1    **Cellular reductase activity in uncultivated *Thiomargarita spp*. assayed using a redox-**

2    **sensitive dye**

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6    Running Head: Redox-sensitive dye assays of *Thiomargarita spp.*

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12  **ABSTRACT**

13  The largest known bacteria, *Thiomargarita spp.,* have yet to be isolated in pure culture, but their

14  large size allows for individual cells to be followed in time course experiments, or to be

15  individually sorted for 'omics-based investigations. Here we report a novel application of a

16  tetrazolium-based dye that measures the flux of reductase production from catabolic pathways to

17  investigate the metabolic activity of individual cells of *Thiomargarita* spp. When coupled to

18  microscopy, staining of the cells with a tetrazolium-formazan dye allows for metabolic responses

19  in *Thiomargarita* spp. to be to be tracked in the absence of observable cell division. Additionally,

20  the metabolic activity of *Thiomargarita* spp. cells can be differentiated from the metabolism of

21  other microbes in specimens that contain adherent bacteria. The results of our redox-dye-based

22  assay suggests that *Thiomargarita* is the most metabolically versatile under anoxic conditions

23  where it appears to express cellular reductase activity in response to the electron donors

24  succinate, acetate, citrate, formate, thiosulfate, $H_2$, and $H_2S$. Under hypoxic conditions, formazan

25  staining results suggest the metabolism of succinate, and likely acetate, citrate, and $H_2S$. Cells

26  incubated under oxic conditions showed the weakest formazan staining response, and then only

27  to $H_2S$, citrate, and perhaps succinate.  These results provide experimental validation of recent

28  genomic studies of *Ca.* Thiomargarita nelsonii that suggest metabolic plasticity and mixotrophic

29  metabolism. The cellular reductase response of bacteria attached to the exteriors of

30  *Thiomargarita* also supports the possibility of trophic interactions between these largest of

31  known bacteria and attached epibionts.

32  **IMPORTANCE**

33  The metabolic potentials of many microorganisms that cannot be grown in the laboratory are

34  known only from genomic data. Genomes of *Thiomargarita* spp. suggest that these largest of

35    known bacteria are mixotrophs, combining lithotrophic metabolisms with organic carbon

36    degradation. Our use of a redox-sensitive tetrazolium dye to query the metabolism of these

37    bacteria provides an independent line of evidence that corroborates the apparent metabolic

38    plasticity of *Thiomargarita* observed in recently produced genomes. Finding new cultivation-

39    independent means of testing genomic results is critical to testing genome-derived hypotheses on

40    the metabolic potentials of uncultivated microorganisms.

41

42    **INTRODUCTION**

43    Sulfide-oxidizing bacteria of the family Beggiatoaceae contain some of the largest known

44    bacteria (1-5), with individual cells of the genus *Thiomargarita spp.* reaching millimetric

45    diameters (3, 5, 6). Dense communities of these organisms on the seafloor comprise some of the

46    most spatially-extensive microbial mat ecosystems on Earth (4, 7). The large sulfur bacteria are

47    primarily chemolithotrophs or mixotrophs that live at interfaces between nitrate or oxygen, and

48    hydrogen sulfide (8-12). These bacteria can have a substantial influence on the biogeochemical

49    cycling of sulfur, nitrogen, phosphorus and carbon in diverse settings (11, 13-15).  Despite their

50    large size and biogeochemical significance, the physiologies and ecologies of these bacteria

51    remain incompletely understood, in part because the large sulfur bacteria are not presently

52    isolated in pure culture.  At present, *Thiomargarita* spp. can only be maintained long-term in

53    their natural sediments, not in axenic isolation or even as mixed communities in defined

54    microbial media. Therefore approaches to studying *Thiomargarita*'s physiology have focused on

55    culture-independent approaches. Schulz and deBeer used microsensors to measure $O_2$ and sulfide

56    gradients influenced by *Thiomargarita* metabolisms (16), and more recently, (meta)genomic and

57    metatranscriptomic approaches have been used to investigate their genetic potential (17, 18) and

58    response to environmental perturbations (19). Here we add to these findings using a novel

59    approach to studying large sulfur bacteria that employs tetrazolium redox dyes.

60    Tetrazolium dyes are widely used for measuring the metabolic activity of cells, including both

61    bacteria (20-22) and eukaryotes (23), growing on a variety of metabolic substrates. In their

62    oxidized form, soluble tetrazolium salts generally result in a colorless or weakly-colored

63    solution. Cellular reductases, such as NADH, reduce tetrazolium salts to an intensely-colored

64    formazan product that can be observed qualitatively or measured quantitatively in a variety of

65    colorimetric metabolic assays (24-26). These dyes can be used to measure catabolic metabolic

66    activity even in the absence of observable cell division. Typically, tetrazolium dyes are applied

67    to bacteria in culture and measured via color response of bulk media and culture in a microplate

68    (27). We sought to apply a tetrazolium dye approach to investigate metabolism in *Thiomargarita*

69    spp. However, *Thiomargarita spp.* are not in pure culture, and these large cells are covered with

70    communities of smaller attached bacteria (12). Our initial attempts to analyze our custom

71    tetrazolium microplate assays via spectroscopy failed to differentiate between the metabolism of

72    *Thiomargarita* and its attached bacteria. We then employed a microscopy-based approach to

73    image tetrazolium color change associated with individual *Thiomargarita* cells, so as to

74    differentiate metabolic responses of *Thiomargarita* spp. from those of attached epibiont cells.

75    Here we report on the cellular reductase response of *Thiomargarita* spp. cells to several organic

76    and inorganic substrates under oxic, hypoxic, and anoxic conditions.

77

78    **MATERIALS AND METHODS**

79    **Sample Collection.** *Thiomargarita* spp. cells were collected from organic-rich sediments on the

80    Atlantic shelf, near Walvis Bay, Namibia (23°00.009' 14°04.117') using a multi-corer on board

81    the R/V *Mirabilis*. All cells used in the experiments were collected from a depth of 1-3 cm depth

82    beneath the sediment/water interface. *Thiomargarita* sp. cells were stored in their host sediments

83    with overlying core-top water in closed 50 ml centrifuge tubes at $4^{o}C$, and protected from direct

84    light for three weeks.

85    **Incubation experiments with tetrazolium staining.** Chains and clusters of *Thiomargarita* sp.

86    cells were rinsed three times in 0.2 µm filtered artificial seawater before being added to an

87    incubation medium in 96-well microplates.  The purpose of these rinse steps was to remove

88    loosely attached smaller bacteria from the exteriors of *Thiomargarita* cells and sheaths. These

89    wash steps did not remove tightly-attached or sheath-embedded epibionts, as confirmed by

90    microscopy and described below.  One *Thiomargarita* cell, cell cluster, or cell chain, was added

91    to each individual well in the 96-well plate. Each substrate treatment contained eight wells with

92    *Thiomargarita* cells, as well as four control wells without *Thiomargarita* cells. Two of these

93    control wells contained 20 µl of the first saltwater bath used to rinse the *Thiomargarita* cells,

94    added to 180 µl of media, as a control that contains cells found loosely attached to

95    *Thiomargarita*.  Wells containing empty diatom frustules picked by pipette from the same

96    samples as the *Thiomargarita* cells were also used as negative controls to monitor color change

97    of xenic biological surfaces from the same environment.  A third control type consisted of empty

98    mucus sheaths that were produced by *Thiomargarita*, but which no longer contained

99    *Thiomargarita* cells. These empty sheaths are common in *Thiomargarita*-rich sediments and can

100   be readily identified by the shape of the chains that is preserved as void space in the sheath

101   material.

102    The incubation medium was designed to maintain metabolizing *Thiomargarita* cells and to

103   provide basic elemental constituents and nutrients based on similar base media recipes for

104    phylogenetically-related taxa (28).  The incubation medium included the following constituents

105    $(l^{-1})$: NaCl, 34 g; $CaCl_2$, 0.112g; $NH_4NO_3$, 0.008g; KCl, 0.5g; $MgSO_4$, 1.46; 20 mL 1M MOPS

106    (3-(*N*-morpholino)propanesulfonic acid) buffer (pH adjusted to 7.8, final conc. 20mM); 1 ml

107    1000x potassium phosphate buffer (pH 7.6); 1 ml 1000x $B_{12}$; 1 ml 1000x vitamin solution, 10 ml

108    100x trace element solution. The vitamin solution contained $(l^{-1})$ 10 mg of riboflavin and 100 mg

109    each of thiamine HCl, thiamine pyrophosphate, L-ascorbic acid, D-Ca-pantothenate, folic acid,

110    biotin, lipoic acid, nicotinic acid, 4–aminobenzoic acid, pyridoxine HCl, thiotic acid,

111    nicotinamide adenine dinucleotide, and inositol dissolved in 100 mL of a 10 mM $KPO_4$ buffer

112    (pH 7). The trace metal solution contained $(l^{-1})$ 0.1 g $FeCl_2 \bullet 2H_2O$, 0.03 g $H_3BO_3$, 0.1 g $MnCl_2$,

113    0.1 g $CoCl_2 \bullet 6H_2O$   1.5 g nitrilotriacetic acid, 0.002 g $NiCl_2 \bullet 6H_2O$, 0.144 g $ZnSO_4 \bullet 7H_2O$,

114    0.036 g $NaMoO_4$, 0.025 g Na-vanadate, 0.010 g $NaSeO_3$ and $NaWO_4 \bullet 2H_2O$. $NaHCO_3$ was

115    added to each base medium for a final concentration of 3mM for the oxic and anoxic stocks, and

116    40 mM for the hypoxic treatments. The medium was sterilized by filtration through 0.22 μm

117    membrane, and the final pH was 7.9-8.0.

118    Potential electron donors acted as experimental variables, and included $H_2$, $H_2S$, thiosulfate,

119    succinate, acetate, citrate, and formate. All electron donors except for $H_2$ and $H_2S$ were supplied

120    at final concentrations of 1mM. $H_2$ was supplied by shaking the plates in a Coy anaerobic

121    chamber containing a 3% $H_2$, 98% $N_2$ atmosphere. $H_2S$ was supplied by the daily addition of 10

122    μl of the freshly-neutralized 4mM sodium sulfide delivered by syringe.  The tetrazolium redox

123    dye mix we used, which is known by the commercial name "Dye H" (Biolog Cat. No 74228)

124    was added to the medium at a final 1x working strength just prior to cell incubation.

125    Microplates were placed on orbital shakers at 50 rpm. One microplate was maintained under

126    benchtop atmospheric conditions, one microplate was placed in a Coy hypoxic chamber with 5%

127  atmospheric level $O_2$ and ~5% total $CO_2$, one microplate was placed in an $H_2$-free anaerobic

128  chamber (NextGen, Vacuum Atmospheres Company, Hawthorne, CA), and one microplate was

129  placed in a Coy anaerobic chamber containing a gas mixture 97% $N_2$, 3% $H_2$. All plates were

130  maintained in plastic containers with an open aperture to allow free exchange of gasses for the

131  week-long duration of the experiments. The plastic chambers that housed the microplates

132  contained moistened paper towels to inhibit plate evaporation. A Unisense oxygen microsensor

133  was used to confirm that $O_2$ was present and wells were well-mixed at depth in oxic treatments,

134  including those that contained $H_2S$.

135

136  *Image processing:* Cells were imaged using an Olympus IX-81 inverted microscope equipped

137  with a long working distance 40x objective (NA 0.6,WD 2.7-4.0), a 17.28 megapixel DP73 color

138  camera. Images were collected using CellSens Dimension (Olympus, Japan) software under

139  constant (manually-set) exposure and white balance settings. ImageJ was used to subtract the

140  background and convert the image to an XYZ color profile. A 40x40 pixel region of interest

141  representing an area of cytoplasm with a low density of sulfur globules was chosen for

142  quantification of dye change in each cell. The average pixel intensity of this region was then

143  measured in the luminance channel (Y). Reciprocal intensity was calculated using the approach

144  described in (29) for quantifying chromogen intensity. Change in intensity relative to time zero

145  was reported and a Student's T-test was used to determine the likelihood that the imaged

146  intensities were distinct from the mean of the controls by chance alone (Table 1). Copying the

147  selection region in ImageJ from image-to-image in the time series for an individual cell ensured

148  that the same area in the cell was measured at each time point.

149  *Genome analysis:* In order to compare our findings to genomic results, Beggiatoaceae genomes

150  that were annotated by the IMG pipeline, were queried using tools available on IMG/ER (version

151  4.570 for this study)(30).  The queried genomes are *Beggiatoa leptomitiformis* D-402 (GOLD

152  analysis project Ga0111282), *Beggiatoa alba* B18LD (Ga0024935), *Candidatus* Maribeggiatoa

153  sp. Orange Guaymas (Ga0010502), *Beggiatoa* sp. PS (Ga0027801), *Beggiatoa* sp. SS

154  (Ga0027802), *Candidatus* Thiomargarita nelsonii Bud S10 (Ga0097846, Ga0063879),

155  *Candidatus* Thiomargarita nelsonii Thio36 (Ga0025452) and *Thioploca ingrica* (Ga0060138).

156  With the exception of the freshwater strains of *Thioploca (31)*  and *Beggiatoa*, all genomes are

157  incomplete genomes generated by multiple displacement amplification of individual cells (17,

158  18, 32, 33).

159

160  **RESULTS AND DISCUSSION**

161  **Tetrazolium dye staining and the metabolism of lithotrophic substrates.** *Thiomargarita* spp.

162  cells exhibited apparent metabolic responses to a variety of substrates, as indicated by formazan

163  staining that was localized within the cell (Figure 1), and that increased in intensity over the

164  duration of the seven-day experiment (Figures 1A,B, Figs. 2-3). Maximum staining generally

165  occurred within 4-6 days (Figs. 2-3).

166  Intense color changes were restricted to the *Thiomargarita* cell itself and did not extend to the

167  sheath **(Figure 1B, C, J)**, except in cases where epibiont bacteria were stained as described

168  below, or in the case of sulfide treatments, also described below. Collapsed or otherwise

169  damaged cells at the time of incubation never showed a color change (Figure 1D), though some

170  cells that did stain early in the experiment were sometimes observed to collapse in the later

171  stages of the experiment. These observations suggest that formazan staining is a good indicator

172   of initial cell viability, but that reductase activity alone was not sufficient to prevent eventual cell

173   collapse in some specimens. Staining of controls, that included cell rinse water (not shown),

174   diatom frustule (Figure 1E), and empty sheaths (Figure 1F) all exhibited very low intensities

175   compared with *Thiomargarita* cell staining (Table 1; Figures 2-3).

176   Sulfur bacteria such as *Thiomargarita* are known for their ability to oxidize $H_2S$ using $O_2$ or

177   nitrate as an electron donor (3, 12). The intense tetrazolium dye staining of cells exposed to $H_2S$

178   under both oxic and anoxic conditions was highly statistically supported  as being distinct from

179   control staining ($p < 0.005$, Student's t-test), while staining under sulfidic hypoxic conditions

180   was marginally statistically distinct from control staining ($p < 0.05$, Student's t-test) (Table 1).

181   The genomes of *Thiomargarita* spp. contain genes for the oxidation of $H_2S$ via either a

182   sulfide:quinone oxidoreductase (SQR), and/or flavocytochrome c (FCC) (17).  In the treatments

183   containing $H_2S$, an orange color change was noted in the media (Fig. 1G). The orange color

184   change, which only occurred in the presence of $H_2S$, was distinguishable from the typical

185   formazan purple color change that occurs within the cells (Fig. 1G). This orange coloration is

186   likely the result of the abiotic reduction of tetrazolium by $H_2S$. Despite the sulfide treatments

187   having a higher background than other treatments, a dark purple color change in the cell could be

188   differentiated from the orange background (Figure 1G, Figure 2C, Table 1).

189   Many sulfur-oxidizing bacteria are also known to be able to oxidize other sulfur-containing

190   substrates such as thiosulfate.  Thiosulfate addition to the experimental wells resulted in a

191   increasingly strong formazan staining response under anoxic conditions ($p < 0.005$, Student's t-

192   test) (Figure 3D). However, significant staining was not observed with thiosulfate addition under

193   oxic and hypoxic conditions (Table 1). Instead, cells were observed to collapse (Fig. 2B). All

194   currently available genomes of Beggiatoaceae contain the genes for thiosulfate oxidation via a

195   partial sox system (*soxABXY*) for the oxidation of thiosulfate to intracellularly stored sulfur

196   granules. (17, 18) Thiosulfate is not as strong a reductant as $H_2S$, which may have left

197   *Thiomargarita* cells susceptible to oxidative stress under oxic and hypoxic conditions. Indeed,

198   cell collapse was common with a variety of metabolic substrates under oxic conditions. Oxic

199   treatments with $H_2S$ were the only exception.

200   In addition to reduced sulfur compounds, some sulfur oxidizing bacteria are also known to use

201   $H_2$ as an electron donor for lithotrophic metabolism (34). Our staining results also showed high

202   statistical support ($p < 0.005$, Student's t-test) for formazan staining relative to controls when the

203   cells were incubated in an anaerobic glove box containing 3% $H_2$ (Figures 1D, 1I, 1J and 3B).

204   Cells incubated without other supplied electron donors in an anoxic chamber without $H_2$ showed

205   no color change (Figures 1H, 3A). Recently, a chemolithotrophic strain of *Beggiatoa* sp., 35Flor,

206   was found to use $H_2$ as an electron donor under oxygenated conditions (35). 35Flor was not

207   observed to oxidize $H_2$ under anaerobic conditions, but *Beggiatoa* sp., 35Flor does not store

208   nitrate to serve as an electron acceptor as *Thiomargarita* does. In addition to the microsensor

209   studies that show $H_2$ consumption by *Beggiatoa* sp. 35Flor, the genomes of *Ca.* T. nelsonii

210   Thio36, and Bud S10 both contain genes for Ni-Fe hydrogenase (17, 18), as do those of other

211   sulfur-oxidizing bacteria (36, 37). The source of $H_2$ to *Thiomargarita* in nature is not clear, but

212   fermentation by epibionts or other bacteria in the environment is a possible source.

213   **Organic acid metabolism.** In addition to their canonical lithotrophic metabolism, some

214   representatives of the Beggiatoaceae are known to metabolize organic acids (38). Organic acid

215   degradation may be a common trait amongst sulfur-oxidizing gammaproteobacteria, since the

216   generation of NADPH through the TCA cycle would coincide with the assimilation organic acids

217   and would thus reduce the energetic costs of carbon fixation (39, 40). The genomes of *Ca.* T.

218    nelsonii Bud S10, Thio36, and other members of the Beggiatoaceae, encode genes for a complete

219    TCA cycle, including NADH dehydrogenase I (17, 18, 32, 33), and while succinate

220    dehydrogenase is not electrochemically favored under most anaerobic conditions, it is tightly

221    coupled with respiratory nitrate and nitrite reduction (41). Nitrate was provided in the medium

222    (100 µM) and nitrate is also stored in the internal nitrate vacuole.

223    All the organic acids tested here yielded positive staining results under anoxic conditions, but

224    staining results with organic acid exposure were more variable under hypoxic and aerobic

225    conditions. We observed a positive staining response with the addition of citrate under oxic,

226    hypoxic, and anoxic conditions, and succinate under hypoxic and anoxic conditions (Table 1).

227    The utilization of exogenous organic acids requires specific inner membrane transporters. The

228    genomes of a few Beggiatoaceae encode putative citrate transporters (Ga0060138_111857,

229    BGP_2612) and a partial putative citrate transporter is in *Ca.* T. nelsonii Thio36

230    (Thi036DRAFT_00068870). Succinate is usually transported by proteins encoded by either one

231    of two three-gene clusters, *dctABD*, where *dctA* is a permease and *dctBD* a two-component

232    system for the activation of transcription (42) or similarly *kgtPSR* (43) or via a tripartite ATP-

233    independent periplasmic transporter (TRAP), *dctPQM* (44). The two available *Thiomargarita*

234    genomes contain a *dctB* gene but lack *dctAD*, although dctB is near the terminal end of a contig

235    in the genome of Bud S10. While freshwater strains of *Beggiatoa* (*B. leptomitiforms* D-402 and

236    *B. alba* B18LD) possess putative *dctPQM* genes, annotated genes for C4–dicarboxylate

237    transporters are lacking in all marine strains. Thus, the capacity to transport succinate via known

238    transporters in marine Beggiatoaceae appears to be lacking. However, the marine strains do

239    possess a number of uncharacterized TRAP transporters that might serve as future targets for

240    characterizing C4-dicarboxylate metabolisms. Acetate is another organic acid that is thought to

241    be metabolized by certain sulfur bacteria. The microelectrode respiration experiments of Schulz

242    and DeBeer showed the stabilization of oxygen/sulfide gradients around *Thiomargarita* when

243    acetate was added to the experiments, although acetate itself was not found to steepen the oxygen

244    gradient (16). Our formazan dye staining results show no statistical support for acetate use under

245    oxic conditions, which is consistent with the results of Schulz and DeBeer. However, our results

246    also show marginal statistical support for acetate metabolism under hypoxic conditions, and

247    strong support for acetate use under anoxic conditions (Table 1, Figure 2D).  Acetate can be

248    metabolized by the tricarboxylic acid and glyoxylate cycles, and is preceded by the activation of

249    acetate by acetate kinase and phosphotransacetylase (45) (known as the *ack-pta* pathway)

250    and/or via an acetyl-CoA synthethase (known as the *acs* pathway). The *ack-pta* pathway occurs

251    in the freshwater strains of the Beggiatoaceae, but not the marine strains. However, almost

252    all Beggiatoaceae possess the *acs* pathway (18) and an acetate/cation symporter (e.g.

253    Ga0063879_05139) (33). These genomic features are consistent with the ability of

254    *Thiomargarita* spp. to take up and metabolize acetate from the environment, but our staining

255    results suggest that this may only occur in *Thiomargarita* spp. under anoxic and perhaps hypoxic

256    conditions.

257    Formate is both a waste product of fermentation and a potential electron donor that can be used

258    for both aerobic and anaerobic respiration (46). The genomes of *Ca.* T. nelsonii Thio36 and

259    *Beggiatoa* PS both contain genes that code for subunits of formate dehydrogenase. In addition to

260    anaerobic respiration, energy-yielding reaction under anaerobic conditions is the formate-assisted

261    cleavage of pyruvate via a pyruvate formate lyase enzyme.  The genomes of both *T. nelsonii*

262    contain three annotated pyruvate formate lyase genes and the gene occurs in most other

263    Beggiatoaceae. The transport of exogenous formate could be mediated by a formate/nitrate

264    transporter (focA), which is present in most marine strains of Beggiatoaceae, including both *T.*

265    *nelsonii* genomes.

266    Although *Thiomargarita* spp. are thought to be more oxygen tolerant than other marine vacuolate

267    sulfur bacteria, such as *Maribeggiatoa* and *Marithioploca* (16), the results presented here suggest

268    that while *Thiomargarita* spp. may tolerate oxygen exposure, their metabolism(s) are most

269    versatile under anoxic conditions. This is perhaps unsurprising given that *Thiomargarita* on the

270    Namibian shelf are primarily found in sediments that are anoxic for much of the year (47).

271    During the collection of samples used in our experiments, no oxygen was detectable in the lower

272    water column as measured by Winkler titration, and in core-top waters as measured using a

273    hand-held oxygen meter. Limited tolerance to $O_2$ is also suggested by recent genomic results for

274    most Beggiatoaceae. All Beggiatoaceae strains have cbb3-type cytochrome c oxidase and some

275    also possess bd-type cytochromes, both of which are specific to hypoxic conditions.  However,

276    both *T. nelsonii* Thio36 and *Beggiatoa* PS possess the more oxygen-tolerant cytochrome c

277    oxidase (*coxABC*, *cyoE*), which suggests greater tolerance in some strains of Beggiatoaceae.

278    However, catalase, which is used to ameliorate oxidative stress, is not present in the genomes of

279    marine Beggiatoaceae, occurring only in the freshwater strains. However, most Beggiatoaceae

280    genomes contain a superoxide dismutase and cytochrome c peroxidase, while most marine

281    strains also possess a desulfoferrodoxin. The enzymes coded for by these genes may provide

282    protection against $O_2$ and reactive oxygen species during periodic exposure to $O_2$. Additionally,

283    $H_2S$, which can scavenge reactive oxygen species (48), may serve as an extracellular antioxidant

284    under conditions in which sulfide is fluxing into oxygenated waters.  Under anoxic conditions,

285    oxidized forms of inorganic nitrogen whether exogenous, or stored within the vacuole, serve as

286    terminal electron acceptors (3, 49). Both *Ca.* Thiomargarita nelsonii Bud S10 (17) and Thio36

287     (18), possess a complete denitrification pathway to include both membrane bound (*nar*) and

288     periplasmic (*nap*) nitrate reductases and the capacity to reduce nitrite to ammonium (*nirBD*).

289     Thus, our staining results are consistent with the genomes and the canonical knowledge of nitrate

290     being used as an electron acceptor by certain members of the Beggiatoaceae.

291     **Epibiont microbial cells.** In our experiments, we undertook imaging of individual formazan-

292     stained *Thiomargarita* cells in order to differentiate *Thiomargarita* metabolism from those of

293     attached bacteria. These attached bacteria exhibited spatially-distinct staining from

294     *Thiomargarita* under the microscope that couldn't be differentiated with a typical microplate

295     assay. Our microscope-based imaging approach had the added benefit of allowing us to observe

296     the discrete staining of epibiont biofilms and filaments associated with *Thiomargarita* (Figure

297     1G, L). In some cases, stained filamentous epibionts could be observed anchored to the

298     *Thiomargarita* cell/sheath (e.g. Figure 1L, arrows). In particular, dense accumulations of

299     filamentous bacteria (Figure 1L) exhibited intense staining when exposed to $H_2$ under anoxic

300     conditions. While filamentous bacteria were observed in some control wells, and in zones of the

301     well distal to *Thiomargarita,* their accumulations were far denser in the vicinity of

302     *Thiomargarita* cells (Figure 1L). This observation suggests the possibility that *Thiomargarita*

303     spp. cells are involved in syntrophic interactions with their epibionts. For example,

304     *Thiomargarita* may supply attached sulfate-reducing bacteria with organic acids or sulfate, while

305     the sulfate-reducing bacteria produce sulfide or other reduced sulfur intermediates that can be

306     oxidized by *Thiomargarita (12)*. In these experiments $H_2$ may have served as an electron donor

307     for sulfate-reducing bacteria that then produced sulfide that was oxidized by the *Thiomargarita*

308     cells. However, as discussed above, *Thiomargarita* spp. has the genetic potential to oxidize $H_2$

309     directly.

310    We also observed under anoxic conditions in the presence of $H_2S$, that small bacteria that stained

311    purple were present throughout the medium, except in clear cell-free zones we observed

312    immediately surrounding *Thiomargarita* cells and chains (Figure 1K, arrows). These zones may

313    represent the inhibition of *Thiomargarita* on other small sulfur-oxidizing bacteria, perhaps

314    through the drawdown of $H_2S$ in the vicinity of the *Thiomargarita* cells.    Additional

315    investigation, including alternative approaches such as gene expression studies, will be needed to

316    further evaluate these interpretations.

317    **Conclusions.** Our observations of reductase-mediated formazan staining in *Thiomargarita* cells

318    exposed to a variety of organic and inorganic substrates are consistent with recent genomic

319    results that suggest metabolic plasticity in *Ca.* Thiomargarita nelsonii. There are however, some

320    limitations and caveats in drawing broad conclusions from our results. It is possible that the

321    incubation media formulations or concentrations we used here, or other aspects of the

322    experimental design, resulted in responses from *Thiomargarita* that do not represent their

323    metabolic activities in nature. Yet the results presented here are broadly consistent with genomic

324    data currently available for *Ca.* T. neslonii, and consistent with the sediment-hosted habitat of

325    these bacteria that is anoxic for much of the year. We used both the spherical *Thiomargarita*

326    cells that are typical of *Thiomargarita namibiensis* and the cylindrical forms that are more

327    typical of Ca. *T. nelsonii* for this study (1). However, the large number of cells used in the

328    experiment with the difficulty in amplifying *Thiomargarita's* 16S rRNA gene due to multiple

329    introns contained therein prevented a detailed phylogenetic characterization of the cells used for

330    the assay (50). As such, we cannot say that our results apply broadly to the multiple candidate

331    *Thiomargarita* species that can sometimes co-occur in sediments off Namibia (1, 51).    While

332    cultivation will ultimately be necessary for rigorous testing of the physiologies of *Thiomargarita*

333  spp., for now, these results provide additional evidence in support of recent genomic and

334  microsensor findings of metabolic versatility and/or mixotrophy in the genus *Thiomargarita* and

335  suggest cultivation approaches that include both reduced sulfur compounds and organic

336  substrates perhaps under extremely low oxygen conditions.

337  Tetrazolium/formazan redox dyes have long been used for studying bacteria, primarily in plate-

338  based assays. Our expansion of the use of these dyes to the microscopic examination of

339  individual stained cells, may be broadly useful for assaying metabolism in mixed communities

340  and other uncultivated microorganisms, and for validating genome-derived assessments of

341  physiological capabilities.

342

343  **FUNDING INFORMATION**

349

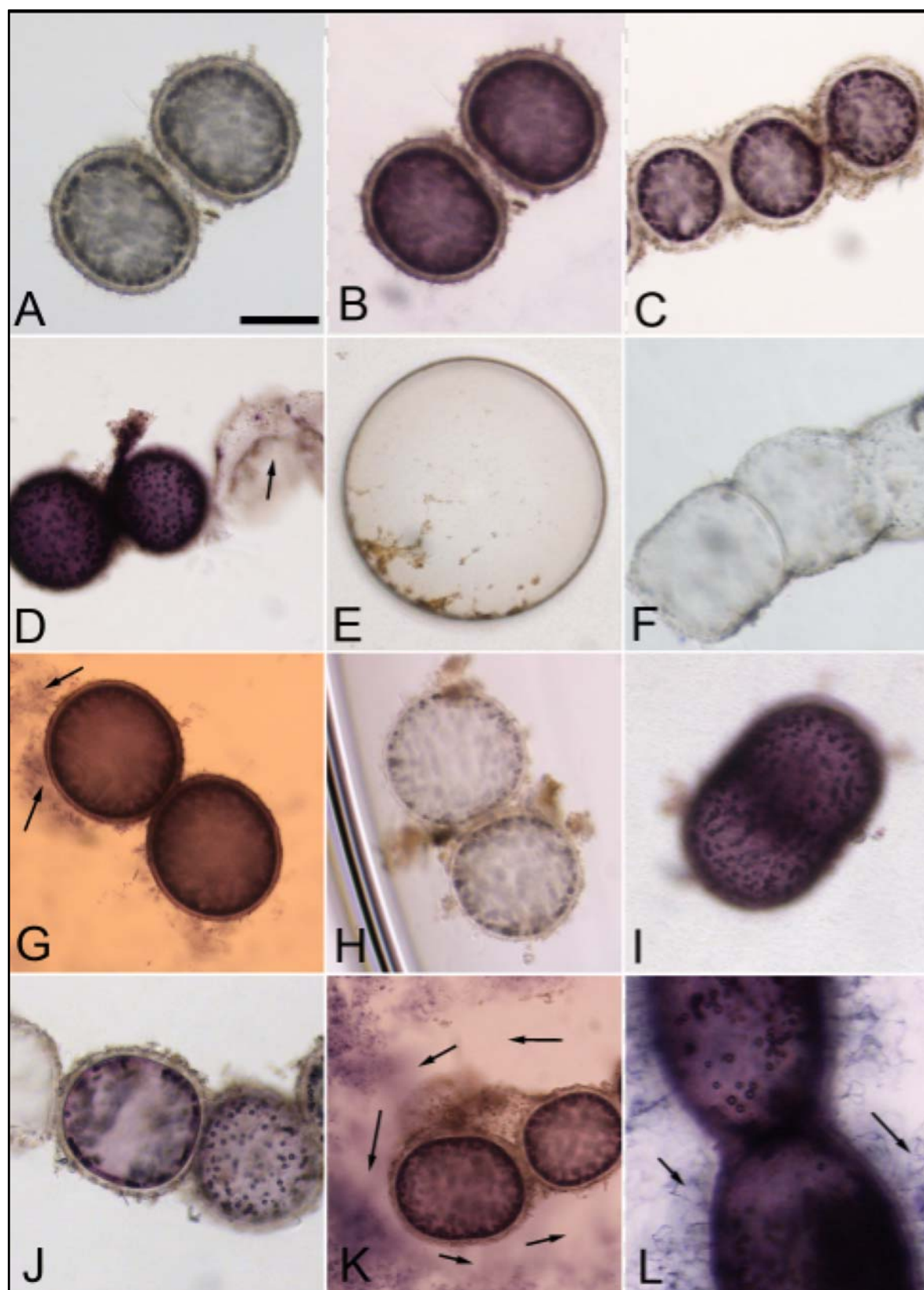350  **ACKNOWLEDGEMENTS**

355  FIGURES

356

357  **Figure 1** – Cellular reductase activity reduces colorless tetrazolium to purple formazan product.

358  (**A**) Initially, *Thiomargarita* spp. cells incubated in the redox dye are colorless and the medium is

359  light green in color. (**B**) Many intact cells under specific treatment conditions, presumably those

360  that are metabolically active, stain a deep purple color and the extracellular medium changes to

361  colorless or light pink, typically within two days. Panel B shows cells incubated under anoxic

362  conditions in the presence of succinate.  Staining of metabolically active cells was spatially

363  separate and distinct from surrounding sheath material (**C**)**.** Collapsed or damaged cells at the

364  time of incubation did not exhibit a color change  (**D**), showing exposure to acetate and hydrogen

365  respectively, under anoxic conditions. Staining of metabolically active cells was distinct in

366  intensity from control diatom frustules (**E**), and control sheath material (**F**).  Under exposure to

367  $H_2S$, the extracellular medium assumed an orange hue that was readily distinguishable from the

368  purple color change in the *Thiomargarita* spp. cells (**G**) and in biofilms of attached epibiont

369  bacteria (arrows). Under anoxic conditions, cells and media showed the most extensive response,

370  with very little color change observed with no additional electron donor added (**H**), and a strong

371  staining response with the addition of other substrates such as $H_2$ under anoxic conditions (**I, J**).

372  In some cases, staining of extracellular bacteria were present as a diffuse stained cloud composed

373  of small cells within the well. A zone characterized by an absence of staining and cells in the

374  immediate vicinity of the *Thiomargarita* cells, suggests some sort of inhibition of these small

375  bacteria (arrows in **K**). In other cases, such as under exposure to thiosulfate under anoxic

376  conditions as shown here, stained filamentous epibionts could be observed anchored to the

377  *Thiomargarita* cell/sheath (**L**).  All images taken at 400x magnification, scale bar in A = ~100
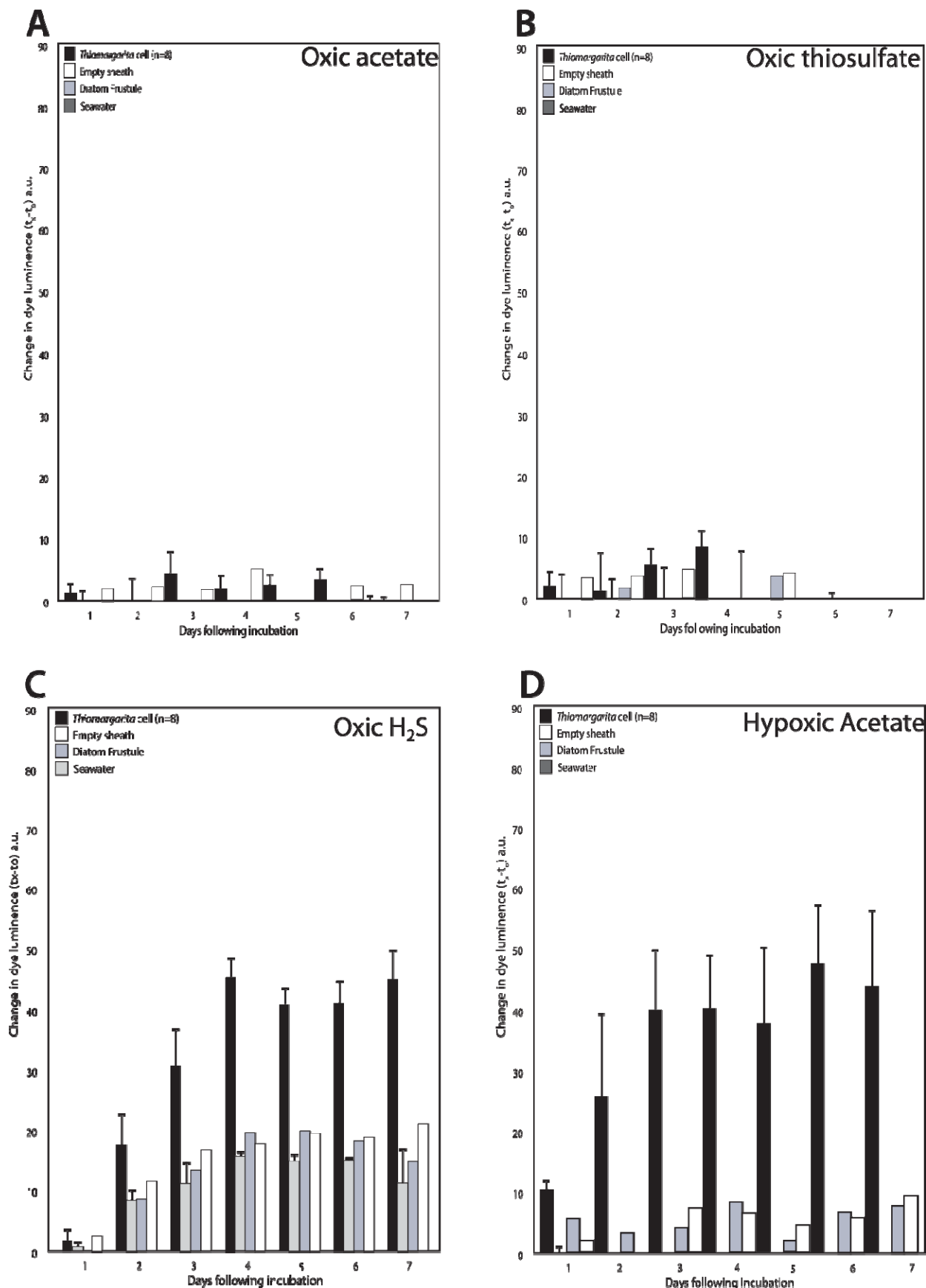
378  μm.

379

380     **Figure 2:** *Thiomargarita* cells exhibited very little staining response to acetate (**A**), thiosulfate

381     (**B**), and other substrates, under oxygenated conditions relative to controls. However, $H_2S$ (**C**)

382     and citrate (not shown), did induce a statistically-significant staining response under oxic

383     conditions. Potentially significant staining was observed under hypoxic conditions in the

384     presence of acetate (**D**), succinate, citrate, and $H_2S$. Plotted here is the mean change in reciprocal

385     intensity of luminance relative to day zero. Error bars indicate one standard error from the mean

386     of the *Thiomargarita* incubations or controls.

387

388

389    **Figure 3:** *Thiomargarita* cells and controls showed a weak response under anoxic conditions

390    with the addition of a substrate that could not be differentiated from controls (**A**). With the

391    addition of $H_2$ (**B**), succinate (**C**), thiosulfate (**D**), $H_2S$, formate, acetate, citrate, and formate (not

392    shown), a statistically-significant staining response was observed under anoxic conditions.

393    Plotted here is the mean change in reciprocal intensity of luminance relative to day zero. Error

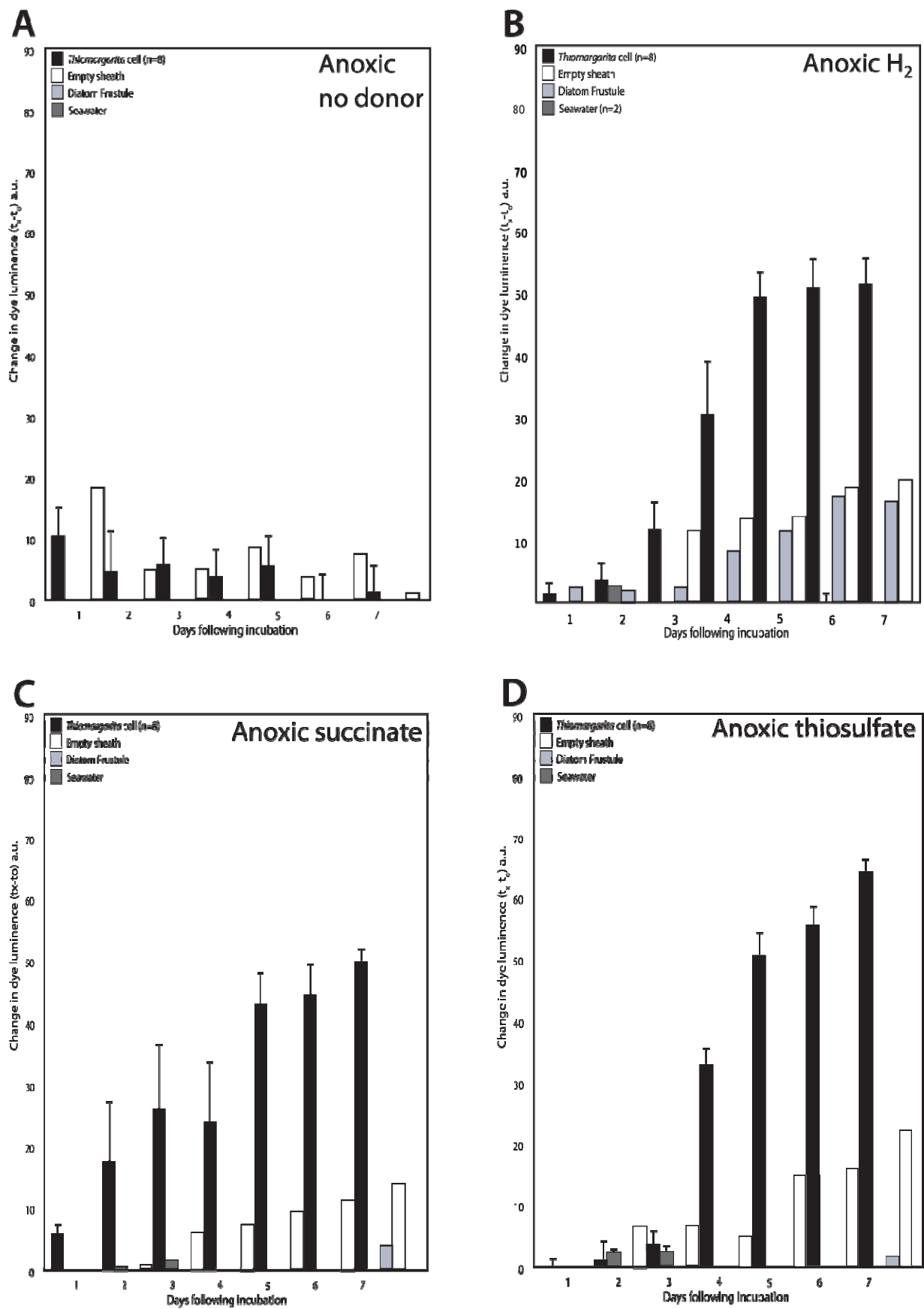394    bars indicate one standard error from the mean of the *Thiomargarita* incubations or controls.

395

396

| Substrate | *Thiomargarita* cells | Sheath control | Diatom control | Water controls |
|---|---|---|---|---|
| **Oxic** | | | | |
| No donor added | 3.503±2.951 (n=8) | 0 | 0 | 0±0.214 (n=2) |
| Succinate | 3.383±2.123 (n=8) | 5.825 | 0.662 | 0±3.246 (n=2) |
| Thiosulfate | All cells collapsed (n=0) | 0 | 0 | 0±4.68 (n=2) |
| Acetate | 0±1.907 (n=8) | 2.652 | 0 | 0±1.829 (n=2) |
| **Citrate** | **13.009±2.108 (n=8)** | 3.993 | 2.878 | 0±2.421 (n=2) |
| Formate | All cells collapsed (n=0) | 0 | 5.672 | 0±0.243 (n=2) |
| **H$_2$S** | **45.307±4.552 (n=8)** | 21.192 | 15.061 | 11.355±3.855(n=2) |
| **Hypoxic** | | | | |
| No donor added | 5.082±2.262 (n=8) | 0 | 0.838 | 2.973±0.577 (n=2) |
| **Succinate** | **24.742±2.081 (n=8)** | 0 | 3.102 | 0±0.438 (n=2) |
| Thiosulfate | All cells collapsed (n=0) | 5.151 | 0.744 | 0±0.293 (n=2) |
| Acetate | <u>44.131±12.107 (n=8)</u> | 9.431 | 7.652 | 0±0.643 (n=2) |
| Citrate | <u>30.562±4.289 (n=4)</u> | 1.579 | 0 | 0±0.422 (n=2) |
| Formate | 4.044±1.599 (n=8) | 3.411 | 4.665 | 0±0.17 (n=2) |
| H$_2$S | <u>61.519±4.487 *(n=6)*</u> | 39.412 | 50.986 | 38.421±7.886 (n=2) |
| **Anoxic** | | | | |
| No donor added | 1.104±4.6 (n=8) | 1.938 | 0 | 0±0.581 (n=2) |
| **Succinate** | **50.732±1.274 (n=3)** | 13.507 | 3.852 | 0±5.616 (n=2) |
| **Thiosulfate** | **73.387±1.954 (n=8)** | 25.755 | 1.857 | 0±0.693 (n=2) |
| **Acetate** | **42.177±3.902 (n=8)** | 12.302 | 4.654 | 0.091±1.096 (n=2) |
| **Citrate** | **37.528±4.031 (n=8)** | 22.271 | 0 | 3.03±0.333 (n=2) |
| **Formate** | **42.234±6.21 (n=8)** | 2.583 | 13.464 | 0.432±0.114 (n=2) |
| **H$_2$S** | **40.927±5.647 (n=8)** | 25.126 | 20.326 | 11.176±2.783 (n=2) |
| **H$_2$** | **61.997± 5.125 (n=8)** | 23.555 | 19.556 | 0±0.49 (n=2) |

397

398    **Table 1:** Mean positive change in the reciprocal intensity of luminance on Day 7 relative to time zero.

399    Treatments that are highly statistically different ($p < 0.005$, Student's t-test) from the mean of the four

400    control samples are indicated in boldface type. Treatments that are marginally statistically different ($p <$

401    $0.05$, Student's t-test) from the mean of the four control samples are indicated in underlined type.

402    Negative values were used to calculate standard error, but are reported in the table as 0 to indicate no

403    positive change.

404

405

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