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Development and Validation of an Analytical Method for Assaying Bacterial Uptake of Methyl Iodide in Estuarine Systems

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ABSTRACT

Methylotrophic bacterial activity has been reported to result in the oxidation of methyl halides (CH_3Xs) in natural waters. However, little is known about microbial degradation of methyl iodide (CH_3I), and it is uncertain whether CH_3I is utilized by methylotrophic bacteria as a sole carbon and energy source. To investigate the breakdown and utilization of CH_3I by methylotrophic bacteria in natural water samples, a rigorous carbon 14 (^{14}C) analytical protocol was developed. This paper describes a reliable and reproducible method using ^{14}C radiolabelled CH_3I as a substrate to examine the potential breakdown, remineralization and uptake of CH_3I in estuarine water samples. Application of the ^{14}C method indicated that there is uptake of CH_3I as a carbon source by methylotrophic bacteria present in water samples from the Tamar Estuary, Plymouth, UK. From the laboratory incubation experiment, up to 17.8% of the CH_3I was oxidized to CO_2 whilst up to 6.7% was incorporated into methylotrophic bacterial protein. The recovery of this analytical approach was ≥97 percent. The detection limit and precision of the method were 0.11 nmol L^{-1} and 5.2%, respectively.

Key Words: ^{14}C Analytical Method; Methyl Iodide; Methylotrophs; Microbial Degradation; Estuaries

INTRODUCTION

Methyl iodide (CH_3I) is the most important marine derived halocarbon in the delivery of iodine to the atmosphere from aquatic environments (Hemann et al. 1987, Moore and Tokarczyk, 1993, Richter and Wallace 2004) as well as the main source to terrestrial regions (Nightingale 2003). The cycling of CH_3I in the aquatic environment involves its production and degradation and is facilitated by both biotic and abiotic processes. The abiotic means of production include photochemical processes (Moore and Zafiriou 1994, Nightingale 2003) and chemical formation (Manley 1994), whilst biotic processes occur mainly through biological production by algae (Gschwend et al. 1985, Manley and Dastoor 1988, Smythe-Wright et al. 2006) which has been proposed to be the main natural source of CH_3I in coastal waters. There is also reported biological production through microbially-mediated activities in marine aggregates (Hughes et al. 2008) as well as aggregates from the estuarine environment (Asare 2007, Asare et al. 2012). Microbially-mediated removal of methyl halides (CH_3Xs) in the aquatic environment through oxidative degradation by methylotrophic bacterial community was proposed and a pathway described by Vannelli et al. (1998) using methyl chloride (CH_3Cl) as the carbon source.

Methylotrophic bacteria involved in the degradation pathways are capable of using organic compounds including CH_3Xs, as a source of carbon (e.g. King and Saltzman 1997, Goodwin et al. 1998, McDonald et al. 2002). It is however uncertain whether CH_3I could be used as a sole carbon and energy source by bacteria, even though it can act as a co-metabolite alongside CH_3Br and CH_3Cl (e.g., Connell Hancock et al. 1998, Schaefer and Oremland 1999). Bacteria may utilize organic compounds in two main ways: (1) for growth in the form of protein and tissue synthesis and (2) for the

production of energy through respiration and oxidation. Hypothetically, if CH_3I substrate (denoted as $^{14}CH_3I_{(i)}$) in aquatic systems is utilized as a carbon source, it may be distributed amongst three target locations: (1) in methylotrophic bacterial cellular biomass as carbon (denoted as ^{14}C), (2) oxidized by methylotrophic bacteria to form CO_2 (denoted as $^{14}CO_2$) and (3) remain unused as CH_3I in the aqueous medium (denoted as $^{14}CH_3I_{(f)}$). From literature, the most probable pathways for the degradation of CH_3I by methylotrophs may involve the utilization of enzymes that are mainly methyltransferases, dehydrogenases, hydrolases and monooxygenases (Vannelli et al. 1998, McDonald et al. 2002, Schäfer et al. 2007). CH_3Xs (including CH_3I) are oxidised by methylotrophs to form formaldehyde by involving various substrate specific enzymes (Inagaki et al. 2004). The resulting formaldehyde may be incorporated directly into cellular biomass or undergo subsequent oxidation to form CO_2. Some methylotrophs are capable of oxidizing all CH_3Xs suggesting the involvement of a common enzyme system (Schaefer and Oremland 1999, Schäfer et al. 2005) in the degradation pathways. In the process of degradation, methyltransferases are used by the methylotrophs to transfer the methyl group (CH_3^+) to a corrinoid group and subsequently to tetrahydrofolate (H_4folate) to form methyl tetrahydrofolate (CH_3-H_4folate) (Vannelli et al. 1999, Studer et al. 2002). CH_3-H_4folate undergoes progressive oxidation steps to form formate (HCOOH) and CO_2 (Vannelli et al. 1999). It is also apparent from literature that most of the known and studied methylotrophs in the aquatic environment prefer CH_3Cl and methyl bromide (CH_3Br) to CH_3I as a sole carbon source (e.g. Connell et al. 1997, Vannelli et al. 1998, Schäfer et al. 2005).

The objective of this study was therefore to develop an analytical method to quantitatively determine the end stages of methylotrophic bacterial utilization of CH_3I as a carbon source in natural water samples through the modification of the methods by Smith and Azam (1992) and Connell et al. (1997).

The analytical method developed by Smith and Azam (1992) determines the bacterial protein synthesis using ^3H-leucine, or ^3H-thymidine, as the radiolabelled carbon source. This is an effective and reliable method in tracking the fraction of radiolabelled carbon that is incorporated in bacterial cells. In addition, a method developed by Connell et al. (1997) in assaying CH_3Br utilization by bacteria can quantitatively isolate CO_2 as the main volatile end-product from the oxidation of [^{14}C] radiolabelled CH_3Xs. The CO_2 is then trapped in a solution containing sodium hydroxide (NaOH), sodium bicarbonate (Na_2CO_3) and strontium chloride hexahydrate ($SrCl_2.6H_2O$) by forming strontium carbonate ($Sr^{14}CO_3$) precipitate through a series of chemical reactions described in equations (1) and (2).

$$^{14}CO_2 + 2NaOH \Rightarrow Na_2{}^{14}CO_3 + H_2O \quad (1)$$

$$Na_2{}^{14}CO_3 + SrCl_2.6H_2O \Rightarrow Sr^{14}CO_3 + 2NaCl + 6H_2O \quad (2)$$

A synergy of the two methods with some modifications could be applicable in assaying CH_3I oxidation and assimilation by methylotrophic bacteria in estuarine waters. No published method for assaying and assessing CH_3I utilization by bacteria in aquatic environments is known to exist. This paper therefore presents an innovative approach to quantify the potential CH_3I breakdown and utilization by bacteria in the estuarine environment.

MATERIALS AND METHODS

Study Area

Natural water samples for this study were obtained from the turbidity maximum zone (TMZ) of the Tamar estuary located in south-west England (50° 26' 35.30"N, -4° 12' 25.42"W) (Figure 1). The Tamar estuary experiences highly dynamic changes physically, chemically and biologically during the transition from the freshwater to the marine environment (Uncles and Lewis 2001). It is characterised by the presence of a strong TMZ (Grabemann et al. 1997) in its upper reaches during summer (Uncles and Stephens 1993) and in the middle reaches during winter due to increased river discharge (Tattersall et al. 2003). These conditions therefore make the Tamar estuary an ideal system for investigating biogeochemical processes in coastal waters. *In situ* temperature of the water samples were maintained during transportation from the field to the laboratory.

First Experimental Design

Due to lack of literature information on microbial utilization of CH_3I, the first experiment was designed to acquire preliminary data on bacterial response to $^{14}CH_3I$ substrate (with respect to minimum concentration levels

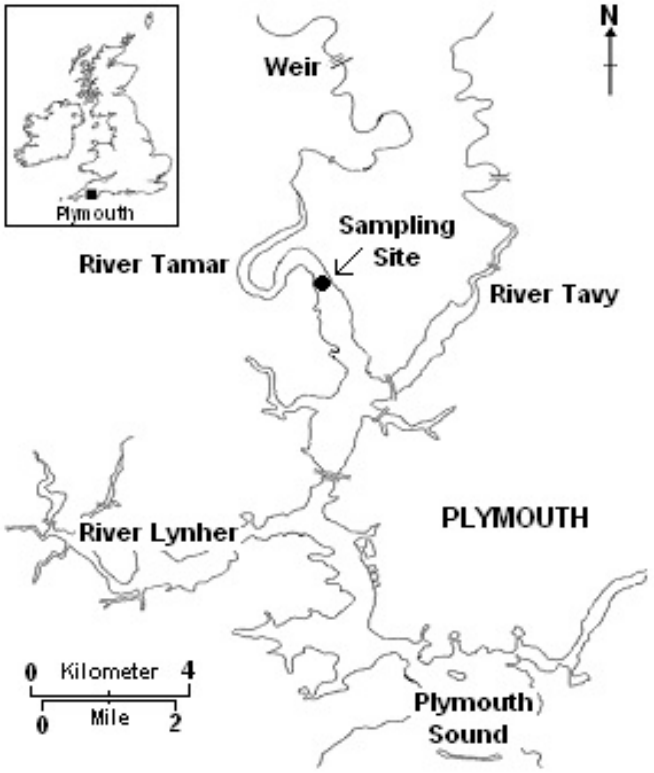

Figure 1. Map of the Tamar estuary showing the turbidity maximum zone as the sampling site

and the sensitivity of the technique). The initial experimental design (Figure 2) was used to carry out two sets of analyses in Experiments 1.1 and 1.2.

Considering the three identified targets of $^{14}CH_3I_{(i)}$ substrate (i.e., ^{14}C, $^{14}CO_2$ and $CH_3I_{(f)}$), stated earlier on, the first experimental design was divided into three separate but continuous stages (I, II and III) to identify and measure the radioactive disintegration of each of the substrate targets recorded using a Liquid Scintillation Counter (Wallac Winspectral 1414).

The experiments began with the addition of different concentrations of $^{14}CH_3I_{(i)}$ to labelled microcentrifuge tubes containing 2 mL of the estuarine water sample (four replicates per sample) and incubated at *in situ* temperatures in complete darkness. Two mL of autoclaved estuarine water sample for each labelled microcentrifuge tube was used as control samples (four replicates per sample). All experiments were conducted with three different substrate concentration levels. During Experiment 1.1, $^{14}CH_3I_{(i)}$ concentrations of 6, 17 and 43 nmol L^{-1} were used for two different incubation durations of 3 and 6 hours respectively whilst in Experiment 1.2, concentrations of 1853, 4475 and 8110 nmol L^{-1} were used.

Stage I: Two millilitres of water samples were pipetted into four replicates microcentrifuge tubes and four control samples tubes. This was immediately followed by the addition of the $^{14}CH_3I_{(i)}$ substrate (volume used depended on the strength of the stock and the required final concentration for each set of samples). The samples were vortex mixed for homogenization and incubated using Thermo-Tote Incubator (Scientific Device Lab Inc.) at *in situ* temperatures for 3 and 6 hr durations in Experiments 1.1 and 1.2 respectively. The samples were centrifuged at 12000 rpm for a period of ten minutes at the end of incubation to separate bacterial cells from the supernatant and obtain ^{14}C of the experiment.

Stage II: One millilitre of supernatant of each replicate from Stage I (containing dissolved $^{14}CO_2$ and $^{14}CH_3I_{(f)}$) was immediately pipetted into a second set of labelled 2 mL microcentrifuge tubes already containing a "Complex"

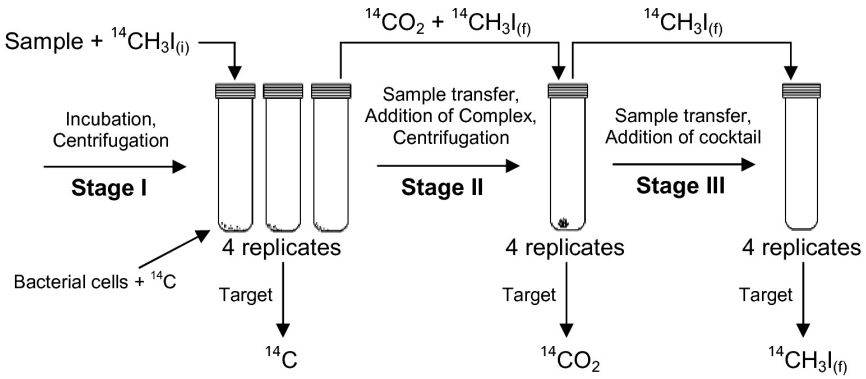

Figure 2 The first experimental design showing the sequence and stage by stage (Stage I to III) assaying of utilized $^{14}CH_3I$ by bacteria present in replicate experimental media

(a mixture of 20 µL NaOH, 100 µL Na_2CO_3 and 500 µL $SrCl_2.6H_2O$). The precipitation of $Sr^{14}CO_3$ pellets occurs through a reaction sequence shown in Equations (1) and (2). By vortex mixing, the reaction is brought to completion. The samples were then centrifuged as in stage I (to separate the $Sr^{14}CO_3$ pellets from the supernatant). $^{14}CO_2$ of the experiment was estimated from $Sr^{14}CO_3$ pellets obtained from this stage.

Stage III: Another 1 mL of supernatant from each replicate at the end of Stage II was again pipetted into a new set of 2-mL labelled microcentrifuge tubes. One mL of Optiphase "HiSafe" 3 scintillation cocktail was added to each sample tube, capped and then vortex mixed to create a gel which was used to estimate $^{14}CH_3I_{(f)}$ of the experiment.

All remaining supernatants at the end of each stage were aspirated from the microcentrifuge tubes after centrifugation and washed with 1.5 mL of Milli-Q water to remove any excess substrate remaining in the tube. One mL of scintillation cocktail was added to each sample tube and all samples kept in the dark for at least thirty minutes to allow for stabilization, before radio-active disintegration of Carbon-14 from each sample tube analysed using liquid scintillation counter.

As a measure of quality control, three replicates for each substrate concentration used for the experiment ($^{14}CH_3I_{(i)}$) were pipetted from the stock into clean micro-centrifuge tubes and 1 mL of scintillation cocktail added and analysed together with the experimental samples. The total amount of radiolabelled CH_3I degraded by the bacterial community was estimated from results of stage I and II samples and the balance checked against the total added amount of CH_3I. Rigourous safety procedures were followed in the handling, application and waste disposal of radiochemicals at each stage.

Second Experimental Design

The second experimental design was derived through evolution and optimisation of the initial experimental design and was necessitated to remedy losses experienced in Experiments 1.1 and 1.2 (see Figure 3).

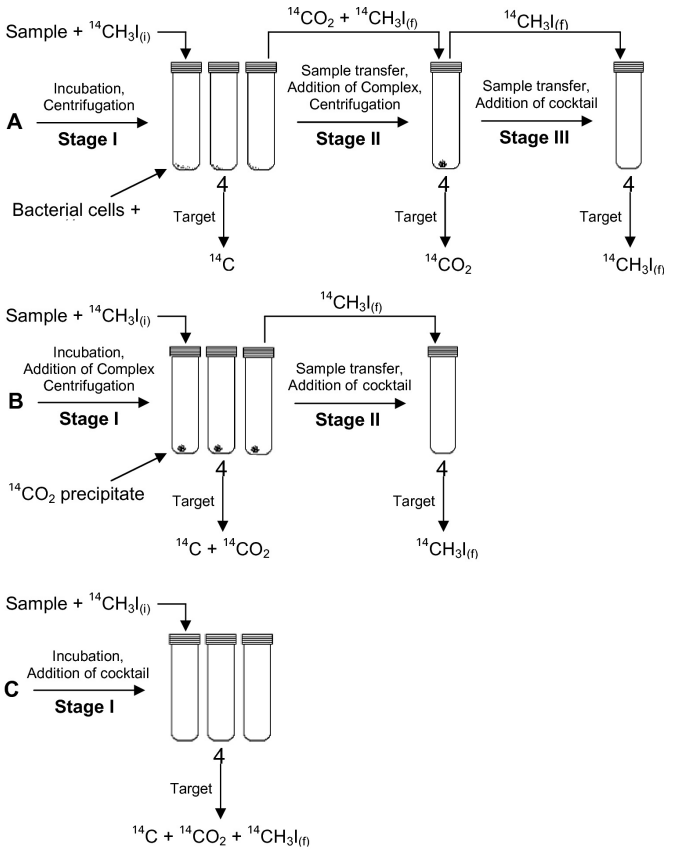

Figure 3 Optimized second experimental design showing the sequence steps (Stage I through III) and sample sets (A, B and C) involved in assaying utilized $^{14}CH_3I$ by bacteria after design modifications

In this optimized design, three sets of samples (A, B and C) were analysed simultaneously during Experiments 2.1 and 2.2. Each set of samples A, B and C was used to estimate a discrete substrate target or a combination of targets. Sample set A was used to analyse for $[^{14}C]$, sample set B for $[^{14}C + {}^{14}CO_2]$ and sample set C for $[^{14}C + {}^{14}CO_2 + {}^{14}CH_3I_{(f)}]$. This approach kept the time spent on analysing each target to the minimum whilst work on all three sets of samples was carried out simultaneously. Incubations were carried out at *in situ* temperatures of 18.4 and 11.6 ºC for Experiments 2.1 and *2.2* respectively for a duration of 1 hour.

Replicates of sample set A were analysed from Stage I to III as described in the first experimental design. With sample set B, two stages instead of three were included in the analyses, following a slightly different procedure compared to that of sample set A. Stage I of sample set B was completed with an immediate addition of 1 mL of the sample to a "Complex". This was followed by an immediate centrifugation to obtain both $^{14}CO_2$ and ^{14}C in a single step. At Stage II, scintillation cocktail was added to 1 mL of supernatant from Stage I in order to obtain $^{14}CH_3I_{(f)}$. With sample set C, replicate analyses involved only a single stage. Scintillation cocktail was immediately added to 1 mL of each sample at the end of the incubation period to obtain the combined concentration of the three substrate targets (^{14}C, $^{14}CO_2$ and $^{14}CH_3I_{(f)}$). To accurate estimate each of the three distribution targets, results from all three sample sets (A, B and C) were applied. ^{14}C was estimated from sample set A, $^{14}CO_2$ from sample set B and $^{14}CH_3I_{(f)}$ from sample set C using the following equations:

$$[^{14}C] = [^{14}C]\ A \quad (3)$$

$$[^{14}CO_2] = [^{14}C + {}^{14}CO_2]\ B - [^{14}C]\ A \quad (4)$$

$$[^{14}CH_3I_{(f)}] = [^{14}C + {}^{14}CO_2 + {}^{14}CH_3I_{(f)}] - [^{14}C + {}^{14}CO_2]\ B \quad (5)$$

Hence,

$$[^{14}C] + [^{14}CO_2] + [^{14}CH_3I_{(f)}] \text{ is expected to be equal to } [^{14}CH_3I_{(i)}] \quad (6)$$

and,

$$[^{14}CH_3I_{(i)}] - \{[^{14}C] + [^{14}CO_2] + [^{14}CH_3I_{(f)}]\} = [^{14}CH_3I_{(i)}]\text{Lost.} \quad (7)$$

To ensure that the developed analytical method is reliable and reproducible, the performance of the Liquid Scintillation Counter (LSC) used in analyzing $^{14}CH_3I$ was determined through the measurement of (1) mean concentration of blanks; (2) the estimation of the detection limit; and (3) the precision (as relative standard deviation (RSD)) with the results provided below.

Mean Blank Concentration = 0.095 ± 0.005 nmol L^{-1}
System Detection Limit = Mean Blank Concentration + (3 x SD)
= 0.095 + (3 x 0.005) = 0.11 nmol L^{-1}
Instrumental RSD = (0.005/0.095) x 100 = 5.2%

RESULTS

Bacterial Methyl Iodide Uptake During Experiment 1.1

The results of Experiment 1.1, indicated an unusually large portion of the $^{14}CH_3I_{(i)}$ (up to 81%) unaccounted for in all the various treatments. However, recorded values ranging between 0.7 and 1.0 nmol L^{-1} in bacterial cells (^{14}C) and 0.2 – 0.3 nmol L^{-1} as oxidised ($^{14}CO_2$) for both the 3-hour and 6-hour incubation durations (Figure 4a-b) highlighted bacterial response to the addition of ^{14}C-radiolabelled CH_3I as substrate.

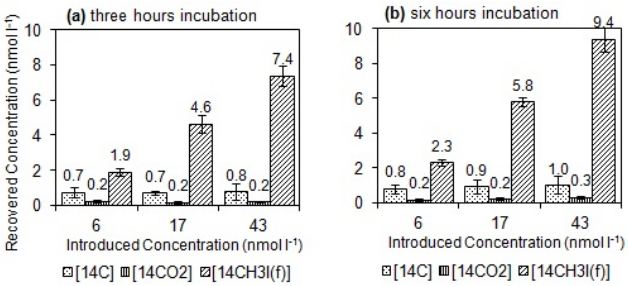

Figure 4. Bacterial CH_3I utilization during (a) three hours and (b) six hours incubation duration using the first experimental design (Experiment 1.1) in order to investigate bacterial response to radiolabelled CH_3I as a substrate upon introduction

However, there were no statistically distinguishable differences (r^2-values from 0.032 to 0.507; p-values from 0.288 to 0.821 based on ANOVA) between bacterial responses for the three concentrations in both the 3-hour and 6-hour durations. Therefore higher substrate concentrations were required to elicit differentiated responses.

Bacterial Methyl Iodide Uptake During Experiment 1.2

From the results of Experiment 1.2 (Figure 5a-b), recorded ^{14}C (in bacterial cells) ranged between 56 – 267 nmol L^{-1} representing 3% of each of the substrate concentrations introduced for the 3-hour and 6-hour incubation durations.

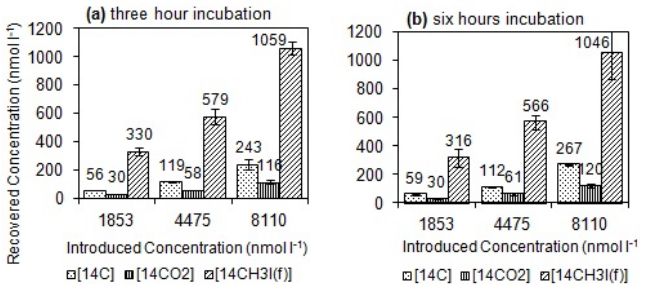

Figure 5. Bacterial response to the three different concentrations of radiolabelled CH_3I substrate for (a) three hours, (b) six hours shown as the recorded concentration for the various substrate 'Targets' (Experiment 1.2)

Observed $^{14}CO_2$ from the same sets of samples ranged between 30 – 120 nmol L^{-1} (see Figure 5a-b) representing between 1 and 2% of the introduced substrate concentrations. However, the fraction of the introduced concentration that could not be accounted for during Experiment 1.2 was still as high (>80%) as that of Experiment 1.1.

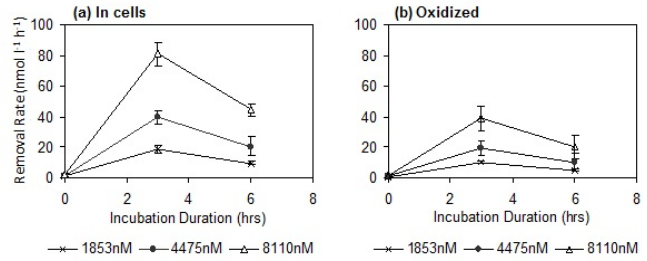

Figure 6. Comparison of the rates of bacterial removal of CH_3I (a) recorded in cells (b) recorded as oxidized in the experimental media upon the introduction of three different CH_3I substrate concentrations [1853, 4475, 8110 nmol L^{-1} (denoted as nM)] and incubated for three different time periods (t = 0, 3, 6 hours respectively) during Experiment 1.2

By comparison, the estimated rate of removal (shown in Figure 6a-b) between the two different durations (including time zero i.e. t = 0, 3 and 6 hours) suggest that optimum bacterial removal of CH_3I substrate occur anywhere between t = 0 and t = 3 hours with an exponential rate of removal.

To establish the ideal incubation duration, an additional experiment was carried out (with the application of four different concentrations between 50 and 400 nmol L^{-1} for durations that range from 0 to 2 hours at 30 minutes intervals). The results presented in Figure 7 indicate that the optimal rate of substrate removal by the methylotrophic bacterial occurred within 90 minutes of incubation (especially with a concentration of 400 nmol L^{-1}).

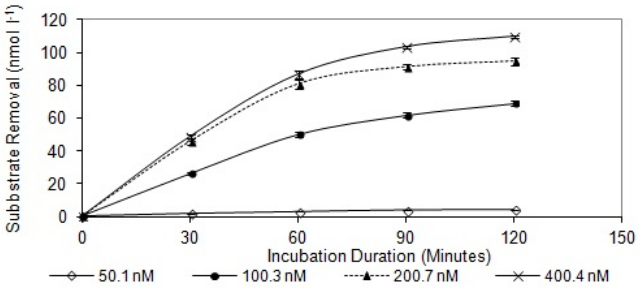

Figure 7. Removal of $^{14}CH_3I$ by methylotrophic bacteria (at different substrate concentrations and incubation durations)

However at 90 minutes the recorded rate of removal was dissimilar in all concentrations used for the incubation (see Figure 7). On the other hand, recorded rate of removal between 200 and 400 nmol L^{-1} substrate concentrations were statistically identical at both 30 minutes (45.8 ± 1.9 nmol L^{-1}, and 48.8 ± 1.0 nmol L^{-1} respectively; *p-value* of 0.028) and 60 minutes of incubation (80.7 ± 2.8 nmol L^{-1}, and 86.6 ± 2.5 nmol L^{-1} respectively; (p-value of 0.001). The ideal incubation duration could therefore be established as 60 minutes maximum.

Bacterial Methyl Iodide Uptake During Experiment 2.1

Using the revised analytical approach (second experimental design), Experiment 2.1 was conducted to address and minimize the losses observed in Experiments 1.1 and 1.2. The results are presented in Figure 8a-b. Through the application of equations 3, 4, 5 and 7, the concentrations of ^{14}C, $^{14}CO_2$, $^{14}CH_3I_{(f)}$ and $^{14}CH_3I_{(i)}$Lost

were estimated to be 148 ± 30, 390 ± 24, 1336 ± 19 and 313 nmol L^{-1} respectively for Experiment 2.1. From this experiment, the recovery of $^{14}CH_3I_{(i)}$ was improved to 86% as against less than 20% in both Experiments 1.1 and 1.2. This is an indication that there was an enhancement of approximately 55% in $^{14}CH_3I_{(i)}$ recovery through a better approach with respect to the revised analytical design although about 14% was still unaccounted for (see Figure 8a-b).

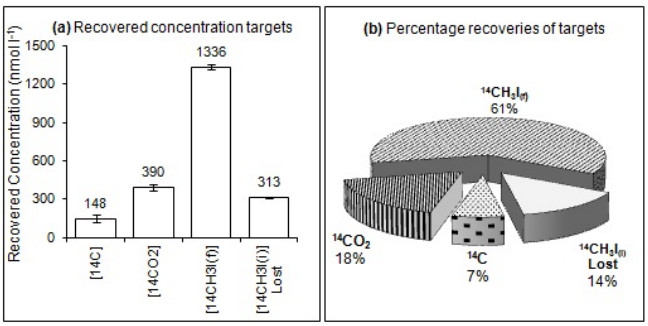

Figure 8. Recovered concentration (a) and percentage (b) of the various substrate 'Targets' during Experiment 2.1

At estimated rates of 148 ± 30 and 390 ± 24 nmol $L^{-1}h^{-1}$, methylotrophic bacterial utilization recorded as ^{14}C and $^{14}CO_2$ during Experiment 2.1 represented 6.7% and 17.8% of the introduced substrate concentration respectively.

Bacterial Methyl Iodide Uptake During Experiment 2.2

The results from Experiment 2.2 (see Figure 9a-b) confirmed and validated the improved recovery of $^{14}CH_3I_{(i)}$ observed in Experiment 2.1. This results indicate that, 20.1 ± 1.6 nmol L^{-1} representing 4% of the introduced concentration was recovered in bacterial cells (^{14}C) whilst 26.2 ± 1.0 nmol L^{-1} (5%) was oxidised ($^{14}CO_2$) by the methylotrophic bacteria present. These concentration figures also represent the rates of degradation (nmol L^{-1} h^{-1}).

The accrued experimental loss was further reduced to 3% in Experiment 2.2 as compared to 14% for Experiment 2.1. This could be attributed to further improvement in personal handling of samples whereby headspaces were kept to the barest minimum during Stage I of sample set A before incubation. Also in sample set B, samples were added to the "Complex" by

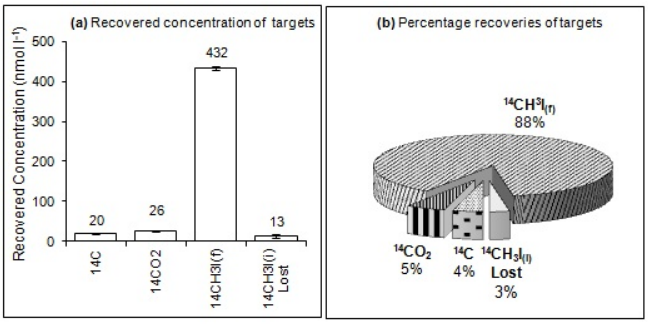

Figure 9. Recovered concentration (a) and percentage (b) of the various substrate 'Targets' during Experiment 2.2

gently decanting with a pipette along the side of the microcentrifuge tubes to reduce agitation in the tubes prior to the formation of strontium carbonate precipitate whilst with sample set C, the addition of cocktail was added right after pipetting samples into the micro-centrifuge tubes. All these practices improved the recovery efficiency of the substrate targets in Experiment 2.2. It may however also be an attribute of the lower *in situ* incubation temperature of 11.6 ºC for Experiment 2.2 as compared to 18.4 ºC for Experiment 2.1 which may have affected the volatility rate of substrate between the two experiments. Further work is however required to confirm these assertions. These outcomes suggest that by reducing the experimental stress such as vortex mixing and centrifugation on the volatile substrate (CH_3I) as shown by the revised analytical approach, losses can be reduced significantly.

DISCUSSION

Observations from Experiment 1.1, revealed no detectable differences in bacterial response to the introduced CH_3I whilst up to 81% of the substrate could not be accounted for using the first experimental design. The absence of statistically significant difference between the bacterial responses could be as a result of low substrate concentrations used. This assumption was proven to be correct during Experiment 1.2 when higher substrate concentrations were used. Unlike results from Experiment 1.1, there were significant statistical differences (r^2-values > 0.998; p-values < 0.0001 from ANOVA) between the recorded concentrations of each substrate target from the three sets of concentrations applied during each incubation period of Experiment 1.2.

However, with the total substrate recovery of Experiment 1.2 ($^{14}C + {}^{14}CO_2 + {}^{14}CH_3I_{(f)}$) representing less than 25% of the added substrate concentration, the problem of substrate loss observed in Experiment 1.1 still persisted in Experiment 1.2 showing a substrate loss of up to 83%. These observed losses in both Experiments 1.1 and 1.2 were most likely due to the loss of $^{14}CH_3I_{(i)}$ and $^{14}CO_2$ during sample transfer between the various stages of the first experimental design.

From the revised analytical approach (second experimental design), substrate losses were significantly reduced in all subsequent experiments. The total percentage recovery was up to 97.3% in Experiment 2.2 with percentage recovery of $^{14}CO_2$ increasing from about 2% in Experiment 1.2 to about 18% in Experiment 2.1. With respect to the unused portion of the substrate ($^{14}CH_3I_{(f)}$), the percentage recovery was 61% during Experiment 2.1 as compared to 18% during Experiments 1.2. These findings imply that about 16% of the 70% concentration loss during the application of the first experimental design was as a result of $^{14}CO_2$ loss. The loss is likely to have occurred during the centrifugation step in Stage I and substrate volatilization during transfer of sample in Stage II. The additional loss of about 43% of $^{14}CH_3I_{(i)}$ during the application of the first experimental design may be attributed to CH_3I volatility coupled with agitations during sample transfer, vortex mixing and centrifugation in Stage I, II and III. Table 1. A summary of recorded CH_3I concentrations (pmol L^{-1}) at the four selected sampling stations along the Tamar Estuary and during the cold) and warm months of the year.

A comparison of percentage recoveries between Experiments 2.1 and 2.2 indicates a 16% reduction in bacterial removal of substrate in the latter experiment. This difference may be as a result of differences in bacterial community structure of the water samples used in the two experiments as well as variations in *in situ* water temperature. The revised analytical approach has shown a significant improvement over the initial analytical approach. Therefore the development of this analytical technique has shown to be effective and reproducible hence could be applied in assessing bacterial utilization of CH_3I in the estuarine environment. During a comprehensive study in the Tamar estuary (Plymouth, UK) with a turbidity maximum zone, the developed analytical method discussed in this paper was successfully applied in the investigation of microbial CH_3I removal in the estuary. Detailed results and interpretation of this survey are presented elsewhere (Asare, unpublished). However, to emphasize the application of the developed method, results presented in Table 1 and Figure 10a-d represent

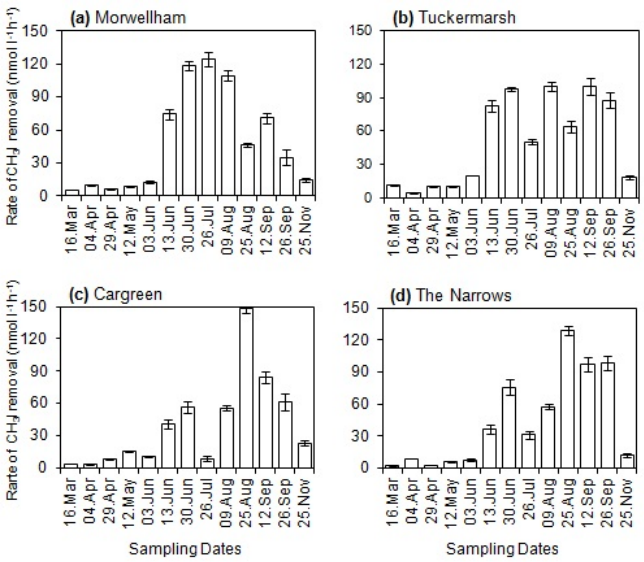

Figure 10. Variation in bacterial CH_3I removal (nmol L^{-1} h^{-1}) at four selected study sites along the Tamar estuary (Plymouth, UK) from the freshwater region (a) Morwellham, (b) Tuckermarsh to the seawater region (c) Cargreen and (d) The Narrows of the estuary

Table 1. A summary of recorded CH_3I concentrations at four selected sampling stations along the Tamar Estuary

Sampling Stations	All Data		Cold Season		Warm Season	
	Mean ± sd	Range	Mean ± sd	Range	Mean ± sd	Range
Morwellham	0.24 ± 0.13	0.06 – 0.49	0.12 ± 0.06	0.06 – 0.17	0.29 ± 0.12	0.16 – 0.49
Tuckermarsh	0.41 ± 0.23	0.09 – 0.73	0.24 ± 0.12	0.14 – 0.38	0.49 ± 0.23	0.09 – 0.73
Cargreen	1.02 ± 0.35	0.46 – 1.43	0.60 ± 0.20	0.46 – 0.82	1.20 ± 0.22	0.91 – 1.43
The Narrows	1.36 ± 0.44	0.65 – 2.04	0.88 ± 0.38	0.65 – 1.31	1.57 ± 0.27	1.28 – 2.04

the spatial and seasonal trends in the rate of removal of CH_3I at four selected study sites along the Tamar estuary (moving from a low salinity region of the estuary, Morwellham and Tuckermarsh, to a high salinity region of the estuary, Cargreen and The Narrows). A strong seasonal variation in the rate of CH_3I removal by methylotrophic bacteria was clearly observed in the Tamar estuary as a result of the temporal variations in bacterial activity.

CONCLUSIONS

The focus of this study was to develop an analytical approach to assay methylotrophic bacterial utilization of CH_3I as a carbon source. The outcome indicates that a reliable and reproducible method using [^{14}C] radiolabelled CH_3I as a substrate has been achieved. Application of the method has also shown that methylotrophic bacteria in estuarine environment are capable of oxidizing CH_3I to CO_2 (up to 17.8%) as well as for protein synthesis (up to 6.7%). Since incubations for all experiments were carried out in complete darkness, photosynthetic production of CH_3I that might affect the results was considered negligible. This assumption is based on the observation that photo-synthetic production of CH_3I in estuarine water samples derived from the TMZ for a laboratory controlled experiment is statistically insignificant (Asare et al. 2012). During the course of this study, the recorded *in situ* concentration of CH_3I in the Tamar estuary was 3.6 x 10^{-3} nmol L^{-1}. This implies that in order for the carried experiments to be quantitatively represented as methylo-trophic bacterial removal of CH_3I in the natural estuarine environment, the CH_3I substrate concentration applied should not have exceeded the recorded *in situ* concentration. However, it is important to note that the level of CH_3I substrate concentration applied to achieve significant and reproducible methylotrophic bacterial responses far exceed the stated *in situ* concentration in the Tamar estuary (to a magnitude of about one hundred thousand fold). Therefore, results from this study could only be a measure of the potential CH_3I removal by methylotrophic bacterial uptake in the natural estuarine environment. This developed analytical procedure could be applicable only in the qualitative interpretation of microbial CH_3I degradation in estuarine systems where the influence of spatial and temporal variations in physicochemical variables on microbial activity is taken into account.

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