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# Microbiology and Ecology of Methylated Amine Metabolism in Marine Ecosystems

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## Abstract

Methylated amines (MAs) are ubiquitous in marine ecosystems, found from surface seawaters to sediment pore waters. These volatile ammonium analogues play important roles in biogeochemical cycles of carbon and nitrogen in the marine water column. They also contribute to the release of climate-active gases, being precursors of the potent greenhouse gas methane through methanogenesis in coastal sediments. Very recently, it also became acknowledged that MAs are important precursors for new particle growth, hence forming cloud condensation nuclei in the marine atmosphere. Microbial metabolism of MAs has been demonstrated in the marine ecosystems for both Archaea and Bacteria. In this chapter, we summarize the latest developments in analytical methods for quantifying MA concentrations in marine surface water and sediments. We discuss the metabolic pathways leading to the formation and degradation of MAs by marine microbes and the novel biochemistry and structural biology of the enzymes for MA transformation. Lastly, we highlight the need for future research towards a better understanding of the microbiology and ecology of oceanic MA cycles.

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## Introduction

Methylated amines (MAs), namely monomethylamine (MMA), dimethylamine (DMA), trimethylamine (TMA) and its oxidation product

TMA *N*-oxide (TMAO), are ubiquitously present throughout marine ecosystems, from surface seawaters to deep sediments (Fitzsimons *et al.*, 1997; Gibb *et al.*, 1999b; Carpenter *et al.*, 2012). Researchers have studied MAs in marine environments for many decades, and various analytical pipelines, such as gas chromatography (GC), high-performance liquid chromatography (HPLC), and ion-exchange chromatography (IC) have been developed for quantifying ambient concentrations of these molecules in the sea, which usually range from nanomolar concentrations (nM) in surface waters to micromolar concentrations ( $\mu$ M) in the sediments. Microbial metabolism of MAs has been of special interest because these compounds can be metabolized by methanogenic Archaea to produce methane. In marine sediments and coastal salt marshes, MAs are the well-known non-competitive substrates for methylotrophic methanogens allowing them to thrive in these high sulfate environments (Oremland *et al.*, 1982; King, 1984). Metabolism of MAs in well-oxygenated marine water columns has been overlooked in the past, however, we now know MAs appear to be important sources of carbon, nitrogen and energy for abundant marine heterotrophic bacteria, particularly members of the *Roseobacter* clade (members of the *Rhodobacteraceae*) and the SAR11 clade (*Pelagibacter* spp.) (Chen *et al.*, 2011; Sun *et al.*, 2011). The recent resurgence of interest in studying the marine MA cycles encompasses efforts towards better

understanding of their atmospheric chemistry, particularly their role in catalysing the formation of new particles in ambient air (e.g. Almeida *et al.*, 2013; Schobesberger *et al.*, 2013). The aim of this chapter is to summarize the current knowledge on analytical methods for measuring ambient concentrations of MAs in the sea, discuss recent advances in understanding the microbiology and ecology of MA metabolism by marine microbes, and highlight the critical directions for future research.

## Methylated amines in marine systems

### Natural concentrations of methylated amines in coastal and open ocean systems

Although MAs are ubiquitous in marine environments and contribute as part of the dissolved organic nitrogen (DON) pool (Gibb *et al.*, 1999b; Carpenter *et al.*, 2012), their *in situ* quantification has proven rather challenging. This is partly due to low natural concentrations of MAs, which typically lie in the nanomolar (nM) range for seawater, while in sediment pore water concentrations can increase to low micromolar ( $\mu\text{M}$ ) levels (Lee and Olson, 1984; Van Neste *et al.*, 1987; Yang *et al.*, 1993; Gibb *et al.*, 1995a, 1999b; Fitzsimons *et al.*, 1997; Hatton and Gibb, 1999) (Table 7.1). Consequently, studies monitoring natural MA concentrations are rare and were mostly performed by a few qualified groups applying custom-made instrumentation (reviewed by Carpenter *et al.*, 2012). Table 7.1 summarizes reports for MA measurements in marine environments.

Interestingly, many studies reporting MA concentrations from sea- or pore water also determined ammonium ( $\text{NH}_4^+$ ), showing that the latter regularly exceeds the dominating MA species by at least one order of magnitude (Fitzsimons *et al.*, 2006; Carpenter *et al.*, 2012). Among MAs, monomethylamine (MMA) often is the most abundant species in seawater samples, e.g. in the Pacific or Atlantic Ocean, the Arabian Sea, the Mediterranean, the Irish Sea, and the English Channel (Van Neste *et al.*, 1987; Abduhl-Rashid, 1990; Gibb, 1994; Gibb *et al.*, 1995a, 1999a,b). According to a study in the Arabian Sea, MMA concentrations in coastal seawater were  $\approx 12$  nM in August to October and  $\approx 22$  nM

in November to December 1994. In contrast, DMA accounted for  $\approx 3.0$  nM and  $\approx 4.2$  nM, while TMA only reached concentrations of  $\approx 0.10$  and  $0.45$  nM, respectively (Gibb *et al.*, 1999b). Comparison with cruise samples taken at offshore sites demonstrated that MA concentrations decreased further away from the coastline. MMA was still the most abundant MA, but its concentrations in offshore samples from the Arabian Sea decreased to  $\approx 6$  nM in August to October and  $\approx 12$  nM in November to December. DMA accounted for  $\approx 2.9$  nM during both seasons and TMA reached  $\approx 0.05$  nM and  $0.13$  nM, respectively (Gibb *et al.*, 1999b).

In coastal areas of the Mediterranean Sea, concentrations were in a similar range with MMA being the dominant MA species (4–38 nM), although TMA was more prevalent and DMA was the least abundant (TMA: 4–22 nM, DMA: 3–15 nM) (Gibb, 1994; Gibb *et al.*, 1995a). Similarly, in the Arabian Sea, MA concentrations also decreased in offshore samples, with both MMA and DMA accounting for  $\leq 9$  nM, and TMA reaching up to 7 nM (Gibb, 1994; Gibb *et al.*, 1995a).

Van Neste and co-workers reported similar concentrations from the coastal Pacific Ocean near Oahu, Hawaii (MMA:  $52 \pm 20$  nM, DMA:  $1.5 \pm 2.0$  nM, TMA:  $12 \pm 3$  nM), whereas concentrations were slightly higher in the Atlantic Ocean along the coast of Massachusetts (MMA:  $200 \pm 58$  nM, DMA:  $8.9 \pm 4.4$  nM, TMA:  $41 \pm 27$  nM) (Van Neste *et al.*, 1987). Concentrations reported by Abdul-Rashid (1990) from both coastal (Port Erin Jetty, Isle of Man) and offshore (Cypris Station, Isle of Man) samples collected in the Irish Sea were even higher than in the Atlantic Ocean samples, although concentrations varied with season being highest for MMA from mid-July until November (coastal up to 619 nM, offshore up to 418 nM). Both DMA and TMA levels were much lower throughout most of the year with DMA reaching  $\leq 110$  nM at the coastal and  $\leq 93$  nM at the offshore site, and TMA accounting for  $\leq 34$  nM and  $\leq 30$  nM, respectively (Abduhl-Rashid, 1990). While MMA dominated in coastal and offshore surface seawater samples, water samples collected from a salt marsh and an anoxic estuary showed highest concentrations of DMA (Flax Pond salt marsh, USA: 15–180 nM; Pettaquamscutt estuary, USA: 10–210 nM) followed by TMA and slightly lower levels of MMA (Flax Pond salt marsh, TMA:

**Table 7.1** Methylated amine concentrations in marine environments from seawater or pore water samples as reported in the literature. Numbers in brackets indicate maximum observed concentrations. For explanations of the methods see the text

Location – sample type	Concentration (nM)					Method	References
	MMA	DMA	TMA	TMAO	EA		
Pacific – Hawaii coastal	52±20	1.5±2.0	12±3	nd	nd	Vacuum distillation + GC-CLD	Van Neste <i>et al.</i> (1987)
Atlantic – Massachusetts coastal	200±58	8.9±4.4	41±27	nd	nd		
Irish Sea – coastal	0-619	0-110	0-34	nd	nd	Microdiffusion + GC-NPD	Abduhl-Rashid (1990)
Irish Sea – offshore	0-418	0-93	0-30	nd	nd		
Flax Pond salt marsh, USA – seawater	10-60	15-180	<3-80	nd	<3-30	Diffusion cell + GC-NPD	Yang <i>et al.</i> (1993)
Flax Pond salt marsh, USA – pore water	587	154	514	nd	98		
Petraquanscutt, USA – anoxic estuary basin	20-22	10-210	5-76	nd	<5-30		
Flax Pond salt marsh, US – seawater	5-40	25-180	10-50	nd	nd	Diffusion cell + GC-NPD	Yang <i>et al.</i> (1994)
Mediterranean – coastal (Gulf of Lions)	18±10.0	12±11.4	10±6.9	nd	nd	FIGD-IC	Gibb (1994)
Mediterranean – offshore	7.5±5.5	4.6±3.0	1.4±1.6	nd	nd		
NW Mediterranean – coastal	4-38	3-15	4-22	nd	nd	FIGD-IC	Gibb <i>et al.</i> (1995a)
NW Mediterranean – offshore	<LoD - 9	<LoD - 9	<LoD - 7	nd	nd		
Tamar and Plym estuary, UK – seawater	37-100	0-49	0-13	nd	nd		
Plymouth Sound, UK – seawater	4-23	13-22	4-17	nd	nd		
Sutton Harbour, UK – seawater	91	<LoD	15	nd	nd		
Arabian Sea – coastal	12±20 (66)	3.0±4.1 (14)	0.10±0.37 (2.0)	nd	nd	FIGD-IC	Gibb <i>et al.</i> (1999b)
	22±13 (55)	4.2±2.8 (14)	0.45±0.81 (4.0)	nd	nd		
Arabian Sea – offshore	6±7 (31)	2.9±2.8 (12)	0.05±0.21 (1.8)	nd	nd		
	12±7 (34)	2.9±1.6 (7)	0.13±0.24 (0.8)	nd	nd		
Arabian Sea – Gulf of Oman	11±9 (32)	2.8±3.1 (11)	0.19±0.42 (1.9)	nd	nd		
Arabian Sea – coastal	16.2 (65.8)	4.54 (13.9)	0.044 (0.44)	nd	nd	FIGD-IC	Gibb <i>et al.</i> (1999a)
	25.6 (49.8)	4.85 (9.95)	0.14 (0.8)	nd	nd		
Arabian Sea – offshore	5.24 (13.9)	3.85 (11.1)	<LoD	nd	nd		
	8.8 (16.5)	2.04 (5.7)	0.036 (0.3)	nd	nd		
Plymouth Sound, UK – seawater	nd	nd	<LoD	3.2	nd	Enzymatic reduction + FIGD-IC	Hatton and Gibb (1999)
Ryder Bay, Antarctica – seawater	nd	nd	<LoD - 4.1	2.1-64	nd		

**Table 7.1** Continued

Location – sample type	Concentration (nM)						Method	References
	MMA	DMA	TMA	TMAO	EA			
Ryder Bay, Antarctica – seawater	12.0 ± 9.1 (36.0)	3.8 ± 3.9 (12.7)	1.6 ± 1.8 (12.0)	15.2 ± 16.6 (76.9)	0.6 ± 0.6 (1.9)	(Enzymatic reduction +) FIGD-IC	Gibb and Hatton (2004)	
Western English Channel – coastal	3	6	20	nd	nd	SPME + GC-NPD	Cree <i>et al.</i> (2018)	
Southern Ocean – offshore	< LOD – 2.6	< LOD – 5.4	1.4–6.9	nd	nd			
Buzzards Bay, USA – coastal pore water	< LOD	48,000	29,000	nd	nd	Steam distillation + GC-CLD	Lee and Olson (1984)	
Buzzards Bay, USA – pore water core	< LOD	1100–1900	400–500	nd	nd			
E Tropical N-Pacific Ocean – offshore pore water	< LOD	100–1300	80–140	nd	nd			
Norsminde Fjord estuary, Denmark – pore water	nd	nd	0–10,000	nd	nd	GC-FID	Sørensen and Glob (1987)	
Flax Pond salt marsh, USA – pore water	1060	190	nd	nd	nd	Steam distillation + RP-HPLC	Wang and Lee (1990)	
Long Island Sound estuary, USA – pore water	870	120	nd	nd	nd			
Peru upwelling region – coastal pore water	650	100	nd	nd	nd			
Flax Pond salt marsh, USA – pore water	100–1700	100–3600	100–600	nd	nd	Diffusion cell + GC-NPD	Wang and Lee (1994)	
Oglet Bay salt marsh, UK – pore water	0–319,000	0–9000	0–50,000	nd	nd	Microdiffusion + GC-NPD	Fitzsimons <i>et al.</i> (1997)	
Burnham Overy Staithe mudflat, UK – pore water	100–1300	100–2800	0–4680	nd	nd	Microdiffusion + GC-NPD	Fitzsimons <i>et al.</i> (2001)	
Thames Estuary, UK – pore water, July	80–130	310–370	30–60	nd	nd	Microdiffusion + GC-NPD	Fitzsimons <i>et al.</i> (2006)	
Thames Estuary, UK – pore water, Nov	980–1440	1200–1850	1400–2230	nd	nd			

CLND, chemiluminescent nitrogen detector; EA, ethylamine; FID, flame ionization detector; FIGD, flow injection gas diffusion; GC, gas chromatography; HPLC, high performance liquid chromatography; IC, ion chromatography; LOD, limit of detection; nd, not determined; NPD, nitrogen-phosphorus detector; RP, reverse phase; SPME, solid phase microextraction.

< 3–80 nM, MMA: 5–60 nM; Pettaquamscutt estuary, TMA: 5–76 nM, MMA: 20–22 nM) (Yang *et al.*, 1993, 1994). Nevertheless, the authors could not explain the observed pattern of high DMA concentrations in the seawater overlying the sediment, as decomposition of marsh grasses and degradation of quaternary amines primarily release TMA as the key intermediate (Yang *et al.*, 1994).

### Natural concentrations of methylated amines in sediment pore water

MA concentrations reported from sediment pore water are at least one order of magnitude higher than those found in seawater (see Table 7.1). Flax Pond salt marsh has been repeatedly sampled for MAs from sediment pore water (Wang and Lee, 1990, 1994; Yang *et al.*, 1993). Concentrations varied depending on sampling depth, and season with the highest concentrations observed in the autumn, which was attributed to the decomposing of the cordgrass *Spartina alterniflora* (Wang and Lee, 1994). DMA was found at highest concentrations in the pore water with concentrations usually < 0.5  $\mu\text{M}$ , while average concentrations for MMA and TMA reached < 0.2  $\mu\text{M}$  (Wang and Lee, 1994). TMA was the most abundant species among MAs in pore water of the Burnham Overy Staithe mudflat, UK, (up to 4.68  $\mu\text{M}$ ) (Fitzsimons *et al.*, 2001) and the Thames Estuary, UK, where also a seasonal pattern was observed (0.03–0.06  $\mu\text{M}$  in July, 1.4–2.23  $\mu\text{M}$  in November) (Fitzsimons *et al.*, 2006). The authors postulated that MAs adsorbed to sediments are released during tidal inundation, which would explain the documented concentrations in the pore water (Fitzsimons *et al.*, 2006).

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### Analytical methods to quantify methylated amine concentration in marine environments

#### The difficulties of determining methylated amine concentrations from marine samples

As described above, several studies have measured MA concentrations in marine environments, but their analyses remain challenging and sample pre-treatments as well as the application of specially designed instrumentation impede data comparability. Low compound concentrations

in natural seawater near the detection limits of analytical instruments (nM range) require tedious pre-concentration approaches such as steam or vacuum distillation, rotary evaporation, concentration in the oven, or the usage of diffusion techniques (static, circular, membrane or microdiffusion, application of diffusion cells) (e.g. Lee and Olson, 1984; Van Neste *et al.*, 1987; Wang and Lee, 1990; Abdul-Rashid *et al.*, 1991; Yang *et al.*, 1993; Fitzsimons *et al.*, 2001, 2006) (see Table 7.1). Consequently, recovery rates after pre-concentration steps are often low and thus influence reproducibility of the data. In addition, the high concentration of inorganic salts in seawater samples interferes with peak resolution. High water solubility and adsorption of MAs, or their polar, volatile nature add additional difficulties to the successful determination of their natural concentrations (Yang *et al.*, 1993; Gibb *et al.*, 1995b). To overcome these limitations a sensitive and reliable method is required that allows the parallel determination of various amines in natural seawater samples. Since the 1980s, several groups started to develop reproducible techniques for measuring ambient concentrations of MAs in seawater, which are summarized below.

#### High-performance liquid chromatography (HPLC)-based methods

The application of high-performance liquid chromatography (HPLC) for the measurement of amines requires a proper derivatization approach. Unfortunately, the lack of a suitable derivatization agent usable for primary, secondary, and tertiary amines in parallel reduces the applicability of HPLC-based methods for amine detection (Mopper and Zika, 1987; Wang and Lee, 1990; Gibb *et al.*, 1995b). Thus, most methods were developed to target amino acids or primary amines, or they require a combination of derivatization approaches (Jacobs, 1987; Mopper and Zika, 1987; Carlucci and Karmas, 1988). Historically, a whole range of detection methods were combined with HPLC such as UV, fluorescence, chemiluminescence excitation, and electrochemical detection (Mellbin and Smith, 1984; Jacobs, 1987; Mopper and Zika, 1987; Carlucci and Karmas, 1988; Wang and Lee, 1990), although not all of these approaches were applied to determine MA concentrations in sea- and pore water samples.

Using a pre-column fluorescence derivatization with *O*-phthalaldehyde and the thiols 2-mercaptoethanol and *N*-acetyl-L-cysteine, Mopper and Zika (1987) measured amino acid and primary amine concentrations in rain and air samples collected during cruises in the Gulf of Mexico, as well as in seawater samples taken in the north-western Atlantic Ocean. Derivatization products were detected by a fluorescence detector after HPLC separation. The authors determined total primary amine concentration to be 4 nM (2-aminoethanol 82.5 mole%, MMA 17.5 mole%) in surface seawater (Mopper and Zika, 1987), but this technique does not allow the detection of secondary and tertiary amines.

In a quantitative derivatization method for amino acids, Henrikson and Meredith (1984) used the reaction of amino acids with phenylisothiocyanate to form phenylthiocarbonyl-amino acid derivatives (Edman degradation). These derivatives produced a fluorescent complex that, after separation by reverse-phase HPLC, could be detected on a fluorescence detector at 254 nm (Henrikson and Meredith, 1984). The method also proved suitable to determine primary and secondary amines such as MMA and DMA in sediment pore water samples (Wang and Lee, 1990). Therefore, pore water samples were first concentrated by steam distillation (Lee and Olson, 1984), followed by addition of phenylisothiocyanate to form phenylthiohydantoin derivatives of MMA and DMA (Wang and Lee, 1990). The products were then analysed by reverse-phase HPLC with fluorescence detection (Wang and Lee, 1990).

### Gas chromatography (GC)-based methods

After pre-concentration, usually by distillation or a diffusion technique (e.g. Lee and Olson, 1984; Van Neste *et al.*, 1987; Abdul-Rashid *et al.*, 1991; Yang *et al.*, 1993; Fitzsimons *et al.*, 2001), gas chromatography (GC), which exploits the volatility of the target compounds, has been widely used for MA detection from environmental samples. Although suffering from its own limitations (summarized by Gibb *et al.*, 1995a,b), GC separation does not require derivatization, and it can be combined with a range of detectors. A concept generally applied in sample preparation exploits the fact that when MAs are exposed to highly basic conditions, they convert to their volatile gaseous forms. For example, TMA

was converted into its gas phase by base addition to specifically analyse TMA in pore water samples, transferred to a cryogenic trap and injected into a gas chromatograph with a flame ionization detector (GC-FID) (Glob and Sørensen, 1987; Sørensen and Glob, 1987).

Alternatively, a chemiluminescent technique originally developed by Lee and Olson (1984) exploits light emission from nitrogen compounds reacting with ozone at high temperature, which can be detected by a chemiluminescent nitrogen detector (CLND) (Lee and Olson, 1984; Van Neste *et al.*, 1987). The method is claimed to be selective for highly volatile nitrogen compounds and was applied to determine MA concentrations in coastal seawaters from Hawaii and Massachusetts, and also in different pore water samples (Lee and Olson, 1984; Van Neste *et al.*, 1987).

One of the more frequently used approaches for the measurement of MAs in seawater samples provides the coupling of a GC to a nitrogen-phosphorus detector (GC-NPD) (Abdul-Rashid *et al.*, 1991; Yang *et al.*, 1993; Fitzsimons *et al.*, 2001). An NPD works similarly to an FID using the electronic conductivity of a hydrogen/air flame combined with an alkali metal salt bead (usually rubidium or caesium chloride). Under heat, ions utilized from the bead interact specifically with nitrogen- or phosphorus-containing compounds and the ion amount is measured at the amplifier (CHROME-DIA Analytical Sciences). Amine determination by GC-NPD requires sample concentration prior to measurement. Dr Cindy Lee's laboratory used a diffusion cell to pre-concentrate amines. Therefore, they combine membrane diffusion with static and circulation diffusion chamber approaches and exploit the ability of amines to protonate or deprotonate with pH changes, which either allows them to diffuse across membranes (deprotonated) or to get trapped (re-protonated) (Yang *et al.*, 1993). Alternatively, Dr Mark Fitzsimons' group uses microdiffusion via a Cavett diffusion flask consisting of a stoppered Quickfit Pyrex Erlenmeyer flask and a small collecting cup at fixed distance from the glass stopper (Abdul-Rashid *et al.*, 1991). The second approach was commonly used to analyse sediment pore waters (Fitzsimons *et al.*, 1997, 2001, 2006).

The newest developments in MA analysis aim to apply more widely available equipment rather than

previous custom-made instrumentation. To specifically target TMA in marine sediments, a cryogenic purge and trap system combined with GC coupled to mass spectrometry (P&T-GC-MS) was applied. Using a stream of inert gas, volatile analytes such as TMA were drawn from the liquid sample phase and trapped cryogenically, while in parallel by a headspace method the isotopic composition of stable carbon isotopes were analysed (Zhuang *et al.*, 2017). Whether or not this method is also applicable to the measurement of MMA and DMA awaits experimental validation, because the detection of compounds with such a small mass to charge ratio ( $m/z$ ) is problematic with most common MS detectors. A suitable alternative approach combines headspace solid phase microextraction (SPME) with GC-NPD (Cree *et al.*, 2018). Here, an SPME fibre was exposed to the headspace above a stirred, basic ( $\text{pH} \approx 13$ ) seawater sample maintained at  $60^\circ\text{C}$  to extract MAs. Upon GC injection, analytes thermally desorbed from the fibre and were detected by an NPD (Cree *et al.*, 2018). MMA, DMA and TMA concentrations from Western English Channel and Southern Ocean seawater samples were thus successfully analysed in lab- and ship-based settings (Cree *et al.*, 2018). As this widely available approach allows the parallel sensitive and robust analysis of MAs and other low-molecular-weight amines, it might encourage further measurements and henceforth improve our understanding of MA cycles in seawater.

### Flow injection gas-diffusion coupled to ion chromatography (FIGD-IC)

Some investigators suggested that ion chromatography (IC) is a good approach for detecting MAs. In addition to the sensitivity of this method, the polar nature of MAs and ammonium ( $\text{NH}_4^+$ ) allows their measurements in parallel in low ionic strength samples such as sea- or pore water without resolution problems sometimes associated with GC separation or the requirement for derivatization (Gibb *et al.*, 1995a,b). However, the samples need pre-concentration to prevent column overloading due to large excess of sodium ( $\text{Na}^+$ ) and potassium ( $\text{K}^+$ ) ions in seawater, and the required sensitivity in the mrange. Similarly to the diffusion cell (Yang *et al.*, 1993), an automated flow injection gas diffusion-ion chromatography (FIGD-IC) system exploits that  $\text{NH}_4^+$  and MAs deprotonate to their volatile gaseous forms

under high pH conditions ( $> 12$ ). In this state the molecules diffuse from the sample stream through a hydrophobic membrane and get re-protonated and trapped after contact with an acidic acceptor stream. Recycling of the stream in the enrichment loop results in analyte enrichment over time, which can then be transferred to the IC for injection (Gibb *et al.*, 1995a,b).

Combination of FIGD-IC with an enzymatic reaction allowed the additional determination of the nitrogenous osmolyte TMAO from seawater. TMAO was reduced to TMA in the presence of the enzyme dimethyl sulfoxide (DMSO) reductase and reducing solutions consisting of flavin mononucleotide (FMN) and ethylenediaminetetraacetic acid (EDTA) (Hatton and Gibb, 1999; Gibb and Hatton, 2004). The authors successfully quantified concentrations of MAs as well as their potential precursor TMAO from seawater samples (Gibb and Hatton, 2004). Although FIGD-IC is a sensitive and powerful approach for measuring MAs, it is not widely used probably because it is technically challenging to assemble the complex combination of the FIGD pre-concentration system to the IC (Gibb *et al.*, 1995a,b).

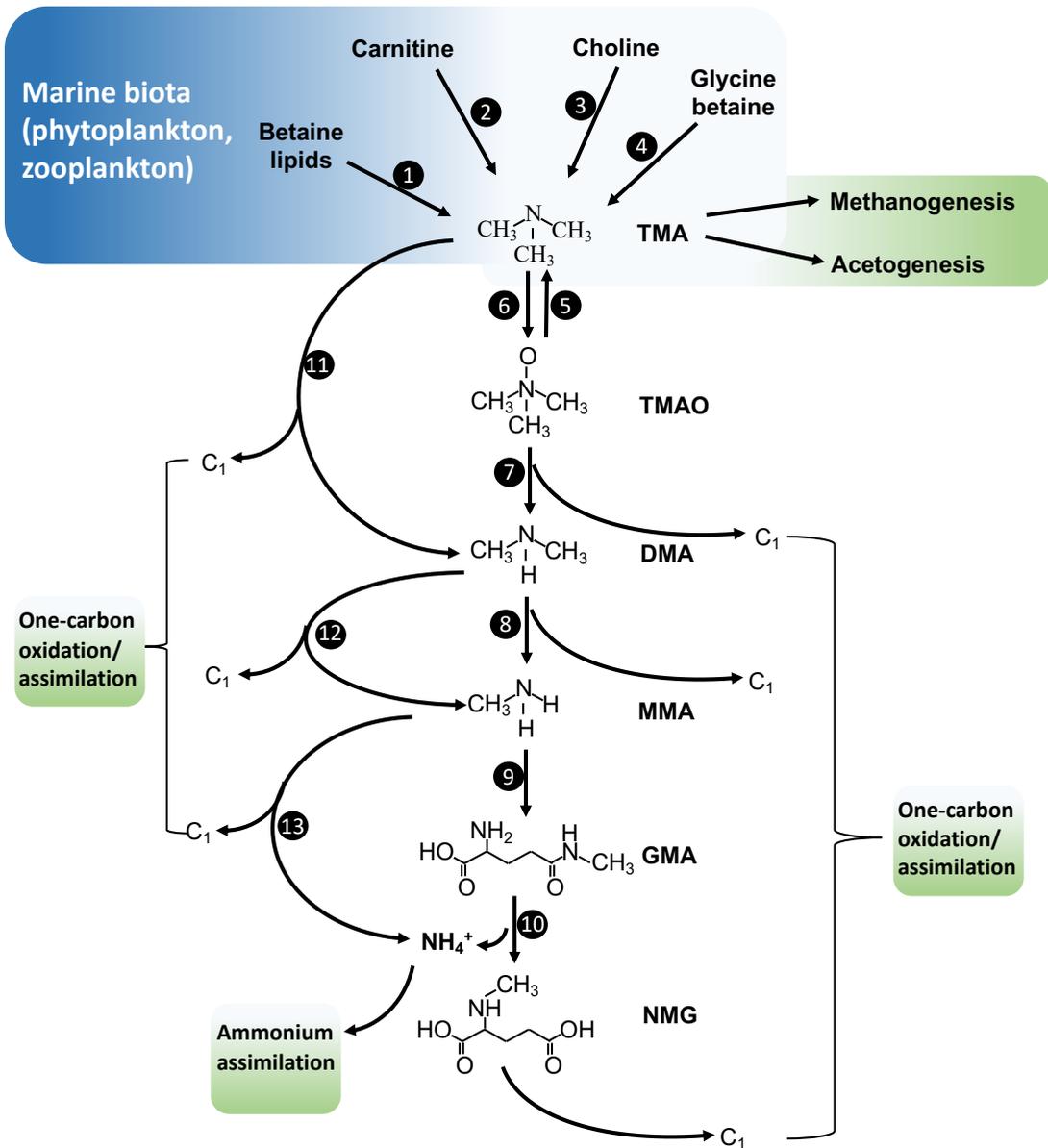
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### Microbial metabolism of methylated amines in marine environments

In the marine environments, MAs can be metabolized by at least four biochemical pathways (Fig. 7.1):

- 1 methylotrophic methanogenesis, which occurs in anaerobic sediments and is conducted by methylotrophic Archaea (Rother, 2010);
- 2 the dehydrogenase pathway, which uses a series of dehydrogenases to sequentially demethylate each methylated amine species while forming formaldehyde as a by-product (Chistoserdova, 2011);
- 3 the aerobic TMA oxidation pathway, which forms TMAO as the key intermediate;
- 4 the TMA-dependent acetogenesis pathway, which is still poorly understood.

We will briefly summarize pathways 1, 2 and 4, but focus primarily on the aerobic TMAO-dependent pathway throughout this section, to reflect its prevalence in marine surface waters.



**Figure 7.1** Methylated amine formation and degradation in marine environments. (1) degradation of betaine lipids; (2) carnitine monooxygenase; (3) choline –TMA lyase; (4) glycine betaine reductase; (5) TMAO reductase; (6) TMA monooxygenase; (7) TMAO demethylase; (8) DMA monooxygenase; (9) GMA synthetase; (10) NMG synthase; (11) TMA dehydrogenase; (12) DMA dehydrogenase; (13) MMA dehydrogenase.  $\text{C}_1$  represents either formaldehyde or its derivatives (e.g. conjugated to tetrahydrofolate).

### Anaerobic methylated amine oxidation by methylotrophic methanogens

In anaerobic habitats methanogenesis comprises the final step in the degradation of organic matter resulting in the production of gaseous methane by methanogenic Archaea belonging to the

*Euryarchaeota* (reviewed in Rother, 2010). Three different pathways exist: hydrogenotrophic, methylotrophic, and acetotrophic methanogenesis. Methylotrophic methanogens, such as the well-studied order of *Methanosarcinales*, can utilize methylated compounds such as methanol, methylamine (MMA, DMA, TMA), and dimethylsulfide (Hippe *et al.*, 1979; Mah, 1980; van der Maarel

and Hansen, 1997). For example, in surface sediment from a salt marsh near Dorset, UK, the turnover time for TMA was 80 days and *Methanosarcinales* were the major methanogens found in the sediment (Parkes *et al.*, 2012). Similarly, we have recently demonstrated that *Methanococoides* are key TMA utilizers in a saltmarsh sediment (Jameson *et al.*, 2019). More recently, a new group of methylotrophic methanogens was described, *Methanomassiliicoccales*, which represent obligate hydrogen-dependent methanogens that can utilize methanol or MAs (Iino *et al.*, 2013). Their capability to grow on MAs has been originally hypothesized from the metatranscriptomic data, followed by *in vivo* validation in the strain *Methanomassiliicoccus luminyensis* B10 (Poulsen *et al.*, 2013; Brugère *et al.*, 2014; Kröninger *et al.*, 2017). Although the known *Methanomassiliicoccales* were isolated from gut and rumen samples, their occurrence in marine habitats is supported by metagenomic and phylogenomic datasets (Paul *et al.*, 2012; Borrel *et al.*, 2013).

### Methylated amine dehydrogenases pathway

Several microbes such as *Hyphomicrobium* and *Methylophaga* use a pathway for sequential demethylation of TMA and DMA through TMA dehydrogenase, DMA dehydrogenase and MMA dehydrogenase. TMA dehydrogenase has been purified from *Methylophaga* sp. strain SK1 and *Methylophilus methylotrophus* (Lim *et al.*, 1986; Kim *et al.*, 2006). TMA dehydrogenase is a homodimeric flavoprotein containing a 4Fe–4S iron–sulfur cluster (Burgess *et al.*, 2008). DMA dehydrogenase is also a homodimeric flavin-containing protein which is closely related to TMA dehydrogenase ( $\approx 60\%$  sequence identity in amino acid sequence) (Yang *et al.*, 1995). In contrast, MMA dehydrogenase is a periplasmic enzyme which requires a specific cofactor, tryptophan tryptophylquinone (TTQ). MMA dehydrogenases have been purified from several bacteria, including *Methylobacterium extorquens* (Eady and Large, 1968) and *Paracoccus versutus* (Haywood *et al.*, 1982), and they are composed of two large and two small subunits MauBA.

### Oxidation pathway involving TMAO as the key intermediate

The third pathway for MA metabolism, involving the oxidation of TMA to TMAO as the key

intermediate, appears to be even more widely distributed across environments (Chen *et al.*, 2011; Nayak and Marx, 2015). In this pathway, TMA is oxidized by a flavin-containing monooxygenase, Tmm, to TMAO, followed by demethylation to DMA by a novel binuclear Zn/Fe metalloprotein, Tdm (Chen *et al.*, 2011; Lidbury *et al.*, 2014; Zhu *et al.*, 2014b, 2016). Both Tmm and Tdm are ubiquitously found in cosmopolitan marine heterotrophs including the *Roseobacter* clade and the SAR11 (*Pelagibacter* spp.) clade (Chen *et al.*, 2011; Lidbury *et al.*, 2014), suggesting a key role of this so-called indirect methylamine oxidation pathway in marine biogeochemical cycles of carbon and nitrogen. We recently also identified the secondary amine oxidase, DMA monooxygenase, in marine *Roseobacter* bacteria (Lidbury *et al.*, 2017). DMA monooxygenase, Dmm, is composed of four subunits, DmmDABC, which catalyse the formation of MMA and formaldehyde from DMA oxidation. Dmm has previously been purified from *Aminobacter aminovorans* (known as *Pseudomonas aminovorans*) by Dr John Dawson's group in the late 1980s (Alberta and Dawson, 1987; Alberta *et al.*, 1989). DMA monooxygenase is a NADPH-dependent haem-containing protein which is sensitive to carbon monoxide (Alberta and Dawson, 1987; Alberta *et al.*, 1989). Recently, the structure of the alpha subunit of Dmm containing the haem-binding domain has been resolved by X-ray crystallography (Ortmayer *et al.*, 2016).

MMA oxidation to formaldehyde and ammonium in this pathway is rather complicated, involving two unusual amino acids, gamma-glutamylmethylamide (GMA) and *N*-methylglutamate (NMG), and three enzymes, GMA synthetase, NMG synthase and NMG dehydrogenase (Anthony, 1982; Chen *et al.*, 2010b; Latypova *et al.*, 2010). GMA synthetase has been purified from *Methylovorus mays* and *Methylophaga* sp. (Kimura *et al.*, 1992; Yamamoto *et al.*, 2008). It is evolutionarily related to bacterial glutamine synthetase and similarly it is likely an oligomer made of 8–12 identical subunits (Kimura *et al.*, 1992). NMG synthase, on the other hand, is still poorly studied in terms of substrate specificity, co-factors and the role of individual subunits, MgsABC (Pollock and Hersh, 1971, 1973; Jorns and Hersh, 1975; Latypova *et al.*, 2010). The last enzyme in this pathway is a multimeric NMG dehydrogenase (MgdABCD),

one of which contains a tetrahydrofolate (THF) binding domain involved in formaldehyde conjugation. This enzyme has only been partially purified from *Aminobacter aminovorans* owing to its attachment to cytoplasmic membranes (Boulton *et al.*, 1980; Chen *et al.*, 2010b; Latypova *et al.*, 2010). Interestingly, in marine environments, this indirect methylamine oxidation pathway seems to play a key role for sequestering nitrogen from methylated compounds; the methyl groups being oxidized by Roseobacters and SAR11 as a supplementary energy source (Chen *et al.*, 2010a; Sun *et al.*, 2011; Lidbury *et al.*, 2015).

### TMA-dependent acetogenesis pathway

It appears that TMA can be also demethylated for acetate formation through an as-yet uncharacterized acetogenesis pathway found in a halophilic homoacetogenic bacterium (Zhilina and Zavarzin, 1990). This bacterium, *Acetohalobium arabaticum*, was isolated from a lagoon near the Sea of Azov and is capable of methylotrophic growth on TMA as a sole carbon source while producing DMA, MMA and acetate. We speculate that this bacterium may use methylamine methyltransferases to channel C1 carbon to the Wood-Ljungdahl pathway for acetogenesis (Wood and Ljungdahl, 1991) but this hypothesis still awaits experimental validation. Interestingly, the methyltransferases encoded by *Acetohalobium arabaticum* are induced by TMA, and they have been shown to contain the unusual amino acid pyrrolysine (Prat *et al.*, 2012). Phylogenetically *Acetohalobium arabaticum* pyrrolysine-containing methylamine methyltransferases emerged within the archaeal groups and it is therefore suggested that these bacterial pyrrolysine-containing methylamine methyltransferases were acquired through horizontal gene transfer (Prat *et al.*, 2012). The distribution of this pathway in marine environments and its ecological significance in biogeochemical cycles of MAs are yet to be carefully studied.

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### Microbial ecology of the metabolism of methylated amines by marine microbes

Thanks to the biochemical characterization of new enzymes involved in methylated amine metabolism using model bacteria over the past decade, it is now

possible to use cultivation-independent approaches to evaluate the distribution, diversity and activity of marine microbes involved in MA metabolism.

### Development of PCR primers targeting functional genes

PCR primers targeting functional genes involved in MA metabolism have been developed in several studies. In a study to characterize TMA and MMA utilizers in coastal surface waters, two PCR primers targeting *tmm* and *gmaS* of marine Roseobacters have been developed (Chen, 2012). These primers have been applied successfully to retrieve *tmm* and *gmaS* sequences from marine Roseobacters, including several groups that are associated with pelagic marine *Roseobacter* clades (e.g. strains HTCC2255, HTCC2083). The *gmaS* PCR primers were subsequently optimized in order to retrieve novel methylamine-metabolizing methylotrophs in Movile Cave (Wischer *et al.*, 2015). In this study, two sets of reverse PCR primers targeting *Alpha-proteobacteria* and *Beta-/Gammaproteobacteria*, respectively, have been designed and optimized.

In order to better understand the diversity of microbes capable of MA metabolism in salt marsh sediments, we have recently also developed PCR primers targeting *tdm* (Lidbury, 2015). These primers were optimized to detect not only alpha-proteobacterial Roseobacters and SAR11, but also *Gammaproteobacteria*. A small clone library was made using DNA extracted from the top few centimetres of a coastal salt marsh sediment located in the UK and we have found several novel *tdm* clades.

PCR primers targeting the large subunit of MMA dehydrogenase (*MauA*) are also available (Neufeld *et al.*, 2007). These primers were designed based on *mauA* from *Paracoccus*, *Methylobacillus*, *Methylophilus*, *Methylobacterium* and *Methylophaga*. These primers have been used successfully to retrieve *mauA* from *Methylophaga* spp. as well as from novel methylamine utilizers in seawater from Station L4 off the coast of Plymouth, UK.

### Omics and single-cell approaches

Taking the advantage of the readily available large-scale oceanic metagenomics/metaproteomics datasets, we have carried out studies to uncover the ecology and to estimate the abundance of MA utilizers in marine ecosystems. In a study by Chen *et al.* (2011), we estimated

that  $\approx 20\%$  of the bacteria in the surface waters of the Global Oceanic Sampling (GOS) Expedition datasets have the genetic potential for TMA oxidation by Tmm. Tmm sequences retrieved from the GOS datasets are primarily phylogenetically assigned to the SAR11 clade or the *Roseobacter* clade, reflecting the dominance of these bacteria in marine surface waters. Similarly, we showed that SAR11 and *Roseobacter* clade bacteria are predominant in the oceanic metagenome datasets for Tdm, GmaS as well as TmoX (encoding a substrate binding protein for TMAO uptake) (Lidbury *et al.*, 2014, 2017). Interestingly, genes encoding the DMA monooxygenase (DmmDABC) are absent in the SAR11 clade bacteria, and metagenomics/metatranscriptomics data analyses provide no support for the presence of the Dmm pathway in the SAR11 clade (Lidbury *et al.*, 2017). It therefore remains to be seen, if and how SAR11 clade bacteria metabolize DMA.

Several studies have used metagenomics/metaproteomics or single cell approaches to link the identity of marine microbes and their function in MA metabolism. Taubert *et al.* (2017) used  $^{15}\text{N}$ -stable isotope probing in combination with metaproteomics to characterize the flow of nitrogen from  $^{15}\text{N}$ -MMA to  $^{15}\text{N}$ -ammonium in coastal seawater from Plymouth. They were able to confirm the uptake of  $^{15}\text{N}$  by *Methylophaga* spp., *Leisingera* spp. as well as a novel gammaproteobacterium that was closely related to strain IMCC2047. Similarly, in an attempt to characterize MMA utilizers in a deep subsurface, Trembath-Reichert *et al.* (2017) combined stable isotope labelling and nanometre scale secondary ion mass spectrometry (Nano-SIMS). The authors showed that MMA was primarily metabolized by heterotrophic bacteria but also, to some extent, by methanogenic Archaea.

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## Conclusions and future directions

Although much progress has been made over the past decades towards a better understanding of MA cycling in marine environments, a number of issues are still remaining unresolved, and these are addressed below.

## Biochemistry and structural biology of enzymes involved in methylated amine metabolism

Several key enzymes in the MA degradation pathways remain challenging for purification and/or crystallization for X-ray analysis. NMG synthase is an intriguing enzyme which could potentially use either MMA or GMA as substrates. This enzyme has so far only been partially purified, and recombinant expression of the genes *mgsABC* in *Escherichia coli* remains problematic. Two of the NMG synthase subunits, MgsB and MgsC have a significant similarity to glutamate synthase domains. Further characterization of this enzyme will help to better understand its evolutionary relationship to glutamate synthase. The membrane-bound NMG dehydrogenase has also been proven difficult for isolation and purification. It shows similarity to the multimeric sarcosine oxidase complex and has a characteristic glycine cleavage T-protein domain (Moriguchi *et al.*, 2010). TMAO demethylase (Tdm) is another interesting enzyme involved in MA degradation. This enzyme catalyses an unusual oxygen-independent oxidative demethylation, probably using the substrate TMAO as the oxygen donor (Zhu *et al.*, 2016). Structural characterization of these novel enzymes will aid in better understanding of their catalytic activities and cofactor requirements.

## Alternative route of DMA catabolism in SAR11 clade bacteria?

SAR11 clade bacteria are numerically abundant in the marine water column and can make up to 50% of bacterioplankton (Giovannoni, 2017). We have demonstrated that Tmm, Tdm and the TMAO transporter protein (TmoX) are functional in catalysing TMA oxidation, TMAO demethylation and TMAO transport, respectively (Chen *et al.*, 2011; Lidbury *et al.*, 2014). The SAR11 bacterium strain HTCC1062 can actively oxidize  $^{14}\text{C}$ -TMA,  $^{14}\text{C}$ -TMAO and  $^{14}\text{C}$ -MMA to  $^{14}\text{CO}_2$  (Sun *et al.*, 2011). It is therefore puzzling that the genomes of SAR11 bacteria do not contain the *dmmDABC* genes required for DMA oxidation (Lidbury *et al.*, 2017). It remains to be established whether TMA and TMAO can be completely mineralized by SAR11 bacteria to obtain both energy and ammonium

from these methylated amines. Our own unpublished data suggest that DMA can be oxidized by at least one strain of SAR11 bacteria, HTCC7211, suggesting the presence of an uncharacterized DMA oxidation pathway in this bacterium.

### Interplay between methylated amine cycles and methylated sulfur cycles

Methylated amine metabolism, in many ways, resembles methylated sulfur (dimethylsulfide-dimethylsulfoniopropionate) cycles in the marine environment. Both methylated amines and methylated sulfurs are likely mainly derived from marine phytoplankton. Of particular interest is the recent finding that TMA monooxygenase from marine bacteria can also oxidize dimethylsulfide (Chen *et al.*, 2011; Lidbury *et al.*, 2016). The apparent  $K_m$  of *Roseobacter* and SAR11 Tmm for TMA and dimethylsulfide is comparable and the Tmm enzyme can completely oxidize dimethylsulfide to dimethylsulfoxide. Oxidation of dimethylsulfide to dimethylsulfoxide in the model *Roseobacter* clade bacterium *Ruegeria pomeroyi* DSS-3 is entirely dependent on the presence of MAs, particularly DMA, which often is the most abundant MA species in surface seawaters. This interesting interplay between marine methylamine and dimethylsulfide metabolism highlights the importance of studying these molecules concomitantly.

### Oceanic sources of methylated amines

The sources of oceanic MAs, particularly in the well-oxygenated surface seawaters remain undetermined. TMA can be generated via anaerobic microbial metabolism through the degradation of quaternary amines, such as choline, glycine betaine and TMAO (Fig. 7.1). Choline is a common dissolved organic nitrogen species which can be released into the marine environment through degradation of phospholipids or conversion from choline sulfate, an osmolyte used by certain seagrasses and coastal plants (Hutchings and Saenger, 1987). Choline degradation to TMA is carried out by an oxygen-sensitive glycol radical containing choline-TMA lyase (Bodea *et al.*, 2016). Glycine betaine and TMAO are both common osmolytes used by many marine organisms, which can be converted to TMA via betaine reductase and TMAO reductase, respectively (Oren, 1990; Seibel and

Walsh, 2002). However, it remains unestablished whether these largely anaerobic processes occur in the oxygenated marine water column and whether they contribute to the formation of MA in surface waters.

Formation of TMA through aerobic processes does occur in microorganisms. For example, we have recently identified a Rieske-containing oxygenase which can generate TMA for the cleavage of carnitine, a common metabolite in mammals (Zhu *et al.*, 2014a). It is therefore plausible that carnitine oxygenase or other similar Rieske oxygenases may contribute to the formation of TMA in surface seawaters. Interestingly, degradation of betaine-containing diacylglycerol lipids may represent an overlooked source of oceanic TMA. Betaine lipids are common in many marine phytoplankton, synthesized in response to phosphorus deficiency, and at least one species of betaine lipids, 1(3),2-Diacylglycerol-3(1)-O-2'-(hydroxymethyl)(*N,N,N*,-trimethyl)- $\beta$ -alanine (DGTA), can be spontaneously degraded to TMA (Vogel *et al.*, 1990; Dembitsky, 1996). It therefore remains to be established whether these aerobic processes are mainly responsible for TMA releases into the water column in marine environments.

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