# Algae from Aiptasia egesta are robust representations of Symbiodiniaceae in the free-living state

**Shumpei Maruyama** Corresp., 1 , **Julia R Unsworth** 2 , **Valeri Sawiccy** 1 , **Virginia M Weis** 1

1 Department of Integrative Biology, Oregon State University, Corvallis, OR, United States

2 Department of Biology, Lewis and Clark College, Portland, OR, United States

Corresponding Author: Shumpei Maruyama
Email address: shumpeim@gmail.com

Many cnidarians rely on their dinoflagellate partners from the family Symbiodiniaceae for their ecological success. Symbiotic species of Symbiodiniaceae have two distinct life stages: inside the host, *in hospite*, and outside the host, *ex hospite*. Several aspects of coral-algal symbiosis can be understood by comparing these two life stages. Most commonly, algae in culture are used in comparative studies to represent the *ex hospite* life stage, however, nutrition becomes a confounding variable for this comparison because algal culture media is nutrient rich, while algae *in hospite* are sampled from hosts maintained in oligotrophic seawater. In contrast to cultured algae, expelled algae may be a more robust representation of the *ex hospite* state, as the host and expelled algae are in the same seawater environment, removing differences in culture media as a confounding variable. Here, we studied the physiology of algae released from the sea anemone *Exaiptasia diaphana* (commonly called Aiptasia), a model system for the study of coral-algal symbiosis . In Aiptasia, algae are released in distinct pellets, referred to as egesta, and we explored its potential as an experimental system to represent Symbiodiniaceae in the *ex hospite* state. Observation under confocal and differential interference contrast microscopy revealed that egesta contained discharged nematocysts, host tissue, and were populated by a diversity of microbes, including protists and cyanobacteria. Further experiments revealed that egesta were released at night. In addition, algae in egesta had a higher mitotic index than algae *in hospite*, were photosynthetically viable for at least 48 hrs after expulsion, and could competently establish symbiosis with aposymbiotic Aiptasia. We then studied the gene expression of nutrient-related genes and studied their expression using qPCR. From the genes tested, we found that algae from egesta closely mirrored gene expression profiles of algae *in hospite* and were dissimilar to those of cultured algae, suggesting that algae from egesta are in a nutritional environment that is similar to their *in hospite* counterparts. Altogether, evidence is provided that algae from Aiptasia egesta are a robust representation of Symbiodiniaceae in the *ex hospite* state and

their use in experiments can improve our understanding of cnidarian-algal symbiosis.

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2    **state**

3    Shumpei Maruyama[1], Julia R. Unsworth[2], Valeri Sawiccy[1], Students of Oregon State

4    University's Z362 Spring 2021[1], Virginia M. Weis[1]

6    [1] Department of Integrative Biology, Oregon State University, Corvallis, OR, United States of

7    America

8    [2] Department of Biology, Lewis & Clark College, Portland, OR, United States of America

11    Corresponding Author:

12    Shumpei Maruyama[1]

13    3029 Cordley Hall, Corvallis, OR, 97330, United States of America

14    Email address: shumpeim@gmail.com

24    **Abstract**

25        Many cnidarians rely on their dinoflagellate partners from the family Symbiodiniaceae

26    for their ecological success. Symbiotic species of Symbiodiniaceae have two distinct life stages:

27    inside the host, *in hospite*, and outside the host, *ex hospite*. Several aspects of coral-algal

28    symbiosis can be understood by comparing these two life stages. Most commonly, algae in

29    culture are used in comparative studies to represent the *ex hospite* life stage, however, nutrition

30    becomes a confounding variable for this comparison because algal culture media is nutrient rich,

31    while algae *in hospite* are sampled from hosts maintained in oligotrophic seawater. In contrast to

32    cultured algae, expelled algae may be a more robust representation of the *ex hospite* state, as the

33    host and expelled algae are in the same seawater environment, removing differences in culture

34    media as a confounding variable. Here, we studied the physiology of algae released from the sea

35    anemone *Exaiptasia diaphana* (commonly called Aiptasia), a model system for the study of

36    coral-algal symbiosis. In Aiptasia, algae are released in distinct pellets, referred to as egesta, and

37    we explored its potential as an experimental system to represent Symbiodiniaceae in the *ex*

38    *hospite* state. Observation under confocal and differential interference contrast microscopy

39    revealed that egesta contained discharged nematocysts, host tissue, and were populated by a

40    diversity of microbes, including protists and cyanobacteria. Further experiments revealed that

41    egesta were released at night. In addition, algae in egesta had a higher mitotic index than algae *in*

42    *hospite*, were photosynthetically viable for at least 48 hrs after expulsion, and could competently

43    establish symbiosis with aposymbiotic Aiptasia. We then studied the gene expression of nutrient-

44    related genes and studied their expression using qPCR. From the genes tested, we found that

45    algae from egesta closely mirrored gene expression profiles of algae *in hospite* and were

46    dissimilar to those of cultured algae, suggesting that algae from egesta are in a nutritional

47   environment that is similar to their *in hospite* counterparts. Altogether, evidence is provided that

48   algae from Aiptasia egesta are a robust representation of Symbiodiniaceae in the *ex hospite* state

49   and their use in experiments can improve our understanding of cnidarian-algal symbiosis.

50

**Introduction**

52       The family Symbiodiniaceae is a diverse group of dinoflagellates, many of which form

53   symbioses with a variety of cnidarian hosts, including corals and sea anemones. In this

54   symbiosis, the algal symbiont is housed within host gastrodermal cells where they provide

55   photosynthate to the host and in return, the host provides inorganic nutrients, protection and a

56   high light environment (Davy, Allemand & Weis, 2012; Marcelino et al., 2013). The two

57   partners must work cooperatively to maintain and regulate symbiosis. For example, the host

58   utilizes carbonic anhydrase and bicarbonate transporters to supply inorganic carbon to the

59   symbiont to maximize photosynthesis (Tansik, Fitt & Hopkinson, 2017; Koch, Verde & Weis,

60   2020). Reciprocally, the symbiont may be modulating the host's immune system to prevent

61   immune destruction or expulsion by the host (Detournay et al., 2012; Mansfield et al., 2017;

62   Jacobovitz et al., 2021; Jinkerson et al., 2022). However, our understanding of the cellular

63   mechanisms involved in maintaining symbiosis remains limited.

64       Studies often compare the biology of Symbiodiniaceae between its two life stages: inside

65   the host, *in hospite* and outside the host, *ex hospite*. In this comparison, algae that are freshly

66   isolated from the host are used to represent algae *in hospite*, while cultured algae are often used

67   to represent algae *ex hospite*. Studies have found that compared to algae in culture, algae *in*

68   *hospite* have lower cell division rates (Smith & Muscatine, 1999; Tivey, Parkinson & Weis,

69   2020) and differential expression of genes that are tightly linked to nutrient limitation (Maor-

70    Landaw, van Oppen & McFadden, 2020; Xiang et al., 2020; Cui et al., 2022). Based on these

71    data, it is commonly hypothesized that the host limits nutrient transfer to the symbiont as a

72    mechanism to maintain a stable population of symbionts (Falkowski et al., 1993; Smith &

73    Muscatine, 1999; Xiang et al., 2020; Tivey, Parkinson & Weis, 2020; Cui et al., 2022). This

74    comparison, however, introduces nutrition as a confounding variable because while algae *in*

75    *hospite* are sampled from hosts maintained in oligotrophic seawater, algae *ex hospite* are sampled

76    from cultures grown in nutrient-rich media. The nutrient-depleted phenotype found in algae *in*

77    *hospite* could therefore not be caused by host-inhibitory mechanisms and instead may simply

78    reflect the low nutrient availability in the water column surrounding hosts (for in-depth

79    discussion, see Maruyama & Weis, 2021). In fact, studies have found that supplementing

80    nutrients to the host, by feeding or by the addition of inorganic nutrients to the water column,

81    will return the algae to a nutrient-enriched phenotype as measured by cell division rates or gene

82    expression profiles, suggesting that the host readily supplies nutrients to their symbionts when

83    available (Hoegh-Guldberg & Smith, 1989; Stambler et al., 1991; Falkowski et al., 1993; Hoegh-

84    Guldberg, 1994; Muller-Parker et al., 1994; Smith & Muscatine, 1999; Ferrier-Pagès et al., 2001;

85    Houlbrèque et al., 2004; Rosset, D'Angelo & Wiedenmann, 2015; Xiang et al., 2020; Tivey,

86    Parkinson & Weis, 2020; Cui et al., 2022). Further work is necessary to tease apart the effects of

87    symbiosis and nutrient availability on algal phenotypes to determine whether the host is limiting

88    nutrient transfer to its symbionts.

89          Multiple transcriptomic studies have found that nutrient-related genes are differentially

90    expressed between algae *in hospite* and those in culture (Maor-Landaw, van Oppen &

91    McFadden, 2020; Xiang et al., 2020; Cui et al., 2022). Examples include ammonium transporter

92    *AMT*, nitrate transporter *NRT*, purine nucleoside permease *NUP*, and nitrate reductase *NR* that

93      are upregulated and glutamine synthetase *GS* that is downregulated in algae *in hospite* compared

94      to those in culture. This gene expression pattern is indicative of nutrient limitation in

95      Symbiodiniaceae and other marine algae (Hildebrand, 2005; Kang et al., 2007; Xiang et al.,

96      2020). Some differentially expressed genes, however, are probably not linked to nutrition, such

97      as the sugar transporter *SWEET1*, that is hypothesized to play a role in transferring photosynthate

98      to the host. Sampling a more robust representation of Symbiodiniaceae in the *ex hospite* state that

99      controls for nutrient availability could confirm whether these differentially regulated genes are

100     reflective of nutrition, the symbiotic state, or both.

101             In cnidarian-algal symbiosis, the host regularly expels viable algae as a homeostatic

102     mechanism to maintain a stable population of symbionts (Steele, 1975; Hoegh-Guldberg,

103     McCloskey & Muscatine, 1987; Hill & Ralph, 2007; Thornhill et al., 2017). In this study, we

104     hypothesized that sampling expelled algae as a representation of the *ex hospite* state in

105     Symbiodiniaceae for comparison with their *in hospite* counterparts could remove nutrition as a

106     confounding variable, as both the host and the expelled algae are in the same oligotrophic

107     seawater environment. Therefore, physiological and/or gene expression differences found

108     between algae *in hospite* and expelled algae in this comparison would be modulated by the host,

109     and not be masked by the high-nutrient state of cultured algae as in the traditional comparison. In

110     the sea anemone *Exaiptasia diaphana* (commonly called Aiptasia), a model system for the study

111     of coral-algal symbiosis, algae are released in distinct, brown pellets, herein referred to as egesta.

112     The brown egesta primarily consist of algae, and are released separately from pellets containing

113     digested food (Steele, 1975). The discrete nature of egesta, with their high abundance of algae,

114     made them a promising experimental system for sampling purposes.

115    To begin the practice of sampling expelled algae in symbiosis studies, we first explored

116    the basic biology of algae from Aiptasia egesta and characterized egesta appearance, timing of

117    egesta expulsion, and characteristics of the algae including mitotic index, algal photophysiology,

118    and ability to initiate symbiosis with new hosts. Then, we studied the gene expression of several

119    nutrient-related genes to determine the nutrient status of algae from egesta. Finally, we discuss

120    several practical considerations of the use of algae from Aiptasia egesta as an experimental

121    model system to represent Symbiodiniaceae in the *ex hospite* state.

122

123    **Materials & Methods**

124    *Animal and algal culture maintenance*

125    Aiptasia (*Exaiptasia diaphana*, clone ID: H2) symbiotic with *Breviolum minutum*

126    (culture ID: SSB01) and SSB01 cultures were used in this study. Animals were maintained in

127    Coralife Instant Ocean (Tempe, AZ, USA) artificial seawater (ASW), and algae were maintained

128    in F/2 media made in filtered artificial seawater (FSW). Animals and algae were housed at 25 °C

129    in a Percival AL-41L4 incubator (Perry, IA, USA) with Zoo Med Laboratories (San Luis Obispo,

130    CA, USA) 10,000k fluorescent lamps set to 40 μmol photons/m$^2$/s using shadecloth on a 12h:12h

131    light:dark cycle. Aposymbiotic anemones were originally bleached using menthol (Matthews *et*

132    *al.*, 2016) and maintained symbiont-free by incubation in the dark for several months before use.

133    Unless otherwise stated, animals were fed three times a week *ad libitum* with freshly hatched

134    brine shrimp and the water was changed three times a week with ASW.

135

136    *Confocal imagery and light microscopy*

137    Live egesta samples were imaged on a Zeiss LSM 780 NLO confocal microscope system

138    (Oberkochen, Germany). Images were taken on three separate channels, with excitation from a

139    405 nm Diode, Argon (488), and 633 nm HeNe. Emission was detected between 410-470 nm,

140    491-572 nm, and 647-722 nm, respectively. Simultaneously, using laser illumination, a

141    transmitted light detector (T-PMT) acquired transmitted light images in bright field. Confocal z-

142    stack images were processed for pseudocoloring and merged to create a maximum intensity

143    projection using ZEN Black (Carl Zeiss AG) and ImageJ software. Separately, differential

144    interference contrast (DIC) images were obtained using an Olympus Vanox-T AH2 microscope

145    to image nematocysts.

146

147    *Time-lapse of egesta release*

148    Sixteen to twenty Aiptasia were transferred to twenty cm diameter glass Carolina

149    Biological (Burlington, NC, USA) culture dishes with one L of ASW. Animals were allowed to

150    settle for at least 24 hrs prior to time-lapse photography. Immediately before imaging began, the

151    culture dish was cleaned with a cotton swab and the water was changed. For time-lapse imaging,

152    a Canon 5D Mark II DSLR with external flash was set to photograph the entire dish hourly for

153    24 hrs. To control for any effect of cleaning on timing of egesta release, two time-lapses

154    (Experiment 1 & 2) began at 16:30 and two other time-lapses (Experiment 3 & 4) began at

155    22:30. Following their original light schedule, lights were set to come on at 7:00 and off at 19:00.

156    Animals were not fed during the experiment. Each image was analyzed with ImageJ using the

157    Cell Counter plugin, and each dark particle in an image were counted as egesta. The number of

158    egesta in each time point was subtracted from the total number of egesta in the previous time

159    point to calculate the quantity of egesta released in the hour. The quantity of egesta was then

160  normalized to anemone number per container to obtain the number of egesta per anemone at

161  each time point.

162

163  *Mitotic Index*

164      Thirty-two Aiptasia were housed individually in two mL FSW in 24-well plates and

165  allowed to settle for 24 hrs. After settlement, each well was cleaned with a cotton swab and the

166  water was changed. Egesta were collected after an additional 24 hrs. Of the 32 original

167  anemones, eight released egesta large enough to be imaged. The eight anemones that released

168  usable egesta were homogenized with a microcentrifuge pestle in 300 µL FSW, spun down at

169  800 x g for five min, and resuspended in twenty µL FSW. Resuspended algae and egesta were

170  then imaged under DIC light microscopy.

171      Images were analyzed using ImageJ with the Cell Counter plugin by students enrolled in

172  Oregon State University's Spring 2021 Z362 Invertebrate Biology laboratory. Following the

173  method of Baghdasarian and Muscatine (2000), individual cells in each image were scored based

174  on presence of a cell wall division plate, and at least 700 cells were counted per sample. Students

175  were tasked to save their cell counter files, and their counts were manually verified by loading

176  the counter files onto the images. The mitotic index was calculated as the percent of cells in the

177  entire population that were undergoing division and paired t-tests were performed for statistical

178  analysis.

179

180  *Algal photophysiology and cell viability*

181      Egesta were collected from Aiptasia less than 24 hrs after last cleaning, to ensure

182  collection of freshly expelled egesta. All collected egesta were pooled together and pelleted at

183   800 x g for five min, the supernatant was removed and the cells resuspended in one mL FSW.

184   The pooled sample was divided into two 500 μL aliquots. One was kept intact, while the other

185   was homogenized using a motorized microcentrifuge pestle. From the homogenized sample,

186   twenty μL was used to quantify cell number using a Thermo Fisher Scientific (Waltham, MA,

187   USA) Countess II automated cell counter. Homogenized and intact egesta samples were then

188   dispensed into twelve well plates in 95 μL (20,000 cells per sample, $n = 5$) and FSW was added

189   up to two mL. Cultured algae (SSB01) was also collected, pelleted at 800 x g for five min and

190   resuspended in FSW. Five culture samples of 20,000 cells were then dispensed in twelve well

191   plates and FSW added up to two mL. All plates were then returned to their original incubator.

192       Maximum quantum yield of photosystem II (Fv/Fm) was obtained using a Light Induced

193   Fluorescence Transient Fast Repetition Rate fluorometer (LIFT-FRRf; Soliense Inc, Santa Cruz,

194   CA). Fv/Fm was measured once a day at 13:00 following dark acclimation for 30 min to ensure

195   measurement of maximum quantum yield. To measure Fv/Fm, excitation was delivered at 475

196   nm wavelength in four phases and fluorescence detected at 685 nm. The first phase was a

197   saturating sequence of 100 flashlets, each lasting 0.7 μs with a 1.5 μs gap. The second phase was

198   a relaxation phase of 80 flashlets beginning with a gap of twenty μs, increasing exponentially

199   until the end of the sequence. The third phase was a sequence of 1600 flashlets lasting two μs

200   each with a 40 μs gap. The final relaxation phase was identical to the second phase. Data were

201   fitted using LIFT software with the three-component exponential model (SEQ_3).

202       To test the ratio of live and dead cells, we used an Evans Blue cell viability dye (Morera

203   & Villanueva, 2009). Approximately ten pellets of egesta less than 24 hrs old were collected and

204   homogenized together using a motorized microcentrifuge pestle in 500 μL of ASW. Cells were

205   then pelleted at 800 x g for five min and the pellet resuspended in 100 μL of FSW. Cells were

206    then stained with 20 µL of Evans Blue dye and incubated for ten min. The sample was imaged

207    under light microscopy and algal cells that internalized the blue dye were counted as dead while

208    cells that were not stained were counted as alive.

209

210    *Inoculation of Aiptasia with algae*

211        Aposymbiotic anemones (n = 9) were plated into 24 well plates in two mL FSW. Plates

212    were placed in their original incubators set on a 12h:12h light:dark cycle for 72 hrs. After

213    incubation in the light, animals were manually checked under fluorescence microscopy to verify

214    aposymbiotic status.

215        Egesta were collected from Aiptasia less than 24 hrs after last cleaning. Samples of both

216    egesta and algae in culture were pelleted at 800 x g for five min and resuspended in 300 µL

217    FSW. To obtain freshly isolated symbionts, one symbiotic anemone was homogenized using a

218    motorized microcentrifuge pestle in 300 µL of FSW. Egesta and cultured algae were also

219    homogenized using a motorized microcentrifuge pestle. All samples were then washed twice by

220    centrifugation at 800 x g for five min and resuspension in two mL FSW. Algal samples were

221    counted using a Countess II automated cell counter, and samples were diluted to 1 x $10^6$ algal

222    cells/mL in FSW.

223        To inoculate aposymbiotic Aiptasia, 1 x $10^5$ algal cells in a 100 µL volume were gently

224    pipetted over anemone mouths. Immediately after the addition of algae, twenty µL of brine

225    shrimp extract was pipetted to anemones to induce a feeding response. Negative controls were

226    fed the brine shrimp extract with no algae added. Anemones were allowed to take up algae for 24

227    hrs. The water was then changed, and anemones were moved to clean wells of a 24 well plate in

228    two mL FSW. After an additional 24 hrs, the anemones were imaged under fluorescence

229    microscopy. Briefly, anemones were relaxed in 0.18 M MgCl$_2$ dissolved in FSW, and images of

230    three tentacles per anemone were taken in several focal depths under brightfield and under the

231    red Filter Set 15 (Carl Zeiss) to visualize algal cell auto-fluorescence using a Zeiss Axio

232    Observer inverted microscope. Each manually constructed z-stack was merged into a single

233    image using Adobe Photoshop. Symbiont density was determined from each image using ImageJ

234    as the number of fluorescent algal cells normalized to two-dimensional tentacle area.

235

236    *Primer design*

237         Target genes were selected based on their function and differential expression between

238    algae in culture and *in hospite* from Xiang et al. (2020). Primer and transcript sequences were

239    obtained from Xiang et al. (2015, 2020) for an ammonium transporter (*AMT*, transcript ID:

240    s6_38207), a nitrate transporter (*NRT*, s6_422), purine nucleoside permease (*NUP*, s6_27864),

241    and nitrate reductase (*NR*, s6_34). New primers were designed for glutamine synthetase (*GS*,

242    s6_5551), photosystem II protein D1 (*psbA*, s6_1009), and a sugar transporter (*SWEET1*,

243    s6_35311). We chose to study the expression of *psbA* because its expression was consistent

244    between algae in culture and *in hospite* (Xiang et al., 2020). Cyclophilin was chosen as an algal

245    housekeeping gene (Rosic et al., 2011) and primers were designed to be specific for *B. minutum*

246    and not amplify Aiptasia cyclophilin. Primer pairs were verified for amplification efficiency of at

247    least 90% by assessing the slope of standard curves generated from quantitative PCR reactions of

248    serial dilutions of template cDNA. Primers sequences are available in the supplementary data.

249

250    *Quantitative PCR*

251       To quantify gene expression, 54 anemones were placed in three separate containers with

252    500 mL ASW (eighteen anemones per container). The average oral disk diameter of the

253    anemones was 5.4 mm (data not shown). Anemones were fed every other day *ad libitum*. Egesta

254    containing algae were collected 24 hrs after feeding, and the container cleaned after egesta were

255    collected. We did not sample egesta containing digested food and they are easy to distinguish

256    due to the pink color of the cartenoids from digested *Artemia* sp. nauplii. To obtain enough

257    material for gene expression studies (approximately $1 \times 10^6$ cells), egesta were collected a total

258    of six times from each container. Immediately after collection, egesta were processed for RNA

259    extraction as follows. First, egesta were homogenized in cold FSW with a motorized

260    microcentrifuge pestle. To completely isolate algae from host cells, the sample was then further

261    homogenized by passing the sample seven times through a three mL syringe fitted with a 23

262    gauge needle. Following protocols from Xiang et al. (2020), the homogenate was then loaded on

263    a 50% isotonic Percoll solution made in FSW and spun for twenty min at 9000 x g. The

264    supernatant containing host material was carefully removed, and the algae were resuspended in

265    one mL cold FSW. The sample was then pelleted at 3,000 x g for five min and the supernatant

266    removed. The pellet was then frozen at -80 °C until further processing.

267       To obtain algae *in hospite*, four random anemones from each container were sampled

268    immediately after the last collection of egesta and processed for RNA extraction following the

269    same procedure as those for egesta, and the algal pellets frozen for at least ten min. Three

270    independent, but identical cultures of SSB01 grown in F/2 culture media were made

271    simultaneously and allowed to grow for three weeks. These cultures were also sampled and

272    followed the same procedure for RNA processing and the algal pellets were frozen for at least

273    ten min.

274       After the final sample of egesta was collected, the frozen samples from each repeated

275   collection were pooled together corresponding to their respective Aiptasia containers. Each

276   frozen freshly isolated, culture, and egesta sample was then resuspended in 300 µL Trizol.

277   Sterilized glass beads were added to the sample, and the sample was homogenized with a bead

278   beater for two min set at 4,000 RPM. The beads were then removed, the samples were

279   centrifuged at 16,000 x g for one min, and the supernatant containing RNA was extracted. The

280   RNA was purified using a Zymo Direct-zol kit following the manufacturer's instructions. RNA

281   samples were further processed using a Turbo DNA-free kit to remove genomic DNA

282   contaminants. Samples were then purified again using a New England Biolabs (Ipswich, MA,

283   USA) Monarch RNA purification kit, followed by a One-Step PCR inhibitor removal kit to

284   remove carbohydrate contaminants. The RNA samples were analyzed for purity and

285   concentration using a Nanodrop.

286       All samples were then diluted to ten ng/µL and cDNA was synthesized from nine µL (90

287   ng total) of RNA in 30 µL reactions using the New England Biolabs Protoscript II cDNA

288   synthesis kit with oligo $d(T)_{23}$ VN primers following the manufacturer's instructions. Quantitave

289   PCR reactions were performed in twenty µL volumes using 1X Power SYBR Green PCR

290   Mastermix, 0.5 µM of each primer pair, and one µL of template cDNA. A BioRad CFX96 Real-

291   Time System was used to carry out reactions using a two-step amplification phase (95 °C/ten

292   min, followed by 40 cycles of 95 °C/ten s and 58 °C/30 s) and then a melting-curve analysis

293   performed to confirm the presence of single amplicons. No-reverse transcription, no-primer, and

294   no-template controls were included as negative controls.

295       For each sample and target gene, $\Delta Ct$ values were calculated by subtracting the $Ct$ value

296   of the target gene from the $Ct$ value of the housekeeping gene (cyclophilin). Then, the $\Delta Ct$ value

297   was subtracted from the mean $\Delta Ct$ value of corresponding genes from cultured algae samples to

298   calculate relative expression $\Delta\Delta Ct$. Fold gene expression was then calculated as $2^{-\Delta\Delta Ct}$ for each

299   gene from each sample. Statistical analyses were conducted on $\Delta\Delta Ct$ values for each gene by

300   ANOVA and post-hoc Tukey's HSD.

301

302   **Results**

303   *Physical characteristics of egesta*

304        Egesta observed under confocal microscopy revealed several autofluorescent features

305   (Figure 1). Chlorophyll autofluorescence was readily apparent from *B. minutum* under 633 nm

306   excitation. Under 488 nm excitation, autofluorescence of putative pyrenoids from *B. minutum*,

307   phycobillins from cyanobacteria, and host tissue were detected (Figure 1A). A signal was also

308   detected under 405 nm excitation within egesta (Figure 1A), and we hypothesize that its origin

309   comes from dead remains of an unidentified ciliate protist that autofluoresced under the same

310   excitation wavelength (Figure 1C). Differential interference contrast microscopy revealed that

311   the surface of egesta contained discharged nematocysts (Figure 1D) and the remains of

312   nematocyst capsules (Figure 1E). Egesta were also colonized by several kinds of unidentified

313   protists and microbes (data not shown). None of the protists were observed to phagocytose *B.*

314   *minutum*.

315

316   *Algal physiology*

317        Egesta were released at night, beginning five to six hrs after lights off and peaking at

318   seven to eight hrs after lights off (Figure 2A). The rate of release slowly decreased until lights

319   were turned on, and release decreased to background levels at the onset of light. The timing of

320  dish cleaning prior to time-lapse imaging had a minor effect on timing of egesta release by

321  delaying the time of peak egesta release by one hr (Figure 2A). We noticed that the size of egesta

322  was not uniform (data not shown) – presumably containing different amounts of algae, but we

323  were unable to quantify the size of egesta and the number of symbionts per egestum in our

324  experiments. Therefore, the time series data reflects only the number of discrete pellets that were

325  released by anemones and is a proxy for the true number of expelled algae.

326      The released algae were alive and biologically active. The mitotic index of algae from

327  egesta was higher than that of algae *in hospite* (Figure 2B; $p = 0.01$, paired t-test), and the Fv/Fm

328  of algae in egesta, disrupted and intact, at time zero was the same as those in symbiotic

329  anemones (Figure 2C). Algae from egesta then declined in Fv/Fm over time, with Fv/Fm

330  remaining relatively high for two days before declining rapidly, with chlorophyll fluorescence

331  becoming unmeasurable by day five. Mechanically disrupting the egesta did not have a

332  significant impact on algal photophysiology. Algae from culture started at a lower Fv/Fm than

333  those from egesta or anemones, but Fv/Fm did not decline over time. Cell staining with Evans

334  Blue dye found that 1.5% (17 out of 1132 total cells) of algae from egesta were dead. Algae from

335  egesta were capable of initiating symbiosis with aposymbiotic Aiptasia, reaching symbiont

336  densities just as high as Aiptasia inoculated with cultured and freshly isolated algae (Figure 2D).

337  Negative controls remained aposymbiotic for the duration of the experiment (data not shown).

338  While freshly isolated symbionts reached the highest density of symbionts, the difference was

339  not significant ($p = 0.06$, Kruskal-Wallis test).

340

341  *Gene expression*

342    Nitrogen transporter and nitrogen metabolism genes, *AMT*, *NRT*, *NUP*, and *NR*, were

343    significantly upregulated in algae from egesta and algae *in hospite* compared to culture (Figure

344    3). Expression levels of these genes were not significantly different between algae from egesta

345    and algae *in hospite*. Glutamine synthetase, *GT*, gene expression showed no significant

346    difference between algal sources. Expression levels of *psbA* and sugar transporter, *SWEET1*,

347    were highest in cultured algae compared to algae from egesta and algae *in hospite*. Overall, with

348    the exception of glutamine synthetase, expression levels of the tested genes were similar between

349    algae from egesta and algae *in hospite* compared to algae in culture.

350

351    **Discussion**

352    *Egesta are sticky communities of microbes*

353    Egesta primarily consisted of *B. minutum* as previously reported (Steele, 1975) and these

354    egesta were released separately from digested food.  In addition, egesta contained host material

355    and a variety of microbes (Figure 1). In sample handling, we noted that egesta were sticky and

356    tended to adhere to plastic surfaces. The stickiness is possibly caused by the presence of fired

357    nematocysts that were present on the egesta surface (Figure 1D, 1E). The presence of

358    nematocysts, both fired and unfired, were previously described in algae-containing egesta from

359    the sebae anemone, *Heteractis crispa* (Alan Verde, Cleveland & Lee, 2015). The authors noted

360    that egesta released by *H. crispa* were consumed by symbiotic anemonefish (Alan Verde,

361    Cleveland & Lee, 2015). It is not known if other animals consume Aiptasia egesta in nature, but

362    if so, they may aid in algal dispersal, as algae often survive digestion (Parker, 1984; Grupstra et

363    al., 2021, 2022).

364

365    *Evidence for the preferential expulsion of dividing algae*

366         The mitotic index of algae was higher in egesta than *in hospite*, a result that agrees with

367    previous findings in Aiptasia and several corals (Baghdasarian & Muscatine, 2000). It was

368    hypothesized by Bhagdasarian and Muscatine (2000) that the host preferentially expels dividing

369    algae. This hypothesis is well-supported from a temporal perspective because the egesta were

370    released at night, and several studies have shown that peak cell division occurs at night in

371    cultured Symbiodiniaceae (Fitt & Trench, 1983; Smith & Muscatine, 1999; Yamashita & Koike,

372    2016; Fujise et al., 2018). In contrast, algal cell division was not elevated at night *in hospite* in

373    Aiptasia (Smith & Muscatine, 1999). In that study, however, the dividing algae may have been

374    expelled by the host prior to sampling, leading to the detection of unchanged cell division at

375    night (Smith & Muscatine, 1999). Measuring the mitotic index of algae from egesta that are

376    released over a diurnal cycle may help complete the picture of algal population dynamics *in*

377    *hospite*.

378         Nutrition may also explain the higher division rates in algae from egesta compared to

379    those *in hospite*. As nutrient availability has been shown to strongly predict cell division rates in

380    Symbiodiniaceae (Smith & Muscatine, 1999; Karako-Lampert et al., 2005; Tivey, Parkinson &

381    Weis, 2020), the host may be preferentially expelling algae that sequester more nutrients for cell

382    division than their more cooperative counterparts that remain undivided *in hospite*. Indeed, in

383    competitive inoculation experiments, the symbiont *Durusdinium trenchii*, known to sequester

384    more nitrogen than *B. minutum* in Aiptasia (Sproles *et al.*, 2020), failed to proliferate in Aiptasia

385    in the presence of the homologous *B. minutum* (Gabay *et al.*, 2019). The preferential expulsion

386    of dividing algae could be a mechanism for the removal of uncooperative symbionts. Future

387    experiments using methods such as stable isotope analysis and NanoSIMS could investigate

388    whether expelled algae sequester more nutrients than their counterparts *in hospite* and whether

389    they continue to sequester more nutrients when establishing symbiosis with new hosts.

390

391    *Timing of algal release varies depending on host taxa*

392            The night-time release of algae in egesta by Aiptasia is not shared by other hosts. In

393    several corals, including *Pocillopora damicornis* and *Acropora digitifera*, expulsion rates

394    increased with the onset of light and peaked in the middle of the day (Stimson & Kinzie, 1991;

395    Koike et al., 2007). Other studies in the coral *Stylophora pistillata*, the soft coral *Xenia*

396    *macrospiculata*, and the giant clam *Tridacna crocea* found no clear diel pattern for algal release

397    (Hoegh-Guldberg, McCloskey & Muscatine, 1987; Umeki et al., 2020). Some corals released

398    algae at night, with peak expulsion rates occuring at night for the corals *Millepora dichotoma*

399    and *Heteroxenis fuscescens* (Hoegh-Guldberg, McCloskey & Muscatine, 1987). These

400    contrasting patterns may reflect the actual differences in patterns of algal release between

401    species, or it could reflect differences in sampling methodology. As peak algal motility occurs at

402    midday for Symbiodiniaceae (Fitt & Trench, 1983; Yamashita & Koike, 2016), some sampling

403    approaches could miss subpopulations of algae in the vessel depending on time of sampling.

404

405    *Algae from egesta are competent symbionts, but are short-lived* ex hospite

406            Algae from egesta were short-lived *ex hospite* compared to their cultured counterparts,

407    possibly due to nutrient limitation and/or the unknown effects of being in a microbial community

408    (Figure 2C). This supports the hypothesis that expelled algae do not form stable populations *ex*

409    *hospite* (Thornhill et al., 2017). However, freshly expelled algae from egesta had high viability

410    and were fully capable of initiating symbiosis with aposymbiotic hosts (Figure 2D). In nature,

411     larval and juvenile recruits probably rely on the continuous release of algae from adult hosts for a

412     source of symbionts (Thornhill et al., 2017). This is supported by studies that found that the

413     presence of adult coral colonies, presumably releasing algae, aided in symbiont acquisition by

414     juvenile corals in aquarium experiments (Nitschke, Davy & Ward, 2016; Ali et al., 2019). In

415     addition, a study found that a giant clam harboring Symbiodiniaceae released viable symbionts

416     that were able to establish symbiosis with *Acropora tenuis* larvae (Umeki et al., 2020). Egesta

417     are also potential vectors for the transmission of the microbiome between adults and recruits. In

418     corals, there is evidence that *Acropora tenuis* and *Pocillopora damicornis* expel beneficial

419     microbes into the seawater after spawning (Ceh, van Keulen & Bourne, 2013). Additional

420     experiments are required to test these hypotheses, particularly in coral recruits.

421

422     *Evidence that algae from egesta have a similar nutritional status to algae* in hospite

423           In general, in our study, gene expression profiles of algae from egesta were more like

424     those of algae *in hospite* than those of algae in culture. Several of the tested genes were nitrogen

425     transporters or metabolizers. Transcriptional regulation of these genes is rapid (less than a day)

426     in *B. minutum* and other marine algae in response to nutrient levels (Hildebrand, 2005; Kang *et*

427     *al.*, 2007; Xiang *et al.*, 2020). As the algae from sampled egesta were 1-2 days old, changes in

428     transcript levels for these genes should have occurred in this experiment if nutrient levels had

429     significantly changed once algae were expelled by their hosts. Our data suggest that algae from

430     egesta have a similar nutritional status to algae *in hospite*, challenging the hypothesis that the

431     host is actively limiting nutrient transport to the symbiont (Maor-Landaw, van Oppen &

432     McFadden, 2020; Xiang et al., 2020; Cui et al., 2022). The identification of nutrient-related

433     genes as differentially expressed in symbiosis studies that used cultured algae to represent the *ex*

434 *hospite* state may instead reflect the nutrient-rich state of algae in culture (Maor-Landaw, van

435 Oppen & McFadden, 2020; Xiang et al., 2020; Cui et al., 2022). In addition, we found that the

436 sugar transporter *SWEET1* (s6_35311), was not differentially regulated with symbiosis,

437 challenging the hypothesis that this gene copy of *SWEET1* functions to transfer photosynthate

438 from the symbiont to the host (Xiang et al., 2020). This current study, however, did not measure

439 algal growth rates in culture – therefore, we were unable to determine the growth phase of the

440 algae, which can have significant effects on algal physiology and gene expression (Droop, 1975;

441 Mansour, Volkman & Blackburn, 2003; Xiang et al., 2020). Nevertheless, we found that algae in

442 culture had the lowest expression rates of nutrient transporters and nutrient metabolizers,

443 indicating a nutrient-replete phenotype compared to algae from egesta and algae *in hospite*.

444 Altogether, these results warrant further testing of gene expression in algae from egesta using

445 high throughput methods to develop a better understanding of symbiosis.

446

447 *Practical considerations for the use of* Aiptasia *egesta in experiments*

448    Based on findings in this study, we argue that it is critical to further explore the biology

449 of expelled algae as a representation of the *ex hospite* state of Symbiodiniaceae to better

450 understand cnidarian-algal symbiosis. To facilitate their use by researchers, we discuss below

451 practical considerations for the use of Aiptasia egesta in experiments.

452    We found that while egesta are primarily composed of Symbiodiniacean algae, they are

453 also populated with microbes, including several protists and cyanobacteria. In our experience,

454 processing of egesta by homogenization and separation by density centrifugation yielded

455 samples of high purity. Collecting ample biological material for use in qPCR and inoculation

456 experiments was difficult. To obtain $1 \times 10^6$ algal cells from egesta, pellets had to be collected

457     six times every other day from approximately twenty anemones with an average oral disk size of

458     5.4 mm. We also found that collection was variable between days and yield was not consistent

459     (data not shown). For collection of egesta, if Aiptasia are housed without water flow, we

460     recommend using a glass pasteur pipet, as the egesta tended to stick to plastic pipets. Egesta can

461     also be mistaken for pedal lacerates, so it is recommended that a dissection microscope be used

462     to aid in identification. It is possible that various factors, such as anemone size, light levels, and

463     feeding, all influence the rate of egesta release, but these variables were not empirically tested.

464     Another consideration is the induction of algal release by mild stress to increase sample biomass,

465     however, the health of the algae may be affected, so this method will require further

466     experimentation.

467

468     **Conclusions**

469         Overall, this study establishes the foundation for using Aiptasia egesta in experiments as

470     a representation of algae in the *ex hospite* state. We found that egesta were released at night, and

471     that algae from egesta had a higher mitotic index than algae *in hospite*, were photosynthetically

472     active but short lived, and could establish symbiosis with new hosts. Finally, we found that algae

473     from egesta had a similar gene expression profile to that of algae *in hospite* compared to algae in

474     culture, warranting future high-throughput studies to determine symbiosis-specific genes in this

475     comparison. Although there are limitations to the use of algae from egesta, their use in

476     experiments alongside algae in culture and algae *in hospite* can provide valuable insight into

477     cnidarian-algal symbiosis.

478

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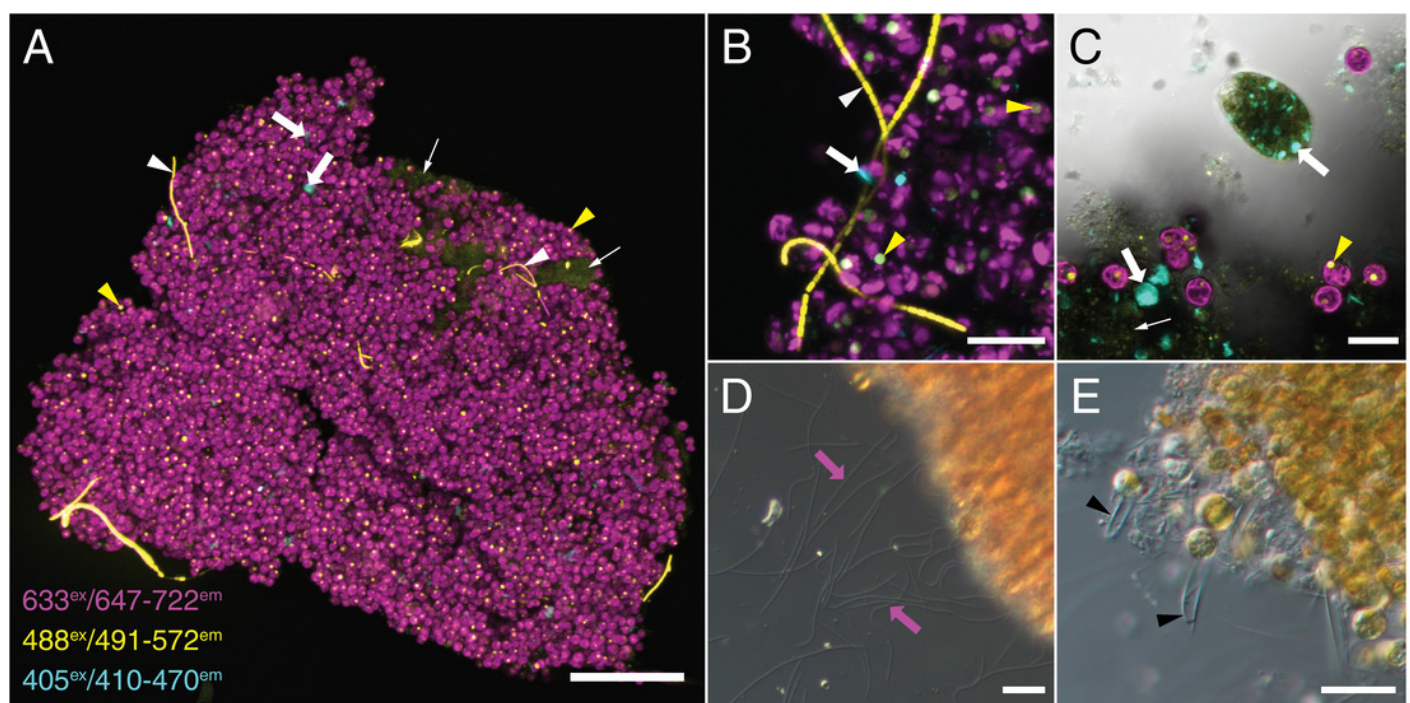
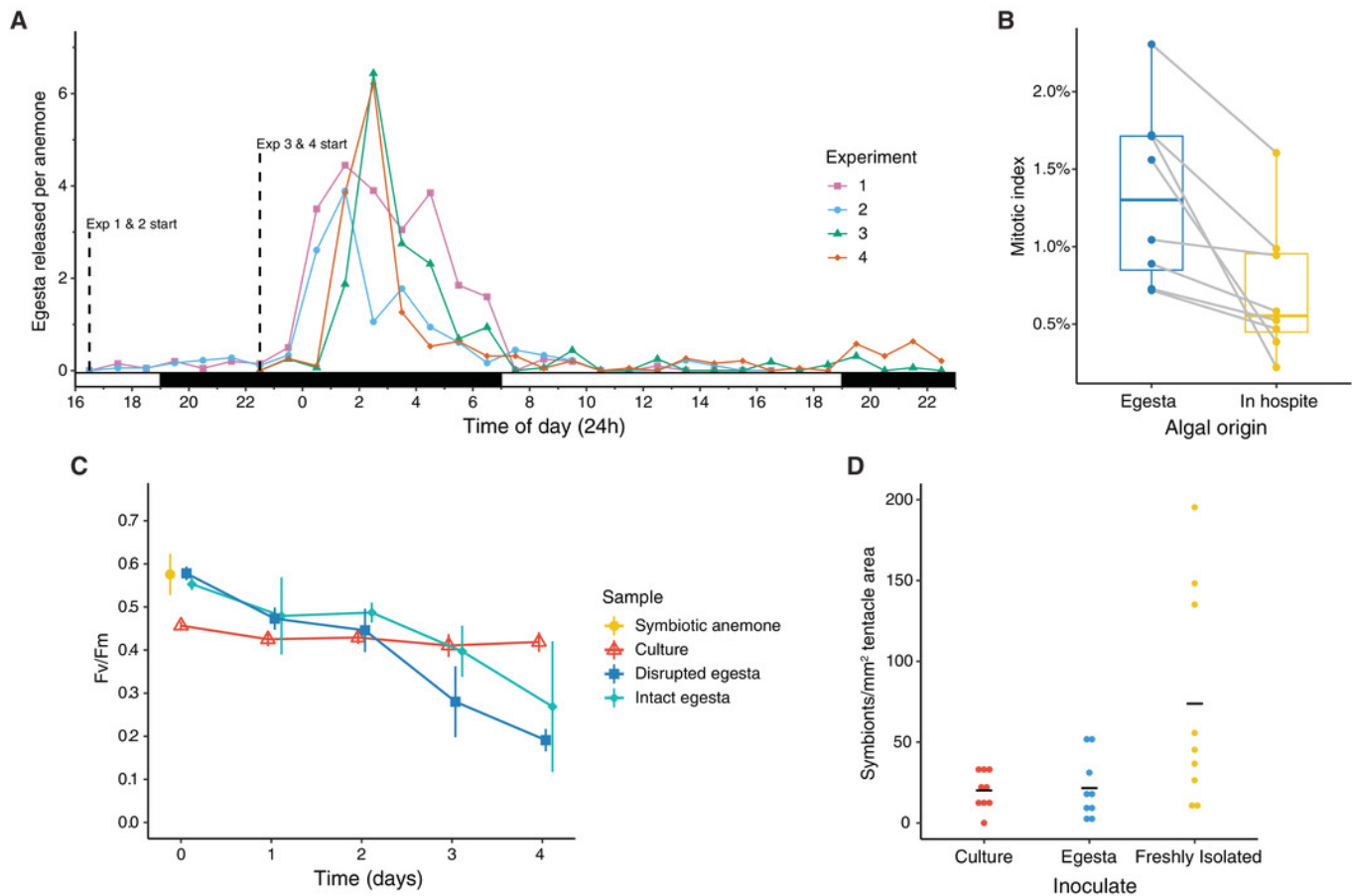
# Figure 1

Confocal and DIC images of whole Aiptasia egesta.

(A-C) Confocal images of whole Aiptasia egesta. Magenta: *Breviolum minutum* chlorophyll is detected with excitation at 633 nm and emission at 647-722 nm. Yellow: Cyanobacteria phycobillin (white arrowhead), host tissue (small white arrow), and *B. minutum* pyrenoids (yellow arrowhead) are detected with excitation at 488 nm and emission at 491-572 nm. Cyan: Pigment from an unknown protist (white arrow) is detected with excitation at 405 nm and emission at 410-470 nm. (D-E) Fired nematocysts (black arrows) and empty capsules (magenta arrowheads) are seen on the superficial surface of egesta under DIC microscopy. Scale bars: (A) 100 μm and (B-E) 20 μm.

# Figure 2

The physiology of algae from egesta.

(A) Amount of egesta released per anemone every hour over 24 hours. Experiments 1 and 2 began at 17:00 and experiments 3 and 4 began at 23:00. Black and white bars on x-axis depict dark and light periods, respectively. (B) The mitotic index of algae in egesta and in the host. Dots indicate individual samples and lines connecting dots indicate paired samples. The difference was statistically significant ($p$ = 0.01, Paired t-test). (C) The photosynthetic health of algae over time as measured by FRRf. Vertical lines indicate standard deviations around the mean. (D) Symbiont density in aposymbiotic Aiptasia inoculated with algae in culture, from egesta, or freshly isolated from hosts. Horizontal lines indicate mean symbiont density. Differences were not statistically significant ($p$ = 0.06, Kruskal-Wallis test).

# Figure 3

Gene expression of nutrient-related, photosynthesis, and sugar transporting genes between cultured algae, algae from egesta, and algae *in hospite.*

With the exception of *GS*, Expression of genes was similar in algae from egesta and algae *in hospite* compared to that of cultured algae. Bars indicate standard deviations around the mean. Transcript IDs are listed under gene abbreviations based on Xiang et al. (2015) . Stars indicate statistically significant differences calculated on ΔΔCt values as determined by ANOVA and post-hoc Tukey's tests. *p < 0.05, **p > 0.01, *** p < .0001, **** p < 0.00001