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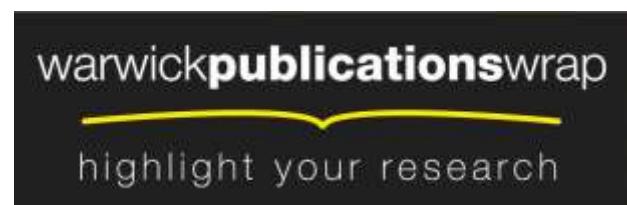
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1 **Trimethylamine and trimethylamine N-oxide are supplementary energy sources for a**
2 **marine heterotrophic bacterium: implications for marine carbon and nitrogen cycling**

3

4 Ian D. E. A. Lidbury¹, J. Colin Murrell², Yin Chen¹

5

6 ¹School of Life Sciences, University of Warwick, CV4 7AL, Coventry, United Kingdom

7 ²School of Environmental Sciences, University of East Anglia, NR4 7TJ, Norwich, United
8 Kingdom

9

10 Correspondence to Dr Y Chen, School of Life Sciences, University of Warwick, UK

11 Email: Y.chen.25@warwick.ac.uk; Phone: 00 44 24 76528976

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13 Running title: TMA/TMAO as energy sources for *R. pomeroyi*

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16

17 **Abstract**

18 Bacteria of the marine *Roseobacter* clade are characterised by their ability to utilise a wide
19 range of organic and inorganic compounds to support growth. Trimethylamine (TMA) and
20 trimethylamine *N*-oxide (TMAO) are methylated amines and form part of the dissolved
21 organic nitrogen pool, the second largest source of nitrogen after N₂ gas, in the oceans. We
22 investigated if the marine heterotrophic bacterium, *Ruegeria pomeroyi* DSS-3, could utilise
23 TMA and TMAO as a supplementary energy source and whether this trait had any beneficial
24 effect on growth. In *R. pomeroyi*, catabolism of TMA and TMAO resulted in the production
25 of intracellular ATP which in turn helped enhance growth rate and growth yield as well as
26 enhancing cell survival during prolonged energy starvation. Furthermore, the simultaneous
27 use of two different exogenous energy sources led to a greater enhancement of
28 chemoorganoheterotrophic growth. The use of TMA and TMAO primarily as an energy
29 source resulted in the remineralisation of nitrogen in the form of ammonium, which could
30 cross feed into another bacterium. This study provides greater insight into the microbial
31 metabolism of methylated amines in the marine environment and how it may affect both
32 nutrient flow within marine surface waters and the flux of these climatically important
33 compounds into the atmosphere.

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35 Keywords: *Ruegeria pomeroyi* DSS-3, methylated amine, C1-metabolism, ammonification

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39 **Introduction**

40 The marine *Roseobacter* clade (MRC) is a monophyletic group (> 87% identity in 16S rRNA
41 genes) of bacteria within the family *Rhodobacteraceae* (Buchan et al, 2005). The MRC are an
42 ecologically significant clade, representing up to 20% of bacterial cells in marine coastal
43 waters (Buchan et al, 2005, Sowell et al, 2011). The use of both ‘omics’ and physiological
44 experimentation has revealed that MRC bacteria harbour an extraordinary ability to
45 metabolise a wide range of substrates to support their growth (Moran et al, 2004, Buchan et
46 al, 2005, Newton et al, 2010). The ecological success of this clade may be in part due to their
47 ability to utilise a variety of metabolic strategies to generate cellular energy, which allows for
48 the more efficient utilisation of carbon (assimilation versus dissimilation) (Sorokin et al,
49 2005, Moran & Miller 2007, Boden et al, 2011b). For these reasons, the MRC bacteria play
50 essential roles in both carbon and sulfur cycling, and more recently, nitrogen cycling (Buchan
51 et al, 2005, Chen et al, 2011) within the marine environment. *Ruegeria pomeroyi* DSS-3
52 (basonym, *Silicibacter pomeroyi* DSS-3) is a member of the MRC which was isolated off the
53 coast of Georgia through enrichment with dimethylsulfoniopropionate (DMSP) (González et
54 al, 2003). The genome of *R. pomeroyi* was sequenced in 2004 (Moran et al, 2004) and this
55 bacterium is now a model organism enabling a better understanding of how and why marine
56 bacteria metabolise a wide range of substrates (Moran et al, 2004, Cunliffe, 2012, Todd et al,
57 2012, Lidbury et al, 2014).

58 Trimethylamine (TMA) and trimethylamine N-oxide (TMAO) form part of the methylated
59 amine (MA) pool found within the marine environment (King, 1984, Gibb et al, 1999, Gibb
60 & Hatton 2004). In the marine environment, TMAO is a compatible osmolyte for a variety of
61 marine biota (Yancey et al, 1982, Treberg et al, 2006) and TMA is produced from the
62 reduction of compatible osmolytes, such as glycine betaine, TMAO and choline (King et al,
63 1984, Arata et al, 1992). TMA production can also occur under aerobic conditions through

64 oxidation of carnitine (Zhu et al, 2014) which may help explain the presence of TMA in
65 oxygenated marine surface waters (Carpenter et al, 2012). Standing concentrations of TMA
66 range from low nanomolar (nM) in coastal and open ocean surface waters to low micromolar
67 (μ M) in the pore water of marine sediments (Gibb et al, 1999, Fitzsimons et al, 2001, Gibb &
68 Hatton, 2004). The ocean: atmospheric flux of MAs is important as they can form aerosols
69 and are precursors for climate-active gases, such as nitrous oxide (Quinn et al, 1988,
70 Carpenter et al, 2012). Furthermore, MAs may represent a significant proportion of the
71 dissolved organic nitrogen (DON) pool (King, 1984, Gibb et al, 1999, Gibb & Hatton, 2004),
72 the second largest sink of nitrogen (N) in the oceans after gaseous nitrogen (N_2) (Capone et
73 al, 2008) and may help bacteria overcome severe competition for N, which is thought to be
74 one of the limiting nutrients for ocean productivity (Zehr & Kudela, 2011).

75 Chen (2012) showed that representatives of the MRC can grow on TMA. Whilst those MRC
76 bacteria harbouring the genes necessary for TMA oxidation could all utilise TMA as a sole N
77 source to support heterotrophic growth, only representatives from the genus *Roseovarius* of
78 the MRC could grow on TMA as a sole carbon (C) source (methylotrophy). All marine
79 bacteria that possess a functional TMA monooxygenase (Tmm) (Chen et al, 2011) and a
80 TMAO demethylase (Tdm) (Lidbury et al, 2014) also have the genes necessary for the
81 complete oxidation of the methyl groups cleaved off during catabolism of TMA (Sun et al,
82 2011, Chen 2012, Halsey et al, 2012). Two different oligotrophic bacteria from the
83 *Alphaproteobacteria* (*Candidatus Pelagibacter ubique* HTCC1062) and *Betaproteobacteria*
84 (*Methylophilales* sp. HTCC2181), respectively, can couple TMAO oxidation to ATP
85 production which results in stimulation of growth (Sun et al, 2011, Halsey et al, 2012),
86 however, these organisms fundamentally differ to members of the MRC. *R. pomeroyi* has the
87 genes required for TMA catabolism (Fig. 1) and can grow on TMA as a N source, but not a
88 sole C source, due to a lack of genes required for C assimilation via the serine cycle (Chen et

89 al, 2011, Chen, 2012). Here we test the hypothesis that the oxidation of MAs is coupled to
90 ATP production, providing an ecophysiological advantage to heterotrophic bacteria. We also
91 test the hypothesis that metabolism of MAs can provide a source of remineralised N in the
92 form of ammonia which can be utilised by another marine bacterium.

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96 **Materials and Methods**

97 **Growth conditions**

98 *Ruegeria pomeroyi* DSS-3 was maintained in the laboratory on marine agar 2216 (Difco,
99 UK). Gentamicin ($10 \mu\text{g ml}^{-1}$) was added to maintain mutant strains *Δtmm::Gm* and
100 *Δtdm::Gm* (Lidbury et al, 2014). For all experiments *R. pomeroyi* (wild-type and mutants)
101 was grown in marine ammonium mineral salts (MAMS) medium (Schäfer, 2007) using
102 glucose as the sole carbon source. MAMS medium was modified from (Schäfer 2007) and
103 contained (per liter): NaCl, 20 g; $(\text{NH}_4)_2\text{SO}_4$, 1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.2 g;
104 $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 2 mg; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 20 mg; KH_2PO_4 , 0.36 g; K_2HPO_4 , 2.34 g; plus 1 ml of
105 SL-10 trace metals solution (Schäfer 2007). Vitamins were prepared as described previously
106 (Chen 2012). Continuous culture work was performed using a glucose-limited (5 mM)
107 chemostat using the methods previously described by (Boden et al, 2011b). To avoid
108 precipitants forming in the medium during autoclaving, NH_4Cl was substituted for
109 $(\text{NH}_4)_2\text{SO}_4$. Steady-state was achieved after 5 dilutions and the dilution rate was set at 0.05 h^{-1}
110 ¹.

111 *Citreicella* sp. SE45 (a gift from Dr. Alison Buchan) was also maintained and grown using
112 the same methods. Both strains were incubated at 30°C on a rotary shaker (150 rpm).
113 *Methylomonas methanica* MC09 (Boden et al, 2011a) was maintained on MAMS plates using
114 methane (5%) as the sole carbon source. For growth experiments, *M. methanica* was grown
115 in MAMS medium using methanol (2 mM) as the sole carbon source and incubated at 25°C.

116 **Determination of biomass (mg dry weight l⁻¹)**

117 *R. pomeroyi* cultures (500 ml) were grown on glucose and ammonium with or without TMA
118 (3 mM) to an $\text{OD}_{540} \sim 1.4$. Cells were diluted to 0, 25, 50, 75 % (n=3) in MAMS and the
119 OD_{540} was recorded prior to filtration onto 0.22 μm nitrocellulose filter pads (Millipore, UK).

120 Cells trapped on the filter pads were washed twice with 15 ml sterile deionised water to
121 remove salts and other debris before being placed in a drying oven at 60°C. Filter pads were
122 repeatedly weighed until a constant weight was achieved. A standard curve was plotted for
123 OD₅₄₀ against dry weight (supplementary Fig. S1). For all conversions of optical density at
124 540 nm (OD₅₄₀) to dry weight, a constant of 1 OD unit at OD₅₄₀ = 254 mg dry weight l⁻¹ was
125 applied.

126 **Variable cell counts of *R. pomeroyi* during carbon/energy starvation**

127 *R. pomeroyi* was grown in MAMS with TMA (3 mM) or TMAO (3 mM) as the sole N source
128 to a final OD₅₄₀ ~0.5. Cells were re-suspended in MAMS with no exogenous C and then
129 aliquoted (20 ml) into 125 ml serum vials (n=3) with either no exogenous C (control), or
130 TMA (1 mM) or TMAO (1 mM). For cell counts, serial dilutions were generated (n=3) and
131 10 µl were spotted (n=3) on ½ YPSS (per litre; 2 g yeast extract, 1.25 g peptone, 20 g sea
132 salts (Sigma-Aldrich) plates and incubated at 30°C. TMA and TMAO were quantified by ion-
133 exchange chromatography as described previously (Lidbury et al, 2014).

134 **Quantification of intracellular ATP concentrations**

135 *R. pomeroyi* wild-type and mutant strains were grown using either TMA or TMAO as the
136 sole nitrogen source and cells were harvested by centrifugation (10 min; 8,000 g) at late
137 exponential phase (1×10^9 cells) and washed twice to remove exogenous C. Cells were re-
138 suspended in MAMS medium minus glucose, given TMA (1 mM), TMAO (1 mM) or no
139 exogenous energy source and then aliquoted (500 µl) into 2 mL microcentrifuge tubes (n=3).
140 Cells were left for 16 hr before adding a further 500 µL of each test compound. After 1 hr,
141 100 µl of cell suspension was mixed with 100 µl of BacTiter Glo cell viability kit (Promega)
142 and incubated for 5 min before recording luciferase activity on a LuminoskanTM Ascent

143 microplate luminometer (Thermo Scientific). A standard curve was generated using ATP
144 standards according to the manufacturer's guidelines.

145 **Co-culture of *R. pomeroyi* and *Methylomonas methanica* MC09**

146 *R. pomeroyi* wild-type and the mutant, *Δtmm::Gm* (Lidbury et al, 2014), was grown using
147 either TMA or TMAO as the sole N source (OD₅₄₀ ~0.3). Cells were re-suspended in fresh
148 medium containing 1 mM methanol. For each strain, triplicate cultures were set up using
149 either TMA or ammonium chloride as the sole N source (1 mM). *M. methanica* was grown
150 using methanol as the C source (2 mM) and ammonia (0.5 mM) as the limiting nutrient until
151 the onset of stationary phase. A 5% (v/v) inoculum of *M. methanica* (~10⁷ cells) was added to
152 each *R. pomeroyi* culture. Co-cultures were incubated at 25°C on a rotary shaker (150 r.p.m.).
153 For *M. methanica* cell counts, serial dilutions were generated (n=3) and 10 µl were spotted
154 (n=3) on MAMS plates with methane as the sole C source and incubated at 25°C.

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159 **Results**160 **TMA and TMAO oxidation increases *R. pomeroyi* growth yields when grown on glucose**

161 *R. pomeroyi* oxidised TMA and TMAO in the presence of both glucose and ammonia in the
162 culture medium (Fig. 2a & 2b). The rate of TMA and TMAO oxidation was greatest through
163 exponential growth but did continue throughout stationary phase when glucose was
164 exhausted from the medium (data not shown). TMA oxidation by wild-type cells resulted in a
165 greater final growth yield ($OD_{540} = 2.91 \pm 0.05$) (Fig. 2a) compared to the mutant, *Δtmm::Gm*
166 ($OD_{540} = 2.063 \pm 0.06$), which was unable to catabolise TMA (Lidbury et al, 2014). TMAO
167 oxidation in wild-type cells (Fig. 2b) also led to an increase in final growth yield ($OD_{540} =$
168 2.46 ± 0.02) compared to the mutant, *Δtdm::Gm* ($OD_{540} = 1.94 \pm 0.07$), which cannot oxidise
169 TMAO (Lidbury et al, 2014). TMA oxidation to TMAO could still function in the *Δtdm::Gm*
170 mutant, resulting in the accumulation of extracellular TMAO in the medium (supplementary
171 Fig. S2).

172 We conducted an initial screen using a plate assay method whereby *R. pomeroyi* was grown
173 on glucose-limited MAMS plates with or without TMA (3 mM). Colonies grew larger in the
174 presence of TMA, suggesting a greater proportion of the glucose was assimilated into
175 biomass (supplementary Fig. S3). We then carried out further experiments to quantify the
176 enhanced growth yield due to the addition of either TMA or TMAO by quantifying dry
177 weight (wt) of *R. pomeroyi* wild-type and the mutants. *R. pomeroyi* was grown in batch
178 culture under glucose-deplete conditions and either supplemented with or without TMA (5
179 mM) or TMAO (5 mM). Wild-type cells grown on glucose alone reached a final biomass of
180 504 ± 14.3 mg dry wt l^{-1} (Fig. 2c) and when supplemented with either TMA or TMAO, a
181 final biomass of 616 ± 8.9 mg dry wt l^{-1} (+22%) and 626 ± 12.6 mg dry wt l^{-1} (+24%) was
182 achieved, respectively. The *Δtmm::Gm* mutant, which cannot catabolise TMA, had no

183 increase in final biomass (519 ± 21.4 mg dry wt L^{-1}) compared to the glucose-only cultures
184 (534 ± 14.3 mg dry wt L^{-1}); however, when supplemented with TMAO, the final biomass was
185 664 ± 13.3 mg dry wt L^{-1} (+24%) (Fig. 2c). Supplementing the $\Delta tdm::Gm$ mutant with either
186 TMA or TMAO did not result in any increase in final biomass (glucose = 489 ± 14.5 ; +TMA
187 = 453 ± 20.6 ; +TMAO = 487 ± 31.7 mg dry wt L^{-1}). When wild-type *R. pomeroyi* cells were
188 grown in a glucose-limited chemostat (dilution rate = 0.05 h^{-1}), we also observed a 30.4%
189 increase in growth yield when supplemented with TMA (5 mM) whilst the growth yield of
190 the mutant, $\Delta tmm::Gm$, did not change (Table 1).

191 *Citreicella* sp. SE45, which was isolated from a salt marsh (USA), is another member of the
192 MRC and can also grow on TMA as a sole N source, but not as a sole C source (Chen 2012).
193 Salt marshes are typified by having high concentrations of MAs, including TMA, derived
194 from the anaerobic degradation of compatible osmolytes such as glycine betaine (King,
195 1984). When *Citreicella* sp. SE45 was grown using glucose-deplete MAMS medium, the
196 addition of TMA led to an increase in final growth yield (supplementary Fig. S4), thus
197 demonstrating that catabolism of TMA can also enhance chemoorganoheterotrophic growth
198 of another closely related bacterium.

199 **TMA increases the growth rate of *R. pomeroyi* when grown on glucose**

200 We also observed a direct correlation between specific growth rates and varying
201 concentrations of TMA in the medium (Fig. 3a). The specific growth rate increased from
202 $0.061 \pm 0.002 (\text{h}^{-1})$ for cells incubated with no TMA to $0.087 \pm 0.003 (\text{h}^{-1})$ for cells incubated
203 with 3 mM TMA. Likewise, the final growth yield increased from 484 ± 10.39 (no TMA) up
204 to 600 ± 8.79 (3 mM TMA) (Fig. 3b). Using intermediate concentrations of TMA (0.5-1 mM)
205 resulted in an intermediate increase in growth rates and growth yields compared to glucose-

206 only cultures. Together, these data confirm that oxidation of MAs can enhance
207 chemoorganoheterotrophic growth on glucose in *R. pomeroyi*.

208 We also observed a synergistic effect of the enhancement of heterotrophic growth when *R.*
209 *pomeroyi* was incubated with two exogenous energy sources (TMA + thiosulfate) during
210 incubations where low concentrations of glucose (100 µM) were stochastically added (every
211 24-48 hrs) four times (400 µM total C). Cells incubated without a supplementary energy
212 source (TMA or thiosulfate) reached a final growth yield of 31.7 ± 1.5 mg dry wt l⁻¹ (Fig. 3c).
213 Cells incubated with either TMA or thiosulfate alone reached a final growth yield of $42.2 \pm$
214 4.7 and 44.3 ± 5.4 mg dry wt ml⁻¹, respectively. Cells incubated with both TMA and
215 thiosulfate reached a final growth yield of 70.8 ± 4.9 mg dry wt ml⁻¹, which equates to over a
216 2-fold increase in biomass.

217 **Oxidation of TMA and TMAO enhances cell survival and viability during energy
218 starvation**

219 *R. pomeroyi* was grown on TMA as a sole N source to induce the enzymes (Fig. 1) involved
220 in MA catabolism, e.g. Tmm, Tdm and GmaS, prior to re-suspension in a fresh minimal
221 medium with no C or energy source. Cells were either supplemented with TMA or TMAO or
222 had no exogenous energy source (control). Both TMA and TMAO were rapidly catabolised
223 over 8 days, although the rate of TMAO catabolism slowed during the final two days (Fig.
224 4a). At the start of energy starvation, the number of viable cells in all cultures was 4.0×10^9
225 cells ml⁻¹ (Fig. 4b). After 4 days, the number of viable cells incubated in the control cultures
226 dropped to 7.4×10^8 , whilst the cell numbers were 2.2×10^9 ml⁻¹ in the presence of TMAO
227 and 1.1×10^9 ml⁻¹ in the presence of TMA, respectively. After 8 days, the number of viable
228 cells from cultures with no exogenous C dropped to 2.9×10^7 ml⁻¹ whilst +TMAO and
229 +TMA cultures had 9.0×10^8 ml⁻¹ and 7.5×10^8 ml⁻¹ cells, respectively. In summary, the

230 number of viable cells surviving periods of energy starvation was an order of magnitude
231 greater when cells were incubated with either TMA or TMAO.

232 To confirm that cells do indeed generate ATP from the oxidation of MAs, cells were energy-
233 starved overnight prior to the addition of either TMA (1 mM) or TMAO (1 mM) and
234 incubated for a further 2 hours. Wild-type cells incubated with either TMA or TMAO had
235 93.6 ± 4.2 and 92.1 ± 7.8 zeptomoles ATP cell $^{-1}$, respectively (Fig. 5) whilst the intracellular
236 concentration of ATP was lower for cells in the no substrate control (58.3 ± 9.7 zeptomoles
237 ATP cell $^{-1}$). Incubating the mutant, *Δtmm::Gm*, with TMA resulted in no increase in
238 intracellular ATP (54 ± 5.3 zeptomoles ATP cell $^{-1}$) compared to the no substrate control (52.2
239 ± 8.1 zeptomoles ATP cell $^{-1}$), whilst incubation with TMAO did result in an increase in
240 intracellular ATP (80.7 ± 4.9 zeptomoles ATP cell $^{-1}$). As expected, incubation with TMA or
241 TMAO did not result in an increase of intracellular ATP concentrations for the *Δtdm::Gm*
242 mutant (control = 56.4 ± 3.4 ; TMA = 55.7 ± 2.1 ; TMAO = 56.1 ± 2.2 zeptomoles ATP cell $^{-1}$).

243 **Metabolism of TMA remineralises nitrogen (ammonification)**

244 As *R. pomeroyi* can metabolise MAs in order to generate energy, we hypothesised that the
245 amine group would undergo remineralisation to ammonia and subsequent cellular release
246 from cells could provide a source of N for other marine microorganisms (Fig. 6a). To test this
247 hypothesis, we designed a co-culture experiment with *R. pomeroyi* and the methylotrophic
248 bacterium, *Methylomonas methanica* MC09 (Boden et al, 2011a). We inoculated a C-starved
249 and N-starved *R. pomeroyi* culture ($\sim 10^8$ ml $^{-1}$ cells) with *M. methanica* ($\sim 10^7$ ml $^{-1}$ cells) and
250 supplied methanol (1 mM) as the only C source in the system as methanol is only utilised by
251 *M. methanica*. Cultures were either supplemented with ammonium chloride (1 mM) or TMA
252 (1 mM) prior to incubation. Incubation of wild-type *R. pomeroyi* with methanol and TMA
253 resulted in no growth whilst TMA was depleted from the medium (data not shown). Addition

254 of ammonium chloride resulted in growth of *M. methanica* when incubated with either wild-
255 type (3.9×10^8) or the *Δtmm::Gm* mutant ($3.3 \times 10^8 \text{ ml}^{-1}$) confirming that *R. pomeroyi* does
256 not inhibit growth of *M. methanica* (Fig. 6b). Wild-type cells of *R. pomeroyi* depleted TMA
257 from the medium, resulting in growth of *M. methanica* ($2.6 \times 10^8 \text{ ml}^{-1}$), however, no growth
258 of *M. methanica* occurred (2.8×10^7) during incubation with the *Δtmm::Gm* mutant of *R.*
259 *pomeroyi*, as a consequence of no TMA degradation during the 9 day incubation period (Fig.
260 6c).

261

262 **Discussion**

263 Methylated one-carbon compounds were originally thought to be substrates primarily for a
264 specialised guild of bacteria, the methylotrophs (Chistoserdova et al, 2009, Chistoserdova
265 2011), however recent evidence has implicated marine heterotrophic bacteria in the
266 catabolism of these compounds (Chen et al, 2011, Sun et al, 2011, Lidbury et al, 2014).
267 Whilst a small percentage of isolates of the MRC can grow on TMA and TMAO as a sole C
268 source, the majority appear to be able to only utilise these compounds as a sole N source,
269 whilst maintaining the genes predicted to be involved in oxidation of the methyl groups
270 (Chen, 2012). We show that *R. pomeroyi* and also *Citreicella* sp. SE45 can oxidise TMA and
271 TMAO to help stimulate growth on an organic substrate. The implications for this are 1)
272 catabolism of MAs results in the more efficient conversion of organic substrates into biomass
273 which provides an ecological advantage to these bacteria (Moran & Miller, 2007); 2) the
274 turnover of MAs in the marine environment is likely to be rapid during times of high primary
275 productivity due to an influx of organic substrates from phytoplankton exudation and cell
276 death; 3) marine heterotrophic bacteria are likely to be an efficient biological sink for these
277 compounds, retarding their flux into the atmosphere; 4) The metabolism of MAs as an energy
278 source results in the remineralisation of MAs to ammonium, which can in turn support the
279 growth of other microbial communities in the environment.

280 The ecological success of the MRC may be in part due to the utilisation of a wide range of
281 both organic and inorganic compounds for the generation of cellular energy. Whilst TMAO
282 oxidation has been shown to provide ATP for *Candidatus Pelagibacter ubique* HTCC1062
283 (SAR11 clade), no effect on the ecophysiology of the bacterium was identified (Sun et al,
284 2011). Our study revealed that TMA and TMAO oxidation could enhance both the growth
285 rate and growth yield of *R. pomeroyi*. This is in agreement with previous work demonstrating
286 that a methylotroph had a higher specific growth rate and higher growth yield as a result of

287 co-oxidation of TMAO alongside its growth on methanol (Halsey et al, 2012). Cells with
288 higher intracellular concentrations of ATP can respond faster to fluxes of organic matter
289 associated with phytoplankton through ATP-mediated transport (Steindler et al, 2011). Both
290 SAR11 and *Roseobacter* cells devote a large amount of resources into the production of
291 ABC-transporter systems to help facilitate the rapid uptake of essential nutrients (Sowell et
292 al, 2008, Sowell et al, 2011, Williams et al, 2012, Gifford et al, 2013). Therefore, bacteria of
293 the MRC and SAR11 clade capable of generating ATP from the catabolism of TMA and
294 TMAO may have an ecological advantage through the efficient scavenging of nutrients in the
295 surface waters. Production of ATP through the oxidation of thiosulfate to sulfate helps
296 *Citreicella thiooxidans* grow more efficiently on organic substrates (Sorokin et al, 2005).
297 This trait is widespread within the MRC (Newton et al, 2010) and *R. pomeroyi* has enhanced
298 growth when incubated with thiosulfate (Moran et al, 2004). In our study, the growth of *R.*
299 *pomeroyi* during additions of glucose was enhanced through the co-catabolism of both TMA
300 and thiosulfate, thus demonstrating how utilisation of multiple exogenous energy sources can
301 enhance growth. Both TMA and thiosulfate are ‘energy rich’ in the sense that they can
302 generate between 7-8 ATP molecules from the oxidation of one TMA or thiosulfate
303 molecule. In contrast, carbon monoxide is a relatively ‘energy poor’ compound, only
304 liberating two electrons, which does not appear to result in an enhancement of growth for *R.*
305 *pomeroyi* (Cunliffe 2012). The utilisation of MAs as a supplementary energy source is
306 consistent with a growing body of data that points towards the success of certain
307 heterotrophic bacterial groups that can generate energy from a wide range of sources,
308 including reduced organic carbon compounds (Eiler, 2006, Moran & Miller, 2007, Boden et
309 al, 2011b, Green et al, 2011, Steindler et al, 2011, Sun et al, 2011).

310 The greater number of viable cells in *R. pomeroyi* cultures incubated with TMA and TMAO
311 is consistent with the notion that exogenous energy sources will be preferentially used instead

312 of endogenous C stores in order to maintain cellular integrity. This also resulted in *R.*
313 *pomeroyi* maintaining higher intracellular ATP concentrations during periods of energy
314 starvation. Representatives of the SAR11 clade and *Vibrio* spp. start to break down and
315 respire endogenous carbon when energy starved and this process is significantly reduced
316 when incubated in the light, through proteorhodopsin mediated energy production (Gómez-
317 Consarnau et al, 2010, Steindler et al, 2011). This results in a greater number of viable cells
318 and also larger, more active cells during periods of energy starvation (Gómez-Consarnau et
319 al, 2010, Steindler et al, 2011).

320 In marine surface waters, primary production is often limited by N availability and this has a
321 direct effect on the amount of organic matter exported to the deep ocean (Eppley & Peterson,
322 1979, Falkowski et al, 1998, Zehr & Kudela 2011). The microbially-mediated
323 remineralisation of N (ammonification) following phytoplankton decomposition has
324 previously been demonstrated in a laboratory study which suggested that this process may
325 occur in seawater (Garber, 1984). Here we demonstrate a ‘proof of concept’ whereby the
326 turnover of TMA resulted in the release of remineralised N in the form of ammonia, which
327 was subsequently taken up by another bacterium and used to support growth. As a number of
328 *Roseobacter* species are frequently associated with phytoplankton blooms (Hahnke et al,
329 2013, González et al, 2000, Buchan et al, 2005, Wagner-Dobler et al, 2009, Nelson et al,
330 2014) we predict that this N remineralisation process may take place with several difference
331 ‘nitrogen rich’ compounds, for example, glycine betaine, choline and carnitine. This process
332 has strong implications for the ‘microbial loop’ which ultimately controls the level of both
333 primary and secondary production in the world’s oceans (Azam et al, 1983). N-rich
334 compounds may represent a source of ammonia in the oceans as the C in these compounds is
335 catabolised to generate energy (Sun et al, 2011, Halsey et al, 2012). This process may reduce
336 the amount of N lost to the sub-photic zone through the sinking of cell debris and particles

337 and may provide a feedback between the phytoplankton and heterotrophic bacteria (Azam et
338 al, 1983, Garber, 1984). Interestingly, in bacteria from the SAR11 clade, N-limitation does
339 not induce any of the genes involved in the catabolism of MAs, whilst energy starvation (in
340 the dark) does induce some (Steindler et al, 2011, Smith et al, 2013). Moreover, an
341 ammonium transporter (SAR_1310) located adjacent to the genes involved in MA catabolism
342 is only induced under nitrogen replete conditions and it has been proposed that this
343 transporter is involved in ammonia export (Smith et al, 2013). All bacteria of the MRC and
344 SAR11 clade capable of utilising MAs have a homolog of the transporter adjacent to genes
345 involved in MA catabolism. Homologs of the putative ammonium exporter related to both the
346 SAR11 clade and MRC are highly expressed in surface waters off the coast of Georgia
347 (Gifford et al, 2013). At this site, genes involved in the catabolism of TMAO are also highly
348 expressed in bacteria related to the SAR11 and MRC clades (Gifford et al, 2013). The
349 function of this proposed ammonium transporter warrants further investigation as it may have
350 a pivotal role in the release of ammonium through remineralisation of organic nitrogen in
351 marine surface waters. Together these data strengthens the hypothesis that MAs are primarily
352 catabolised to generate cellular energy which in turn remineralises ammonium through
353 methylamine oxidation.

354 In summary, catabolism of MAs by a heterotrophic bacterium enhances
355 chemoorganoheterotrophic growth as well as enhancing the survival of energy starved cells.
356 In turn, this liberates inorganic N (ammonification) which can be subsequently used by other
357 microbes. As there are no data regarding *in situ* residence times and turnover rates of MAs in
358 the surface waters of the oceans, our recent findings may help to predict the likely fate of
359 these compounds in which rapid microbial consumption of MAs may present an oceanic sink
360 and retard their flux from the oceans to the atmosphere.

361

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368 **Conflict of interest**

369 The authors declare no conflict of interest.

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372 Supplementary information is available at ISMEJ's website

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- 500

501 **Figure Legends**

502 **Figure 1.** Proposed model for methylated amine catabolism in the marine bacterium
503 *Ruegeria pomeroyi* DSS-3. Text in brackets denotes the locus tag of the corresponding gene
504 in *R. pomeroyi*. Abbreviations: TMA, trimethylamine; TMAO, trimethylamine *N*-oxide;
505 DMA, dimethylamine; MMA, monomethylamine; GMA, gamma-glutamylmethyamide;
506 NMG, *N*-methylglutamate; CH₂=H₄F, 5,10-methylene tetrahydrofolate; CO₂, carbon dioxide.
507 TmoXWV, ATP-dependent TMAO transporter (Lidbury et al., 2014).

508

509 **Figure 2.** **(a)** Catabolism of TMA during growth of *R. pomeroyi* wild-type (grey circles) and
510 the *Δtmm::Gm* mutant (white circles) on glucose and ammonium. TMA in the culture
511 medium was quantified throughout growth for both wild-type (grey diamonds) and the
512 mutant (white diamonds). Note that the y axis is not presented as a logarithmic scale. **(b)**
513 Catabolism of TMAO during growth of *R. pomeroyi* wild-type (white) and the *Δtdm::Gm*
514 mutant (grey circles) on glucose and ammonium. TMAO in the culture medium was
515 quantified throughout growth for both wild-type (grey diamonds) and the mutant (white
516 diamonds). Note that the y axis is not presented as a logarithmic scale. **(c)** Final growth yields
517 of *R. pomeroyi* wild-type and mutant strains, *Δtmm::Gm* and *Δtdm::Gm*, grown on glucose
518 and ammonium (black bars) and supplemented with either 5 mM TMA (white bars) or 5 mM
519 TMAO (grey bars). Error bars denote standard deviation. Results presented are the mean of
520 triplicate cultures.

521

522 **Figure 3.** A comparison of the specific growth rates **(a)** and final growth yields **(b)** of the
523 wild-type *R. pomeroyi* grown on glucose and ammonium when supplemented with increasing
524 concentrations of TMA, using a starting inoculum that was pre-incubated with TMA (24
525 hours). **(c)** The final growth yield of *R. pomeroyi* after 7 days during which four additions of

526 glucose (100 μ M) were added every 24-48 hrs. Cultures were incubated with TMA (2 mM)
527 or thiosulfate (2 mM) or both and the same concentrations were added every 48 hours. Error
528 bars denote standard deviation. Results presented are the mean of triplicate cultures.

529

530 **Figure 4.** (a) Quantification of TMA (white squares) and TMAO (grey squares) during
531 incubations with energy-starved *R. pomeroyi* cells. (b) Quantification of viable cells in
532 carbon and energy-starved *R. pomeroyi* cultures incubated with either no exogenous carbon
533 (black circles), TMA (white circles) or TMAO (grey circles). Error bars denote standard
534 deviation. Results presented are the mean of triplicate cultures.

535

536 **Figure 5.** Quantification of intracellular ATP concentrations from *R. pomeroyi* cultures
537 energy-starved for 18 hrs prior to incubation for a further two hrs with either 1 mM TMA
538 (white bars), 1 mM TMAO (grey bars) or no exogenous carbon source (black bars). Error
539 bars denote standard deviation. Results presented are the mean of triplicate cultures.

540

541 **Figure 6.** (a) Schematic diagram of the flow of nitrogen in a co-culture system involving *R.*
542 *pomeroyi* and *Methylomonas methanica* MC09. Ammonia liberated from the catabolism of
543 TMA can be used by another bacterium to support its growth. Abbreviations: NH_4^+ ;
544 ammonium. (b) The cell count of *Methylomonas methanica* MC09 after incubation for 9 days
545 with either *R. pomeroyi* wild-type (A) or *Δtmm::Gm* mutant (A') and supplemented with
546 either ammonium chloride (1 mM) or TMA (1 mM). (c) Quantification of TMA during
547 incubation with wild-type (white triangles) or *Δtmm::Gm* mutant (grey triangles). Error bars
548 denote standard deviation. Results presented are the mean of triplicate cultures.

549

Figure 1.

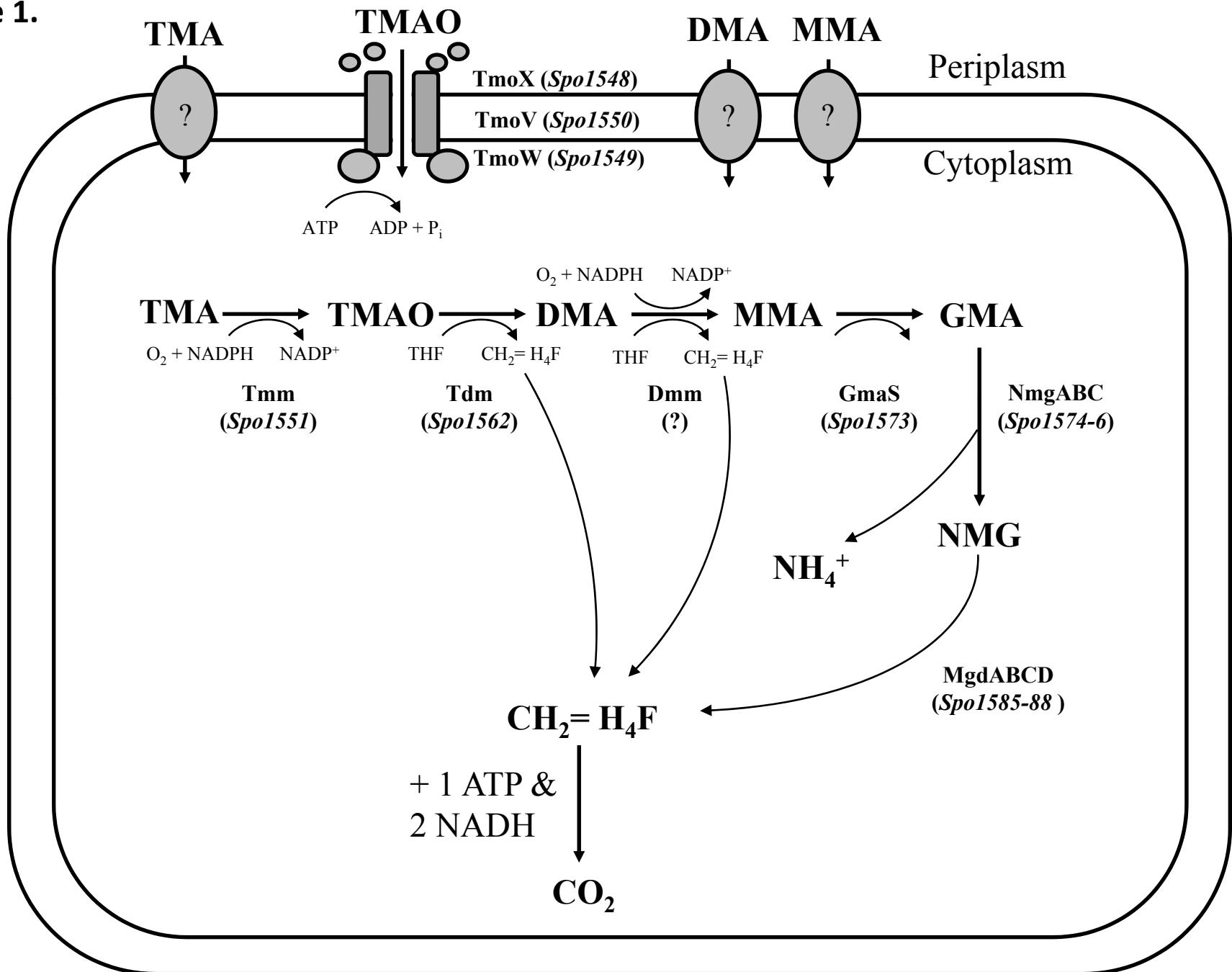
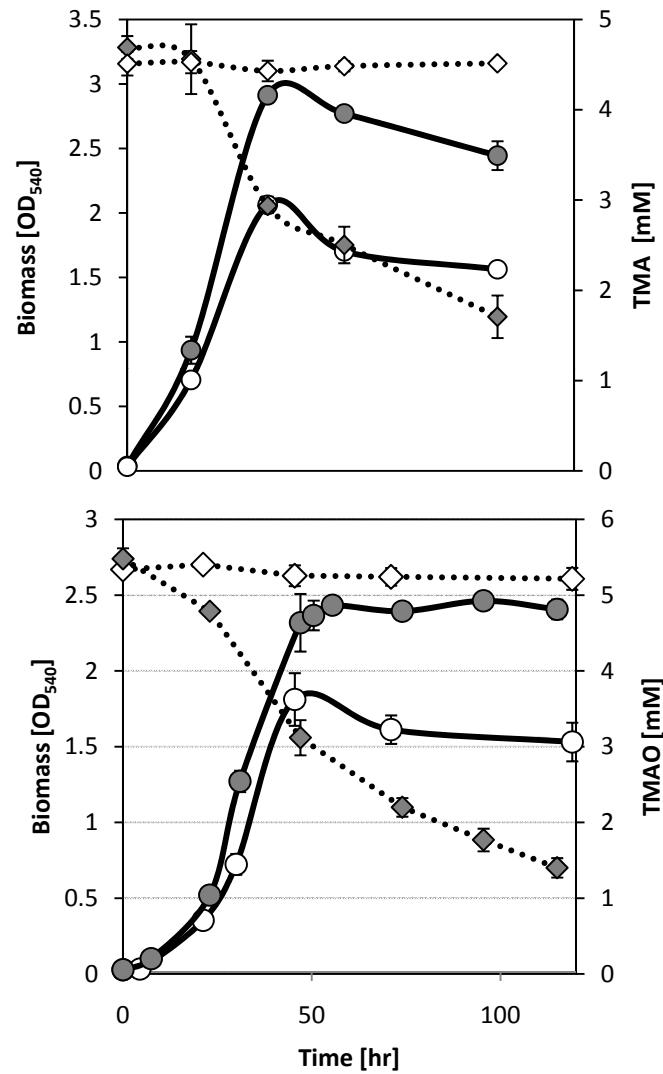


Figure 2.

a)



b)

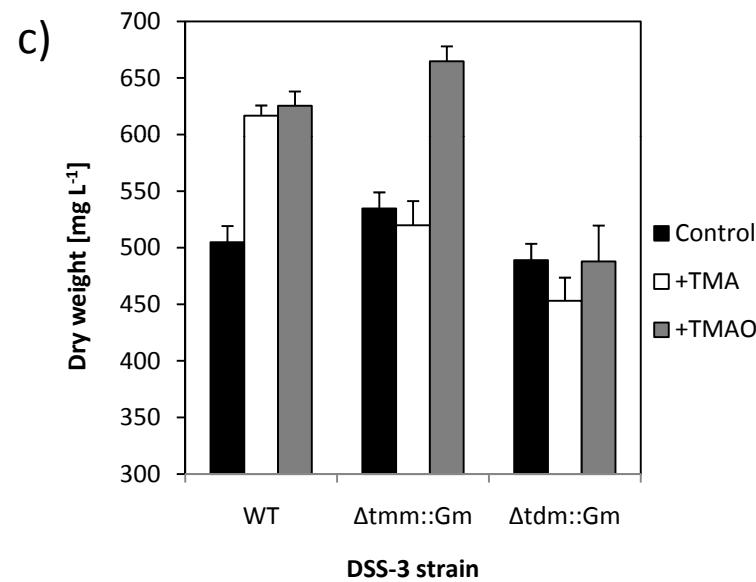


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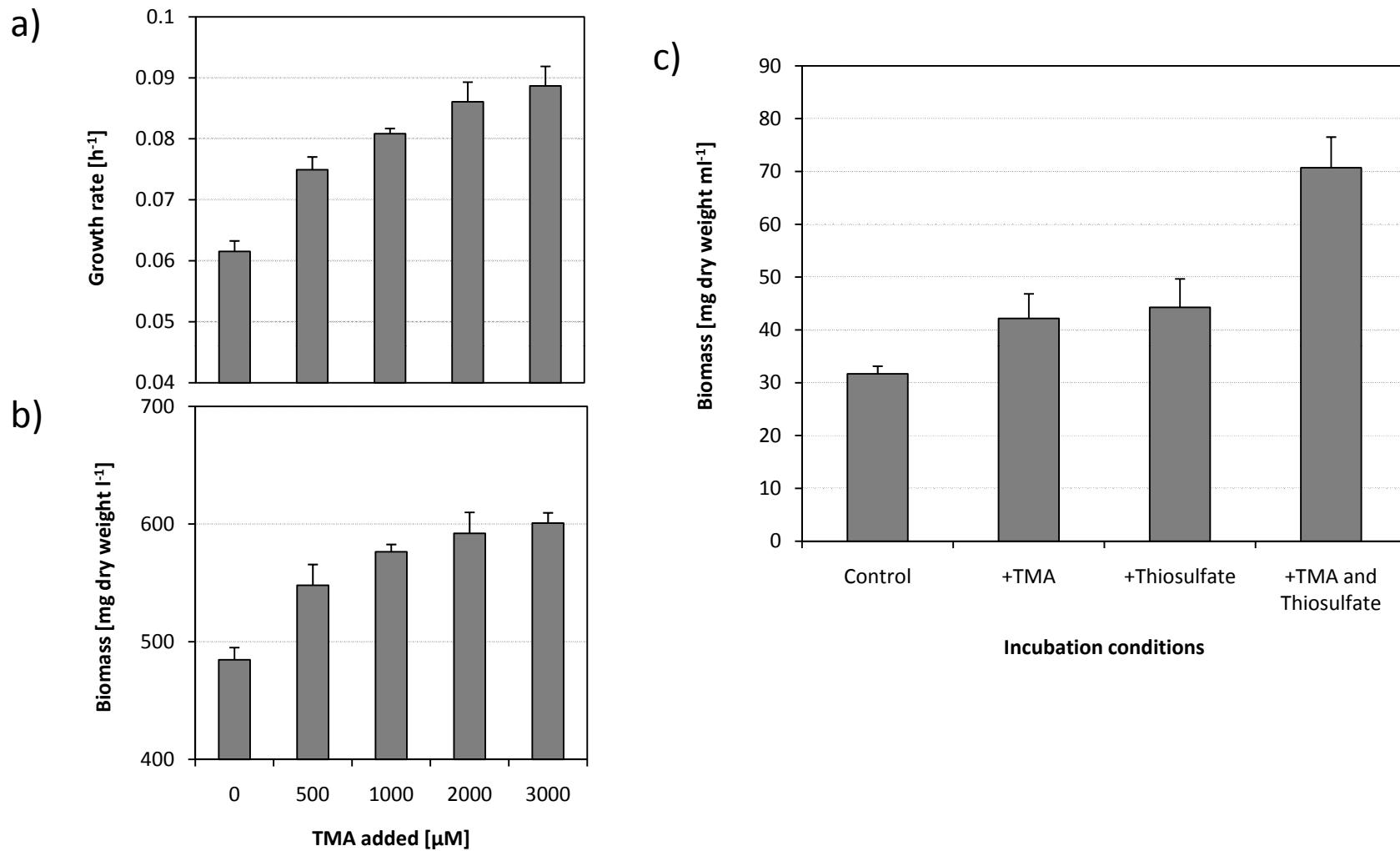


Figure 4.

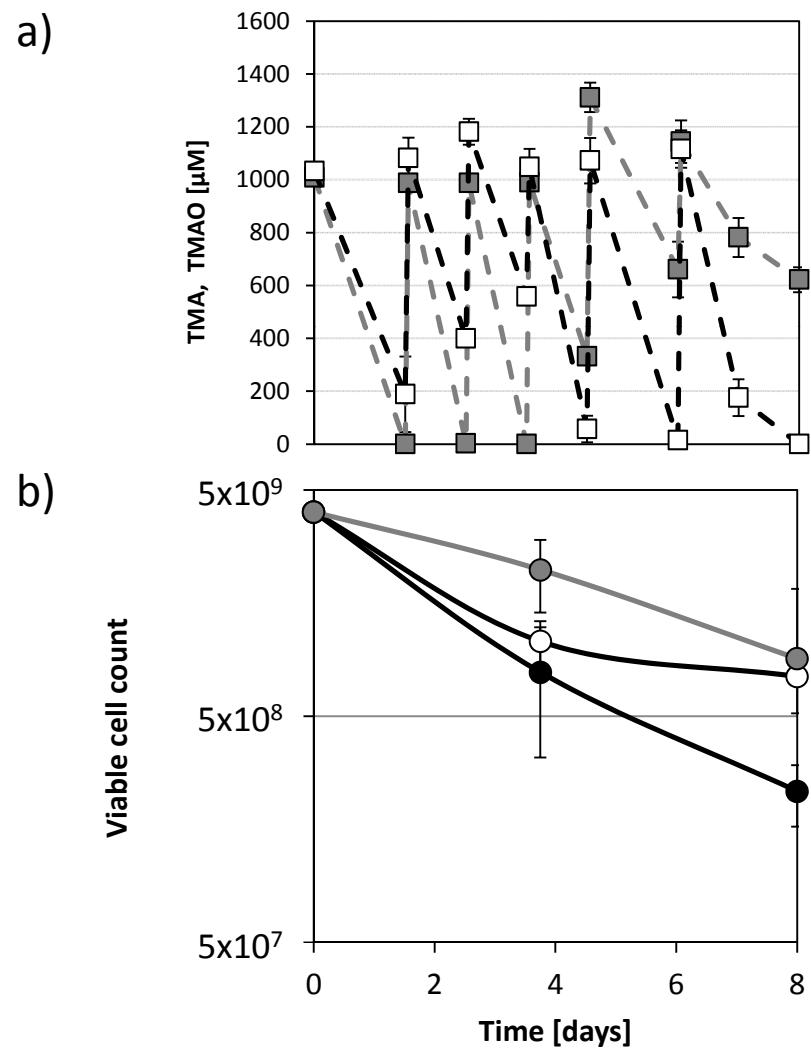


Figure 5.

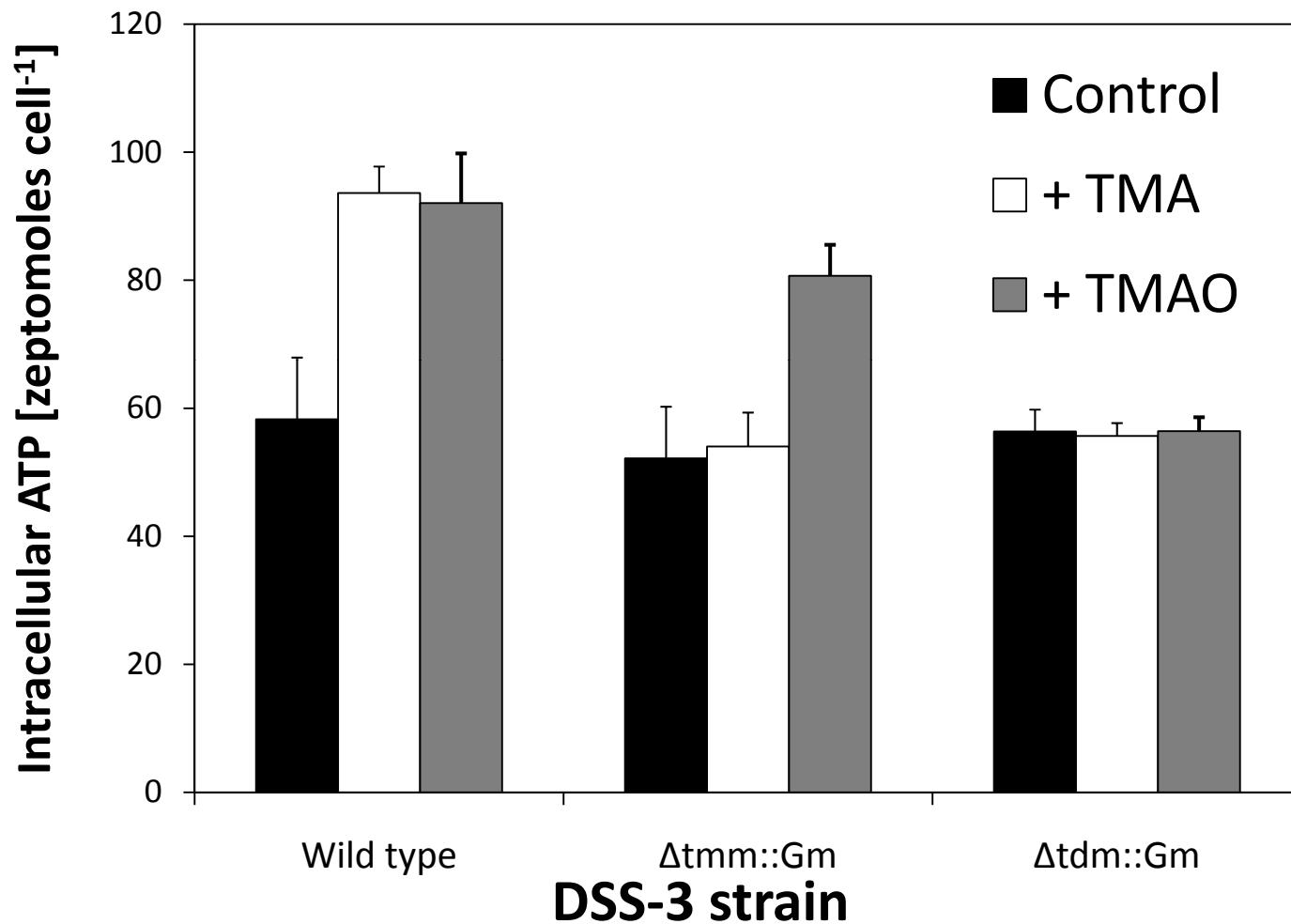


Figure 6.

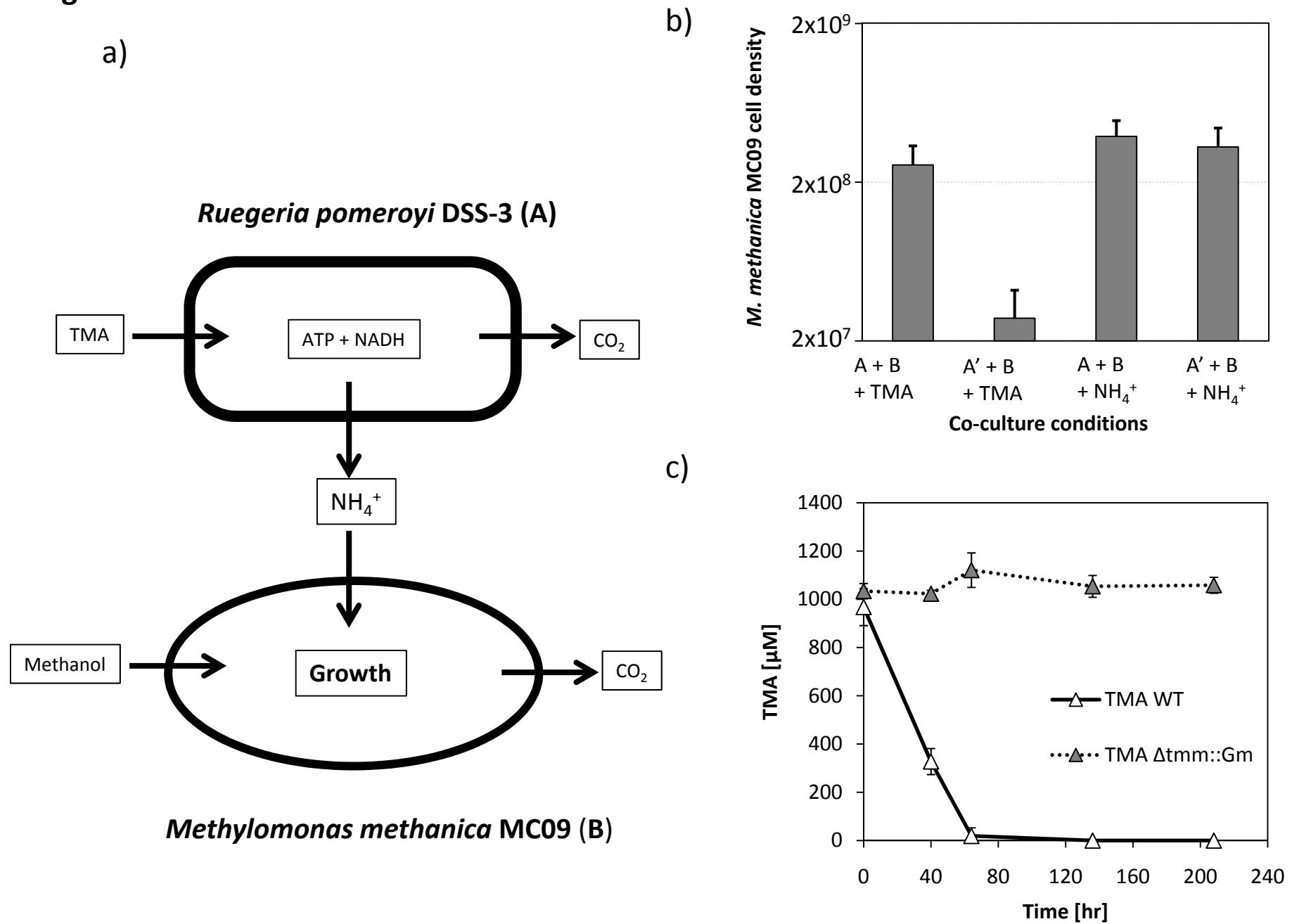


Table 1. Growth yields for *R. pomeroyi* strains grown in a glucose-limited chemostat at a growth rate of 0.05 h^{-1} +/- TMA (5 mM).

Strain		g dry biomass mol⁻¹ glucose	g dry biomass mol⁻¹ carbon	% difference with TMA	TMA remaining (mM)
Wild-type	- TMA	48.77	8.13	-	-
	+ TMA	63.61	10.60	30.4	2.2
<i>Δtmm::Gm</i>	- TMA	48.42	8.07	-	-
	+ TMA	48.08	8.01	-	5