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ORIGINAL ARTICLE

Methane oxidation and methane driven redox process during sequential reduction of a flooded soil ecosystem

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Abstract A laboratory incubation study conducted to assess the temporal variation of CH_4 oxidation during soil reduction processes in a flooded soil ecosystem. A classical sequence of microbial terminal electron accepting process observed following NO_3^- reduction, Fe^{3+} reduction, SO_4^{2-} reduction and CH_4 production in flooded soil incubated under initial aerobic and helium-flushed anaerobic conditions. CH_4 oxidation in the slurries was influenced by microbial redox process during slurry reduction. Under aerobic headspace condition, CH_4 oxidation rate (k) was stimulated by 29 % during 5 days (NO_3^- reduction) and 32 % during both 10 days (Fe^{3+}) and 20 days (early SO_4^{2-} reduction) over unreduced slurry. CH_4 oxidation was inhibited at the later methanogenic period. Contrastingly, CH_4 oxidation activity in anaerobic incubated slurries was characterized with prolonged lag phase and lower CH_4 oxidation. Higher CH_4 oxidation rate in aerobically incubated flooded soil was related to high abundance of methanotrophs (r=0.994, p<0.01) and ammonium oxidizers population (r=0.184, p<0.05). Effect of electron donors NH_4^+, Fe^{2+}, S^{2-} on CH_4 oxidation assayed to define the interaction between reduced inorganic species and methane oxidation. The electron donors stimulated CH_4 oxidation as well as increased the abundance of methanotrophic microbial population except S^{2-} which inhibited the methanotrophic activity by affecting methane oxidizing bacterial population. Our result confirmed the complex interaction between methane-oxidizing microbial groups and redox species during sequential reduction processes of a flooded soil ecosystem.

Keywords Methane · Oxidation · Methanotrophs · Soil · Redox process

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Introduction

Microbially mediated methane (CH_4) oxidation plays a major role in reducing global atmospheric CH_4 , and annually about 10–40 Tg atmospheric CH_4 is consumed by methane-oxidizing bacteria (Hardy and King 2001; Reeburg 1993, 2003; Roslev and King 1995). Microbial CH_4 oxidation has been reported to occur at significant rates in many natural ecosystems, and soils can act as sinks for CH_4 from the atmosphere (Boetius et al. 2000; Börjesson et al. 2001; Conrad and Routhfuss 1991; Hütsch and Powlson 1994; Kightley and Cooper 1995; Suwanwaree and Robertson 2005). Therefore, the biological CH_4 oxidation process is important to minimize global climate change and there is need for extensive research to characterize methanotrphic activity in various ecosystems for possible application to reduce atmospheric greenhouse gases (GHG). CH_4 is produced under anaerobic condition from flooded rice fields, while its oxidation takes place under aerobic conditions. So far, most of the studies characterizing the methane oxidation rate are restricted to upland aerobic soil ecosystems, and limited information is available to support our understanding of a flooded soil ecosystem (Bronson and Mosier 1994; Conrad and Routhfuss 1991; Del Grosso et al. 2000; Mohanty et al. 2006). Soil moisture is important to regulate CH_4 oxidation (Mancinelli 1995), either by affecting diffusion of the gas phase (Striegl 1993) or affecting soil methanotroph processes by osmotic stress (Schnell and King 1996). In wet soils, CH_4 oxidation decreases with high soil moisture (Adamsen and King 1993;

Keller and Reiners 1994; Steudler et al. 1989; Whalen and Reeburgh 1990), but at low moistures CH_4 oxidation is not highly correlated with moisture content (Castro et al. 1995; Dunfield and Knowles 1995; Mosier et al. 1996). Typically, in very dry soils such as in deserts, CH_4 oxidation is higher after precipitation (Strieg et al. 1992). In such soils, osmotic stress may limit activity of CH_4-oxidizing bacteria more than diffusion of gases through the soil (Schnell and King 1996). Few studies have revealed that water addition to soil can stimulate CH_4 oxidation, and methanotrophic activity maxima can be attained at intermediate soil moistures (Czepiel et al. 1995; Torn and Harte 1996). It has been projected that climate change will affect the water distribution globally and increasing temperature will lead to more wetlands (Davidson and Janssens 2006; Walther et al. 2002). Many upland soils will remain flooded and this may influence the GHG footprint by affecting both methanogenic and methanotrophic bacteria.

In flooded rice soil, CH_4 oxidation activity varies with cropping period (Dannenberg and Conrad 1999). Under flooded condition anaerobes are predominantly active and reduce aerobic microbial process. However, flooded soil does not necessarily result in the development of a uniformly reduced profile. A thin, oxidized surface horizon overlying a deep, reduced horizon is formed due to the dissolved oxygen from the overlying floodwater diffusing across the surface water–soil interface and in soils planted with rice, the rhizosphere is oxidized because of the delivery of O_2 into roots (Bodelier and Frenzel 1999; Bosse and Frenzel 1997; Patrick and Engler 1974). In periodically submerged soil, anaerobic microbial redox processes takes place by sequential reduction of inorganic electron acceptors such as oxygen, nitrate, manganese (IV), iron (III), sulfate and CO_2. The sequence of a reduction processes is best described by the thermodynamic theory, which predicts preferential reduction of available electron acceptors with the most positive redox potential (Ponnamperuma 1972; Zehnder and Stumm 1988). Many studies have investigated the impact of oxidized electron acceptors on methanogens in flooded rice soil (Bond and Lovley 2002; Kumaraswamy and Sethunathan 2001). Anaerobes like denitrifiers, dissimilatory iron reducers, sulfate reducers, and methanogenic bacteria are active in presence of high input of labile organic material in anaerobic layer and they often compete for common reduced carbon sources (Carucci et al. 1999; Paul et al. 1989; Tiedje et al. 1983). In flooded soil ecosystems, CH_4 oxidation activity is affected due to O_2 limitation, and along with a predominance of reduced species (Van Bodegom et al. 2001; Henckel et al. 2000) under such conditions, CH_4 oxidation has been reported at less-reduced sites through NO_3^-, Fe^{3+} and SO_4^{2-} reduction (Miura et al. 1992; Murase and Kimura 1996). Anaerobic CH_4 oxidation is s poorly understood process because the microorganisms capable of performing this process have not been characterized from soil. The present study was undertaken to examine the microbial processes involved in CH_4 oxidation in vertisol under flooded conditions. Experiments were carried out to define (1) CH_4 oxidation in flooded soil during sequential reduction of terminal electron acceptors, (2) the role of redox metabolites on methanotrophic activity, and (3) changes in the population of methane-consuming methanotrophs during the sequential reduction process. Our result provided information on the microbial-mediated processes in flooded soil ecosystems for a deeper understanding on the methanotrophic activity, complex interaction processes with chemical attributes of soil, and the methanotrophs involved in CH_4 oxidation.

Materials and methods

Soil sample

Soils samples were collected from the experimental fields of Indian Institute of Soil Science (IISS), Bhopal, Madhya Pradesh, India (23°18′N latitude, 77°24′E longitude and 485 m above mean sea level). The soil is a heavy clayey vertisol (typic Haplustert), and the experimental site was characterized with organic carbon (5.7 g kg^{-1}), available N (225 mg kg^{-1}) and available P (2.6 mg kg^{-1}) but high in available K (230 $mgkg^{-1}$). The textural composition of soil was: sand 15.2 %, silt 30.3 %, clay 54.5 %, electrical conductivity (EC) 0.43 dS m^{-1}, and pH7.5. After collection, the soils were air dried under shade and after breaking the clods were passed through a 2-mm mesh sieve and stored in air-tight polyethylene bags at room temperature in the laboratory.

Slurry preparation and sampling strategies

The incubation experiment was carried out with a 10 g portion of soil placed in 130-ml presterilized serum bottles and closed with neoprene septa. Soils were moistened with sterile distilled water at a ratio of 1:2.5; i.e., for 10 g soil, 25 ml of sterile distilled water were added. Bottles were divided into two sets, one with ambient air (aerobic) and other with helium (He) in the head space (anaerobic). Stimulated during incubation by flushing head space with He for 30 min. Pure CH_4 was injected into the head space and bottles were kept at 30±2 °C in an incubator with intermittent shaking for a period of 8 h each day using a rotary shaker. At a given time intervals, gas samples were withdrawn from the headspace after vigorously shaking the soil incubation bottles by hand to allow equilibration between the liquid and gas phase. Slurry subsamples were collected from the incubated bottles during the terminal electron accepting period, particularly during NO_3

reduction, Fe^{3+} reduction, SO_4^{2-} reduction, and the methanogenesis period. The experiment was carried out by preparing numerous incubation bottles in parallel, and all the measurements were made on three replicates.

Chemical analysis

Concentration of electron acceptors in slurry samples over incubation were carried out by wet chemical analysis. NO_3^- content in slurry samples were estimated after extraction with $CaSO_4$ and reaction by phenol disulphonic acid method (Jackson 1958). SO_4 was estimated using $Ca(H_2PO_4)_2$ as extractant and turbidometric analysis (Searle 1979). Reduced Fe^{2+} in slurry was determined by extraction of slurry samples with 0.5 N HCl and ferrozine assay (Weber et al. 2006).

CH_4 oxidation

Head space of the incubated bottles were injected with 5 ml pure (100 %) CH_4 to provide 2100 μmol of CH_4 g^{-1} air dried soil. Head space CH_4 concentration was measured at regular interval. After each day sampling, the headspace was replaced with an equivalent amount of high purity He to maintain the pressure equilibrium. The CH_4 concentration in the headspace of serum bottles was analyzed in a Shimadzu GC-17A gas chromatograph equipped with FID and a Porapak N column. The column and detector were maintained at 60 °C and 100 °C respectively. The gas samples were injected through injection port of an on-column injector. The GC was calibrated before and after each set of measurement using different standard concentration of CH_4 in N_2 (Sigma gasses, N. Delhi, India) as primary standard. Under these conditions, the retention time of CH_4 was 1.15 min and the minimum detectable limit was 0.5 μl ml^{-1}.

Electron donors influence on methane oxidation

In a follow-up experiment, soil slurry samples were incubated with different electron donors to understand the CH_4 - driven redox process in the soil. Briefly, 10-g portions of soil samples placed in a presterilized 130-ml serum bottle were held under flooded condition by adding sterile distilled water at 1:2 volume ratio. Soil slurries were then amended with a freshly prepared aqueous solution of different inorganic electron donor redox species (as unamended control, NH_4Cl, $FeCl_2$, and N_2S) separately. After closing with butyl rubber stoppers, a set of incubation vessels was flushed with helium (He) for 30 min, and another set was prepared with ambient air in the headspace, respectively. The headspace of all the serum bottles was injected with 5 ml pure methane. Then, the incubation bottles were kept in the dark in an incubator, with intermittent shaking on a rotary shaker for a period of 8 h on each day, at 30±2 °C for 10 days. CH_4 concentration in the headspace of the serum bottles was analyzed each day until 10 days, in a Shimadzu 17A gas chromatograph. A similar set-up of parallel incubation was carried out with sterile soil to link the role of microbes in the redox process. Mean values from the three replicate observations of each treatment at every sampling period were presented.

Microbiological analysis

Methane oxidizers with soluble methane monooxygenase (sMMO) activity from soils were enumerated as described by (Graham et al. 1992). Triplicate plates for each dilution were incubated in vacuum desiccators under the atmosphere of CH_4 (5 %) air mixture by replenishing the headspace atmosphere with CH_4 on every 4 days, for 30 days in an incubator. The colonies that developed a colored complex with naphthalene and O-dianisidine (tetrazotized) were counted positive for CH_4 oxidizers with sMMO. Cultivable ammonium oxidizing bacteria were enumerated by the MPN method (Schmidt and Belser 1982). Appropriate dilutions of suspensions of soils from different treatments were incubated with ammonium medium for 4 weeks. The number of positive tubes in each of the appropriate dilutions was scored and the NH_4^+ - oxidizing population was estimated (Adhya et al. 1996).

Statistical analysis

We performed data analysis to estimate the mean and standard deviation of three replicated samples using Excel software (Microsoft Office, 2007). The Pearson coefficient was estimated to define the correlation between variables and parameters of study. Correlation analyses were performed using R statistical software (R version 2.15.1).

Results

Microbial redox process

The reduction process in the vertisol incubated under aerobic and anaerobic conditions followed the classical sequential reduction process. The microbial-mediated redox process in flooded soil was initiated with NO_3^- reduction, followed by Fe^{3+}, SO_4^{2-} reduction and CH_4 production sequentially. Soil under the anaerobic environment exhibited faster and higher redox metabolic activity than ambient-incubated slurry samples. Under both conditions of head space, NO_3^- reduction started immediately after incubation. Nitrate was present at a concentration of 743 μM initially, and it reduced to 200 μM and 42 μM after 5 days of incubation, and subsequently reduced below the detection limit after 10 days (Fig. 1). Reduced Fe^{3+} content of soil was estimated as equivalent

Fe^{2+} produced over incubation. In both incubation conditions, Fe^{3+} reduction was significantly stimulated during the initial 5 days. Under anaerobic conditions, Fe^{3+} reduction reached maximum at 20 days and then steadily increased to 38.56 μM at 60 days, while in aerobic slurries Fe^{3+} reduction was slow and progressed until 60 days, producing an equivalent of 34 μM Fe^{2+}. SO_4^{2-}content in the soil was 86.43 μM and its reduction started significantly after 20 days and during 5 to 10 days of incubation under the aerobic and anaerobic incubation conditions, respectively. At 60 days, SO_4^{2-} content was very low: only 3 μM was estimated from the ambient incubated slurry, while in the anaerobic slurry its level was below detection. Once SO_4 reduction started, methanogenesis took place in both the conditions; however, CH_4 production was higher in the anaerobic slurry than in the aerobically incubated samples. CH_4 production was initiated after 20 days in anaerobic slurry, while in aerobic incubation methanogenesis started after 40 days of incubation. At 60 days, CH_4 production in the headspace was quantified as 2.86 and 23.50 μg g^{-1} soil in both aerobic and anaerobic slurries, respectively.

CH_4 oxidation during sequential reduction

CH_4 oxidation activity in slurries incubated under the two incubations exhibited differentially over the incubation period. The CH_4 oxidation curves in Figs. 2 and 3 were consistent with the assumption of progressive growth of methane oxidizing bacteria (MOB) populations, a larger population resulting in faster CH_4 oxidation. CH_4 oxidation activity was characterized by calculating the apparent rate constant (k) from the logarithm of the CH_4 concentration in the headspace (Mohanty et al. 2007) during the rapid decline period of incubation (Table 1). The rate k is expressed as μg CH_4 oxidized g^{-1} soil d^{-1} during the period of incubation. In general, the initial aerobic condition stimulated CH_4 oxidation activity in slurries more than the anaerobic incubations. Under aerobic conditions, CH_4 oxidation started soon after incubation and headspace CH_4 was completely oxidized over 12 days (Fig. 2). Interestingly, slurries of 5 days, 10 days and 20 days old exhibited a higher CH_4 oxidation rate (k) than the unreduced fresh slurry samples by stimulating 28.60 %, 32. 25 %, and 32.14 %, respectively. CH_4 oxidation activity was significantly inhibited on slurries of 40 days and 60 days. The CH_4 oxidation curve revealed no apparent effect on the lag phase of CH_4 oxidation in aerobically incubated slurries. Contrastingly, slurries incubated under anaerobic condition were characterized by a lower CH_4 oxidation rate and a prolonged lag period (Fig. 3). CH_4 oxidation rate k also decreased in all the slurry samples, irrespective of reductive period, unlike the unincubated control (Table 1). In fact, incomplete CH_4 oxidation was observed in highly reduced slurries (40 days and 60 days). After 12 days, partial CH_4 oxidation observed with 19 % and 48 % residual CH_4 in the headspace of 40-day and 60-day slurries. The lag phase of CH_4 oxidation was more pronounced in the 40-day and 60-day samples by 3 days and 7 days, respectively.

Role of electron donors on methane oxidation

Electron donors influenced CH_4 oxidation in slurry samples incubated under aerobic conditions, but not under anaerobic condition. Under anaerobic conditions, headspace CH_4 concentration remained unchanged and was at par with that of the initial CH_4 concentration over 10 days of incubation, irrespective of treatments (data not shown). Under ambient aerobic headspace conditions, the electron donors (including the NH_4^+, Fe^{2+}) stimulated microbial CH_4 oxidation process except Na_2S (Fig. 4) than that of unamended control.

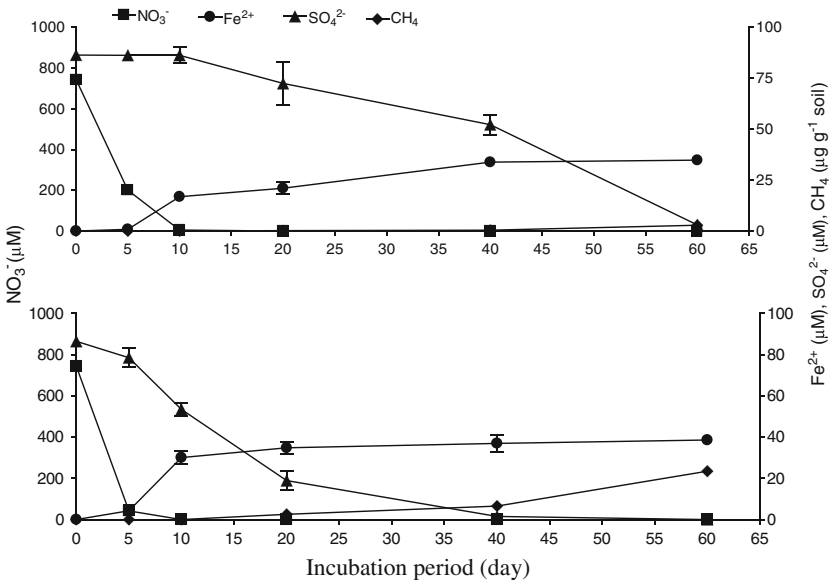

Fig. 1 Redox metabolism during sequential reduction processes in slurry incubated under aerobic (*top*) and anaerobic (*bottom*) conditions. NO_3^- (*black square*), Fe^{2+} (*black circle*), SO_4^{2-} (*black up-pointing triangle*) and CH_4 (*black diamond suit*) were estimated at regular intervals from the incubated slurry samples to determine the time course of soil reduction process. Each data point represents the mean±SD of triplicate slurry samples

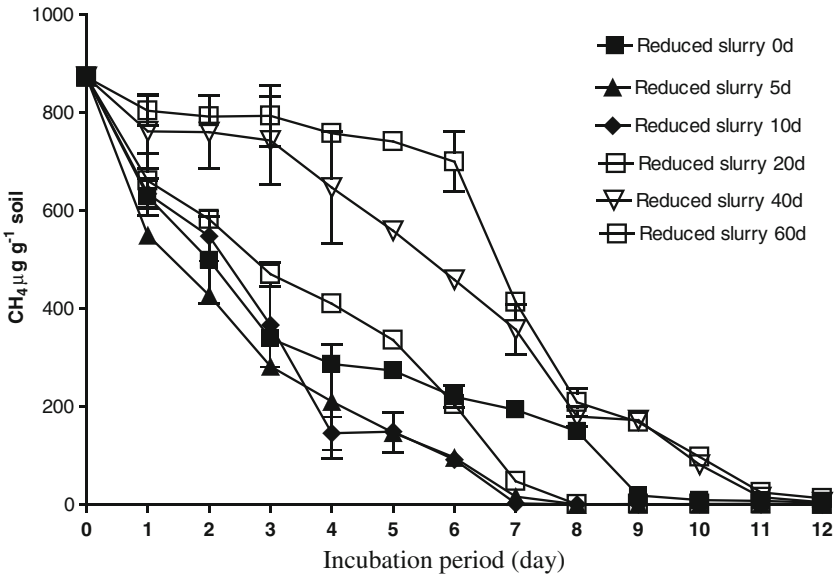

Fig. 2 Methane oxidation potential of slurry samples during different reduction periods under aerobic conditions, i.e., with ambient air in the head space. Each data point represents the mean±SD of triplicate slurries

Stimulation in CH_4 oxidation by both NH_4^+ and Fe^{2+} electron donor was observed during the initial 4–5 days of incubation. S^{2-} , on the other hand, exhibited inhibition of the methane oxidation process during the initial period of incubation. The rate constant (k) derived from the exponential decrease period of headspace CH_4 (Table 3) revealed that electron donors NH_4^+ and Fe^{2+} stimulated k by nearly 70 %, while the electron donor S^{2-} inhibited the rate by 21 % relative to the unamended control slurry.

Microbial population dynamics

Dynamic alterations of microbial population of aerobic methanotrophs and ammonium oxidizers in soil following sequential reduction after flooding were also monitored. Methane-oxidizing bacterial population varied in slurries incubated under aerobic and He flushed anaerobic conditions (Table 2). Sequential soil reduction processes affected the population of methanotrophs differentially under both incubation set-ups. The methanotrophic bacterial population was higher in slurries incubated in aerobic conditions than in the anaerobic conditions. In aerobic slurries, methanotroph populations doubled during the reduction period of 5 days and increased slowly to the highest number at 10 days. Subsequent incubation further decreased methane oxidizer populations by 83 % at 60 days. In anaerobically incubated slurry, methanotroph populations reduced drastically. During 5 days and 10 days, their population decreased to 29 % and 63 %, respectively, compared to the freshly prepared unincubated slurry samples. Further, their population decreased significantly by 89 % and 97 %, respectively, during 40 days and 60 days of flooding. The population of cultivable

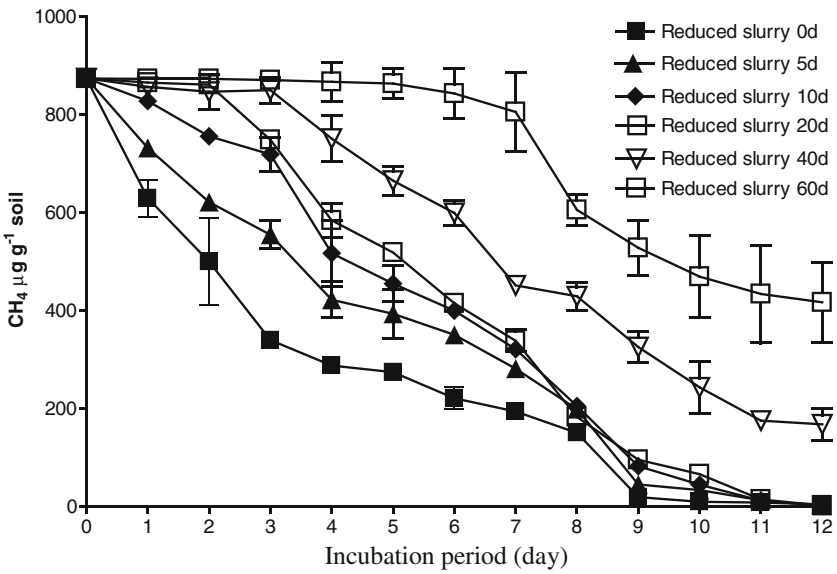

Fig. 3 Methane oxidation potential of slurry samples during different reduction periods under anaerobic conditions, i.e., with pure He in the head space. Each data point represents the mean±SD of triplicate slurries

Table 1 CH_4 oxidation activity of soil incubated under aerobic and anaerobic conditions. Rate constant k is the slope of log scale CH_4 concentration over time during the rapid decline incubation period

Incubation condition	Day of incubation	Apparent rate constant k (μg CH_4 day^{-1} g^{-1} soil)
Aerobic	0	0.46±0.05
	5	0.64±0.02
	10	0.68±0.01
	20	0.68±0.01
	40	0.38±0.01
	60	0.32±0.02
Anaerobic	0	0.46±0.05
	5	0.44±0.02
	10	0.44±0.01
	20	0.42±0.01
	40	0.15±0.02
	60	0.07±0.01

Table 2 Changes in the population of methane oxidizing bacteria and ammonium oxidizing bacteria in flooded soil incubated under aerobic and anaerobic conditions. Microbial population estimated after complete oxidation of head space CH_4. For all samples n=3. Values are means and standard deviations

Incubation period (day)	Methanotrophs with soluble methane monooxygenase (smmo) (10^4CFU g^{-1} soil)		Ammonium oxidizers (10^3 MPN g^{-1} soil)	
	Aerobic	Anaerobic	Aerobic	Anaerobic
0	11.66±1.52	11.66±1.52	0.86±0.10	0.86±0.10
5	21.66±2.51	8.33±3.51	0.43±0.05	0.11±0.01
10	28.33±2.88	4.33±0.60	0.30±0.07	0.06±0.02
20	22.00±2.64	2.66±1.15	0.12±0.03	0.04±0.01
40	7.00±2.64	1.33±0.57	0.05±0.05	0.01±0.00
60	2.00±1.00	0.35±0.26	0.02±0.01	0.01±0.00

ammonium-oxidizing bacteria was initially low, and reduced further following incubations. Ammonium oxidizer populations (Table 2) during sequential soil reduction processes were more pronounced in anaerobially incubated flooded soil.

Discussion

We found that soil samples incubated under flooded conditions followed sequential reduction of terminal electron acceptors. It is well known that under flooded conditions soil undergoes microbially mediated anaerobic respiratory redox processes with alternative electron acceptors being sequentially reduced in the order of NO_3^-, Fe^{3+}, SO_4^{2-} and CO_2 (Delaune and Patrick 1972; Froelich et al. 1979). Slurries incubated anaerobically had pronounced microbial soil reduction activity due to absence of headspace O_2 that often diffuses to increase dissolved O_2 content (Hamilton et al. 1995). The observed sequential reduction process reflects that homogeneity of soil samples maintained during incubation with similar micro- or macroaggregates and carbon content, providing uniform microsites suitable for various functional microbial groups (Chow et al. 2002; Tanji et al. 2003). However, we also found the overlapping redox process in anaerobially maintained slurry samples during the reduction processes. Fe^{3+}, SO_4^{2-} reduction, and methanogenesis were taking place simultaneously. It is presumed that microbial groups responsible for specific redox processes also tend to overlap, leading to unclear temporal distinction

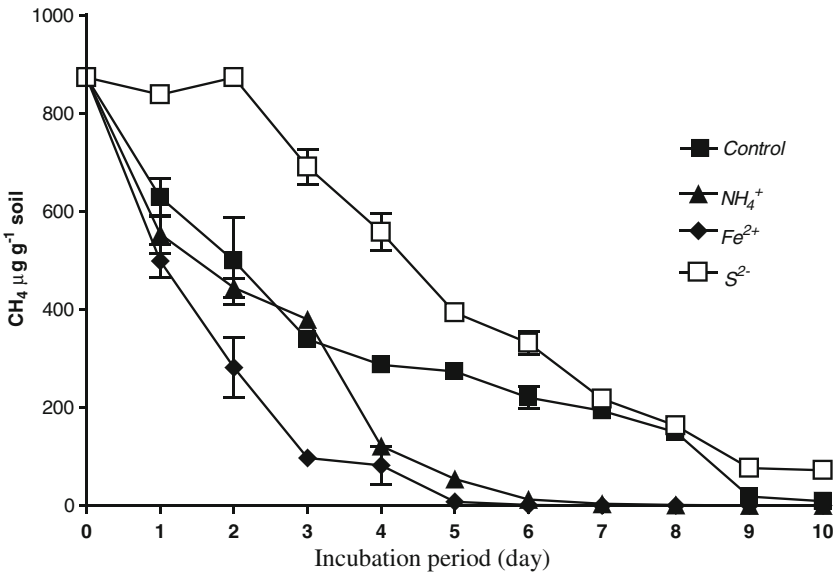

Fig. 4 Effect of electron donors on CH_4 oxidation in soil incubated under flooded condition. Slurry samples were amended with electron donors (NH_4Cl, $FeCl_2$, Na_2S) at 0.1 M. Each data point represent mean and SD of three replicate observations

in the activity of NO_3^- reducers, Fe^{3+} reducers, SO_4^{2-} reducers, and CH_4 producers in that many species of anaerobes that reduce other terminal electron acceptors are capable of Fe^{3+} reduction. It is reported that many NO_3^- reducers are Fe^{3+} reducers, and even some SO_4^{2-} reducers are methanogens which also can reduce Fe^{3+} (Coleman 1993; Lovley et al. 1993). The main objective of this research was to define CH_4 oxidation in a flooded soil system's reductive phases, and our results showed that the microbial CH_4 oxidation process was differentially activated. We found that CH_4 oxidation activity was higher during early reductive phases when NO_3^- and Fe^{3+} reduction was taking place, but CH_4 oxidation activity was inhibited in slurries in the later period of SO_4 reduction as well as methanogenesis. CH_4 oxidation is an aerobic processes and O_2 concentration mostly regulates methanotrophs (Frenzel et al. 1992; Holzapfel-Pschorn et al. 1985; Mohanty et al. 2006). In saturated flooded soil, CH_4, O_2, NH_4 are proximal factors controlling CH_4 oxidation (Schimel et al. 1993). Populations of methanotrophs and ammonium oxidizers were highly correlated with the CH_4 oxidation rate (k) indicating that methanotrophs mediated the CH_4 oxidation process during the sequential reduction processes. Pearson's product moment analysis revealed significant correlations between the methanotrophic bacterial population and the rate constant k for both ambient (r=0.994, p=3.579e-09) and anaerobic conditions (r=0.703, p=0.001128). Comparatively, ammonium oxidizers were less significantly correlated to k under aerobic (r=0.184, p=0.4634) and anaerobic (r=0.441, p=0.0666) conditions. A similar significant correlation between methanotrophic bacterial population and CH_4 oxidation rate k has been found earlier (Adhya et al. 2000; Bharati et al. 2000). Significant correlations between methanotrophs and ammonium oxidizers were found in the slurry samples incubated under ambient (r=0.15, p=0.436) and anaerobic (r=0.773, p=0.00016) conditions. This indicated that both the microbial groups are influenced by the redox metabolites in the flooded soil ecosystem.

To understand the processes regulating CH_4 oxidation during reduction phases, follow-up experiments were conducted with amendment of electron donors. Slurry samples were amended with NH_4Cl, $FeCl_2$, Na_2S, and CH_4 oxidation rate and methane-consuming microbial population change was estimated under both ambient headspace and He-flushed anaerobic conditions, as described before. A parallel experiment also carried out using the sterile autoclaved soil to find if the CH_4 oxidation process was microbially mediated. Results were explanatory, and to our observation CH_4 oxidation in flooded soil was stimulated by amendment of the electron donors NH_4^+, and Fe^{2+} (Fig. 4). Electron donor S^{2-} (Na_2S), on the other hand, inhibited the CH_4 oxidation process. The rate constant of CH_4 oxidation was stimulated by 90 % in both NH_4^+ and Fe^{2+} amendments, while S^{2-} inhibited the k value by 32 % over the unamended control (Table 3). The methane-consuming bacterial population was differentially enumerated in the different electron donors. Slurries amended with NH_4^+ and Fe^{2+} stimulated the methane-oxidizing bacterial population by about 2 and 3 times, while S^{2-} was inhibited by 10 times compared to the unamended control (Table 3). CH_4 oxidation rates were not stimulated in slurry samples incubated under anaerobic conditions (data not shown). No CH_4 oxidation was found in sterile soil, which revealed that the decrease in headspace concentration was mediated by methane oxidizers, and the electron donors indeed stimulated methanotrophic bacterial activity. Pearson's product moment analysis revealed a significant positive correlation (r=0.925, p=1.602e-05) between k and the population of methanotrophs in the slurry samples. Significant correlation revealed that electron donors regulate CH_4 oxidation process by influencing the methanotrophic bacterial population.

Our study defined a mechanistic process of electron donors on the growth and activity of methanotrophs under reduced flooded soil ecosystem. NH_4^+ stimulates CH_4 oxidation by favoring methane and ammonium oxidizers (Bedard and Knowles 1989; Bodelier and Frenzel 1999). NO_2 produced during NH_4^+ oxidation also found to favor Type I, methylomicrobium album over type II methanotrophs (Nyerges et al. 2010). The effect of iron ions on particulate methane monooxygenase has been studied with *Methylosinus trichosporium* OB3b and the metal ions, ferric, ferrous and cupric ions stimulated activity, as these ions are essential for enzymatic activity (Takeguchi et al. 1999). The influence of iron on methane monooxygenase activity can be explained as some of the enzyme preparations of pMMO contain iron, and it has been proposed that the active site is a dinuclear iron center (Smith et al. 2011). Even the soluble sMMO active site is a carboxylate-bridged di-iron centre and the catalytic cycle has been studied extensively (Rosenzweig 2008). It is well known that under flooded condition the common inorganic redox species, NO_3^-, SO_4^{2-} and CO_2 are soluble while Fe^{3+}

Table 3 CH_4 consumption potential (k) and methane oxidizing bacterial population (CFU) in slurries incubated with different electron donors (NH_4Cl, $FeCl_2$, Na_2S). For all samples n=3. Values are means and standard deviations

Electron donors (added to slurry)	Rate constant k (μg CH_4 g^{-1} $soil^{-1}$)	Methanotrophs population (10^4 CFU g^{-1} soil)
None	0.46±0.05	11.66±1.52
NH_4^+	0.78±0.02	24.00±5.29
Fe^{2+}	0.77±0.02	28.00±6.56
S^{2-}	0.36±0.04	1.67±1.15

and Mn^{4+} are largely solid and capable of recycling and redistribution, effective for higher enzyme activity (Ratering and Schnell 2000; Zhang et al. 2009). However, in the case of electron donors like S^{2-}, the inhibition of CH_4 consumption might have been caused due to its toxicity effect and also by the reaction of S^{2-} with metal species to form metal sulphides (MS^-) (Elliott et al. 1998; Rittle et al. 1995). Under anaerobic conditions, MS^- gets precipitated, thus the FeS^- immobilized to reoxidize. MS- are extremely low in solubility (Hao et al. 1996; Moore et al. 1988; Zhang and Millero 1994), formation of MS^- minimizes its reaction with headspace O_2 resulted into lower CH_4 oxidation rate. MS^- are known for their inhibition effect on soil microorganisms important for biogeochemical cycling like nitrifiers (Joye and Hollibaugh 1995) and anaerobes (McCartney and Oleszkiewicz 1991). No reports have been published on the impact of sulfides or MS^- on methanotrophs; therefore, further investigation on the impact of reduced S species on methanotrophic activity are needed to define the mechanistic process of interaction.

Conclusion

CH_4 oxidation in flooded soil is microbially mediated, and it varies with soil reduction processes. Ambient O_2 in flooded soil ecosystems plays an important role in the differential response of methane oxidizers and the redox process. CH_4 oxidation is stimulated during the early phase of flooding, particularly during NO_3^- and Fe^{3+} reduction, and is inhibited during SO_4^{2-} reduction and the methanogenic period. Reduced inorganic electron donors like NH_4^+, Fe^{2+} stimulate CH_4 oxidation by stimulating the microbial groups involved in CH_4 oxidation. The results of the present study provide evidence of complex microbial interactions during sequential redox processes in the presence of CH_4. Soil parameters like nitrogen content and iron minerals can influence CH_4 oxidation during flooding. On account of the future climatic scenario with elevated global temperatures, higher precipitation, and increased areas of wetter land, we conclude that temporal variation of the CH_4 oxidation activity in a flooded soil ecosystem should be taken into account. However, there is need for further studies to understand the detailed mechanism of the interaction between redox metabolic processes, anaerobes, and methane oxidizers, in order to predict ecosystem effects and to mitigate global climate change.

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