the ocular surface.
368   *Drug Design, Development and Therapy* **9**:3741. 10.2147/DDDT.S84370

369 Nakakoshi M, Nishioka H, and Katayama E. 2011. New versatile staining reagents for biological
370   transmission electron microscopy that substitute for uranyl acetate. *Journal of Electron*
371   *Microscopy* **60**:401-407. 10.1093/jmicro/dfr084

372 Petersen JM, and Dubilier N. 2009. Methanotrophic symbioses in marine invertebrates. *Environmental*
373   *Microbiology Reports* **1**:319-335. 10.1111/j.1758-2229.2009.00081.x

374 Przysiezniak J, and Spencer AN. 1989. Primary culture of identified neurones from a cnidarian. *Journal of*
375   *Experimental Biology* **142**:97-113.

376 Salvenmoser W, Egger B, Achatz JG, Ladurner P, and Hess MW. 2010. Chapter 14 - Electron Microscopy
377   of Flatworms: Standard and Cryo-Preparation Methods. In: Thomas M-R, ed. *Methods in Cell*
378   *Biology*: Academic Press, 307-330.

379 Schieber NL, Nixon SJ, Webb RI, Oorschot VMJ, and Parton RG. 2010. Modern Approaches for
380   Ultrastructural Analysis of the Zebrafish Embryo. In: Müller-Reichert T, ed. *Methods in Cell*
381   *Biology - Electron Microscopy of Model Systems*. San Diego: Elsevier Academic Press, 425-442.

382 Schliwa M, and van Blerkom J. 1981. Structural interaction of cytoskeletal components. *Journal of Cell*
383   *Biology* **90**:222-235. 10.1083/jcb.90.1.222
384 Sridharan G, and Shankar AA. 2012. Toluidine blue: A review of its chemistry and clinical utility. *Journal*
385   *of Oral and Maxillofacial Pathology : JOMFP* **16**:251-255. 10.4103/0973-029x.99081
386 Stieglmeier M, Klingl A, Alves RJ, Rittmann SK, Melcher M, Leisch N, and Schleper C. 2014.
387   *Nitrososphaera viennensis* sp. nov., an aerobic and mesophilic ammonia-oxidizing archaeon
388   from soil and member of the archaeal phylum Thaumarchaeota. *International Journal of*
389   *Systematic and Evolutionary Microbiology* **64**:2783-2752. 10.1099/ijs.0.063172-0

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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*.

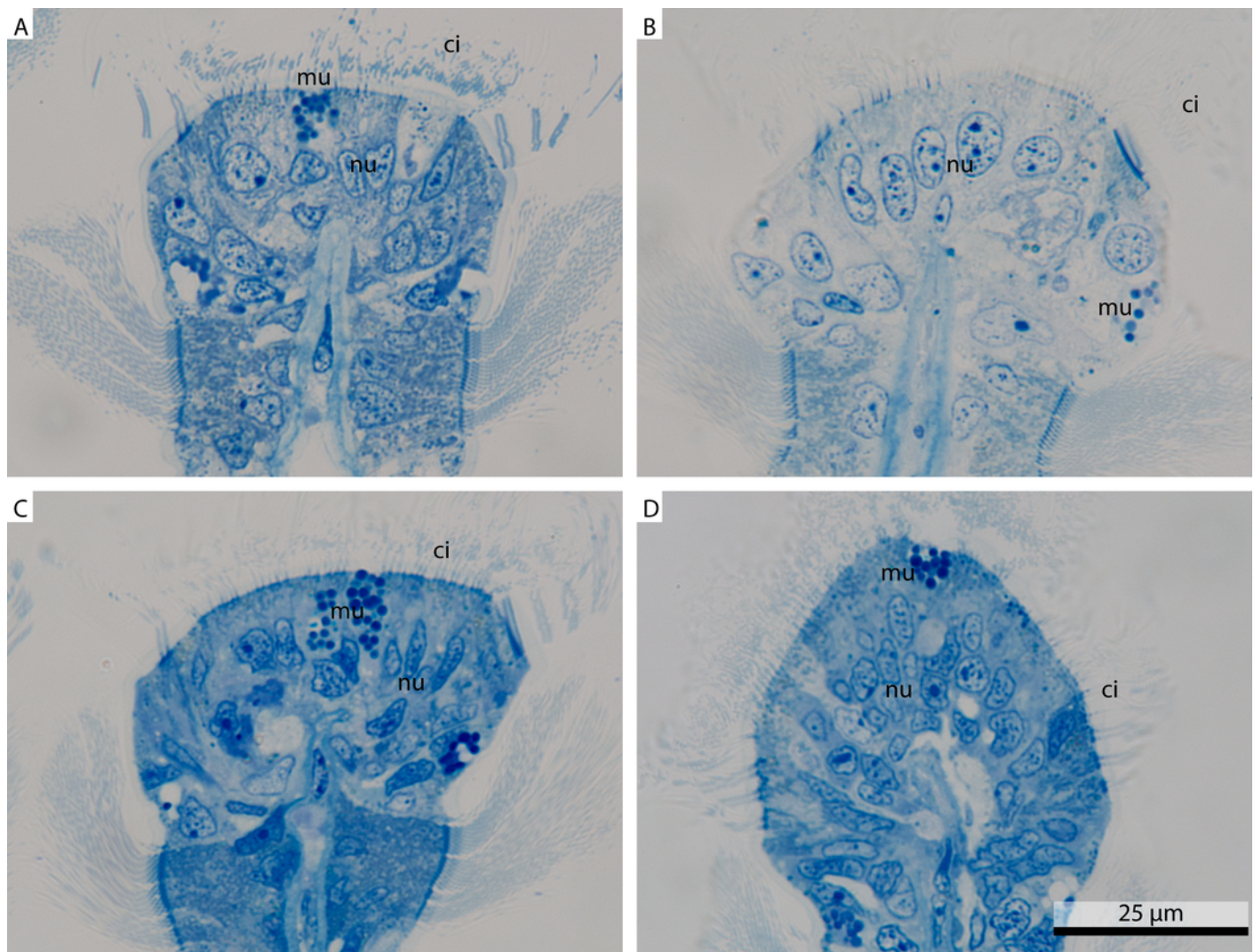
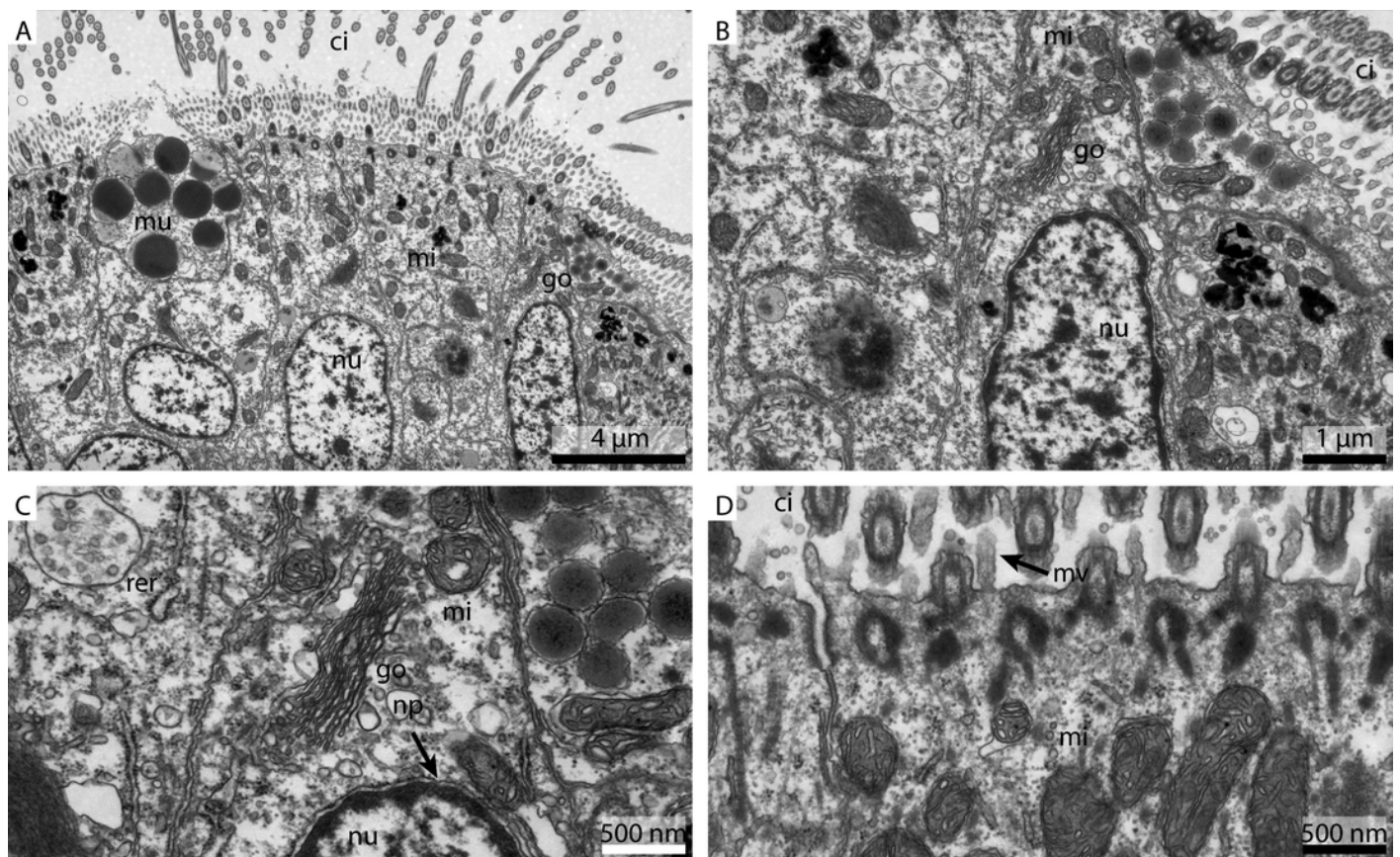
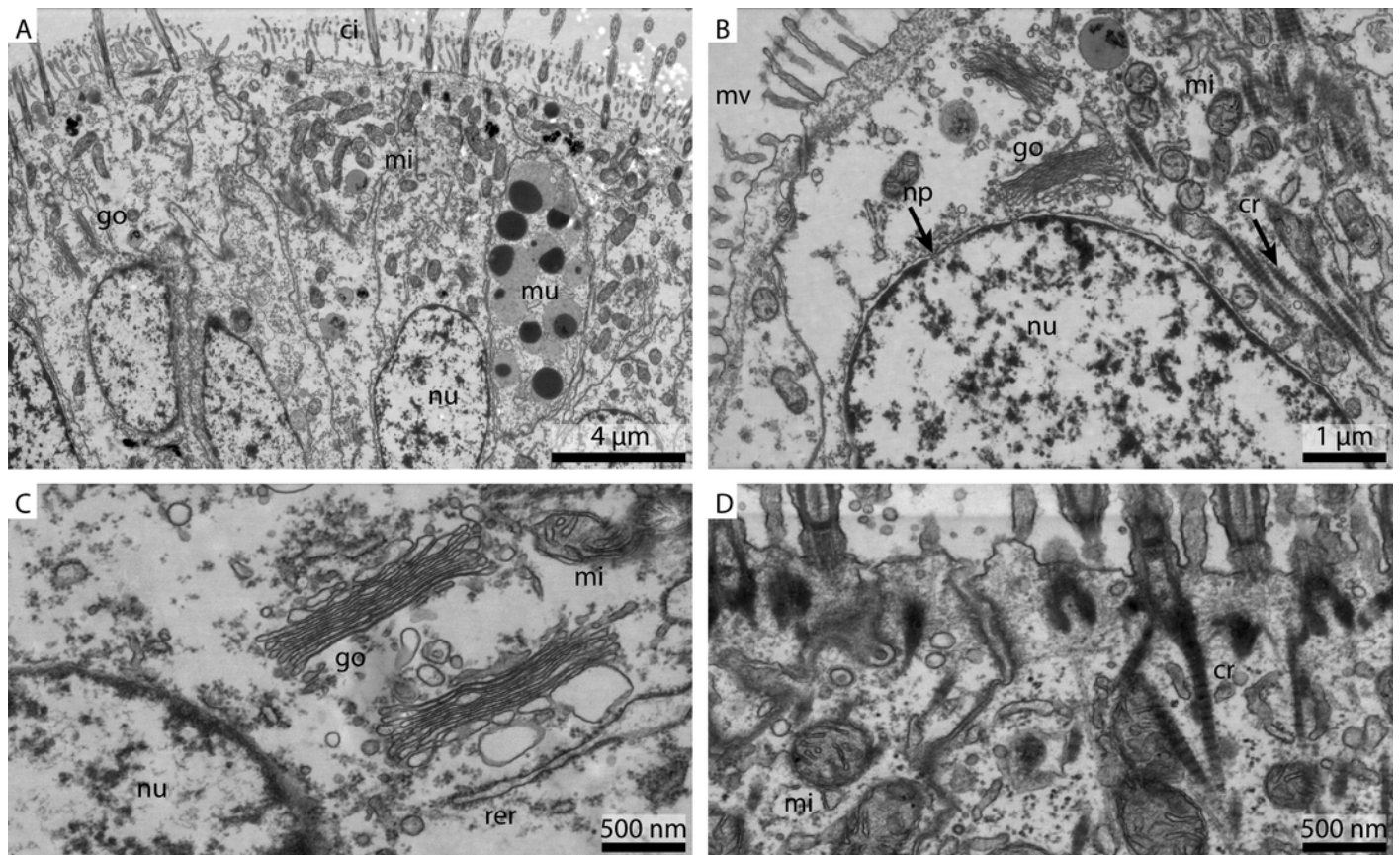
Figure 1: Overview of four differently fixed *Mytilus edulis* gills. All images show transverse sections through gill filaments focusing on the ciliated frontal surface. (A) shows a seawater-GA fixed sample, (B) a marCaco fixed sample, (C) a marPHEM fixed sample and (D) a marPBS fixed sample. ci = cilia,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

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.

4

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

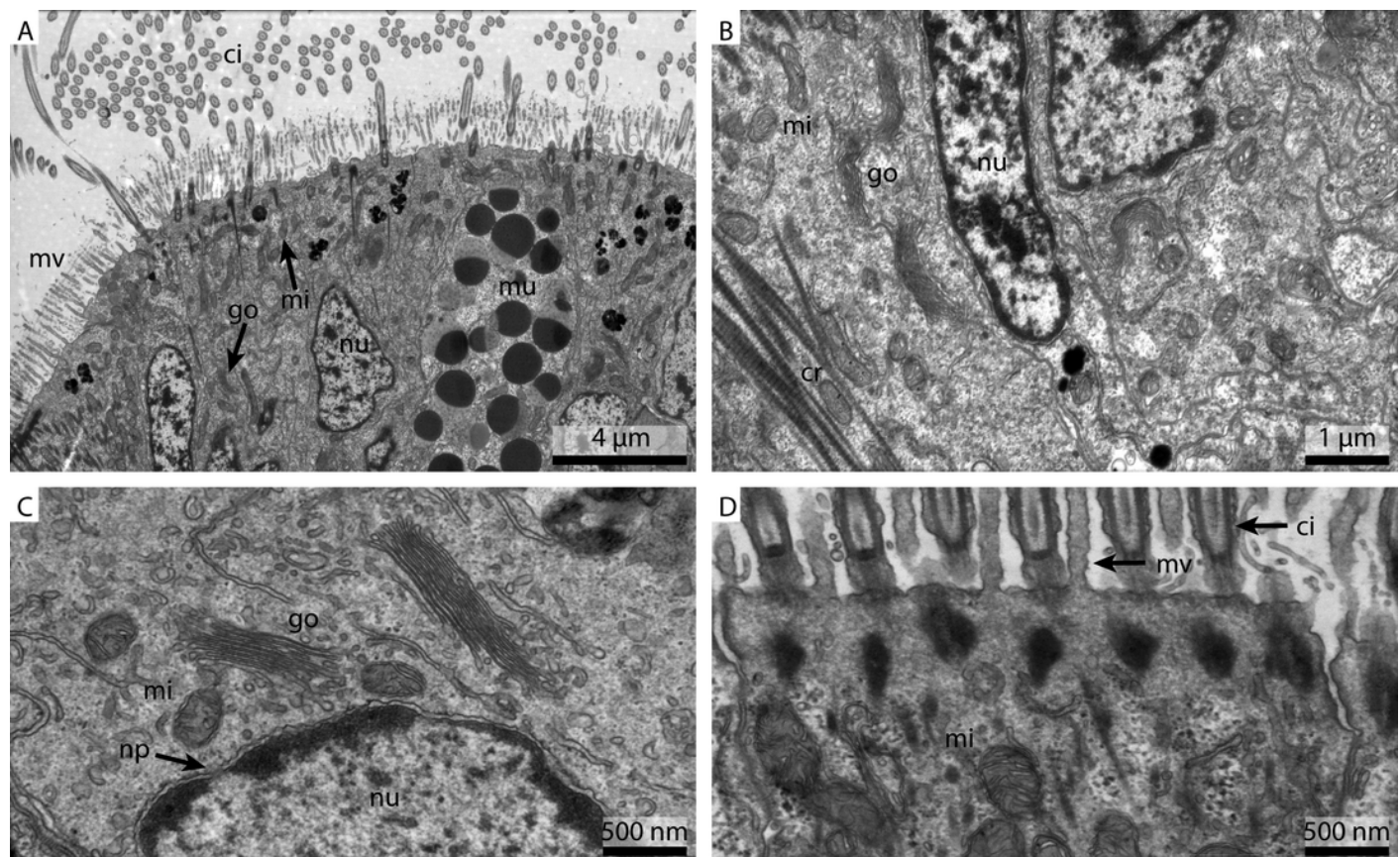
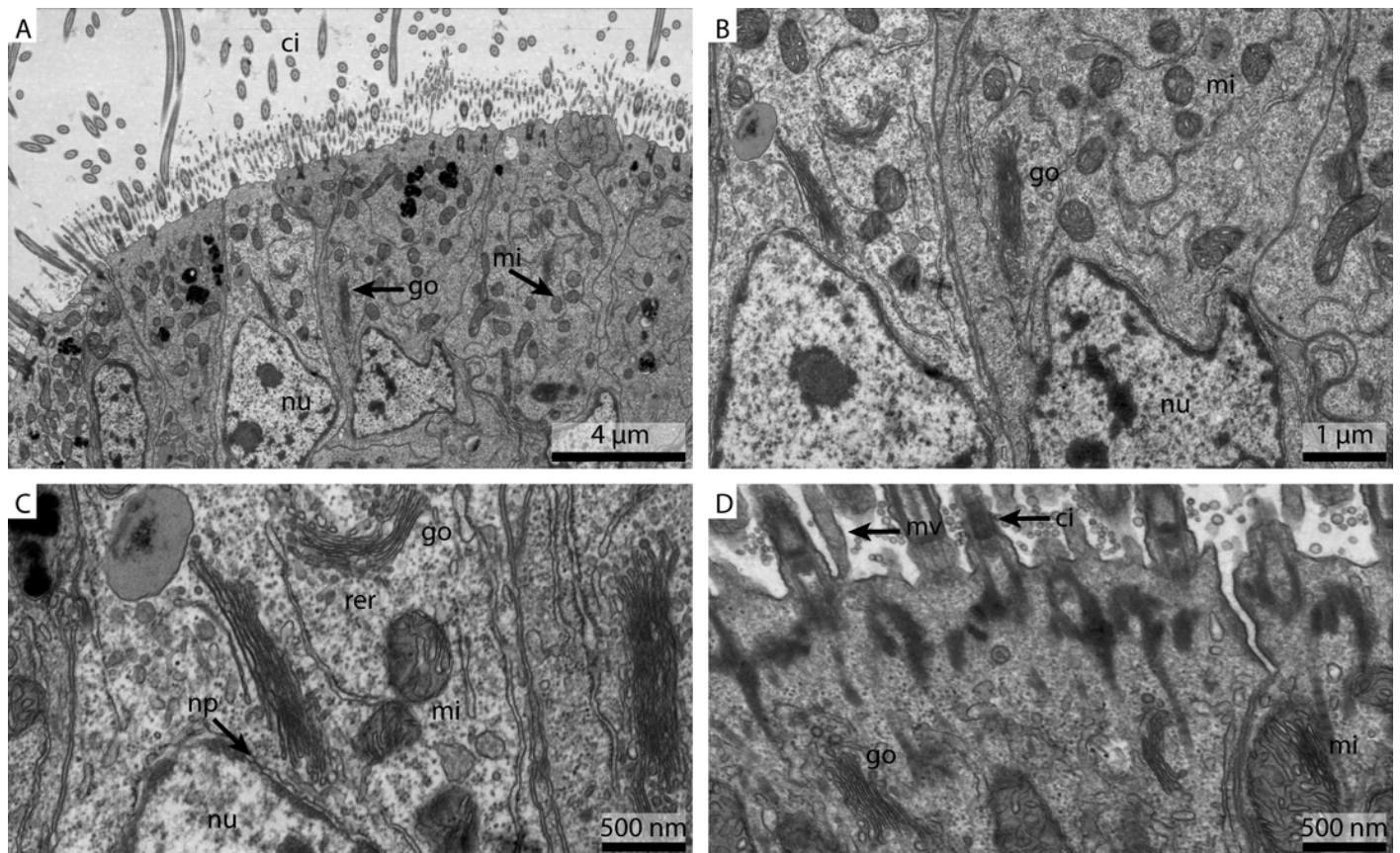
Figure 4: Ultrastructure of marPHEM fixed gill cell of *Mytilus edulis* (A) shows an overview of the cells of the ciliated frontal surface, (B) is a higher magnification of the same, (C) shows the nucleus in higher magnification and (D) shows details of the cell surface. go = Golgi apparatus, nu = nucleus,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

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.