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

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Abstract

Bacteria of the marine *Roseobacter* clade are characterised by their ability to utilise a wide range of organic and inorganic compounds to support growth. Trimethylamine (TMA) and trimethylamine *N*-oxide (TMAO) are methylated amines and form part of the dissolved organic nitrogen pool, the second largest source of nitrogen after N_2 gas, in the oceans. We investigated if the marine heterotrophic bacterium, *Ruegeria pomeroyi* DSS-3, could utilise TMA and TMAO as a supplementary energy source and whether this trait had any beneficial effect on growth. In *R. pomeroyi*, catabolism of TMA and TMAO resulted in the production of intracellular ATP which in turn helped enhance growth rate and growth yield as well as enhancing cell survival during prolonged energy starvation. Furthermore, the simultaneous use of two different exogenous energy sources led to a greater enhancement of chemoorganoheterotrophic growth. The use of TMA and TMAO primarily as an energy source resulted in the remineralisation of nitrogen in the form of ammonium, which could cross feed into another bacterium. This study provides greater insight into the microbial metabolism of methylated amines in the marine environment and how it may affect both nutrient flow within marine surface waters and the flux of these climatically important compounds into the atmosphere.

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

Introduction

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

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

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

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

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

Materials and Methods

Growth conditions

Ruegeria pomeroyi DSS-3 was maintained in the laboratory on marine agar 2216 (Difco, UK). Gentamicin (10 μg ml^{-1}) was added to maintain mutant strains *Δtmm::Gm* and *Δtdm::Gm* (Lidbury et al, 2014). For all experiments *R. pomeroyi* (wild-type and mutants) was grown in marine ammonium mineral salts (MAMS) medium (Schäfer, 2007) using glucose as the sole carbon source. MAMS medium was modified from (Schäfer 2007) and contained (per liter): NaCl, 20 g; $(NH_4)_2SO_4$, 1 g; $MgSO_4 \cdot 7H_2O$, 1 g; $CaCl_2 \cdot 2H_2O$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 2 mg; $Na_2MoO_4 \cdot 2H_2O$, 20 mg; KH_2PO_4, 0.36 g; K_2HPO_4, 2.34 g; plus 1 ml of SL-10 trace metals solution (Schäfer 2007). Vitamins were prepared as described previously (Chen 2012). Continuous culture work was performed using a glucose-limited (5 mM) chemostat using the methods previously described by (Boden et al, 2011b). To avoid precipitants forming in the medium during autoclaving, NH_4Cl was substituted for $(NH_4)_2SO_4$. Steady-state was achieved after 5 dilutions and the dilution rate was set at 0.05 h^{-1}.

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

Determination of biomass (mg dry weight l^{-1})

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

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

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

R. pomeroyi was grown in MAMS with TMA (3 mM) or TMAO (3 mM) as the sole N source to a final OD_{540} ~0.5. Cells were re-suspended in MAMS with no exogenous C and then aliquoted (20 ml) into 125 ml serum vials (n=3) with either no exogenous C (control), or TMA (1 mM) or TMAO (1 mM). For cell counts, serial dilutions were generated (n=3) and 10 µl were spotted (n=3) on ½ YPSS (per litre; 2 g yeast extract, 1.25 g peptone, 20 g sea salts (Sigma-Aldrich) plates and incubated at 30^{o}C. TMA and TMAO were quantified by ion-exchange chromatography as described previously (Lidbury et al, 2014).

Quantification of intracellular ATP concentrations

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

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

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

R. pomeroyi wild-type and the mutant, *Δtmm::Gm* (Lidbury et al, 2014), was grown using either TMA or TMAO as the sole N source (OD_{540} ~0.3). Cells were re-suspended in fresh medium containing 1 mM methanol. For each strain, triplicate cultures were set up using either TMA or ammonium chloride as the sole N source (1 mM). *M. methanica* was grown using methanol as the C source (2 mM) and ammonia (0.5 mM) as the limiting nutrient until the onset of stationary phase. A 5% (v/v) inoculum of *M. methanica* (~10^7 cells) was added to each *R. pomeroyi* culture. Co-cultures were incubated at 25^{o}C on a rotary shaker (150 r.p.m.). For *M. methanica* cell counts, serial dilutions were generated (n=3) and 10 µl were spotted (n=3) on MAMS plates with methane as the sole C source and incubated at 25^{o}C.

Results

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

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

We conducted an initial screen using a plate assay method whereby *R. pomeroyi* was grown on glucose-limited MAMS plates with or without TMA (3 mM). Colonies grew larger in the presence of TMA, suggesting a greater proportion of the glucose was assimilated into biomass (supplementary Fig. S3). We then carried out further experiments to quantify the enhanced growth yield due to the addition of either TMA or TMAO by quantifying dry weight (wt) of *R. pomeroyi* wild-type and the mutants. *R. pomeroyi* was grown in batch culture under glucose-deplete conditions and either supplemented with or without TMA (5 mM) or TMAO (5 mM). Wild-type cells grown on glucose alone reached a final biomass of 504 ± 14.3 mg dry wt l^{-1} (Fig. 2c) and when supplemented with either TMA or TMAO, a final biomass of 616 ± 8.9 mg dry wt l^{-1} (+22%) and 626 ± 12.6 mg dry wt l^{-1} (+24%) was 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 *Δtdm::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, *Δtmm::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 ± 0.002 (h^{-1}) for cells incubated with no TMA to 0.087 ± 0.003 (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-

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

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

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

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

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

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

Metabolism of TMA remineralises nitrogen (ammonification)

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

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

Discussion

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

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

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

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

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

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

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

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

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Conflict of interest

The authors declare no conflict of interest.

Supplementary information is available at ISMEJ's website

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Figure Legends

Figure 1. Proposed model for methylated amine catabolism in the marine bacterium *Ruegeria pomeroyi* DSS-3. Text in brackets denotes the locus tag of the corresponding gene in *R. pomeroyi*. Abbreviations: TMA, trimethylamine; TMAO, trimethylamine *N*-oxide; DMA, dimethylamine; MMA, monomethylamine; GMA, gamma-glutamylmethylamide; NMG, *N*-methylglutamate; $CH_2=H_4F$, 5,10-methylene tetrahydrofolate; CO_2, carbon dioxide. TmoXWV, ATP-dependent TMAO transporter (Lidbury et al., 2014).

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

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

526 glucose (100 μM) 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.

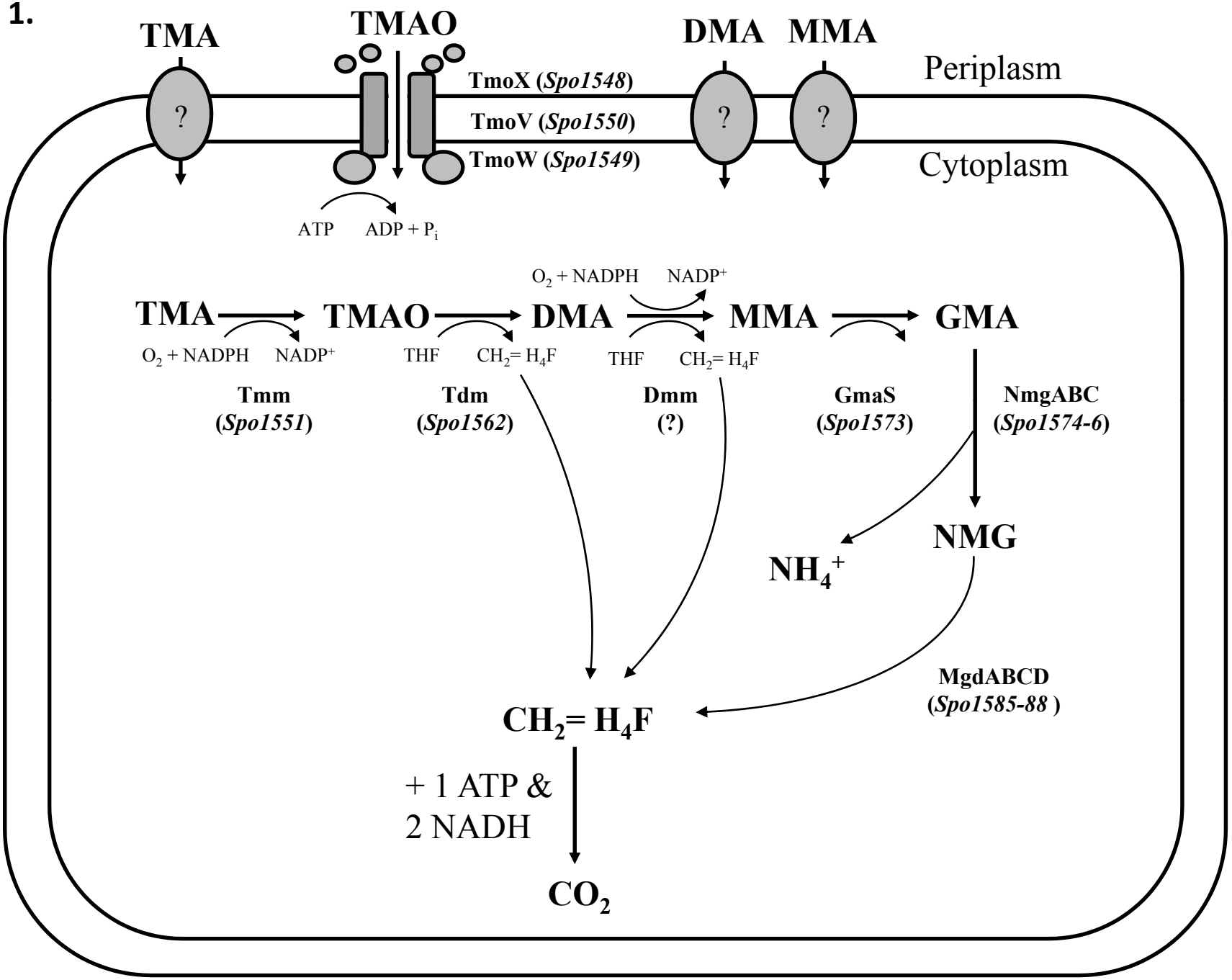
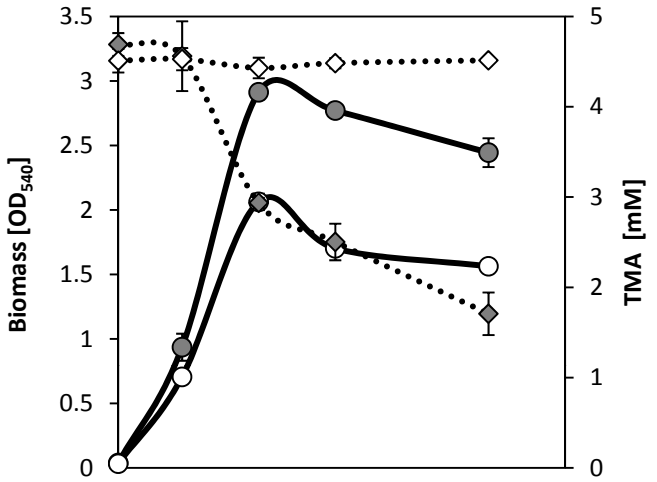
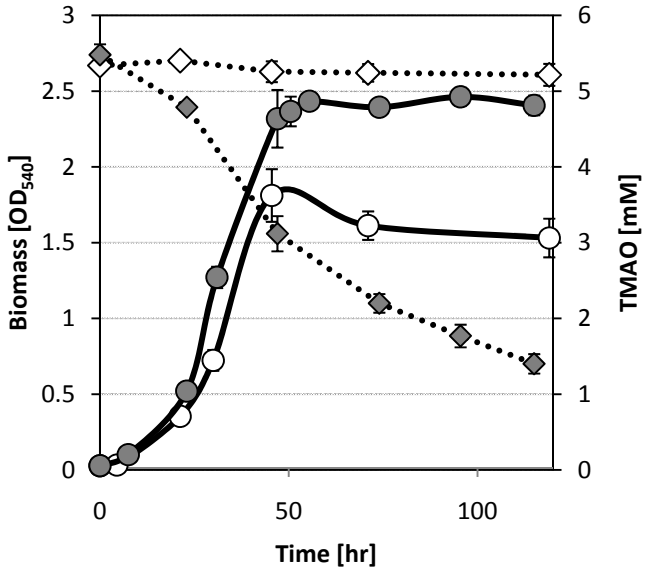

TMA
TMAO
DMA
MMA
Periplasm
Cytoplasm
TmoX (Spo1548)
TmoV (Spo1550)
TmoW (Spo1549)
?
ATP
ADP + Pi
O2 + NADPH
NADP+
THF
CH2= H4F
Tmm (Spo1551)
Tdm (Spo1562)
Dmm (?)
GmaS (Spo1573)
NmgABC (Spo1574-6)
GMA
NMG
NH4+
MgdABCD (Spo1585-88)
CH2= H4F
+ 1 ATP &
2 NADH
CO2

Figure 2.

a)


Biomass [OD540]
TMA [mM]
3.5
3
2.5
2
1.5
1
0.5
0
5
4
3
2
1
0

b)


Biomass [OD540]
TMAO [mM]
3
2.5
2
1.5
1
0.5
0
6
5
4
3
2
1
0
0
50
100
Time [hr]

c)

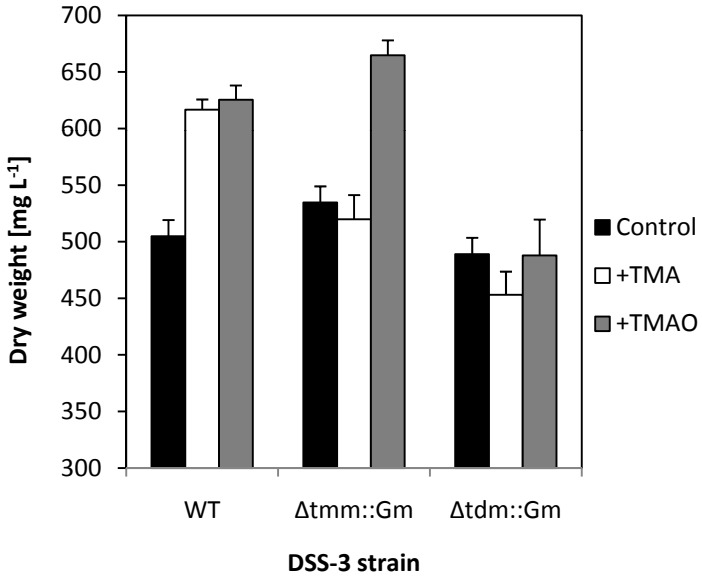

Dry weight [mg L-1]
700
650
600
550
500
450
400
350
300
WT
Δtmm::Gm
Δtdm::Gm
DSS-3 strain
Control
+TMA
+TMAO

Figure 3.

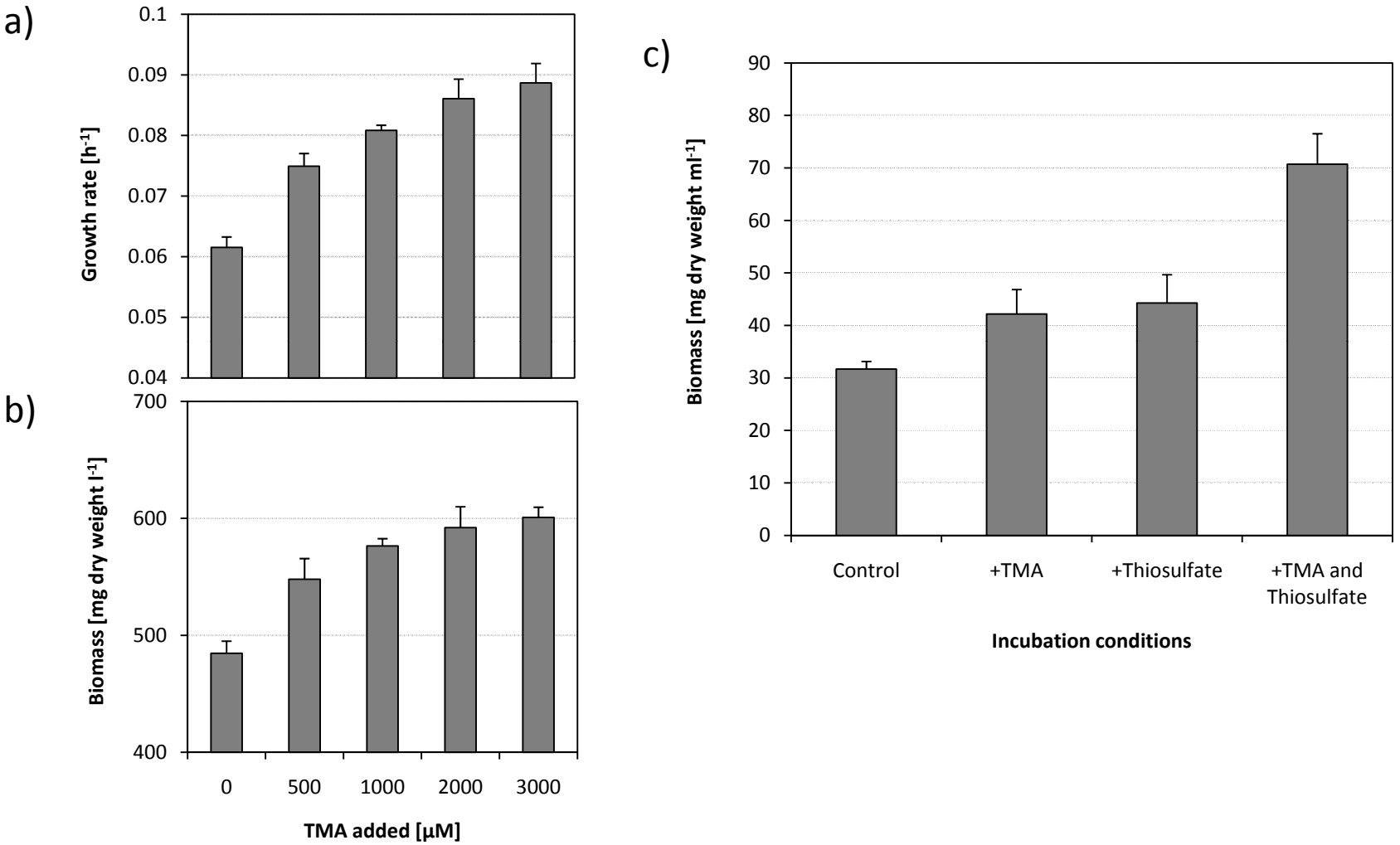

a)
b)
c)
Growth rate [h-1]
0.1
0.09
0.08
0.07
0.06
0.05
0.04
Biomass [mg dry weight l-1]
700
600
500
400
0
500
1000
2000
3000
TMA added [µM]
Biomass [mg dry weight ml-1]
90
80
70
60
50
40
30
20
10
0
Control
+TMA
+Thiosulfate
+TMA and Thiosulfate
Incubation conditions

Figure 4.

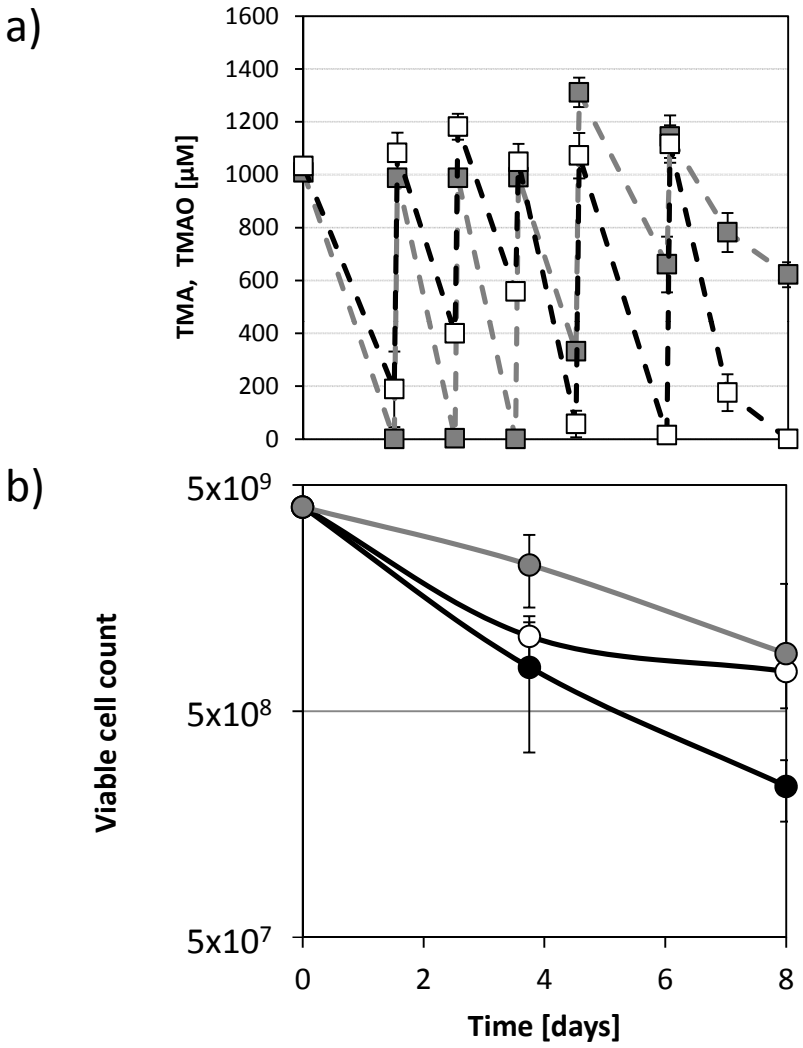

a)
1600
1400
1200
1000
800
600
400
200
0
TMA, TMAO [µM]
b)
5x10^9
5x10^8
5x10^7
Viable cell count
0
2
4
6
8
Time [days]

Figure 5.

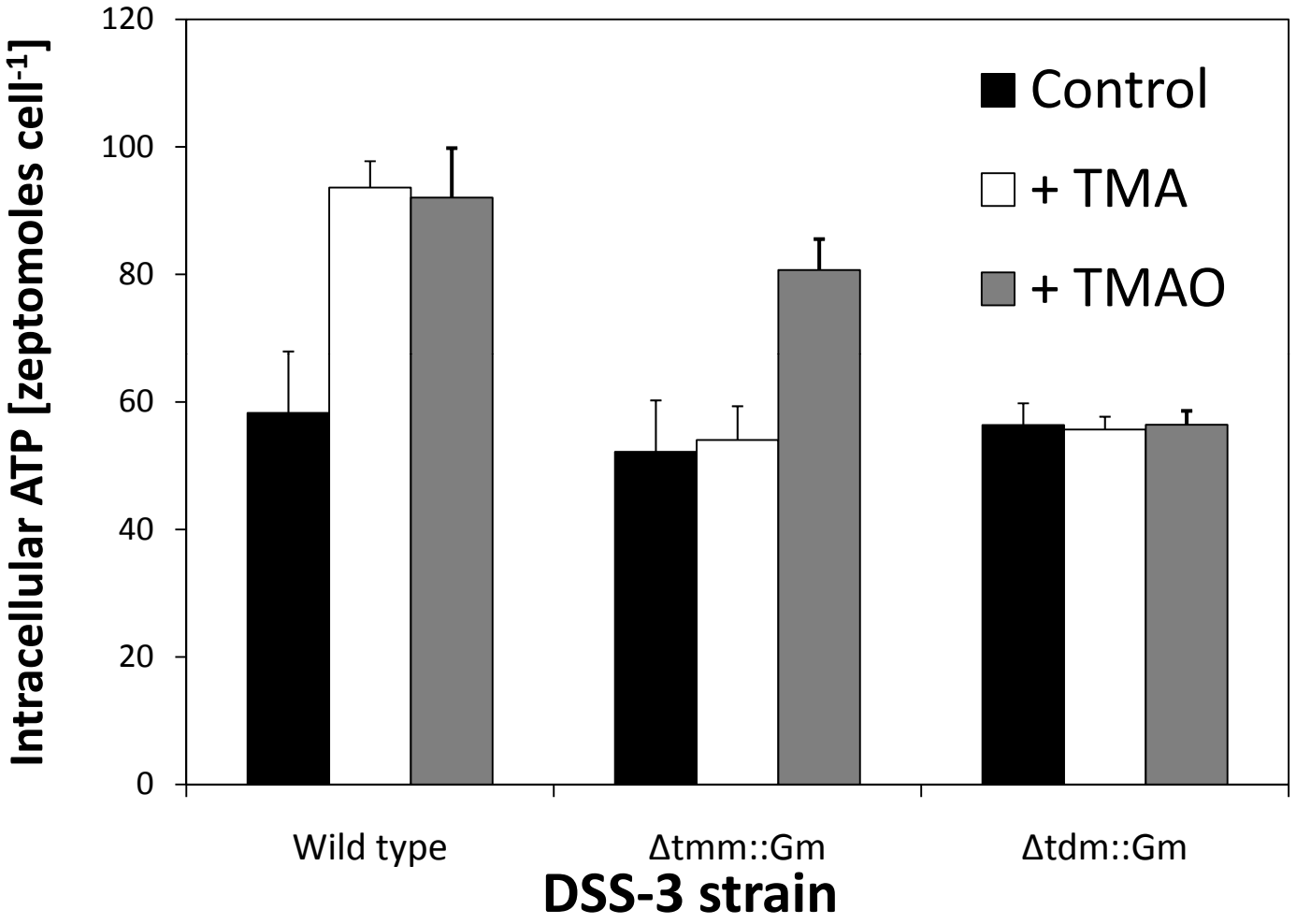
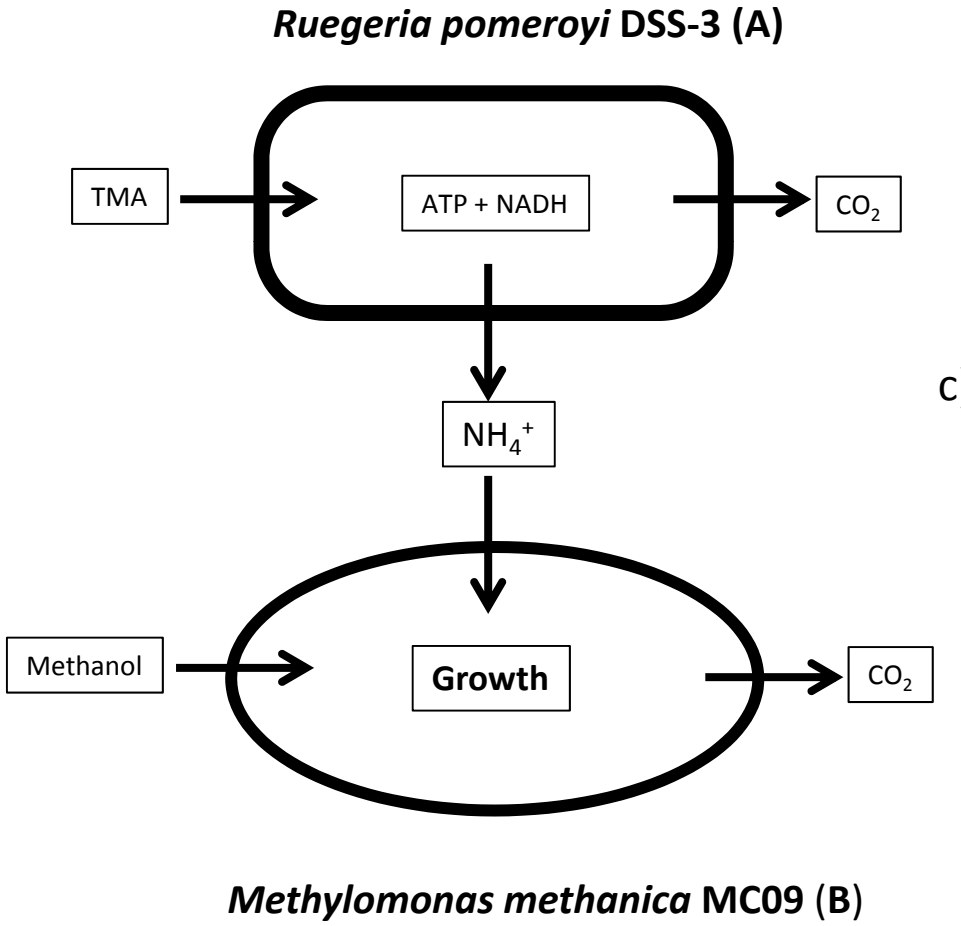
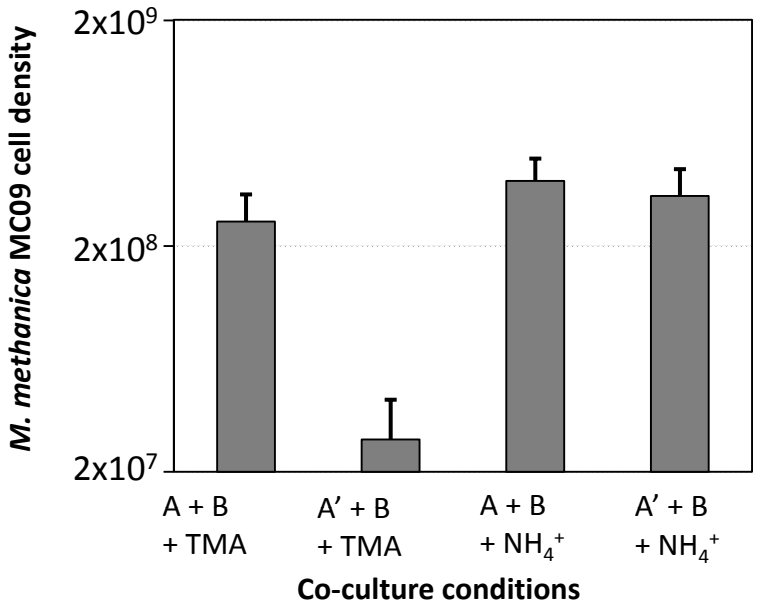

Intracellular ATP [zeptomoles cell-1]
120
100
80
60
40
20
0
Control
+ TMA
+ TMAO
Wild type
Δtmm::Gm
Δtdm::Gm
DSS-3 strain

Figure 6.

a)


Ruegeria pomeroyi DSS-3 (A)
TMA
ATP + NADH
CO_2
NH_4^+
Methanol
Growth
CO_2
Methylomonas methanica MC09 (B)

b)


M. methanica MC09 cell density
$2x10^9$
$2x10^8$
$2x10^7$
A + B + TMA
A' + B + TMA
A + B + NH_4^+
A' + B + NH_4^+
Co-culture conditions

c)

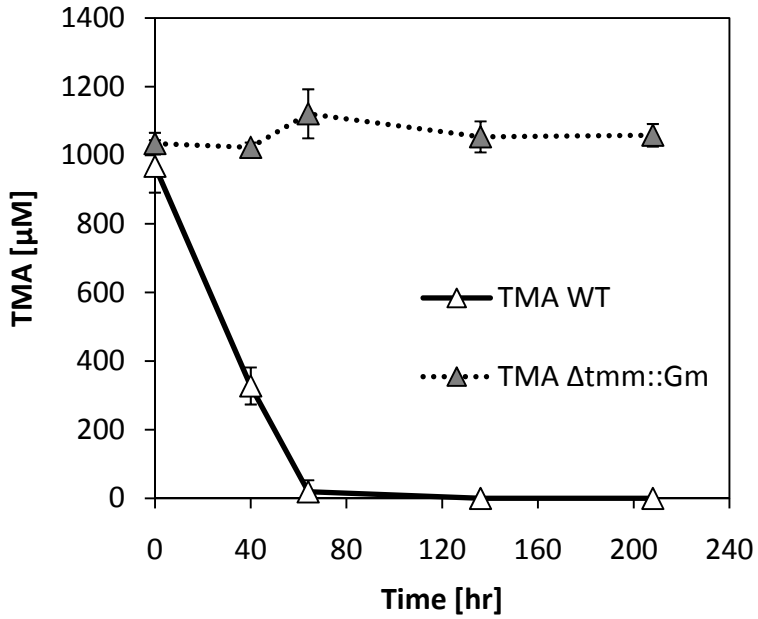

TMA [µM]
1400
1200
1000
800
600
400
200
0
TMA WT
TMA Δtmm::Gm
0
40
80
120
160
200
240
Time [hr]

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
Δtmm::Gm	- TMA	48.42	8.07	-	-
	+ TMA	48.08	8.01	-	5