MICROBIAL ECOLOGY OF METHANE EMISSION IN RICE AGROECOSYSTEM: A REVIEW

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Abstract. Methane has profound impact on the physico-chemical properties in atmosphere leading to global climate change. Out of the various sources of CH_4, rice fields are the most significant contributors. The processes involved in the emission of CH_4 from rice fields to the atmosphere include CH_4 production (methanogenesis) in the soil by methanogens, methane oxidation (methanotrophy) by methanotrophs and vertical transfer of CH_4 via plant transport and diffusion or ebullition. In the overall methane dynamics rice plants act as : a) source of methanogenic substrate, b) conduit for CH_4 through well developed system of inter cellular air space (aerenchyma), and c) potential methane oxidizing micro-habitat in the rhizosphere by diffusing oxygen which favour the growth and multiplication of methanotrophs. Apart from mechanistic uncertainties, there are several other uncertainties in the estimation of CH_4 flux. Methane dynamics in the paddy field is controlled by a complex set of parameters linking the biological and physical characteristics of soil environment like temperature, carbon source, Eh, pH, soil microbes and properties of rice plants, etc. It has now become possible to isolate, detect and characterize the methanogens and methanotrophs by using molecular biological tools like PCR, FISH, etc. techniques. The apparent half saturation constant (K_m) and maximum oxidation rate (V_{max}) are distinctive parameters which determine the ability of bacteria to survive on atmospheric methane.
Keywords. *methane; methanotrophs; methanotrophy; methanogens; molecular tools*

Introduction

Atmospheric methane (CH_4) is a potent greenhouse gas with high absorption potential for infrared radiation. Methane is present at about 1.8 ppmV in the atmosphere [135]. During the last 20 years, its concentration has been increasing, on an average at the rate of 0.8% y^{-1} [125]. Due to this, CH_4 is of great concern as a greenhouse gas. Although the tropospheric CH_4 concentration is very low as compared to CO_2 (357 ppmV), methane accounts for 15 to 20% of global warming [71]. The total annual source strength of all methane emissions from anthropogenic origin is estimated to be 550 Tg [133]. Major sources of this input include natural wetlands, rice fields, enteric fermentation in animals, termites and land fills. The contribution from rice cultivation is estimated to range from 20-100 Tg CH_4 y^{-1} with an average of 60 Tg CH_4 y^{-1} [71]. The biogenic methane is mostly produced by methanogenic archaea (methanogens) in anaerobic environments i.e. sediments and flooded rice fields [59]. Each year methanogens produce about 400 million metric tons of CH_4 [49]. Recent studies have shown that methane is not only produced in anoxic rice fields soil but also directly from the roots of rice plants which are inhabited by a methanogenic community different from that in the rice field soil [92].

According to a current estimate, rice production will need to expand by around 70% over the next 25 years to support the growing human population [39]. For this, intensified global fertilizer application will be essential, and this will exacerbate the methane problem. It is projected that the methane emission from rice cultivation may increase from the 1990 level of 97 Tg y^{-1} to 145 Tg y^{-1} by 2025 [5]. India is an important rice producing country, comprising 28.6% of world rice cultivated area [65]. During recent years, studies on methane emission from Indian rice fields have focused on the influence of soil type, season, water regime, organic inputs, fertilizers, rice cultivars and agrochemicals [111]. Using the baseline scenario, annual methane emissions for China, India, Indonesia, Philippines and Thailand were calculated to be 3.73, 2.14, 1.65, 0.14 and 0.18 Tg CH_4 y^{-1}, respectively [106].

Chemical and biological processes consume methane in the global methane cycle. The only known biological sink for atmospheric methane is its oxidation in aerobic soils by methanotrophic bacteria, this may contribute up to 10-20% to the total methane destruction [128], or between 15 and 45 Tg CH_4 y^{-1} [71]. Methanotrophs oxidize CH_4 with the help of methane monooxygenase (MMO) enzyme. These bacteria are classified into three groups (Type-I, Type-II and Type –X) based on the pathways used for assimilation of formaldehyde and other physiological and morphological features [58]. An enormous effort is being made worldwide by microbial ecologists to isolate, detect and characterize methanotrophs and methanogens in different rice ecosystems by using molecular biological tools and techniques [18, 46, 57]. Methanotrophy is an aerobic process [52], but in marine sediments and in some saline inland waters it could be anaerobic [36]. The apparent half saturation constant (K_m), and maximum rate (V_{max}) of CH_4 oxidation are characteristic parameters, which determine the ability of methanotrophs to grow on atmospheric CH_4 [31]. Several workers have reported that methane oxidation occurs in rice microcosm [56], wetland rice [40, 158] and dryland rice fields [44, 45]. Methane oxidation in rhizospheric soil is considered as an important sink for CH_4 [44, 45, 56]. Therefore, the knowledge of several environmental factors (e.g. temperature, fertilizer inputs, crop phenology and soil moisture) that can provide feedback on the capacity of soil to oxidize atmospheric CH_4, may have significant consequences on the global atmospheric CH_4 budget.

This review presents an overview of the underlying microbial basis for production, oxidation and emission of methane in paddy fields under the influence of several environmental factors. Molecular ecological approaches for the isolation, detection and characterization of methanogens and methanotrophs are also described.

Production of methane

Methanogens

Methanogens are strictly anaerobic unicellular organisms originally thought to be bacteria but now recognized as belonging to a separate phylogenetic domain, the *archae* [53]. Phenotypic characteristics of methanogenic bacteria are listed in Table 1. 16S rRNA analysis suggested that methanogens can be categorized under three groups. Group I comprises *Methanobacterium* and *Methanobrevibacter,* Group II contains *Methanococcus,* and Group III comprises the genera including *Methanospirillum* and *Methanosarcina* [53]. They proliferate in anaerobic fresh water environments, such as sediments and the digestive tract of animals [147].

Table 1. *Characteristics of methanogenic and methanotrophic bacteria (Source: [53, 58, 74, 101]).*

Characteristics	Methanogens	Methanotrophs
Cell form	rods, cocci, spirilla, filamentous, sarcina	rods, cocci, vibrios
Gram stain reaction	Gram +/–	Gram –
Classification	Archaebacteria	Eubacteria
Cell wall	pseudomureine, protein, heteropolysaccharide	peptidoglycon
Metabolism	anaerobic	aerobic
Energy and carbon source	H_2+CO_2; H_2+methanol; formate; methylamines; methanol, acetate	methane; methanol; dimethyl-ether, methyl formate, dimethyl carbonate
Catabolic products	CH_4 or CH_4+CO_2	CO_2
TCA cycle	Incomplete	Incomplete (Type-I) or complete (Type-II)
Carbon assimilation pathways	TCA cycle, gluconeogenesis	ribulose monophosphate pathways (Type-I) or serine pathways (Type-II)
Resting cells	-	cysts (Type-I) or exospore (Type-II)
GC content mol%	26-60	50-62.5
Typical species	*Methanobacterium bryanthii* *Methanobrevibacter smithii* *Methanomicrobium mobile* *Methanogenium cariaci*	*Methylosinus trichosporium* *Methylomonas methanica* *Methylocystis minimus* *Methylobacter albus*

In these habitats, methanogens play an important role in the degradation of complex organic compounds. Most methanogens are mesophilic, able to function in temperature ranging from 20 to 40^0C [147]. They are also found in extreme environments like hydrothermal vents where they thrive at temperatures above 100^0C. Methanogens mainly use acetate (contributes about 80% to CH_4 production) as a carbon substrate but other substrate like H_2/CO_2 and formats also contribute 10-30% to CH_4 production [27]. All methanogens use NH_4^+ as a nitrogen source, although the ability to fix molecular nitrogen and the *nif* gene is present in all the three orders (*Methanobacteriales, Methanococcales and Methanomicrobiales*) of methanogens [120].

Methanogenesis

Methane is produced in the anaerobic layers of paddy soil by bacterial decomposition of organic matter [39]. The organic matter converted to CH_4 is derived mainly from plant-borne material, and organic manure [35], if applied. The anaerobic degradation of organic matter involves four main steps: a) hydrolysis of polymers by hydrolytic organisms, b) acid formation from simple organic compound by fermentative bacteria, c) acetate formation from metabolites of fermentations by homoacetogenic or syntrophic bacteria, and d) CH_4 formation from H_2/CO_2, acetate, simple methylated

compounds or alcohols and CO_2 [163]. CH_4 is produced in rice fields after the sequential reduction of O_2, nitrate, manganese, iron and sulphate, which serve as electron acceptors for oxidation of organic matter to CO_2 [164]. Methanogenesis from all substrates requires a number of unique coenzymes, some of which are exclusively found in methanogens [98]. At least nine methanogen-specific enzymes are involved in the pathway of methane formation from H_2 and CO_2 [140]. In paddy soil, acetate and H_2 are the two main intermediate precursors for CH_4 formation [162].

Methanogenesis from H₂/CO₂

The first step of the pathway comprises the binding of CO_2 to methanofuran (MFR) and its H_2 dependent reduction to formyl-MF (first stable intermediate compound of the pathway). Formation of this complex is catalyzed by *formylmethanofuran dehydrogenase*. Further, the formyl moiety of the formyl-MF is subsequently transferred to tetrahydromethanopterin (H_4MPT), which with H_4MPT *formyl transferase,* formes methenyl-H_4MPT, while reduces to methylene-H_4MPT and then to methyl-H_4MPT. In both reactions, reduced coenzyme F_{420} serves as the reductant. The F_{420}-dependent reduction of methenyl-H_4MPT is reversible and is catalyzed by *methylene H_4MPT dehydrogenase*. The reversible F_{420} H_2-dependent reduction of methylene - H_4MPT to methyl-H_4MPT is catalyzed by *methylene-H_4MPT reductase.* In the next step of CO_2 reduction pathway, the methyl group is transferred from N-5-methyl H_4MPT to coenzyme M (2-mercaptoethane sulfonate) giving rise to methyl coenzyme M(methyl-CoM). The reduction of methyl CoM is catalysed by the methyl-CoM-reductase. This reaction involves two unique coenzymes. The first one is N-7-mercaptoheptanoylthreonine phosphate (HS-HTP) which acts as electron donor in the reduction of methyl CoM giving rise to CH_4 and mixed disulfide of HS-CoM and HS-HTP (CoM-S-S-HTP). The second coenzyme involved in this reaction is factor F_{430} (a nickel porphinoid) which is the characteristic prosthetic group of the methyl reductase [16, 74, 140].

Methanogenesis from acetate

Methanogenesis from acetate starts with its activation to acetyl-CoA. *Methanosarcina* and *Methanothrix* use different ways of acetate activation. The former organism takes advantage of *acetate kinase* and *phosphotans acetylase* whereas the later makes use of *acetyl-CoA synthatase.* All three enzymes are soluble and oxygen insensitive. Further breakdown of *acetyl- CoA* catalyzes the cleavage of *acetyl-CoA,* giving rise to a methyl group, a carbonyl group and CoA, all of which are transiently bound to the enzyme. In a further step, the Co-*dehydrogenase* complex catalyzes the oxidation of the carbonyl group. The CO_2 is thereby formed and CoA is released from the enzyme, where the methyl group is transferred to a corrinoid-Fe-S protein. This complex catalyzes not only the cleavage of *acetyl-CoA* and oxidation of the carbonyl group but in addition, the transfer of the methyl moiety to H_4MPT. Further pathway from methyl H_4MPT to CH_4 takes advantage of the pattern similar to that discussed for the utilization of the CO_2/H_2 [16, 98].

Thermodynamics of CH₄ production

The process of methane production in paddy fields is thermodynamically exergonic [163]. The Gibbs free energy (ΔG) for the process is mainly a function of the acetate concentration and H_2-partial pressure. A pre-requisite for early methane production

seems to be a sufficiently high H_2-partial pressure that corresponds to ΔG of H_2 dependent methanogenesis (hydrogenotrophic) of less than about -23 kJ mol^{-1} CH_4 [162]. The time until the onset of CH_4 production and the magnitude of production is a function of the quantity of easily degradable organic matter, reducible Fe(III) and sulfate [162]. Methanogens are energetically limited by availability of their substrates H_2 and acetate as long as iron or sulfate reducers are able to compete for them [1]. The methanogens have to compete for available substrates with other anaerobic bacteria, namely the nitrate, manganese, ferric iron and sulfate reducers. The competition for carbon substrates in general follows thermodynamic rules: nitrate reducers outcompete the other anaerobic bacteria for the substrates [149]. Several studies have reported different ΔG values for methanogenesis in various paddy fields. For Italian paddy fields the values of ΔG for methane production were found to be -31.6 to 34.8 kJ mol^{-1} CH_4 [1] and -24 to -38 kJ mol^{-1} CH_4 [27]. Peters and Conrad [123] found that ΔG ranged from -25 to -50 kJ mol^{-1} CH_4 for German rice fields. Cultures of *Methanobacterium bryantii* required ΔG values of less than -30 kJ mol^{-1} CH_4 for CH_4 production [31]. Kral et al. [86], reported that for cultures of other methanogens ΔG values varied between -32 and -60 kJ mol^{-1} CH_4 for hydrogenotrophic methanogenesis. Yao and Conrad [162] calculated the ΔG for acetate dependent methanogenesis (acetoclastic) and concluded that unlike H_2-dependent methanogenesis acetate based process was apparently not under thermodynamic control. They found that CH_4 production was less at ΔG equal to -26 kJ mol^{-1} CH_4 and was the average at ΔG equal to - 29 kJ mol^{-1} CH_4. Roy et al. [130] have suggested that early CH_4 production is due to H_2-dependent methanogenesis and that acetate-dependent methanogenesis only starts later when sulfate and Fe(III) have been reduced in paddy soils. Further, methane production from H_2/CO_2 is not started before fermentation has increased the H_2 partial pressure to an amply high value to allow exergonic production of CH_4 at a ΔG of less than about - 26 kJ mol^{-1} CH_4 [130, 162].

Methanogenic population and CH$_4$ emission

The mechanism of methanogenesis in paddy fields worldwide has been investigated in detail. However, information regarding methanogenic population size in rice fields is limited. Rajagopal et al. [126] were the first to carry out isolation and characterization of methanogens from Louisiana paddy fields and reported the presence of two *Methanobacterium*-like strains and two *Methanosarcina*-like strains. Joulian et al. [76] recorded the methanogenic populations from the paddy fields of France, the Philippines, and USA. Their results of the classic counts of methanogenes, and strains isolated and identified by l6S rRNA gene sequencing, suggested the dominance of *Methanobacterium spp.* and *Methano-sarcina spp.* among the culturable organisms. Reichardt et al. [129] revealed that methanogens were abundant in root extracts of mature rice plants. Methanogens are also exist on the rhizoplane of the rice plants [92]. Fetzer et al. [50] isolated four genera (*Methanobacterium, Methano-sarcin, Methanobrevibacter* and *Methanoculleus*) from Italian rice fields. According to Asakawa et al. [6] there are only two strains (*Methanobrevibacter arboriphilus* and *Methanosarcina mazeii*) of methanogens in rice fields which have been identified to the species level. Adachi [2], isolated *Methanobacterium* and *Methanobrevibacter spp.* from subtropical Japanese rice fields. Kudo et al. [87] reported the presence of *Methanosarcina, Methanogenium, Methanosaeta* and *Methanoculleus*-like organisms in rice paddy fields of Japan by using a molecular retrival approach with archael small

subunit (SSU) rRNA encoding gene (rDNA) sequences. Results of investigations on methanogenic population size in various types of rice fields are summarized in Table 2.

Table 2. *Population size of methanogens and methanotrophs in various rice fields.*

Ecozones/location	Methanogens	References	Methanotrophs	References
Rice fields (Australia, France, Philippines, USA, Trinidad)	$<10^1$–2.3×10^6 cells g^{-1} dw	[75]	1.5×10^3–3.5×10^7 cells g^{-1} dw	[75]
Rice fields (Zhejiang, China)	4.6×10^3–1.3×10^7 CFU g^{-1} dw	[108]	3.0×10^6-2.3×10^8 CFU g^{-1} dw	[108]
Rice fields (Beijing, China)	1.4×10^5 – 2.3×10^5 g^{-1} dw	[64]	-	-
Rice fields (Germany)	-	-	3.7×10^4–3.8×10^7 bacteria g^{-1} dw	[13]
Rice fields (Cuttack, India	$0.2 – 2.8 \times 10^5$ MPNg^{-1} soil	[90]	4.2×10^4–5.2×10^6 CFU g^{-1} dw	[89]
Rice fields (Cuttack, India)	2.4–6.1×10^6 MPN g^{-1} dw	[88]	-	-
Rice fields (Cuttack, India)	$5.4 – 7.5 \times 10^3$ cells g^{-1} dw	[15]	-	-
Dryland Rice fields (Varanasi, India)	-	-	5.3×10^6–7.4×10^7 cells g^{-1} soil	[42]
Wetland Rice fields (Varanasi, India)	-	-	2.6×10^6–5.0×10^7 cells g^{-1} soil	[43]
Rice fields (Northern Italy)	-	-	4.2×10^6–2.3×10^7 cells g^{-1} dw	[10]
Rice fields (Narthern Italy)	-	-	4.0×10^5-2.0×10^8 cells g^{-1} dw	[56]
Rice fields (Japan)	5.2×10^4–1.1×10^6 cells g^{-1} dw	[151]	1×10^4–1×10^7 CFUg^{-1} dw	[157]

Table 3. *Variation of estimates of global methane emission (Tg CH$_4$ y^{-1}) from rice fields during 1963-1998.*

CH$_4$ emission Tg CH$_4$ y^{-1}	References
190	[85]
280	[48]
59	[28]
95	[78]
35-59	[138]
120-200	[32]
70-170	[62]
142-190	[14]
47-145	[136]
25-60	[116]

Table 3. continued from page 6.

CH$_4$ emission Tg CH$_4$ y^{-1}	References
20-100	[68]
66	[79]
50	[114]
20-150	[124]
60-105	[70]
20-100	[71]
25-54	[132]
30-50	[117]
50-80	[93]

The first measurements of CH$_4$ emission from paddy fields were conducted in California by Cicerone and Shetter [28].This was followed by extensive studies in Spain [138], China [26], USA [133], Japan [157], Philippines [115], and India [110, 139], but the estimates of methane emissions vary widely, e.g., from 18.0 to 27.1 mg CH$_4$ m^{-2} h^{-1} in Indonesia [119], 19.5 to 32.2 mg CH$_4$ m^{-2} h^{-1} in Thailand [160] and 19 to 79 mg CH$_4$ m^{-2} h^{-1} in Philippines; CH$_4$ emission from temperate rice fields is relatively lower (6.67 and 18.25 mg CH$_4$ m^{-2} h^{-1}) [136]. These field experiments varied widely in the methodologies (closed chamber with automatic or manual sampling devices), sampling frequencies (continuous or sporadic sampling), observation periods (one season or several consecutive years) and sampling field designs (randomized plots or single fields). Recent estimates indicated that methane release (m^{-2}y^{-1}) from different rice ecosystems follows the order deepwater> irrigated> rainfed rice [115]. The distinction among irrigated, rainfed, and deepwater rice fields is a common feature of the available statistics of rice cultivated area. A specific assessment of these ecosystems will, therefore, directly improve the accuracy of regional and global estimates of the methane source strength [67]. The estimates on global CH$_4$ emission from paddy fields, however, have varied over time period, mainly depending on approach, technique and database used for extrapolation. Table 3 shows reported estimates of global methane emission rates from various rice fields during 1963-1998. These have varied from as low as 12 Tg CH$_4$ y^{-1} to as high as 200 Tg CH$_4$ y^{-1}. This is largely due to the lack of uniformity in the methods used for collecting field data, location of experimental sites and sesional variations.This problem was addressed by Khalil and Shearer [79] who developed an inventory of direct flux measurements from a number of studies and modified the information from Matthews et al. [105] on the duration of growing seasons to estimate global and regional annual methane emission rates. They arrived at a figure for the global CH$_4$ emission rate of 66 Tg CH$_4$ y^{-1}.

Pathways of methane emission

The net amount of CH$_4$ emitted from soil to the atmosphere is the balance of two opposite processes - production and oxidation. Methane, the product of methanogenesis, escapes to the atmosphere from soil via aerobic interfaces where CH$_4$ oxidation takes place. There are three pathways of CH$_4$-transport into the atmosphere – molecular diffusion, ebullition and plant transport (Fig. 1).

In the temperate rice fields more than 90% of the CH$_4$ is emitted through plant transport [136] while in the tropical rice fields, significant amounts of CH$_4$ may evolve

by ebullition (gas transport via gas bubbles) in particular during the early period of the season and in the case of high organic input [38].

Ebullition is also the common and significant mechanism of CH_4 flux in natural wetlands [155]. According to Sass et al. [131], ebullition can play significant role in CH_4 transport under high organic fertilization. If soil is unvegetated or plant aerenchyma is not yet well-developed, ebullition plays a major role in CH_4 emission [22] but it occurs only at surface layer and its rate is regulated by CH_4 concentration, temperature, soil porosity and plant aerenchyma [94].
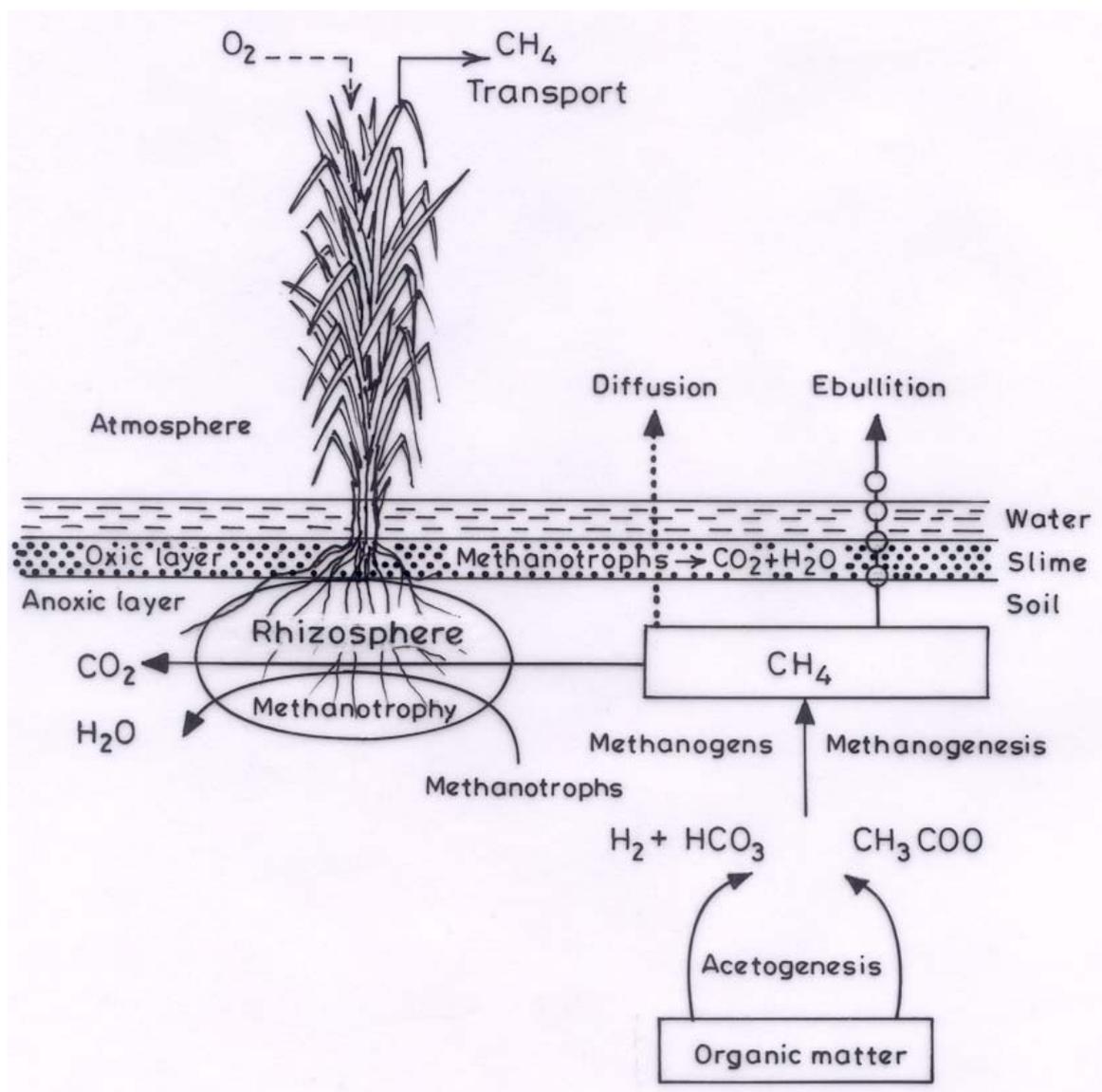
Figure 1. *Conceptual schematic diagram of methane production, oxidation and emission from paddy field.*

Diffusion of CH_4 across the flooded soil and overlying water of the rice field to the atmosphere is a function of surface-water concentration of CH_4, wind speed and CH_4 supply to the surface water [137]. CH_4 diffusion through the soil is a very slow process because the 'diffusion rate' of gaseous CH_4 is very low in liquid phase (about 104 times

slower than diffusion through the gas phase), therefore, it hardly contributes to the total CH_4 flux [8].

Plant mediated transport is the primary mechanism for the CH_4 emission from paddy fields, and contributes 60-90% to the total CH_4 flux [154]. Methane in the soil-water surrounding the roots dissolves into the surface-water of the roots, diffuses into the cell-wall water of root epidermis cells, and then diffuses through the cell-wall water of the root-cortex, depending upon the concentration gradient between the soil-water surrounding the roots and the lysigenous inter-cellular spaces in the roots [118]. Methane is then gasified in the root cortex and transported to the shoots via lysigenous intercellular spaces and aerenchyma. Eventually, CH_4 is released primarily through the micropores in the leaf sheath of the lower leaf position and also through the stomata in the leaf blade [118].

Factors affecting methane emission

Methane emission from paddy fields is controlled by a complex set of parameters linking the physical and biological characteristics of soil environments with specific agricultural practices. Methane production depends on the soil organic carbon content and quality, texture, Eh, pH, Fe content, sulfate content and salinity and application of fertilizers, etc.

Soil pH. Eh and texture

Methane production in flooded rice soils is very sensitive to pH with an optimum range between 6.7 and 7.1 [152]. Effect of soil pH on CH_4 production varied by about two orders of magnitude in four different Indian soils but was found to be maximum at pH around 8.2 [121].

Yagi and Minami [161] reported that values of redox potential (Eh) varied from -100 to -200 mV for the initiation of CH_4 production in paddy soils. Masscheleyn et al. [104] incubated rice soil under controlled redox levels ranging between -250 and +500 mV. They found the threshold for methane production to be -150 mV. Some suggested that soils containing greater amounts of readily decomposable organic substrates (acetate, formate, methanol, methylated amines, etc.) and low amounts of electron acceptors (Fe^{3+}, Mn_4^+, NO_3^-, SO_4^{2-}) are likely to show high production of CH_4. According to sequential oxidation -reduction order, molecular O_2 is the first to be reduced at an Eh of about +30 mV followed by NO_3^- and Mn_4^+ at 250 mV, Fe^{3+} at + 125 mV and SO_4^{2-} at -150 mV (Patrick, 1981). Subsequent to SO_4^{2-} reduction, methanogens will start producing methane [8].

As texture determines various physico-chemical properties of soil, it could influence CH_4 production indirectly. Jackel et al. [72] found that rates of CH_4 production increased when the aggregate size of the soil increased. A negative co-relationship between CH_4 emission and clay content was reported by Sass and Fisher [133]. Seasonal CH_4 emissions indicated a negative relationship to clay content for Texan paddy soils [133].

Temperature

Methane emission is much more responsive to temperature. Temperature not only has an effect on methane production itself but also has an effect on the decomposition of

organic materials from which the methanogenic substrates are produced [27]. The influence of temperature on CH_4 production rates has been reported for several rice ecosystems [121, 145]. Wassman et al. [156] observed a faster development of CH_4 production rate and higher maximum value with increasing temperatures between 25 and 35°C. Hattori et al. [59] recorded optimum temperature of 40°C for CH_4 production in Japanies paddy fields due to dominance of methanogenic population at this temperature.

Growth period and crop phenology

Wassmann et al. [154] recorded lower CH_4 fluxes in the early growth period of rice plant, which increased gradually during mid to late season and dropped to very low level before or after harvest. Jermsawatdipong et al. [73] found that more than 50% of CH_4 was emitted in the first half of the growth period in Thailand rice fields, while CH_4 emissions in Japanese rice fields occurred mainly in the second-half of the growth period [81]. Jermsawatdipong et al. [73] argued that the high temperatures from the beginning of rice growth in the tropics caused the main decomposition stage of soil and applied organic materials to shift to early growth stage which resulted in active CH_4 production from the very beginning of rice growth. Seiler [138] observed maximum CH_4 emission at the end of heading and flowering stage off rice plants in Spain.

Flowering period is generally considered as the peak period for methane emission. The peak emission value remains for a period of 10-15 days in the crop duration of 90-100 days. According to Holzapfel-Pschorn et al. [62] this period emits 90% of the total, methane during the whole crop season, because the biomass of rice crop increases gradually, reaching the maximum weight by flowering. Up to 50 % of the total methane emission from rice fields can be due to root exudation [35]. Methane emission decreases after flowering because the rate of photosynthesis declines after the commencement of grain development and hence the supply of available assimilates for methane production decreases [142].

Diurnal and seasonal variations

Emission rates of CH_4 generally increase rapidly after sunrise, reach a peak in the early afternoon then decline rapidly and level off at night. Methane emission rates during the early and late phase of plant growth varied with a distinct maximum in the early afternoon, while this variation pattern is less pronounced in the middle stage of plant growth [8]. Buendia et al. [21] reported that diurnal patterns of CH_4 fluxes are relatively similar across study sites in same climates and depend on crop phenology. Three seasonal maxima were found in Italy, the first shortly after flooding, the second during the vegetative growth stage and third during the grain filling and maturity stage of rice plants [136].

Rice cultivars, organic manures and crop residues

Rice cultivars have received high research priority because high yielding rice cultivars with low CH_4 emission rates can be easily extended to farmer's fields without any additional input and management practices [151]. Wang et. al. [153] argued that, cultivars influence the CH_4 emission by providing the soil with root exudates, decaying root tissues and leaf littre while Aulakh et al. [7] found significant variations in methane

transport capacity of different rice cultivars. In Korean rice cultivars, the CH_4 flux among the rice varieties ranged from 36.9 g CH_4 m^{-2} to 76.0 g CH_4 m^{-2} [141]. The amendment of organic matter (cattle manure, pig manure, chicken manure, etc.) to a flooded rice field, increases CH_4, production. It reduces the soil Eh and provides carbon to methanogens. Organic materials influence the CH_4 formation through change in qualitative and quantitative properties of soil.

Fertilizers

Numerous studies have revealed the impact of chemical fertilizers on CH_4 emissions [3, 136, 139]. The effect of fertilizers on CH_4 emission depends on rate, type and mode of applications. Urea application enhances CH_4 fluxes over the growth season possibly by increasing soil pH following urea hydrolysis and the drop in redox potential, which stimulates methanogenic activities [152].

Lindau [95] reported decrease in CH_4 emission rate with ammonium nitrate application due to competitive inhibition of nitrate reduction in favour of methane production. Under field conditions, the application of sulphate based fertilizers such as $(NH_4)_2$ SO_4 and $CaSO_4$ have reduced CH_4 emission [24] and application of K_2HPO_4 enhances the CH_4 emission [4].

Methane oxidation

Methanotrophs

Methanotrophs (gram negative, aerobic bacteria belonging to the subset of a physiological group of bacteria known as methylotrophs) oxidize CH_4 via methanemonooxygenase (MMO) enzyme (Table 1). These bacteria are classified into three groups: Type-I, Type-II and Type-X. According to Conrad [29], all the methanotrophs that have so far been isolated and described belong to the Proteobacteria, of the γ sub-class (Type I) or ∝ sub-class (Type II). The Type I group is represented by the *Methylomonas, Methylocaldum, Methylosphaera, Methylomicrobium* and *Methylobacter*. The Type-II comprises *Methylosystis* and *Methylosinus*. The members of the genus *Methylococcus* occupy an intermediate position and have been kept in to a separate group Type-X [58]. By using molecular ecology techniques, it has become clear that methanotrophs are ubiquitous in nature and well adopted to high or low temperature, pH and salanity [148]. Henckel et al. [61] found that both Type-I and Type-II methanotrophs were stimulated in rice fields with unsaturated water content. Bodelier et al. [18] reported that Type II methanotrophs dominated in unplanted, unfertilized soils and the presence of rice plant was an essential factor for Type-I methanotrophs to proliferate. Methanotrophic bacteria are present in the aerobic soil layer, rhizosphere [42, 56, 75] and on the roots and stem bases of flooded rice plants [158]. The physiology, biochemistry and ecology of methanotrophic bacteria have been recently reviewed [29, 41].

Methanotrophy

The oxidation of methane by methanotrophs is initiated by methane monooxygenase (MMO) enzyme. The MMO occurs in two forms: as a membrane bound particulate form (sMMO) in all types of methanotrophs, and as a soluble form in Type-II and Type-

X methanotrophs [102]. Results of comprehensive studies on the structure, function and regulation of the MMO have been recently presented by Murrell et al. [113].

According to Conrad [29], the biggest problem for the energy metabolism is the activation of the relatively inert CH_4 molecule. The activation is achieved in the initial step by the MMO which converts CH_4, O_2 and reducing equivalents to methanol and H_2O, i.e.:

$$CH_4 + O_2 + 2NAD(P) \xrightarrow{MMO} CH_3OH + H_2O + 2\,NAD(P)$$

The reducing equivalents are supplied by the subsequent dehydrogenation steps, e.g. the conversion of methanol to formaldehyde to formate to CO_2 in which a total of 6 electrons are liberated [29].

Two main CH_4 oxidation pathways, catabolic and anabolic are present in methanotrophs [34]. Catabolic pathway is purely enzymatic through which methanotrophs oxidize methane to CO_2 via methanol, formaldehyde, and formate catalyzed by the enzymes: methane monooxygenase, methanoldehydrogenase, formaldehyde dehydrogenase and formate dehydrogenase, respectively. In this pathway energy is released but carbon is not incorporated into cellular biomass. The anabolic pathway may be further divided into two sub-pathways, ribulose monophosphate pathway and serine pathway. In both these pathways carbon of methane is incorporated in cellular biomass at the level of formaldehyde. Carboxylic acids and amino acids are the intermediary products of the serine pathway. Serine transhydroxy-methylase is the initiater enzyme of this pathway. On the other hand, phosphoglycerated sugars are intermediary products in ribulose monophosphate pathway. RuMP pathway is a cyclic pathway in which fixation is followed by cleavage and rearrangement reactions. Type-I and Type-X methanotrophs follow RuMP pathway whereas serine pathway is followed by Type-II and Type-X methanotrophs [58].

Methanotrophs population and methane oxidation in paddy soils

In rice fields all type of methanotrophs have been detected [52]. From Italian paddy soils two strains of Type-II methanotrophs were isolated [56]. There is a large variation in number of methanotrophs on rice roots, e.g. < 0.1 MOB mm^{-2} to > 120 MOB mm^{-2} of root surface and in the soil (Table 2). It has been reported that the population size of methanotrophs depends upon location of experimental site, concentration of CH_4 in the soil [10] and concentration of NH_4^+-N [42, 75]. Rhizosphere has several orders of magnitude higher MOB population than bulk soil [42, 43, 56]. Population size of MOB in rice planted microcosm, rhisoplane, dryland and flooded rice soils as well as endorhizospheric population in rice roots increase with time [20].

Most quantitative data upon methanotrophic population size rely on MPN methods. The limitations of this method are well known. The medium may be selective for certain strains; cells may be in unculturable state; resting and active cells can not be differentiated and microcolonies may be counted instead of single cells [52]. Considering these limitations, the current state of knowledge about population size of methonotrophs from various paddy fields is given in Table 2.

Uptake of atmospheric CH_4 through biological oxidation has been reported in a variety of rice-agroecosystems (Table 4). First evidence of plant associated CH_4 oxidation came from studies with microcosms [52]. Several scientists estimated the amount of CH_4 that is oxidized in association with rice plants and compared with overall CH_4 oxidation [20, 56]. From the review of the available data of CH_4 uptake,

Minami et al. [109] estimated the total terrestrial CH_4 consumption to be between 7 and 78 Tg y^{-1}. Although, rice field is an important source of CH_4, the data of CH_4 oxidation by unflooded paddy soil after harvest could be important for the CH_4 global budget [145].

Table 4. *Methane oxidation rates in a variety of rice fields.*

Site	CH_4 oxidation	Sources
Rice field (Panama)	0.1-1.5 mg CH_4 $m^{-2}d^{-1}$	[77]
Paddy field (Italy)	0.1-7.6 nmol CH_4 $h^{-1}cm^{-2}$	[30]
Paddy field (USA)	170-460 mg CH_4 $m^{-2}d^{-1}$	[54]
Paddy field (Italy)	0.3-162.5 nmol CH_4 $h^{-1}g^{-1}dw$	[12]
Paddy field (USA)	126.2-348.5 ng CH_4 $h^{-1}g^{-1}$	[153]
Paddy field (China)	0.7-1.4 µmol CH_4 $h^{-1}g^{-1}dw$	[17]
Paddy field (Japan)	9.5-18.8 µg CH_4 g^{-1} root d^{-1}	[151]
Paddy field (China)	54.0-892.0 ng CH_4 $g^{-1}h^{-1}$	[23]
Paddy field (Italy)	284-810 nmol CH_4 $h^{-1}gfw^{-1}$	[72]
Paddy field (Italy)	0.1-0.2 µmol CH_4 g^{-1} dwh^{-1}	[51]

Gilbert and Frenzel [56] found that the greater part of the CH_4 produced in paddy soil is probably oxidized either in the surface layer of the paddy soil or in the rhizosphere of rice plants. Thurlow et al. [145] showed that unflooded paddy soils after drainage practices, are able to act as sink of CH_4 and vary in their ability to oxidize it depending on the soil temperature and atmospheric CH_4 concentrations. We have found that rhizospheric soil oxidized greater amount of CH_4 (dryland 64-86% ; flooded rice soil 46 to 64%) as compared to bulk and bare soils [40, 45]. Denier and Neue [37] reported that CH_4 emission from rice plants one week before panicle initiation increased by 40% if CH_4 oxidation in the rhizosphere was blocked.

Kinetics of methane oxidation

The apparent half saturation constant (K_m), and maximum oxidation rate (V_{max}) of CH_4 oxidation are characteristic parameters which determine the ability of methanotrophs to grow on atmospheric methane. The CH_4 concentration is a key determinant of $K_{m(app)}$ but this could be mediated through the MMO enzyme, the methanotrophs or the bacterial community as a whole [47].

A model recently proposed by Koch [84] suggest that $K_{m(app)}$ may change in response to the dynamics of substrate utilization as determined by coupling between transport, growth and internal substrate pools. The general model may be useful when applied to methanotrophs, because the first product of methane oxidation, methanol, is sometime excreted and affects the kinetics of MMO [84]. Affinity of methanotrophs for CH_4 varies with growth conditions [45, 47]. According to King [83] the V_{max} of root associated CH_4 oxidation varied largely with season, indicating quantitative and/or qualitative changes in methanotrophic communities.

Recent studies revealed that there are two types of CH_4 oxidizers present in the soil. One population, having a high affinity for CH_4, typically has K_m in the range 1000 nM CH_4, and the other population, having a high affinity for CH_4, has K_m in the range of 30 to 60 nM CH_4 [13]. These methanotrophs typically occur in upland soils that consume atmospheric methane [11], but can also be activated in the paddy soils [10]. However,

the atmospheric CH_4 oxidation has hardly been studied in irrigated rice fields soil. Henckel and Conrad [61] found that moisturised air dried paddy soil does not oxidize CH_4 at atmospheric concentration unless it has been pre-incubated under elevated CH_4 concentration. A decreasing trend of K_m and V_{max} with decreasing CH_4 uptake rate along the soil depth was reported by Wang et al. [153].

Dubey et al. [44, 45] have found that K_m and V_{max} values for CH_4 oxidation in dryland/flooded rice fields decreased from rhizosphere to bulk to bare soil in confirmity with the decreasing CH_4 oxidation activity. Variations in kinetic parameters (K_m and V_{max}) for different rice fields are shown in Table 5. Bender and Conrad [11] have stated that different K_m values may indicate the existence of different types of methanotrophs in soils. According to Conrad [29] type II methanotrophs, which are frequently found in soils, are able to adopt to CH_4 concentration by changing their K_m. This difference could be due to differences in species composition and/or due to conditioning of methanotrophs under different soil microhabitats. According to King [82] all the methanotrophs that have been isolated from soil thus far do not possess the required kinetic properties. These methanotrophs have an ecological niche that is characterized not by atmospheric CH_4 oxidation but by oxidation of relatively high CH_4 concentration that emerge in the proximity of CH_4 production sites i.e. wetlands [128].

Factors affecting methane oxidation
Temperature

Although microbial community is expected to respond to changes in temperature, there are contradictory reports regarding its effect on methane oxidation. Whalen et al. [159] showed that CH_4 consumption increased with increasing temperature (5-20°C) under high CH_4 (103ppm) amended atmosphere. Bender and Conrad [13] reported a linear response in the temperature range of 20-35°C, but 13-38% of the maximum activity remain even at 0°C. A weak relationship between CH_4 uptake and soil temperature suggests that an abiotic process, such as diffusion of CH_4 or O_2 can be a controlling factor for CH_4 uptake.

Methane concentration and soil moisture

Methanotrophs are highly sensitive to variation in CH_4 concentration in atmosphere [11]. CH_4 concentration affects the rate of consumption both directly or indirectly. Enhanced concentration of CH_4 increases the number of methanotrophs) which have a significant role in methanotrophy [103]. Bender and Conrad [13] reported that increase of microbial methane oxidation activity and number of methanotrophs at CH_4 mixing ratios exceeding about 100-1000 μl $CH_4 l^{-1}$. The threshold value below which no CH_4 uptake occurs is much lower for soils than for sediments. For example, 2-3 ppm and < 0.1 to 0.4 ppm threshold values have been reported for sediments and soils, respectively [19]. The maximal CH_4 oxidation rates are probably determined by the magnitude of the supply of CH_4 to the zone of oxidation [82].

Early studies have shown that methane oxidation was sensitive to desiccation and dramatically decreased at soil moisture below at 20 % WHC [13, 72]. The low solubility of CH_4 in water enhances this effect, mainly at low limiting CH_4 concentrations.

Oxygen availability

Oxygen availability depends upon soil porosity. As the porosity increases, a decreased volume of water is distributed in pore volume, decreasing the water film thickness. This increases the rate of substrate (CH_4) delivery to the methanotrophs for oxidation [102]. Methanotrophs in the rice rhizosphere do not have to compete for methane with microbial or chemical compounds, although there is a strong sink of methane by methane transport. However, intensive competition for oxygen occurs. The available values for $K_{(app)}$ for O_2 and CH_4 indicated that uptake of both substrates is saturated at concentrations of ≥ 10 μM [82].

Table 5. *Observed kinetic parameters of CH_4 oxidation.*

Ecozone/location	K_m	V_{max}	Reference
Rice field (Italy)	4.0μM	0.1μ mol g^{-1} dw h^{-1}	[20]
Rice field (Italy)	4.1-165μg	1.2-12.5μ g h^{-1} g^{-1}dw	[153]
Rice field (Italy)	56.0-186.0nM	-	[47]
Rice field (Italy)	16.8nM	839 n M g dw^{-1} h^{-1}	[72]
Dryland rice field (India)	4.8-81.6μ g g^{-1}dw	0.05-0.61μ g h^{-1} g^{-1}dw	[45]
Flooded rice field (India)	6.2-81.1μ g g^{-1}dw	0.03-0.41μ g h^{-1} g^{-1}dw	[40]

Nitrogenous compounds and soil pH

Inorganic N influences CH_4 oxidation due to shifts in the population structure and the kinetics of methanotrophs [45]. This may affect the threshold value for CH_4 oxidation [82]. NO_3^--N fertilization did not affect the CH_4 consumption but NH_4^-+N fertilization completely ceased CH_4 oxidation [66]. Nitrite was found to inhibit CH_4 oxidation in the cultures of *Methylomonas albus BG8* and *M. trichosperium OB3b* [83]. Recently it was shown that methane oxidation is stimulated by increased nitrogen availability due to unquantified nitrogen limitation of methanotrophs [18]. However, the most solidly substantiated explanation for ammonium inhibition of methane oxidation is competitive inhibition at the enzyme level. This occurs because, at the molecular scale, methane and ammonium are similar in size and structure [135]. As a result, the enzyme MMO can bind to ammonium ion and react with it. Because the possibility of competitive inhibition is fundamental to the biochemistry of methane oxidation, it was generally thought that inhibition should occur in paddy fields as well as in upland systems [135].

In a pasture soil, CH_4 oxidation at pH 6.3 was greater than at pH 5.6 and was completely inhibited at pH 4.8-5.1 [66]. In certain other soils, oxidation has been reported at pH as low as 3.2 [143]. In general, low pH has an inhibitory effect on methane consumption although the mechanism responsible for this effect is not fully known [66].

Inhibitors for CH_4 production and oxidation

An inhibitor specific to either methanogens or methanotrophs would be useful for distinguishing which of these organisms is responsible for CH_4 production and oxidation in environments in which such activities occur. A variety of chemicals used in agriculture such as pesticides and herbicides and nitrification inhibitors, are known to affect microbial processes. It is well established that CH_4 production is inhibited by acetylene, aminopurine, ammoniumthiosulphate, carbofurane, calcium carbide (capsulated), DDT, dicyandiamide, methyle chloride, methyle fluoride, nitrapyrine, pyridine, organochlorine and sodium azide and CH_4-acetylene, bromoxynil, dicyandiamide, DDT, 2,4,-D, ethylene, hexachlorocyclohexane, hydrazine, methomyle, nitrapyrine, phenylalanine, sodium thiosulphate, threonine and thiourea [9, 25, 147]. Nitrification inhibitors such as acetylene and nitrapyrin can inhibit the growth of nitrifiers, methanogens and methanotrophs [107]. Lindau et al. [96] found that CH_4 emissions from rice fields decreased by 35% and 14% following the application of encapsulated calcium carbide and dicyandiamide, respectively. Topp [146], found that the pesticides, bromoxynil, methomyl and nitrapyrin were inhibitory to CH_4 oxidation at 50 g l^{-1}. Sathpathy et al. [134] reported that the application of HCH (organochlorine insecticide) to flooded rice soils reduced the production and emission of CH_4. Kumarswamy et al. [89] have revealed that carbofuran inhibited net CH_4 production when applied at low rates (5-10 mg g^{-1} soil), but stimulated it when applied at a rate of 100 mg g^{-1} soil. Chan and Parkin [25] have recommended the use of acetylene and ethylene for inhibition of CH_4 oxidation and methyl chloride for inhibition of methanogenesis.

Approach for Methanogens/Methanotrophs Detection at Molecular Ecological Level

Effective representation of the diversity towards desired functional gene is one of the major concerns of microbial ecology of any ecosystem. A preliminary attempt has been reported by Dubey et al. [46] where, by cluster analysis of ARDRA pattern, it has been shown that different types of methanotrophs dominate in rhizosphere and non-rhizosphere soils of tropical rice ecosystems. The growing demand of paddy with increasing population is an emerging issue and the scenario results in the over exposure of soil microflora with pesticide residues. As discussed earlier pesticides play a major role at the physiological level for the expression of key enzymes in methane production and its further utilization as a substrate. The question, what are the emerging criteria that decide the course of evolution for these bacteria, which play key role in maintaining the greenhouse gas methane in the ecosystem and for habitats exposed to such residues, is likely to trigger a lot of interest. Studies are required to compare the performance of diverse strains from such habitats followed by molecular analysis of key genes to understand which is the most susceptible genotype for such stresses. These studies are

likely to generate the tracking probes for the same target genes but having substituted primary coding sequences that will help evaluating the impact on soil methane cycle and its relation to pesticide residues. Similarly, other parameters such as application of fertilizers and accumulation of inorganic compounds in soil will lead to modified soil chemistry which in turn will be changing the overall microbial community structure vis-à-vis their relation in density and survival of methane oxidizing bacteria. Therefore, parallel tracking tools would have to be designed to assess the existing microbial population using selected markers which could act as biomarker for associated biogeochemical cycles.

Several approaches have been adopted in molecular ecological studies for the detection and characterization of methanogens and methanotrophs in the various ecosystems [46, 112]. These are schematically shown in Figure 2. The first approach is indirect and relies on enrichment and/or isolation with subsequent characterization of methanogens and methanotrophs. The second approach is direct and relies on polymerase chain reaction (PCR) technique and use of phylogenetic and functional gene probes (Table 6) for the molecular analysis of these microbes in the environmental samples without the prerequisite of their cultivation. At each stage, in both approaches, molecular biological analysis of the key genes (*pmoA, mmoX, mxaF* for methanotrophs, *mcr, mtd, mth, mrt, frh* for methanogens and 16S r RNA) can be carried out and comparison can be made to see if the same organism that grows in culture derived from the sample is representative of what is actually present in the environmental sample (as revealed by DNA analysis).

Several other approaches have also been used to study methanogens and methanotrophs. Phospholipid analysis has been used successfully for the detection of these microbes in the wetland ecosystem [144].

Table 6. *Sequences of oligonucleotides commonly used as PCR primers and probes for molecular analysis of methanogens and methanotrophs.*

Primers /Probes	Targets	Sequences (5´ → 3´)	Tm (ºC)	References
PRIMERS				
A 189 f	pmoA ; pMMO/AMO	GGN GAC TGG GAC TTC TGG	56	[63]
A 682 r	pmoA ; pMMO/AMO	GAA SGC NGA GAA GAA SGC	56	[63]
882 f	mmoX ; sMMO$^+$Methylotrophs	GGC TCC AAG TTC AAG GTC AG	55	[99
1403 r	mmoX ; sMMO$^+$Methylotrophs	TGG CAC TCG TAG CGC TCCGGCTCG	55	[99]
1003 f	mxaF; All Methylotrophs	GCG GCA CCA ACT GGG GCT GGT	55	[100]
1561 r	mxaF; All Methylotrophs	GGG CAG CAT GAA GGG CTC CC	55	[100]
ME1 f	mcrA; methanogens	GCM ATG CAR ATH GGW ATG TC	50	[60]
ME2 r	mcrA; methanogens	TCA TKG CRT AGT TDG GRT AGT	50	[60]
Probes				
MSMX 860	Methanosarcinaceae	GGC TCG CTT CAC GGC TTC CCT	54	[127]
MS821	Methanosarcina spp.	CGC CAT GCC TGA CAC CTA GCG AGC	54	[127]
MC1109	Methanococcaceae	GCA ACA TAG GGC ACC GG TCT	47	[127]
MB310	Methanobacteriaceae	CTT GTC TCA GGT TCC ATC TCC G	52	[127]
MG1200	Methanomicrobiales	CGG ATA ATT CGG GGC ATG CTG	45	[127]

This technique relies on the fact that methanotrophs contain unusual fatty acids (16:1 and 18:1 derivaties). Phospholipid analysis is, however, limited due to small database

on fatty acid profiles from methanotrophs [113] and the relatively high cost of equipment used in this analysis.
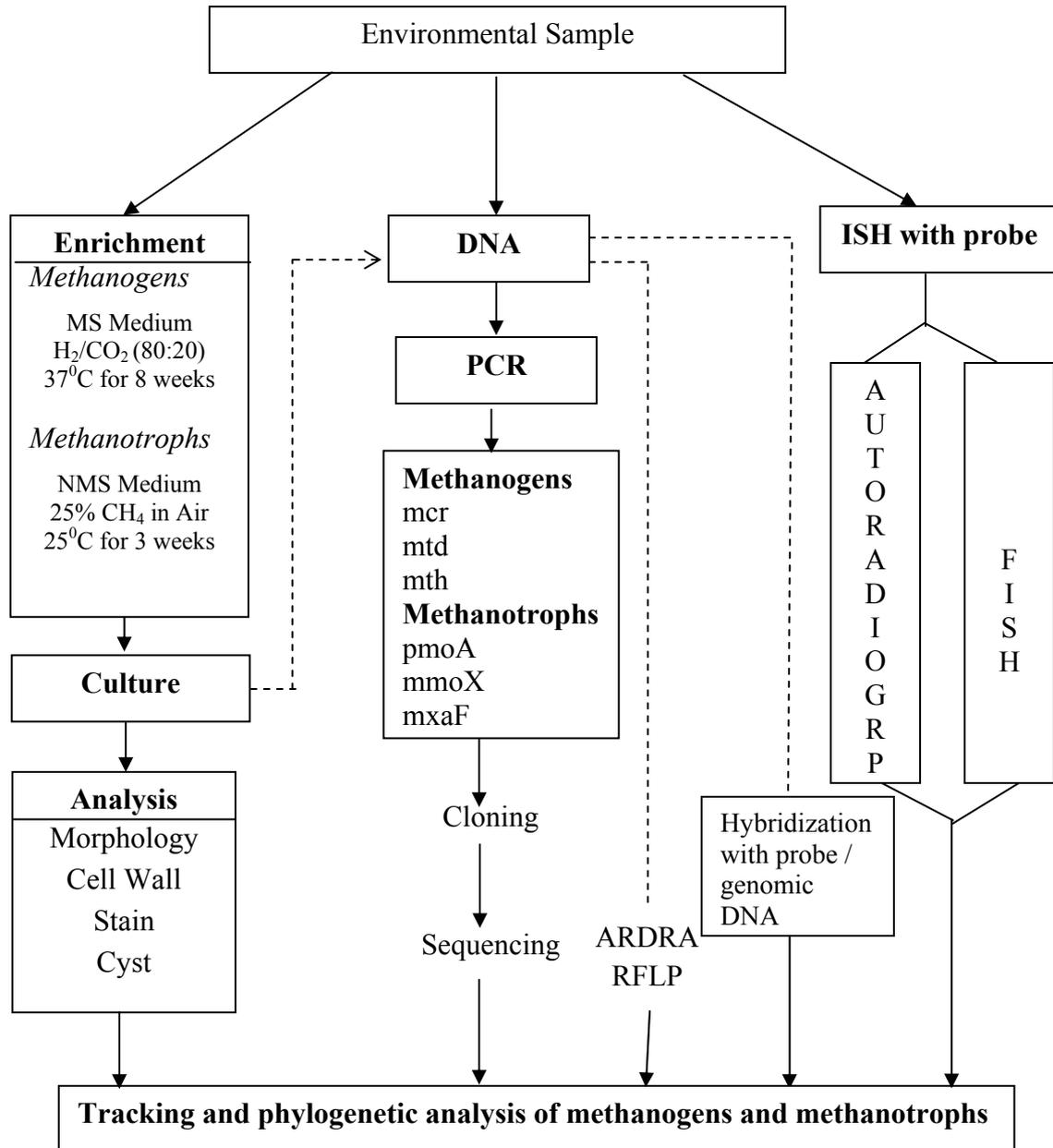
Figure 2. *Generalized scheme of molecular anlysis for detection and phylogenetic study of methanogens and methanotrophs*

The detection and characterization of these microbes can also be attempted using antibodies either to whole cells of culturable methanogens/methanotrophs or to key enzymes such as MMO, MDH (methanotrophs) and MCR, MTD (methanogens). One of the most powerful tools in the molecular ecology is FISH (fluorescence in situ hybridization). FISH allows the specific detection and enumeration of methanogens/ methanotrophs directly in the natural habitats without cultivation. Grobkopf et al. [57] detected most of the archaeal group members (e.g. *Methanosarcinaceae,*

Methanosaetaceae, Methanomicrobiaceae, Methanobacteriaceae, *RC-I* to *RC-IV* and *RC-VI*) on rice plant roots by using FISH.

Conclusions

Methane is an important greenhouse gas and it affects the chemistry of the atmosphere. The ecological role of methanogens and methanotrophs in the methane dynamics in rice fields is still unclear. Current information is insufficient for the development of technology and strategy for reduction in methane emission from rice field at regional and global levels. Knowledge of comparative genomics and proteomics of methanogens and methanotrophs will contribute to the deciphering their population structure and existing mechanisms of methane emission in paddy fields.

It has now become possible to isolate, detect and characterize these microbes by using molecular biological tools like PCR, FISH, etc. techniques. Knowledge of structure and function of methanogens and methanotrophs communities will be beneficial for understanding the microbial ecology of methane to control the CH_4 turnover in rice soils.

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