Minireview

Microbial Trimethylamine Metabolism in Marine Environments

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Running title: Microbial TMA metabolism

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Originality-Significance Statement: We identify the key aspects of originality and significance that place the work within the top 10% of current research in environmental microbiology.

Summary:

Trimethylamine (TMA) is common in marine environments. Although the presence of this compound in the oceans has been known for a long time, unlike the mammalian gastrointestinal tract, where TMA metabolism by microorganisms has been studied intensely, many questions remain unanswered about the microbial metabolism of marine TMA. This mini-review summarizes what is currently known about the sources and fate of TMA in marine environments and the different pathways and enzymes involved in TMA metabolism in marine bacteria. This review also raises several questions about microbial TMA metabolism in the marine environments, and proposes potential directions for future studies.

Introduction

Trimethylamine (TMA) is one of the several methylated amines that are ubiquitous in marine systems (King, 1984a; Gibb et al., 1999a; Gibb and Hatton, 2004) and make up an important component of the oceanic carbon and nitrogen pools (Gibb and Hatton, 2004; Chen *et al*., 2011; Carpenter *et al*., 2012) (Figure. 1). TMA has the characteristic odor of rotting fish. It was found to be produced during fish spoilage in the 1930s (Beatty, 1938) and later was recognized as a malodorous pollutant (Sandberg and Ahring, 1992; Rappert and Muller, 2005). TMA is a precursor of other methylated amines, for example trimethylamine *N*-oxide (TMAO), through an oxidation pathway that will be explained further down. TMAO is a common osmolyte used by many marine biota to regulate osmotic pressure and stabilize proteins against denaturation (Barrett and Kwan, 1985; Seibel and Walsh, 2002).

TMA first attracted the interest of biogeochemists because it is an important precursor for methane formation in a variety of marine environments (Figure. 1). Under anoxic conditions, up to 90% of the methane from salt marsh sediment or slurries can be attributed to microbial conversion of TMA from the degradation of quaternary amine precursors (Oremland et al., 1982). A similar finding by an independent research group also found that 35 to 61% of total methane in surface sediments of an intertidal mud flat could result from TMA metabolism (King *et al*., 1983, King GM 1984a). TMA is a component of marine aerosols. Along with other methylated amines (*e.g*. dimethylamine (DMA) and monomethylamine (MMA)), it is emitted from surface seawater into the atmosphere, which can actively affect the climate system (Carpenter

et al., 2012; Lidbury *et al*., 2017) (Figure.1).

Currently, there are two major challenges for biogeochemists in the study of oceanic methylated amines: the first is to identify the major sources of these compounds - what is the source of marine TMA? The second challenge concerns the fate of this compound - who are the major TMA consumers in marine environments? In this review, we first summarize what is known about the distribution of oceanic TMA and discuss the significance and sources of this compound in marine environments. We then describe three different metabolic pathways of TMA degradation and the enzymes that catalyze the reactions. Finally, we discuss the potential issues and challenges in the study of TMA metabolism and conclude by highlighting opportunities for future research directions.

Sources of marine TMA

It was not until the 1990s, when highly sensitive analytical techniques became available for the measurement of methylated amines, that researchers gained the capability to quantify TMA in the oceans reliably and accurately. Ocean-scale research revealed that concentrations of TMA range from nanomolar (nM) to micromolar (μM), depending on the marine environment sampled (Table 1). In surface water, TMA concentrations are low. Unlike DMA and MMA, TMA concentrations in seawater had no seasonal pattern and did not correlate with the abundance of diatoms nor mesozooplankton grazing activities (Gibb *et al*., 1999b). However, methylated amines can be strongly adsorbed to marine sediments, particularly to those with a high organic

content (Wang and Lee, 1990), which may help to explain the elevated TMA concentrations observed in marine sediments (Table 1).

Although the importance of TMA in the global carbon and nitrogen cycle is being recognized (Lee and Olson, 1984; Gibb and Hatton, 2004; Chen et al., 2011), the sources of this compound in marine ecosystems are not well established. Many marine plants and animals have been found to contain high concentrations of methylated amines (Wang and Lee, 1994; Calderón *et al.*, 2007). Hence, one hypothesis is that TMA is directly released from tissues during excretion or decay of marine organisms. It has been reported that TMA is commonly found in marine algae (Fujiwara-Arasaki and Mino, 1972; Smith, 1975) and TMA production is associated with annual senescence and production of marsh grass in salt marsh sediments (Wang and Lee, 1990). Wang and Lee experimentally demonstrated that the plant *Spartina alterniflora* gets decomposed to release amines, especially TMA, to salt marsh sediments (Wang and Lee, 1994). Fish, benthic animals and phytoplankton also contain high concentrations of methylated amines and could be important sources of oceanic TMA, either by direct release or through decomposition (Shewan, 1951; Budd and Spencer, 1968; Barrett and Kwan, 1985; Wang and Lee, 1994).

Another potential source of TMA is degradative pathways that form TMA as an intermediate or end-product. Potential TMA sources include common organic compounds such as the compatible solutes (osmolytes) TMAO, glycine betaine, and choline, which are abundant in marine eukaryotic cells (Ikawa and Taylor, 1973; King, 1984a; Oren, 1990; López-Caballero et al., 2001; Treberg et al., 2006). These

compounds can be transformed to produce TMA by a TMAO reductase (TorA), a glycine betaine reductase (GrdH) or a choline-TMA lyase (CutC), respectively. Metagenomic studies have shown that the *grdH* gene is present in marine environments but at low abundance. The *cutC* gene is more prevalent in anaerobic marine sediments. Among these functional genes, the *torA* gene is the most abundant in both open ocean and marine sediment datasets, which implies that TMA formation from the TMAO reduction pathway is prevalent and important in the oceans (Jameson *et al*., 2016).

TMA production can also occur under aerobic conditions through oxidation of carnitine (Unemoto et al., 1966; Rebouche and Seim, 1998; Zhu et al., 2014), which may explain the presence of TMA in oxygenated marine surface waters (Carpenter *et al*., 2012). Notably, but rarely studied, TMA can be produced from the betaine-containing lipid diacylglyceryl hydroxymethyl *N*,*N*,*N*-trimethyl-β-alanine (DGTA) by a spontaneous deamination process (Vogel *et al*., 1990). DGTA is widely distributed in marine phytoplankton (Araki *et al*., 1991; Cañavate *et al*., 2016).

The metabolic fate of marine TMA

The study of TMA metabolism has been primarily focused on methylotrophic bacteria and methanogens (Hippe *et al*., 1979). These microorganisms can use methylated amines as their carbon, nitrogen and energy sources (Chistoserdova *et al*., 2009; Chistoserdova, 2011). Generally, there are four different pathways for microbial metabolism of TMA: acetogenesis pathway, methanogenesis pathway, the dehydrogenase pathway and the aerobic oxidation pathway. The bacterium *Acetohalobium* is capable of demethylating TMA to an equimolar amount of acetate

along with less amounts of DMA and MMA via anaerobic acetogenesis (Zhilina and Zavarzin, 1990), although not much is known on the genes/enzymes involved. TMA-dependent acetogenesis has been rarely studied; therefore we will mainly describe the other three pathways, which are depicted in Figure 2.

TMA-dependent methanogenesis

A number of investigations have shown that TMA can be a significant source of methane in a variety of marine systems (Oremland *et al*., 1982; Oremland and Polcin, 1982; King *et al*., 1983; Summons *et al*., 1998). Oremland *et al.*, and King *et al.*, showed that the addition of TMA to marine sediments stimulates the production of methane (Oremland et al., 1982; King et al., 1983; King, 1984b). Several novel strains of methylotrophic methanogens have been isolated from anoxic marine sediments which can catabolize TMA to produce methane (Singh *et al*., 2005). Although the phenomenon of methanogenesis from TMA has been observed for many years in anoxic oceans (Hippe *et al*., 1979; Sowers *et al*., 1984; Siegert *et al*., 2011), the featured species and metabolic process in marine environments are still unclear.

Notably, in similar anaerobic environments, such as the gastrointestinal tract of ruminants and sewage sludge digesters, TMA has been shown to be a significant substrate for methylotrophic methanogens (Neill *et al*., 1978; Mah and Kuhn, 1984; Zinder *et al*., 1985; Zhilina and Zavarzin, 1987). Methanogenesis is a metabolic process driven by obligate anaerobic *Archaea*. Methanogens, such as members of the order *Methanomassiliicoccales* and *Methanosarcinales*, can use methyl groups from

142 TMA to firstly produce methyl-coenzyme M (methyl-CoM) by the concerted action of
143 two methyltransferases: TMA methyltransferase and coenzyme M methyltransferase
144 (Ferguson and Krzycki, 1997; Bose et al., 2008). Methyl-CoM is subsequently
145 converted into methane, CO_2 and ammonia by a methyl-CoM reductase (MCR), the
146 key enzyme of methanogenesis (Figure 2A) (Friedrich, 2005; Kröninger et al., 2017).
147 Recent bioinformatics analyses of metagenomes and metatranscriptomes provided
148 further evidence of the TMA-dependent methanogenesis pathway in marine
149 environments. For example, the alpha-subunit of MCR (*mcrA*) was detected from
150 sediment samples of the Western Mediterranean Sea by PCR amplification.
151 Phylogenetic analysis revealed the presence of diverse methanogen communities
152 distributed along the different geochemical zonations, including those from known
153 TMA-utilizers e.g. *Methanococcoides* and *Methanosarcina* (Zhuang *et al*., 2018).
154 Similarly, metatranscriptomic data from anoxic sediment in the Baltic Sea revealed that
155 *mcrA* transcripts affiliated to *Methanosarcina* were highly abundant, suggesting a role
156 of TMA-dependent methanogenesis in the sediment (Thureborn *et al*., 2016).
157
158 **Anaerobic TMA dehydrogenase pathway**
159 The second pathway of TMA degradation involves the direct dehydrogenation of
160 TMA to form DMA and formaldehyde, catalyzed by a TMA dehydrogenase (TMADH)
161 (Colby and Zatman, 1973; Kasprzak *et al.*, 1983; Yang *et al*., 1995). In some
162 methylotrophs, DMA is further demethylated to MMA and then ammonia by a series
163 of dehydrogenase enzymes: DMA dehydrogenase (DMADH) and MMA

dehydrogenase (MMADH), with each step simultaneously forming the side-product formaldehyde (Figure 2B) (Asatoor and Simeshoff, 1965; Colby and Zatman, 1973; Barrett and Kwan, 1985; Chistoserdova, 2011). The whole pathway is energetically favorable and oxygen is not required for these processes. However, this energy-saving pathway seems not to be important in marine microorganisms, since little evidence for these dehydrogenases have been found in marine metagenomic data. Instead, pathways for aerobic TMA degradation by bacterioplankton, which are discussed below, have been intensively studied.

Aerobic TMA oxidation pathway

This pathway involves the oxygenation of TMA to TMAO, which is further catabolized to DMA, MMA, ammonia and formaldehyde (Figure 2C). The initial step of conversion of TMA to TMAO is mediated by a TMA monooxygenase (Tmm). Tmm is a flavin-dependent enzyme. Bacterial Tmm was first identified and characterized in the soil bacterium *Methylocella silvestris* (Dunfield et al., 2003; Chen et al., 2011). Enzymatic activity assays showed that the marine *Roseobacter* clade (*Roseovarius* sp. 217 and *Ruegeria pomeroyi* DSS-3) and SAR11 clade (HTCC1002 and HTCC7211), two of the most abundant bacterioplankton groups in the surface ocean, also have Tmm enzymes to catabolize TMA oxidation (Chen *et al.*, 2011). Metagenomic evidence revealed that most marine bacterioplankton possess TMA monooxygenase, leading to the estimate that about 20% of the bacteria in the surface ocean contain this gene (Chen *et al*., 2011). This suggests that aerobic TMA degradation is the major pathway for

TMA utilization in the marine environment, especially in the oxygen-rich surface water.

Most recently, the molecular mechanism of TMA oxygenation by marine bacterial Tmm was elucidated (Li *et al*., 2016). There are two half-reactions (reductive and oxidative) in the catalytic process. In the first half-reaction, flavin adenine dinucleotide (FAD) is reduced by nicotinamide adenine dinucleotide phosphate (NADPH), and an intermediate C4a-hydroperoxyflavin is formed. In the second half-reaction, this intermediate attracts TMA to the catalytic pocket. TMA binding to the catalytic site of Tmm causes a conformational change in $NADP^+$, which shuts off the substrate entrance and exposes C4a-hydroperoxyflavin to TMA, thereby starting the oxidative half-reaction (Li *et al*., 2016).

After oxidation, the oxygenated form, TMAO, is further demethylated to yield DMA and formaldehyde by a TMAO demethylase (Tdm) (Chen *et al*., 2011; Lidbury *et al*., 2014; Lidbury *et al*., 2015). Tdm was first proposed and partially purified from *Bacillus* (Myers and Zatman, 1971) and methylotrophs such as *Pseudomonas aminovorans* (Large, 1971; Boulton *et al*., 1974) and *Hyphomicrobium* spp. (Meiberg *et al*., 1980; Barrett and Kwan, 1985). Recently, Tdm has been demonstrated to occur in abundant marine heterotrophic bacteria as well (Chen *et al*., 2011; Lidbury *et al*., 2014; Lidbury *et al*., 2015). Although the enzyme can be purified from aerobic bacteria, Tdm is oxygen-independent and is not affected in aerobic or anaerobic conditions (Large, 1971). This enzyme is strongly activated by Zn^{2+} and Fe^{2+} metal cofactors (Zhu *et al*., 2016).

Conversion of DMA to MMA by a secondary amine monooxygenase has been

proposed for a long while (Alberta and Dawson, 1987; Alberta *et al*., 1989). The enzymology of this protein was also first characterized from *P. aminovorans* by spectroscopic analysis (Alberta *et al*., 1989) and was later known as a heme-dependent oxidative *N*-demethylase with a heme-dependent Per-ARNT-Sim (PAS)-domain (Ortmayer *et al*., 2016). This particular PAS enzyme is a heterotetramer, and requires NADPH in the DMA catabolic pathway (Ortmayer *et al*., 2016) .

Only recently did a study confirm that the gene *dmmDABC* encodes a functional DMA monooxygenase (Dmm) in *R. pomeroyi* DSS-3 for DMA demethylation (Lidbury *et al.*, 2017), which fills a gap and completes the marine DMA degradation pathway. The genes encoding DmmDABC are widely distributed in the marine *Roseobacter* clade, whereas they are absent from the genomes of some important marine bacterial taxa, including all representatives of the SAR11 clade. This would explain why the abundance of the gene cluster *dmmDABC* was much lower in marine metagenomics data than the other relative genes involved in degradation of methylated amines (Lidbury *et al*., 2017).

Concluding remarks and future prospects

Although the significance of marine TMA is recognized, the sources, fluxes and fates of this compound in the ocean are still not fully understood. The development of better analytical methods for the *in situ* quantification of methylated amines remains a challenging problem (Lee and Olson, 1984; Abdul-Rashid *et al*., 1991; Yang *et al*., 1993). A recent improvement by Zhuang et al., (2017) used a method combining a purge and trap system coupled with gas chromatography-mass spectrometry (P&T-GC-MS).

This method quantifies TMA in one analytical step, requires small volumes (5 mL) of porewater or sediment samples, and can simultaneously measure the stable carbon isotopic composition in the solid phase of marine sediments (Zhuang *et al*., 2017). More recently, Cree *et al.* reported another method to determine dissolved methylated amines in seawater samples. Methylated amines converted to the gaseous phase were analyzed by coupling headspace solid phase microextraction (SPME) and gas chromatography coupled with a nitrogen–phosphorus detector (GC-NPD) (Cree *et al.*, 2018). This method provides lower detection limits and is more suitable for measuring methylated amines at low-nM level in marine environments. Compared to the P&T-GC-MS system, SPME-GC-NPD has better sensitivity to the low-molecular weight amines, but requires a larger sampling volume (1L). During the SPME extraction process, maintaining the thermostat and homogeneity of seawater samples is particularly important. Although keeping the equilibrium of one sample in the study is available, operating parallel extractions from multiple large volume samples under the same conditions may be difficult to control. The possible solution would be to combine the purge and trap system with the SPME extraction, which could create a constant equilibrium between aqueous phase and gaseous phase with less interference from temperature variations. In addition, the introduction of inert gas flow could potentially improve the recoveries of methylated amines to achieve a better sensitivity and more accurate measurements. With the development in methodology, more information on *in situ* concentrations of methylated amines is likely to become available in the near future, contributing to a better understanding of TMA biogeochemistry.

In nature, some microorganisms have been found to possess pathways for both the aerobic and anaerobic degradation of TMA. This raises two questions: why do some microbes require two metabolic pathways, and are these two pathways independent or related? *Paracoccus* sp. Strain T231 can use two different enzymes, Tmm and TMADH, to initialize the degradation of TMA in aerobic and anaerobic metabolism, respectively (Kim *et al*., 2001). When grown aerobically on TMA, enzyme activities of Tmm, Tdm, Dmm and MMA monooxygenase from cell-free extract are detected. When grown anaerobically on TMA and nitrate, enzyme activities of TMADH and DMADH from the cell-free extract are detected (Kim *et al.*, 2001). In contrast, in aerobic metabolism, both Tmm and TMADH can be used to initialize the oxidation of TMA in *Pseudomonas putida* ATCC 12633 (Liffourrena *et al*., 2010).

TMA metabolism of *Hyphomicrobium* is more complicated. This microorganism is commonly found in soil and fresh water (Harder and Attwood, 1978) and is able to oxidize TMA by TMADH under both aerobic and anaerobic conditions in the presence of nitrate (Meiberg and Harder, 1978). For the two known pathways of DMA demethylation to MMA, oxygen and TMA availability are the key regulatory factors. The enzyme Dmm is strictly dependent on oxygen as a substrate. Dmm activity was undetectable when oxygen was absent in the medium, and was expressed immediately when oxygen was provided. Although the activity of DMADH is independent of oxygen, the synthesis of DMADH in *Hyphomicrobium* X was inhibited by high oxygen tensions, and lowering the oxygen tension relieved this inhibition (Meiberg *et al*., 1980). In addition, TMA concentrations were proposed to regulate DMADH activity. During

the initial stage of cell growth on TMA, a high concentration of TMA acts as a potent competitive inhibitor for DMADH, and the product DMA accumulates in the medium. As TMA is degraded and the concentration decreases, DMADH is upregulated, which allows for the subsequent catabolism of DMA (Meiberg and Harder, 1979; Meiberg *et al*., 1980). Overall, these regulatory properties could provide this microorganism with a selective advantage over competitors in habitats where oxygen and TMA concentrations fluctuate.

In marine environments, knowledge of microbial TMA catabolism is limited to a few studies. Genome analysis of heterotrophic bacteria that are abundant in marine surface water (i.e. the *Roseobacter* and SAR11 clade) revealed gene clusters only for aerobic TMA catabolism and the physiological experiments confirmed oxidative degradation of TMA via TMAO as the key intermediate (Chen *et al*., 2011; Sun *et al*., 2011). Metagenomic data from global ocean sampling have also shown an abundance of the *tmm gene* and low frequency of *dmm* (Lidbury *et al*., 2017), implying the adaptation of dominant plankton groups to oxygen and the significance of the TMA oxygenation pathway in marine surface water. However, due to the lack of metagenomic data in hypoxic zones, whether anaerobic TMA degradation occurs under low oxygen conditions of the water column is still unknown. Up till now, only a few marine bacterial species, such as a methylotrophic bacterium *Methylophaga* sp. strain SK1 and some denitrifying bacteria isolated from coastal sediments (Kim *et al*., 2003), have been found to contain both TMADH and Tmm metabolic pathways for TMA degradation (Choi *et al*., 2003; Kim *et al*., 2006; Chen *et al*., 2011). However, the

regulation of the anaerobic dehydrogenase pathway and the aerobic TMA oxidation pathway in these marine microorganisms is poorly characterized, which limits the understanding of the adaption of these marine bacteria to their surrounding habitats and their ecological significant. All of these questions remain to be explored, and will likely be the focus of future research.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Tables:

Table 1. Concentration of TMA in marine environments as reported in the literature

TMA concentration	Source/Location	References
12 ± 3.0 nM	Pacific—Hawaii coastal	(Van Neste *et al*., 1987)
41 ± 27 nM	Atlantic—Massachusetts coastal	
1.4 ± 1.6 nM	Offshore, Mediterranean	(Gibb, 1994; Gibb *et al*., 1999b)
10 ± 6.9 nM	Costal, Mediterranean	
< 4 nM	Arabian Sea	(Gibb *et al*., 1999b)
1.6 ± 1.8 nM	Antarctic coastal waters	(Gibb and Hatton, 2004)
< 3-80 nM	Flax Pond seawater, New York	(Yang *et al*., 1993)
20 nM	Western English Channel	(Cree *et al*., 2018)
1.4-6.9 nM	Southern Ocean	
0 - 4.7µM	Porewater of East Anglian Estuary sediments	(Fitzsimons *et al*., 2001)
0 - 50 µM	Porewater of Oglet Bay sediments	(Fitzsimons *et al*., 1997)
0 - 15 µM	Porewater of Norsminde Fjord Estuary sediments	(Glob and Sørensen, 1987)
0.6 µM	Porewater of Flax Pond salt marsh	(Wang and Lee, 1990, 1994)

Figure legends:

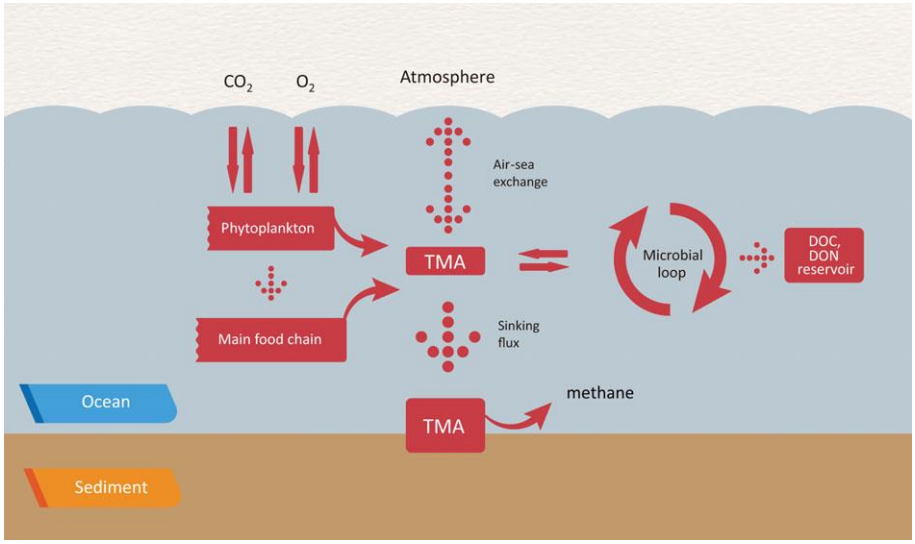

Figure 1. Diagram of marine biogeochemical cycles of TMA. DOC: dissolved organic carbon; DON: dissolved organic nitrogen; TMA: trimethylamine.

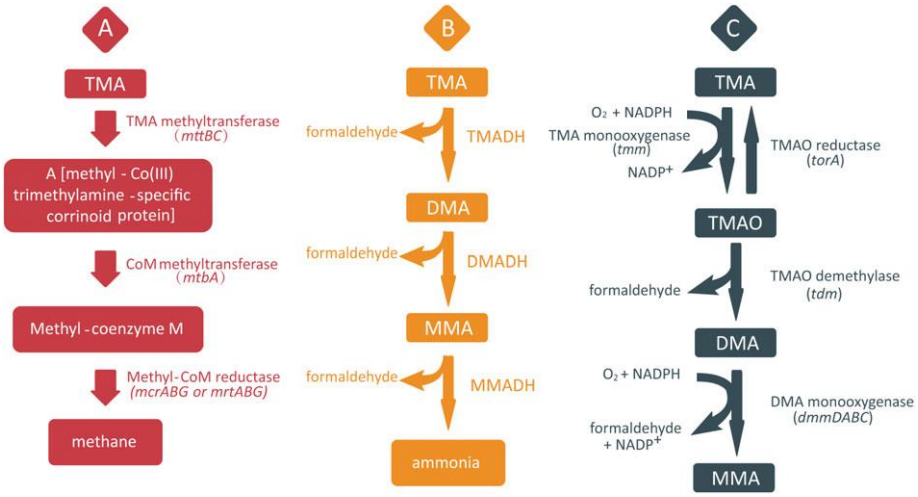

Figure 2. Proposed three main TMA metabolic pathways in marine microbes. A) Methanogenesis; B) Anaerobic TMA dehydrogenase pathway; C) Aerobic TMA oxidation pathway. DMA: dimethylamine; MMA: monomethylamine; TMA: trimethylamine; TMAO: trimethylamine *N*-oxide; $NADP^+$: nicotinamide adenine dinucleotide phosphate; NADPH: reduced form of nicotinamide adenine dinucleotide phosphate. TMADH: trimethylamine dehydrogenase; DMADH: dimethylamine dehydrogenase; MMADH: monomethylamine dehydrogenase.

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