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ORIGINAL ARTICLE

# 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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**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 (MA) 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 to 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 MAs 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.**
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## 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 and Miller, 2007; Boden *et al.*, 2011b). For these reasons, the MRC bacteria have essential roles in both carbon and sulphur 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 dimethylsulphoniopropionate (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 and 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, 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

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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 and 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 pool (King, 1984; Gibb *et al.*, 1999; Gibb and 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 and Kudela, 2011).

Chen (2012) showed that representatives of the MRC can grow on TMA. While 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 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 from members of the MRC. *R. pomeroyi* has the genes required for TMA catabolism (Figure 1) and can grow on TMA as a N source, but not on 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*

*R. pomeroyi* DSS-3 was maintained in the laboratory on marine agar 2216 (Difco, Sparks, MD, USA). Gentamicin ($10\,\mu g\,ml^{-1}$) 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

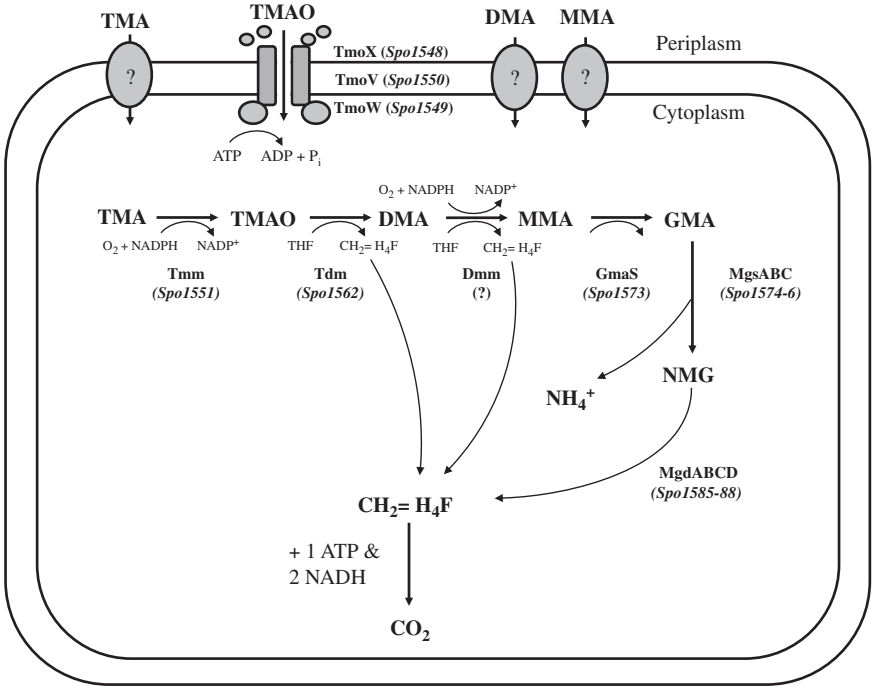


**Figure 1** Proposed model for methylated amine catabolism in the marine bacterium *Ruegeria pomeroyi* DSS-3. Text in brackets denotes the locus tag of the corresponding gene in *R. pomeroyi*. $CH_2 = H_4F$, 5,10-methylene tetrahydrofolate; $CO_2$, carbon dioxide; DMA, dimethylamine; Dmm, dimethylamine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

was modified from Schäfer (2007) and contained (per litre): 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 five 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 r.p.m). *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 optical density at 540 nm($OD_{540}$) ~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, Darmstadt, Germany). 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 °C. Filter pads were repeatedly weighed until a constant weight was achieved. A standard curve was plotted for $OD_{540}$ against dry weight (Supplementary Figure S1). For all conversions of $OD_{540}$ to dry weight, a constant of 1 OD unit at $OD_{540}=254$ mg dry weight (wt) $l^{-1}$ 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, Gillingham, UK) plates and incubated at 30 °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; 8000 g) at late exponential phase ($1 \times 10^9$ 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 h before adding a further 500 μl of each test compound. After 1 h, 100 μl of cell suspension was mixed with 100 μl of BacTiter Glo cell viability kit (Promega, Fitchburg, WI, USA) and incubated for 5 min before recording luciferase activity on a Luminoskan Ascent microplate luminometer (Thermo Scientific, Waltham, MA, USA). 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), were 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 °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 °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 (Figures 2a and b). The rate of TMA and TMAO oxidation was greatest through exponential growth, but did continue throughout the 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 ($OD_{540}=2.91\pm0.05$; Figure 2a) compared to the mutant, *Δtmm::Gm* ($OD_{540}=2.063\pm0.06$), which was unable to catabolise TMA (Lidbury *et al.*, 2014). TMAO oxidation in wild-type cells (Figure 2b) also led to an increase in final growth yield ($OD_{540}=2.46\pm0.02$) compared to the mutant, *Δtdm::Gm* ($OD_{540}=1.94\pm0.07$), which cannot oxidise TMAO (Lidbury *et al.*, 2014). TMA oxidation to TMAO could still function in the *Δtdm::Gm* mutant,

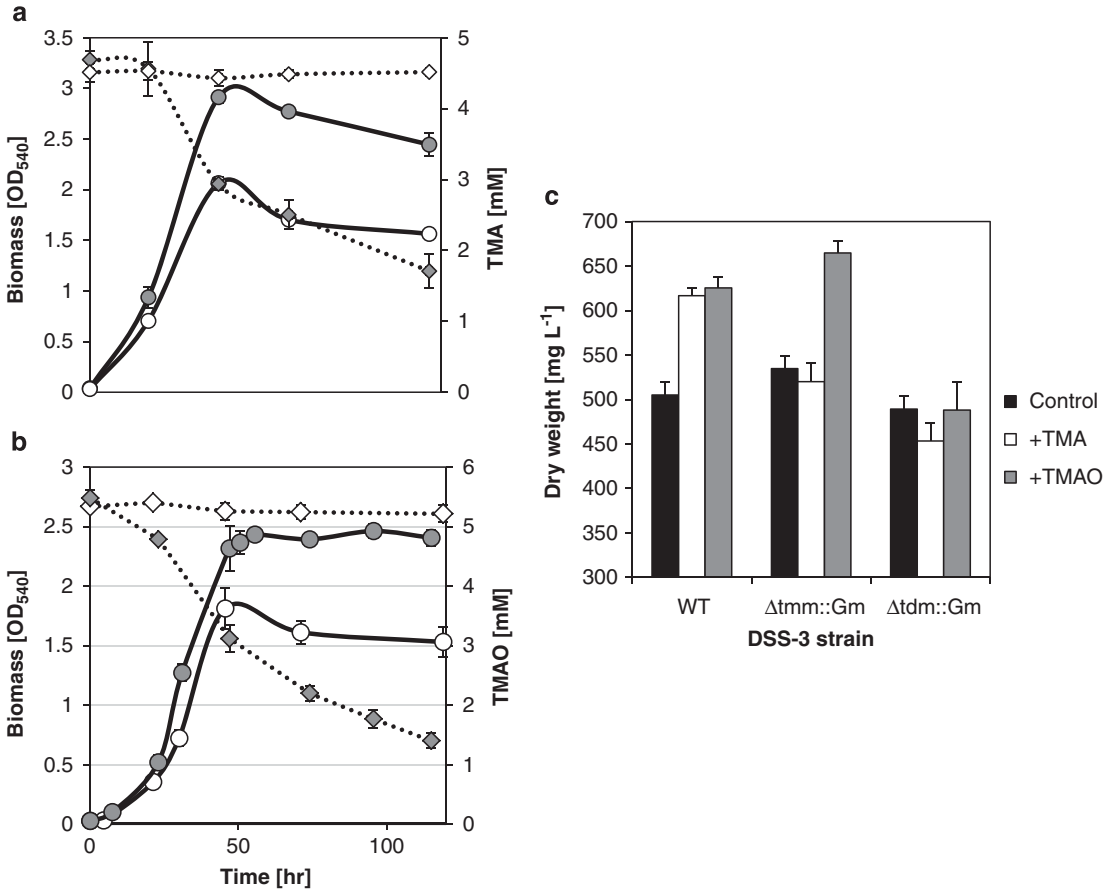


**Figure 2** (**a**) Catabolism of TMA during growth of *R. pomeroyi* wild type (grey circles) and the *Δtmm::Gm* mutant (white circles) on glucose and ammonium. TMA in the culture medium was quantified throughout growth for both wild type (grey diamonds) and the mutant (white diamonds). Note that the *y* axis is not presented as a logarithmic scale. (**b**) Catabolism of TMAO during growth of *R. pomeroyi* wild type (grey circles) and the *Δtdm::Gm* mutant (white 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 s.d. Results presented are the mean of triplicate cultures.

resulting in the accumulation of extracellular TMAO in the medium (Supplementary Figure 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 the biomass (Supplementary Figure 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 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 $504 \pm 14.3$ mg dry wt $l^{-1}$ (Figure 2c) and when supplemented with either TMA or TMAO, a final biomass of $616 \pm 8.9$ mg dry wt $l^{-1}$ ($+22\%$) and $626 \pm 12.6$ mg dry wt $l^{-1}$ ($+24\%$) was achieved, respectively. The *Δtmm::Gm* mutant, which cannot catabolise TMA, had no increase in final biomass ($519 \pm 21.4$ mg dry wt $l^{-1}$) compared with the glucose-only cultures ($534 \pm 14.3$ mg dry wt $l^{-1}$); however, when supplemented with TMAO, the final biomass was $664 \pm 13.3$ mg dry wt $l^{-1}$ ($+24\%$; Figure 2c). Supplementing the *Δtdm::Gm* mutant with either TMA or TMAO did not result in any increase in final biomass (glucose $= 489 \pm 14.5$; $+$ TMA $= 453 \pm 20.6$; $+$TMAO $= 487 \pm 31.7$ mg dry wt $l^{-1}$). When wild-type *R. pomeroyi* cells were grown in a glucose-limited chemostat (dilution rate $= 0.05$ $h^{-1}$), we also observed a 30.4% increase in growth yield when supplemented with TMA (5 mM), whereas the growth yield of the mutant, *Δtmm::Gm,* did not change (Table 1).

*Citreicella* sp. SE45, which was isolated from a salt marsh (USA), is another member of the MRC and can also grow on TMA as a sole N source, but not as a sole C source (Chen, 2012). Salt marshes are typified by having high concentrations of MAs, including TMA, derived from the anaerobic degradation of compatible osmolytes such as glycine

**Table 1** Growth yields for *R. pomeroyi* strains grown in a glucose-limited chemostat at a growth rate of $0.05\,h^{-1}$ with or without TMA (5 mM)

| *Strain* | *Gram dry biomass per mol glucose* | *Gram dry biomass mol 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 |

Abbreviation: TMA, trimethylamine.

betaine (King, 1984). When *Citreicella* sp. SE45 was grown using glucose-deplete MAMS medium, the addition of TMA led to an increase in final growth yield (Supplementary Figure S4), thus demonstrating that catabolism of TMA can also enhance chemoorganoheterotrophic growth of another closely related bacterium.

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

We also observed a direct correlation between specific growth rates and varying concentrations of TMA in the medium (Figure 3a). The specific growth rate increased from $0.061 \pm 0.002$ ($h^{-1}$) for cells incubated with no TMA to $0.087 \pm 0.003$ ($h^{-1}$) for cells incubated with 3 mM TMA. Likewise, the final growth yield increased from $484 \pm 10.39$ (no TMA) up to $600 \pm 8.79$ (3 mM TMA; Figure 3b). Using intermediate concentrations of TMA (0.5–1 mM) resulted in an intermediate increase in growth rates and growth yields compared with 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 + thiosulphate) during incubations where low concentrations of glucose (100 μM) were stochastically added (every 24–48 h) four times (400 μM total C). Cells incubated without a supplementary energy source (TMA or thiosulphate) reached a final growth yield of $31.7 \pm 1.5$ mg dry wt $l^{-1}$ (Figure 3c). Cells incubated with either TMA or thiosulphate alone reached a final growth yield of $42.2 \pm 4.7$ and $44.3 \pm 5.4$ mg dry wt $ml^{-1}$, respectively. Cells incubated with both TMA and thiosulphate reached a final growth yield of $70.8 \pm 4.9$ mg dry wt $ml^{-1}$, which equates to over a twofold 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 (Figure 1) involved in MA catabolism, for example, 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 2 days (Figure 4a). At the start of energy starvation, the number of viable cells in all cultures was $4.0 \times 10^9$ cells $ml^{-1}$ (Figure 4b). After 4 days, the number of viable cells incubated in the control cultures decreased to $7.4 \times 10^8$, whereas the cell numbers were $2.2 \times 10^9$ $ml^{-1}$ in the presence of TMAO and $1.1 \times 10^9$ $ml^{-1}$ in the presence of TMA, respectively. After 8 days, the number of viable cells from cultures with no exogenous C decreased to $2.9 \times 10^7$ $ml^{-1}$, whereas +TMAO and +TMA cultures had $9.0 \times 10^8$ and $7.5 \times 10^8$ $ml^{-1}$ 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 h. Wild-type cells incubated with either TMA or TMAO had $93.6 \pm 4.2$ and $92.1 \pm 7.8$ zeptomoles ATP per cell, respectively (Figure 5), whereas the intracellular concentration of ATP was lower for cells in the no substrate control ($58.3 \pm 9.7$ zeptomoles ATP per cell). Incubating the mutant, *Δtmm::Gm*, with TMA resulted in no increase in intracellular ATP ($54 \pm 5.3$ zeptomoles ATP per cell) compared with the no substrate control ($52.2 \pm 8.1$ zeptomoles ATP per cell), whereas incubation with TMAO did result in an increase in intracellular ATP ($80.7 \pm 4.9$ zeptomoles ATP per cell). As expected, incubation with TMA or TMAO did not result in an increase of intracellular ATP concentrations for the *Δtdm::Gm* mutant (control = $56.4 \pm 3.4$; TMA = $55.7 \pm 2.1$; TMAO = $56.1 \pm 2.2$ zeptomoles ATP per cell).

*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 (Figure 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 per cells) with *M. methanica* (~$10^7$ ml per 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

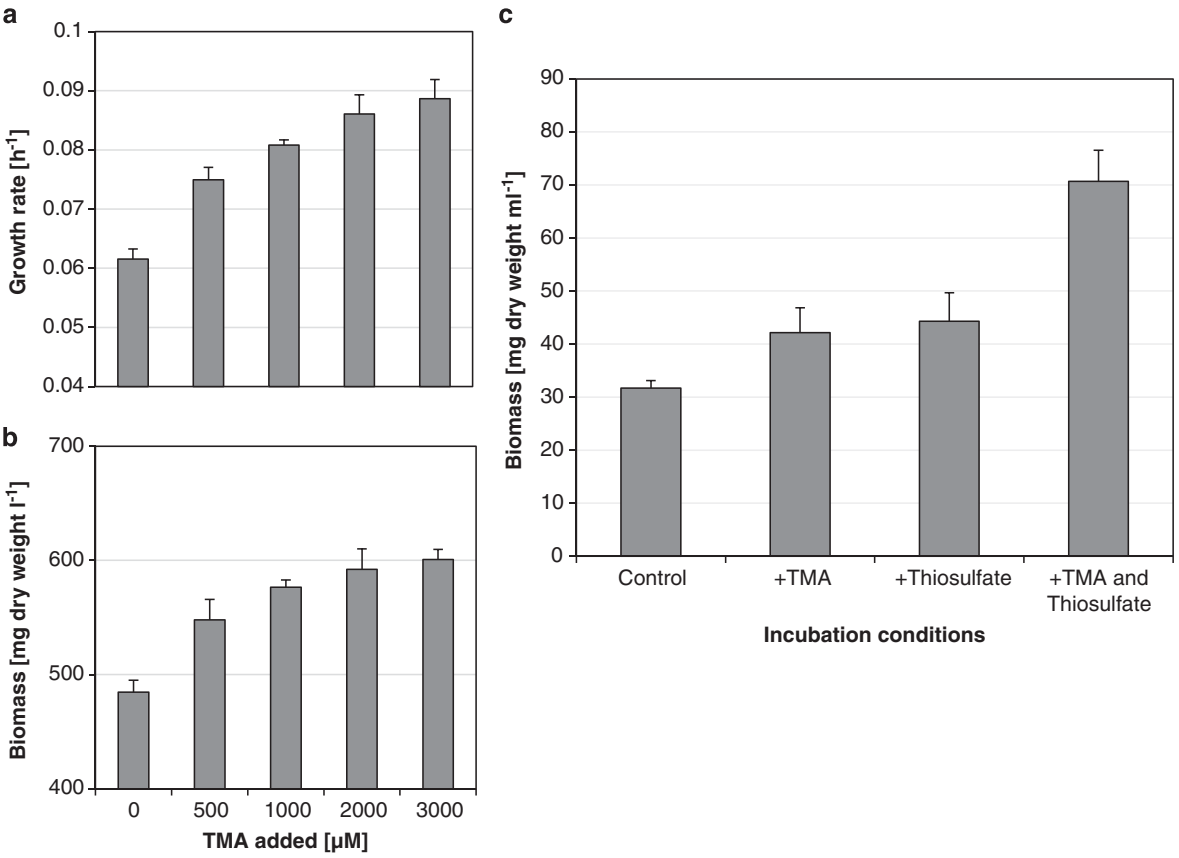


**Figure 3** A comparison of the specific growth rates (**a**) and final growth yields (**b**) of the wild-type *R. pomeroyi* grown on glucose and ammonium when supplemented with increasing concentrations of TMA, using a starting inoculum that was pre-incubated with TMA (24 h). (**c**) The final growth yield of *R. pomeroyi* after 7 days during which four additions of glucose (100 μM) were added every 24–48 h. Cultures were incubated with TMA (2 mM) or thiosulphate (2 mM) or both and the same concentrations were added every 48 h. Error bars denote s.d. Results presented are the mean of triplicate cultures.

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, while 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 ($3.9 \times 10^8$ ml$^{-1}$) or the *Δtmm::Gm* mutant ($3.3 \times 10^8$ ml$^{-1}$), confirming that *R. pomeroyi* does not inhibit growth of *M. methanica* (Figure 6b). Wild-type cells of *R. pomeroyi* depleted TMA from the medium, resulting in growth of *M. methanica* ($2.6 \times 10^8$ ml$^{-1}$); however, no growth of *M. methanica* occurred ($2.8 \times 10^7$ ml$^{-1}$) during incubation with the *Δtmm::Gm* mutant of *R. pomeroyi*, as a consequence of no TMA degradation during the 9-day incubation period (Figure 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). Although 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, while 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 and 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

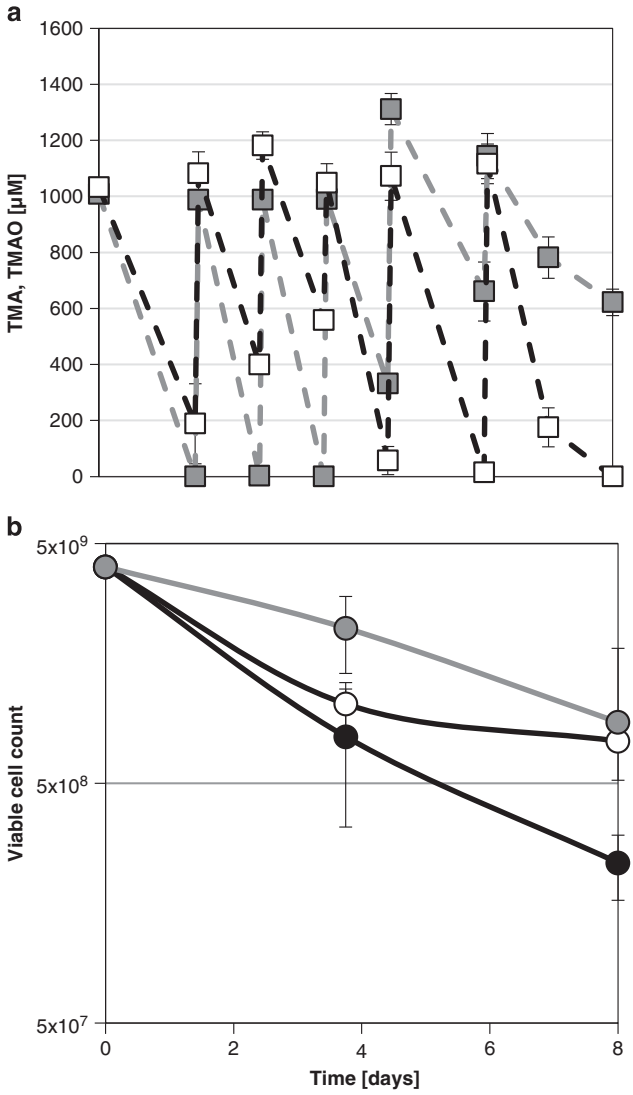


**Figure 4** (**a**) Quantification of TMA (white squares) and TMAO (grey squares) during incubations with energy-starved *R. pomeroyi* cells. (**b**) Quantification of viable cells in carbon and energy-starved *R. pomeroyi* cultures incubated with either no exogenous carbon (black circles), TMA (white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

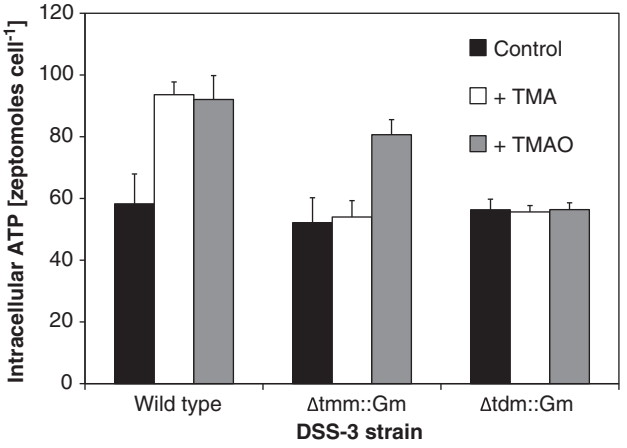


**Figure 5** Quantification of intracellular ATP concentrations from *R. pomeroyi* cultures energy-starved for 18 h before incubation for a further 2 h with either 1 mM TMA (white bars), 1 mM TMAO (grey bars) or no exogenous carbon source (black bars). Error bars denote s.d. Results presented are the mean of triplicate cultures.

and inorganic compounds for the generation of cellular energy. Although 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; 2011; Williams *et al.*, 2012; Gifford *et al.*, 2013). Therefore, bacteria of the MRC and SAR11 clades 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. The production of ATP through the oxidation of thiosulphate to sulphate 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 thiosulphate (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 thiosulphate, thus demonstrating how utilisation of multiple exogenous energy sources can enhance growth. Both TMA and thiosulphate are 'energy rich', in the sense that they can generate between seven and eight ATP molecules from the oxidation of one TMA or thiosulphate 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 and 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

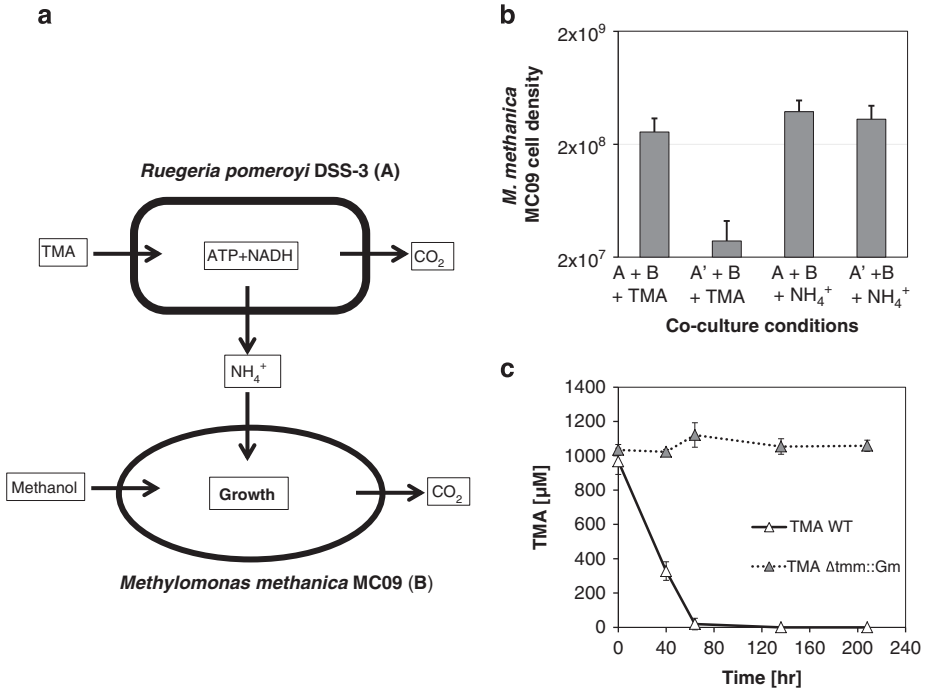


**Figure 6** (**a**) Schematic diagram of the flow of nitrogen in a co-culture system involving *R. pomeroyi* and *Methylomonas methanica* MC09. Ammonia liberated from the catabolism of TMA can be used by another bacterium to support its growth. (**b**) The cell count of *Methylomonas methanica* MC09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

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 and Peterson, 1979; Falkowski *et al.*, 1998; Zehr and 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 MRC species are frequently associated with phytoplankton blooms (González *et al.*, 2000; Buchan *et al.*, 2005; Wagner-Dobler *et al.*, 2009; Hahnke *et al.*, 2013; Nelson *et al.*, 2014), we predict that this N remineralisation process may take place with several different '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, while 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 clades capable of utilising MAs have a homologue of the transporter adjacent to genes involved in MA catabolism. Homologues of

the putative ammonium exporter related to both SAR11 and MRC clades are highly expressed in surface waters of 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 strengthen 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) that 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.

## Conflict of Interest

The authors declare no conflict of interest.

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