Differential Effects of Nitrogenous Fertilizers on Methane-Consuming Microbes in Rice Field and Forest Soils†‡

Santosh R. Mohanty,1§ Paul L. E. Bodelier,2* Virgilio Floris,2 and Ralf Conrad1

Max Planck Institute for Terrestrial Microbiology, Department of Biogeochemistry, Karl-von-Frisch-Str., 35043 Marburg, Germany,1 and Netherlands Institute of Ecology (NIOO-KNAW), Centre for Limnology, Department of Microbial Wetland Ecology, Rijkstraatweg 6, 3631 AC Nieuwersluis, The Netherlands2

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The impact of environmental perturbation (e.g., nitrogenous fertilizers) on the dynamics of methane fluxes from soils and wetland systems is poorly understood. Results of fertilizer studies are often contradictory, even within similar ecosystems. In the present study the hypothesis of whether these contradictory results may be explained by the composition of the methane-consuming microbial community and hence whether methanotrophic diversity affects methane fluxes was investigated. To this end, rice field and forest soils were incubated in microcosms and supplemented with different nitrogenous fertilizers and methane concentrations. By labeling the methane with 13C, diversity and function could be coupled by analyses of phospholipid-derivative fatty acids (PLFA) extracted from the soils at different time points during incubation. In both rice field and forest soils, the activity as well as the growth rate of methane-consuming bacteria was affected differentially. For type I methanotrophs, fertilizer application stimulated the consumption of methane and the subsequent growth, while type II methanotrophs were generally inhibited. Terminal restriction fragment length polymorphism analyses of the pmoA gene supported the PLFA results. Multivariate analyses of stable-isotope-probing PLFA profiles indicated that in forest and rice field soils, Methylocystis (type II) species were affected by fertilization. The type I methanotrophs active in forest soils (Methylomicrobium/Methyllosarcina related) differed from the active species in rice field soils (Methylobacter/Methylomonas related). Our results provide a case example showing that microbial community structure indeed matters, especially when assessing and predicting the impact of environmental change on biodiversity loss and ecosystem functioning.

The loss of biodiversity from ecosystems and the potential consequences in terms of ecosystem functioning and stability have been central issues for environmental sciences in the last decade (35). Microbial communities comprise much of Earth’s biodiversity and have a critical role in ecosystem functioning (42, 43) by collectively determining the biogeochemical processes that regulate the Earth system. Nevertheless, microorganisms have not been considered in the ongoing debate about global biodiversity loss and global change.

This neglect is probably due to the huge diversity and associated functional redundancy within microbial communities (42), which make it unlikely that losing a single bacterial species will result in altered ecosystem functioning. Changes in microbial community composition in concordance with changes in the functions that the microorganisms catalyze have been observed for specific groups of low diversity catalyzing a very narrow range of biogeochemical functions, such as ammonia-oxidizing bacteria (23, 44). However, in these and other cases separate community members could not be linked to the functions they catalyze, and therefore a direct link between diversity and biogeochemical functioning cannot be made. The latter is possible with bacteria that consume methane.

Methane-consuming microbes play a vital role in global warming issues, as they are the only biological sink for methane. In dry upland soils (e.g., forest and grassland) they account for approximately 6% of the global sink strength of atmospheric methane, and in wetlands they attenuate the source strength by 10 to 30% (26, 33). Methane-oxidizing bacteria (MOB) utilize methane as their sole carbon and energy source and are subdivided into types I and II on the basis of phylogeny, physiology, morphology, and biochemistry (8, 18). There are 12 recognized genera within the Alphaproteobacteria (type II MOB/MOB) and Gammaproteobacteria (type I (8, 22). A very distinct characteristic of these bacteria is the presence of specific phospholipid ester-linked fatty acids (PLFA) which differentiate them from each other (type I, C16:1ω5c and C16:1ω6c; type II, C18:1ω7c) and also from all other organisms (9). Tracing the C of methane (which these organisms use as the sole carbon and energy source) back into these PLFA by using stable isotope (13C) (6, 36) or radioisotope (14C) (3) labeling enables linkage of the biogeochemical function of these bacteria with their phylogeny and community composition. The activity of MOB is regulated mainly by the availability of oxygen and methane and the presence of mineral nitrogen (2, 18). The effect of nitrogen, mainly supplemented in the form of inorganic fertilizer or by atmospheric deposition, has been intensively investigated since nitrogen was found to be inhibitory to methane consumption in forest soils (40) 2 decades ago. Many studies conducted since then have shown
conflicting results. Inhibition, stimulation, and no effect were observed in upland soils (e.g., forest and grassland soils) as well as in wetland soils (e.g., rice paddies and freshwater marshes). Several mechanisms of inhibition or stimulation by nitrogen have been proposed (2), but none of them has been proven experimentally.

In this study we tested whether the diverse fertilizer effects may be explained by different compositions of the methane-oxidizing community in different soils and by changes to them effected by nitrogen addition (38). For this purpose, we incubated forest and rice field soils with different nitrogenous fertilizers and at different methane concentrations and assessed the growth and activity of the methane-oxidizing microbiota by analyzing PLFA concentrations and measuring the incorporation of $^{13}$CH$_4$ into the various PLFAs.

**MATERIALS AND METHODS**

Soils. The rice field soil originated from a rice field in Vercelli, Italy. The soil was collected from a drained field on 18 April 1999 before flooding. This soil had a maximum water-holding capacity corresponding to a gravimetric water content of 47% ± 1% (w/w) (n = 5) (19). The estimated C and N contents (percent dry weight) in the rice field soil were 1.8 ± 0.13 and 0.15 ± 0.01 (n = 4), respectively. This soil has repeatedly been used in many microbial ecological investigations (see, e.g., references 10, 21, and 32), and detailed soil characteristics have been described previously (39). The forest soil was collected from a 5- to 20-cm soil depth from a forest near Marburg, Germany (51°00′00″N, 9°50.625″E). The site was located on a slope in a deciduous forest consisting mainly of beech (Fagus sylvatica) and oak (Quercus robur). The soil type was a cambisol with Ah (2 to 6 cm), Bv (6 to 28 cm), and sandstone C horizons. The water pH values of the organic Ah horizon and the mineral subsoil were 3.8 and 4.3, respectively. Detailed characteristics of this forest soil have been described elsewhere (20, 29).

Both soils were air-dried and stored at room temperature. Soils were homogenized by sieving through 2-mm mesh. The incubations described here were carried out in 2001 and 2002.

Soil incubations. About 100 g air-dried soil was incubated in 1,000-ml Erlenmeyer flasks that were closed with silicone septa. Soils were moistened and mixed well with sterile water to maintain a water-holding capacity of 40%. These flasks represented the controls. In the case of fertilized soils, the water to moisten the soils was supplemented with either NH$_4$Cl or KNO$_3$, corresponding to 3 mmol (100 ml) of nutrient solution per kg soil, which is equal to 60 kg N per ha. Half of all soil incubations were supplemented with methane to maintain a low concentration (1,000 ppmv by volume [ppmv]), and the other half was supplemented to maintain a high concentration (10,000 ppmv). The $^{12}$CH$_4$ was spiked with $^{13}$CH$_4$ (99%; Cambridge Isotope laboratory, MA) at a mixing ratio of 97% $^{12}$CH$_4$ to 3% $^{13}$CH$_4$. Tubes were sealed with silicone septa to prevent diffuse leakage of $^{13}$CH$_4$ into the various PLFAs.

Analyzing PLFA concentrations and measuring the incorpora-
tion of $^{13}$CH$_4$ into the various PLFAs.

Soils of the same type were supplemented with either NH$_4$Cl or KNO$_3$, corresponding to 30 ml of nutrient solution per kg soil, which is equal to 60 kg N per ha. Soils were moistened and mixed well with sterile water to maintain a water-holding capacity of 40%. These flasks represented the controls. In the case of fertilized soils, the water to moisten the soils was supplemented with either NH$_4$Cl or KNO$_3$, corresponding to 30 ml of nutrient solution per kg soil, which is equal to 60 kg N per ha. Half of all soil incubations were supplemented with methane to maintain a low concentration (1,000 ppmv by volume [ppmv]), and the other half was supplemented to maintain a high concentration (10,000 ppmv). The $^{12}$CH$_4$ was spiked with $^{13}$CH$_4$ (99%; Cambridge Isotope laboratory, MA) at a mixing ratio of 97% $^{12}$CH$_4$ to 3% $^{13}$CH$_4$. Tubes were sealed with silicone septa to prevent diffuse leakage of $^{13}$CH$_4$ into the various PLFAs.

Isotope laboratory, MA) at a mixing ratio of 97% $^{12}$CH$_4$ to 3% $^{13}$CH$_4$. Tubes were sealed with silicone septa to prevent diffuse leakage of $^{13}$CH$_4$ into the various PLFAs.
type II it was assumed that 49% of the total PLFA content is C18:1ω9c, using a conversion factor from dry weight to cell numbers of 0.075 × 10^12. Growth rates were calculated using cell numbers according to the formula (ln Xf – ln Xi)/f (τ2 – τ1).

Statistical analyses. (i) Effects of methane concentration and N amendment. Effects of methane concentration and nitrogen amendment on intrinsic growth rates and type-specific 13C incorporation in forest soil samples were tested using a factorial two-way analysis of variance (ANOVA). Effects of methane concentration, nitrogen amendment, and incubation time on intrinsic growth rates and type-specific 13C incorporation in rice field soil samples as well as on the relative abundance of restriction fragment 245 bp in forest and rice field soil samples were tested using a factorial three-way ANOVA. Before all ANOVAs the data were checked for normality (by plots of standard deviations versus means) and for homogeneity of variances (by Levene's test). If necessary, the data were transformed to meet the assumptions of the ANOVAs. All analyses were performed using the STATISTICA software package version 6.1 (Statsoft Inc., Tulsa, OK).

(ii) Multivariate analyses of SIP profiles. SIP 13C-PLFA profiles of forest and rice field soil samples were compared to profiles of methanotrophic cultures to identify active MOB in these samples, as defined by the most similar profile available from cultivated MOB. A prerequisite for making these comparisons, however, is that only label incorporation in type I- and type II-related PLFAs separately can be determined. Otherwise, a mixed profile of type I and II species would be compared to profiles from separate cultures. Since in soil SIP profiles there are PLFAs that occur in both type I and type II MOB, a selection of PLFA that were regarded as occurring predominantly in type II (>0.1%) but not in type I (<0.1%) and vice versa was made. The selected PLFAs for type I MOB were C14:0, C16:0, C16:1ω7c, C16:1ω6c, C16:1ω9c, C16:1ω5c, C16:1ω5t, C17:0 cyc, and C17:1ω6c. The selected PLFAs used for type II MOB were C15:0, C17:0, C17:1ω7c, C17:1ω6c, C16:0, C16:1ω7c, C16:1ω6c, C16:1ω9c, C16:1ω5c, and C18:2ω6c. (The question mark in the last PLFA indicates that the precise isomeric structure and double bond position are not known and are still under investigation [P. L. E. Bodelier, unpublished data]). The total 13C uptake taken in these selected PLFAs was used to calculate the relative uptake in all individual PLFAs. These relative PLFA concentration profiles of soil samples and known cultures formed the matrix which was used for cluster analyses and nonmetric multidimensional scaling (MDS) analyses. The inputs of cluster analyses as well as MDS were Bray-Curtis similarity matrices which were log(x + 1) transformed to even out the contributions of very rare and very dominant PLFA. Clustering was done using the group average linking routine. The MDS analysis results in a two-dimensional plot where the distance between samples indicates the similarity of these samples to other samples in the plot. The accuracy of the two-dimensional representation is indicated by the “stress” value (Kruskall’s stress formula). Stress values of <0.1 indicate a good ordination with no prospect of a misleading interpretation. Stress values of <0.2 still give a good two-dimensional representation, but not too much reliance should be put on the detail. In this case, other methods of representation should be used in parallel, such as clustering analyses. All clustering and MDS analyses were performed using the Primer-E software (Plymouth Marine Laboratory, Plymouth, United Kingdom). All theoretical aspects of the cluster and MDS analyses used have been described previously (11).

RESULTS

Gross methane consumption. The consumption of methane by both forest and rice field soils was found to be stimulated by the application of both ammonium and nitrate at an elevated methane concentration after addition of N fertilizer (Table 1). Only nitrate inhibited methane consumption in forest soils at a low methane concentration.

Type-specific biomass and growth rates. Methanotrophic biomass was assessed by extracting and quantifying the specific PLFA C16:1ω9c and C16:1ω7c (characteristic of type I MOB) and C18:1ω9c and C18:1ω5c (characteristic of type II MOB). At a low methane concentration (1,000 ppmv), the biomass increase of type II MOB (i.e., C18:1ω9c) in forest soil was delayed during the first 20 days of incubation when ammonium or nitrate fertilizer was applied, whereas type I biomass increased from the start of the incubation (Fig. 1). At an elevated methane concentration (10,000 ppmv), biomass accumulation of type II was repressed after 25 days of incubation with ammonium and nitrate, while

<table>
<thead>
<tr>
<th>CH4 concn</th>
<th>N treatment</th>
<th>Methane consumption (nmol CH4 g^-1 day^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Forest soil</td>
<td>Rice field soil</td>
</tr>
<tr>
<td>1,000</td>
<td>No N</td>
<td>156.56 ± 7.71 bd</td>
</tr>
<tr>
<td></td>
<td>NH4+</td>
<td>148.31 ± 6.45 abc</td>
</tr>
<tr>
<td></td>
<td>NO3-</td>
<td>133.47 ± 5.08 a</td>
</tr>
<tr>
<td>10,000</td>
<td>No N</td>
<td>858.12 ± 40.07 a</td>
</tr>
<tr>
<td></td>
<td>NH4+</td>
<td>1,342.55 ± 26.32 b</td>
</tr>
<tr>
<td></td>
<td>NO3-</td>
<td>1,253.60 ± 13.31 b</td>
</tr>
</tbody>
</table>

*Values are means and standard deviations for three replicate incubations. Significant differences between means for the two soils are indicated for each methane concentration separately by different letters (Tukey’s test, P < 0.05).
istic of Methylocystis species (16), confirmed the PLFA data and demonstrated again a negative effect of fertilizer application in both rice field and forest soils on type II MOB (Fig. 6; see Table S5 in the supplemental material). For the active type I MOB, the PLFA-SIP data showed that forest soils differed from rice field soils. The active type I methanotrophs in forest soil were most closely related to representatives of the genera Methylomicrobium/Methylosarcina (Fig. 4A and B) while in rice field soil representatives of the genera Methylomobacter/Methylomonas (Fig. 5A and B) were the most related to the soil SIP profiles. N amendment obviously stimulated a specific part of the type I community and did so differentially in the two types of soils.

### DISCUSSION

The consumption of methane of both forest and rice field soils was stimulated by both ammonium and nitrate fertilizer application. For rice field soil this is consistent with earlier field studies on rice field soils (3), where a strong positive correlation between application of N fertilizer and methane oxidation was observed (32). The immediate stimulating effect in these studies was suggested to be the consequence of an immediate response of the methane-consuming enzyme machinery (2) rather than community growth following the relief of N limitation. A similar response was observed in our study. However, for forest soils this stimulation is rather surprising and has not yet been observed. Usually forest soils have been found to consume atmospheric methane, a process that is particularly sensitive to inhibition by fertilizer application and carried out by an as-yet-uncultivated group of methanotrophs (29). In our study the forest soil was exposed to higher methane concentrations supposedly favoring the “low-affinity” methane oxidation carried out by most methanotrophs available in culture (28). It is striking, however, that even in the forest soils at hand these low-affinity methanotrophs become active immediately upon exposure to elevated methane levels, a situation which can occur in forest soils when the soils get wet as a result of heavy rainfall. However, in other studies methane consumption by low-affinity MOB at high methane concentrations was inhibited (37). As suggested earlier (38), many of these discrepancies with respect to fertilizer effects on methane oxidation may be explained by assuming that not all community members are affected in the same way, making MOB diversity

### TABLE 2: Effect of methane concentration and N amendment on intrinsic in situ growth rates of type I and II MOB in forest soil incubations

<table>
<thead>
<tr>
<th>CH₄ concn (ppmv)</th>
<th>N treatment</th>
<th>Type I</th>
<th>Type II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Growth rate (day⁻¹)</td>
<td></td>
</tr>
<tr>
<td>1,000</td>
<td>No N</td>
<td>0.020 ± 0.004</td>
<td>0.029 ± 0.010</td>
</tr>
<tr>
<td></td>
<td>NH₄⁺</td>
<td>0.017 ± 0.001</td>
<td>0.012 ± 0.008</td>
</tr>
<tr>
<td></td>
<td>NO₃⁻</td>
<td>0.022 ± 0.007</td>
<td>0.032 ± 0.020</td>
</tr>
<tr>
<td>10,000</td>
<td>No N</td>
<td>0.031 ± 0.002</td>
<td>0.036 ± 0.005</td>
</tr>
<tr>
<td></td>
<td>NH₄⁺</td>
<td>0.033 ± 0.002</td>
<td>0.013 ± 0.010</td>
</tr>
<tr>
<td></td>
<td>NO₃⁻</td>
<td>0.043 ± 0.008</td>
<td>0.034 ± 0.014</td>
</tr>
</tbody>
</table>

* Growth rates were calculated using cell numbers calculated from PLFA C₁₅:1ω₅c (type I) and C₁₈:1ω₅c (type II). For all samples n = 3. Values are means and standard deviations.
an explanatory variable in methane dynamics. That is actually what we observed in this study.

Growth rates as well as methane incorporation were generally stimulated in type I MOB, whereas these parameters were inhibited in type II MOB, by nitrogenous fertilizers. The growth rates themselves are unique data, since actual growth rates in soils are hardly available for microbes. The rates we measured represent doubling times of 20 to 60 days, which in the case of the growth rates observed at 1,000 ppmv are in the same range as those of MOB cultures growing under methane-limited conditions (27). The rates at 10,000 ppmv methane, however, are orders of magnitude lower than those of laboratory cultures grown under the same conditions (27), indicating some other limitation (e.g., N) or grazing by protozoa.

Surprisingly, the methane concentration did not affect the growth rate of type II MOB. It is generally assumed that type II methane oxidizers, in comparison to type I methanotrophs, thrive especially in environments with high methane and low oxygen concentrations (8, 18), a fact that is certainly not supported by our results with forest soil. Very recently, some Methylocystis spp. were shown to be very oligotrophic with respect to methane, which also indicates that no generalizations can be made about the effect of methane concentration on type II MOB (27).

With respect to the effects of nitrogenous fertilizers on methane consumption, also no generalizations can be made. The general idea until now was that type I MOB profit much more from the presence of mineral nitrogen than type II MOB, possibly because of the ability of type II MOB to fix molecular nitrogen (2, 3, 16). However, the data presented on forest and

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![Graph showing total 13C incorporation in MOB-specific PLFA in forest soil after 48 days of incubations.](image)

**FIG. 2.** Total 13C incorporation in MOB-specific PLFA in forest soil after 48 days of incubations. Table SA3 in the supplemental material shows the statistical analyses of the effects of methane concentration and N amendment on 13C incorporation.

![Graph showing total 13C incorporation in MOB-specific PLFA in rice field soil incubations.](image)

**FIG. 3.** Total 13C incorporation in MOB-specific PLFA in rice field soil incubations. Incubations were performed using 10,000 ppmv methane and were supplemented with or without ammonium. Table SA4 in the supplemental material shows the statistical analyses.
rice field soil incubation clearly demonstrate that methanoxidizing microbial species belonging to type I or II were differentially affected by the availability of nitrogen. Type II MOB were repressed by the presence of mineral N, especially ammonium. This could be an effect of competition for N between types I and II in the incubations. However, for rice field soil the effect of ammonium is immediate (Fig. 3), while the T-RFLP analyses demonstrate that in forest soil the inhibition also acts at the beginning of the incubation (Fig. 6).

The SIP-PLFA profiles enable us to pinpoint at the genus level which organisms were actually active in the incubations. The genera identified by our SIP-PLFA approach have already been detected in rice field and forest soils by using molecular approaches such as denaturing gradient gel electrophoresis, cloning, and real-time PCR (7, 15, 19, 21, 29, 30). In forest soil the observed type I genera are most certainly not the dominant members of the methane-consuming community. However, they are present and can become active and grow, as was the case in our incubations, where apparently representatives of the genera *Methylobacter/Methylomonas* (rice field soil) and *Methylomonas/Methylosarcina* (forest soil) were dominant in methane consumption over other type I genera. Similarly, we know from this study that the type II genus *Methylocystis* is repressed by N addition. There is as yet no evidence that this also holds true for other MOB genera such as *Methylocaldum, Methylococcus, Methylosinus, Methylocella*, and *Methylocapsa* and the uncultured soil clusters USC3 and USC7 (29), which can dominate the soil methanotrophic community (31). This makes the picture of the effects of nitrogenous fertilizers on MOB communities far less “black and white” than has been assumed until now.

The nature of the effect (i.e., stimulation or inhibition) obviously depends on the community composition and hence on the biodiversity of the MOB present. We hypothesize that methane consumption in soil or sediment with a predominance of type I MOB will not be affected by fertilizer application, while methane uptake by a soil or sediment with a predominance of type II will be inhibited. Moreover, differentiation can also be expected within type I and II representatives. Clear examples of habitats with a dominance of a specific type of
MOB are acid peat, dominated by Methylocella and Methylocystis (12); periodically water-saturated gleyic soils, with a dominance of Methylocystis and USC\textsubscript{G} (28); and lake sediments, dominated by Methylomonas (1, 14). A major determinant of the outcome of fertilizer effects will also be the methane concentration in the respective environment, because this determines the active MOB type. In periodically water-saturated upland soils, type I MOB participated in methane consumption only from 500 ppm methane upward (28), while type II MOB consumed at lower methane concentrations. With respect to the latter, we have to keep in mind that in the case of forest soils, the effects in our study were observed at a far higher methane concentration than that to which MOB in dry forest soils are normally exposed. Hence, we cannot say whether this differential effect will also act at atmospheric methane concentrations. However, the bacteria that consume atmospheric methane in forest and other upland soils are still not identified or in culture (29) or phenotypically characterized. Nevertheless, we know from culture studies that Methylocystis and other MOB were present and activated immediately upon exposure to elevated methane, a situation which could emerge when methane availability in forest or other upland soils is elevated due to flooding or increased precipitation, as predicted for future climatic conditions (26).

However, our study demonstrated that the composition of the methanotrophic microbial community apparently affected methane fluxes and hence that microbial diversity has to be taken into account in the global biodiversity debate. Change in land use and global environmental change are both factors that may provoke shifts in methane-consuming microbial communities (24). The latter may have consequences for the flux that the microbes catalyze. However, to confirm this, extensive eco-physiological experiments have to be carried out with pure cultures and with experimental systems differing in MOB diversity. Fertilizer addition studies have to be carried out in habitats with a dominance of either type of MOB, where methane consumption has to be linked with diversity by using, e.g., stable isotope probing of PLFA, DNA, or RNA.

FIG. 5. Multivariate statistical analyses of SIP \textsuperscript{13}CH\textsubscript{4}-PLFA profiles of the MOB community in rice field soil samples. The inputs of the MDS (A and C) and the cluster analyses (B and D) were PLFA profiles of MOB cultures (expressed as percentage of total PLFA content) and of SIP profiles of soil samples (expressed as percentage of \textsuperscript{13}C incorporated in separate PLFAs of the total PLFA \textsuperscript{13}C uptake). The two-dimensional distances between samples in the MDS graph show the relative similarity between samples. Stress values of the MDS plots were 0.13 (A) and 0.10 (C). For the cluster analyses, the profiles were transformed $[\log(x+1)]$ before the Bray-Curtis similarity matrix was established. The clustering was done using the group average linking method (11).
In conclusion, it can be said that biodiversity conservation and land use policy makers should also consider prokaryotic diversity, which is apparently important for certain ecosystem functions, such as methane oxidation in soil.

ACKNOWLEDGMENTS

S. R. Mohanty and P. L. E. Bodelier contributed equally to this work.

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REFERENCES


FIG. 6. Relative abundance of terminal restriction fragment 245, which is representative of type II MOB, as affected by incubation time, methane concentration, and N amendment in forest and rice field soils. Relative abundance is expressed as the percentage of the total fluorescence recorded per sample. All points represent the means and standard errors for three replicate samples. The statistical evaluation of the data is presented in Table S4A5 in the supplemental material.