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Bacterial and Archaeal Communities Influence on Methane Production

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Animal manure is a low cost substrate suitable for biofuels production, such as hydrogen and methane, via anaerobic fermentation (Kyazze et al., 2007). Previous studies attributed the higher biogas yield from certain types of manure to the presence of a native microflora (Yeole and Ranande, 1992) and/or a higher or better carbon-nitrogen (C/N) ratio (Fulford, 1988). Probably the main difference between the different types of dung is related to the specific food the animal eat, which changes the overall C content and C/N ratio. We measured the C/N ratio and the native microflora of dung collected during one year. We related it to the biogas yield during anaerobic fermentation both for lactating and non-lactating buffaloes manure. The results showed that the buffalo manure was in both cases plenty of carbohydrates with a C percentage content based on the dry sample weight of 36.2 ± 2.0 % and 31.4 ± 1.5 %, respectively. The C/N ratio was 18.6 ± 1.5 % and 20.6 ± 2.9 %, respectively, and it changed during the different seasons because of the fluctuating N content. Indeed we found an acceptable correlation (R = 0.75) between the average environmental temperature and the C/N ratio of lactating buffalo manure. Neither the C or N content nor the C/N ratio appeared to be strongly correlated to the biogas production. The manure was fermented in batch reactors at 37 °C and pH = 6.0 after different pretreatments of the collected manure.
The analysis of the microbial community was carried out using denaturing gradient gel electrophoresis (DGGE) to generate fingerprints of 16S rRNA genes (Carillo et al., 2012). The sequences analysis revealed that the Eubacterial community during fermentation in batch reactors was much more taxon rich and diversified than the Archeal community. The study of Archeal community instead showed the presence of only three main methanogen strains very active in methane production and largely predominant.

1. Introduction

The decree of 15 March 2012 committed Italy on the definition and qualification of regional objectives, called "burden sharing", for the renewable energy production. It has implemented the Directive 2009/28/CE which established that about 17 % of Italy's final energy consumption should be covered by renewable sources by 2020. This goal is easily attainable since Italy has a huge energy renewable potential arising also from the anaerobic digestion of residual biomasses. The wastes from the agricultural sector, industrial food processing and livestock can be considered as a resource rather than as unmanageable waste, whose landfilling, non compliant with EU directives, also contributes to increase greenhouse effect (Pietrangeli et al. 2013). In particular, animal manure is a low cost substrate rich in carbohydrates, suitable for biofuel production, such as CH_4 and H_2. The swine manure appears to be the most suitable for biogas production for the presence of a particular native microflora and/or to the high carbon/nitrogen (C/N) ratio. In fact, literature data demonstrated that pig manure produced more gas per unit weight as compared with the cow dung (Muyiiya and Kasisira, 2009). Probably the main difference between the two types of dung is in the specific food the animals are forced to eat which changes the overall carbon content and carbon-

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nitrogen ratio. Notwithstanding, we have chosen to study the potentiality of biogas production using buffaloes dung. In some regions of southern Italy, like Campania, the Mediterranean water buffaloes (*Bubalus bubalis*, subsp. River) have increased due to the demand for the products obtained only from buffalo milk (Borghese, 2010); being their management exclusively intensive, this production system tends to produce more manure than it can be used as fertilizer on nearby cropland. Each year, an adult buffalo can produce 4-6 tonnes of wet manure plus additional urine (Nanda and Nakao, 2003). This manure is usually "distributed to a small, local landmass resulting in soil accumulation and runoff of phosphorus, nitrogen, and other pollutants" (Koneswaran and Nierenberg, 2008). The manure from buffalo could become a valuable source of biogas instead of a pollutant whose disposal in Campania represents an additional environmental problem. For this reason a study of the potential use of this type of biomass as substrate for biogas production has been assessed checking preliminary if the different ways of feeding lactating and dry buffaloes could affect the carbon-nitrogen ratio and/or the native microflora and biogas yield.

2. Materials and Methods

The present study was carried out between May 2010 and February 2013 on Mediterranean water buffalo (*Bubalus bubalis*, subsp. River) manure collected in a commercial buffalo farm located in the municipality of Villa Literno, Province of Caserta, Italy, during seven experimental campaigns. The animals were divided in two groups, the first was composed by lactating buffaloes (LB, with a number of heads between 120-150), the second by dry buffaloes (DB, with a number of heads between 170-200). Milk production in lactating buffaloes was sustained by an average daily intake of dry matter (DM) around 17 kg/head/day with an higher energy (from 0.85 to 0.95 Milk Feed Units MFU/kg DM) and protein concentration (14-16 % crude protein on DM), based on maize and other silages, cereal grains, soya, alfalfa or "graminaceae" hay and by-products, while the dry matter intake of the dry buffaloes was around 11 kg/head/day, with a lower energy (around 0.63 MFU/kg DM) and protein (7-8 % crude protein on DM) (Bartocci et al., 2002). The output energy content of the forage being estimated by NIRS method (Chase, 1981) as net energy for milk cow production (MFU = 7.24 MJ). Buffaloes were kept loose in paddocks close to the milking room. The cleaning of the paddocks was done every morning: the movement and stocking of dung was mechanized. The manure was collected early in the morning during the cleaning of paddocks. Three samples for each type of manure were collected each time by a small sterile plastic shovel in sterile polypropylene lab containers and transported to the lab where they were stored at 4 °C. Samples of manure were used to determine fresh and dry weights. Dry weight was determined upon oven dehydration at 105 °C (until steady weight). The average values were determined from the three different samples for each type of manure. Carbon and nitrogen content were determined by combustion in an Elemental Analyzer EA1110 (Carlo Erba Strumentazioni, Italy) (Friis et al., 1998). Percentage composition of C, N and C/N values of dung from different animals (found in literature, see Table 1) was compared with LB and DB dung values obtained sperimentally (LB and DB dung values are mean of seven experimental campaigns, n = 3 for each campaign). A heat map in Table 1 summarizes the differences among C, N and C/N values. Logarithm base 2 (Log_2) ratios were calculated for each value by dividing animal dung by LB values to estimate the differences. Log_2 ratios are visualized according to the scale bar in the figure below Table 1. The analysis of the microbial community was carried out using denaturing gradient gel electrophoresis (DGGE) to generate fingerprints of 16S rRNA genes (Carillo et al., 2012). Nucleotide sequences (Operational Taxonomic Units, OTU) were compared to the GenBank-NCBI nucleotide database using the BLAST network service as described in Ciarmiello et al. (2013).

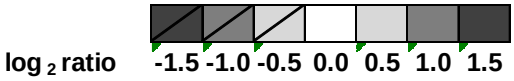
The fresh wet manure was gently mixed with bidistilled water, with a weight ratio of 30/70, and the mixture gently stirred and acidified, with a solution of HCl 1 M, to obtain the desired pH values. The typical pH value of the original water/manure solution is around 8.0 and it is corrected to 6.0. Subsequently, the water/manure solution was homogenized to simulate a stressing mechanical treatment and finally filtrate with a Büchner funnel. The anaerobic fermentation process was performed in batch mode in bottles filled with 80 mL of manure mixture, flushed with oxygen free nitrogen to assure anaerobiosis and finally placed in an incubator at a controlled temperature of 37 °C to start the fermentation process. No continuous mixing was applied, and the bottles were simply hand shaken every day. The composition of the gas in the bottle headspace was analysed by a MicroGC Agilent 3000 (S.R.A. Instruments, France) gas chromatograph equipped with two capillary columns: a MolSieve 5A and a Poraplot U and TCD detectors for separating H_2, O_2, N_2, CH_4 and CO_2 measurements (Carillo et al., 2012). At least three chromatographic runs were performed for each gas composition measurement.

3. Results and Discussion

Manure from different animals can show different C/N ratios (Table 1), it is really surprising, when data presented in literature are compared, to discover that the C/N ratios of same species can be very different. The reason is that composition of animals faecal matter can vary by subspecies and seasonality, but above all in dependence on the physiological state and/or the diet which they are subject. LB and DB manure was in both cases plenty of carbohydrates with a percentage C content based on the dry sample weight of 36.2 ± 2.0 % and 31.4 ± 1.5 %, respectively. The C/N ratio was 18.6 ± 1.5 % and 20.4 ± 2.9 %, respectively, and it changed during the different seasons because of the fluctuating N content (Figure 1). Indeed we found a good correlation (R = 0.75) between the average environmental temperature and the C/N ratio of LB manure, but neither the C or N content nor the C/N ratio appear to be strongly correlated to the biogas production (not shown).

Table 1: Percentage composition of C, N and C/N values of dung from different animals compared with LB dung. For details see Materials and Methods

Dung from	C	N	C/N	Reference	Dung/LB dung C	Dung/LB dung N	Dung/LB dung C/N
LB	36.2	2.0	18.6				
DB	31.4	1.5	20.4				
Buffalo	33.8	1.1	30.2	Yasin and Wasim, 2011			
Chicken	37.4	1.9	19.5	Uzodinma and Ofoefule, 2009			
Chicken	46.0	6.3	7.3	Barnett, 1978			
Chicken	35.7	3.7	9.7	Maramba, 1978			
Cow	30.6	1.7	18.0	Barnett, 1978			
Cow	24.8	1.2	20.0	Dhanya et al., 2009			
Cow	35.8	1.8	19.9	Maramba, 1978			
Cow	42.4	1.5	28.3	Uzodinma and Ofoefule, 2009			
Duck	21.9	0.8	27.4	Maramba, 1978			
Hog	38.4	2.8	13.7	Maramba, 1978			
Horse	57.5	2.3	25.0	Barnett, 1978			
Pig	25.9	0.9	21.5	Uzodinma and Ofoefule, 2009			
Rabbit	44.5	1.8	25.3	Uzodinma and Ofoefule, 2009			



The LB and DB manure were fermented in batch reactors at 37 °C and pH = 6.0 and the average methane composition during the experimental tests was 3.33 ± 0.74 mL_N/g wet manure and 3.14 ± 0.37 mL_N/g wet manure, respectively. Therefore no significant difference was found in the production of biogas from buffaloes fed with different fodder. But while the LB dung showed the capability to produce methane up to about 4.11 mL_N/g wet manure in some experimental campaigns, the DB dung showed more constant biogas production, whit maximal value of about 3.50 mL_N/g wet manure.

In figure 2 is shown the PCR and DGGE study of LB bacterial and archeal microflora. Twenty-one different fragments (gel bands) of similar size were eluted and sequenced after reamplification from DGGE gel. Search by GenBank database using the BLAST network service, showed that 19 of 21 had high identity (84–98 %) to V3 16S-rRNA gene sequences belonging to previously characterized microbes (Table 2). 15 of 21 of them were bacteria residing in waste digesters, anaerobic sediments, rumina or fecal samples Only the sequences 8 and 15 showed low identity to other 16S-rRNA gene sequences present in the nucleotide databases, resulting still unknown. 7 of the 16 eubacterial 16S-rRNA gene sequences could be assigned to the phylum Firmicutes, five to the phylum Bacteroidetes, and five sequences (17-21) could be assigned to the phylum Euryarchaeota.

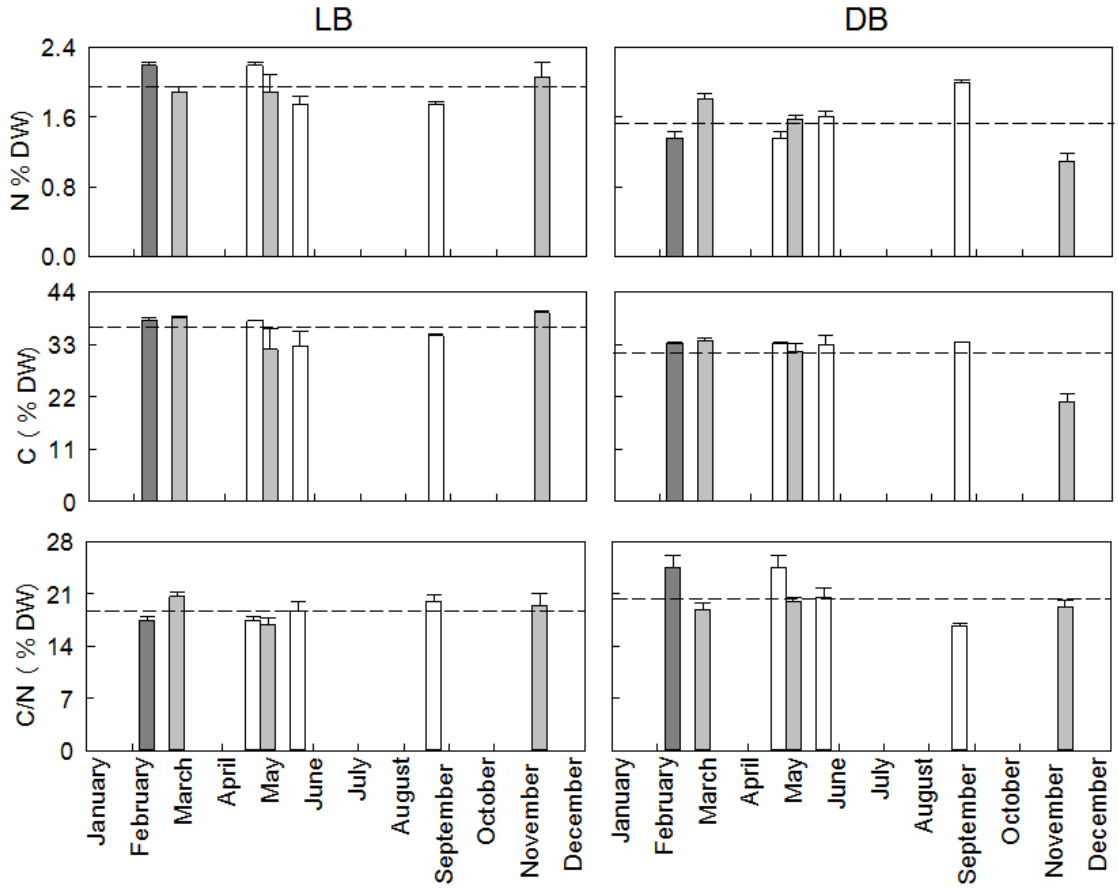


Figure 1: C, N and C/N values for LB and DB obtained during different periods of three consecutive years (2011, 2012, 2013) compared with their average value (- - -). Values are mean ± SD (n = 3)

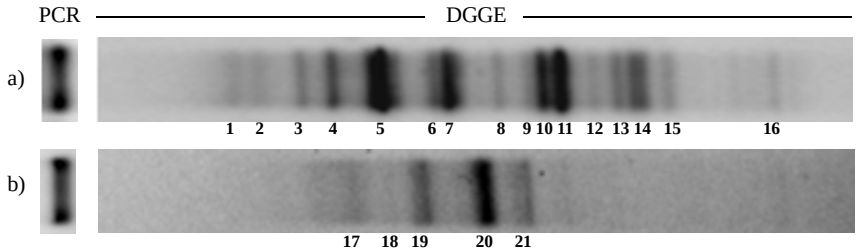


Figure 2: PCR (Polymerase Chain Reaction) and DGGE (Denaturing Gradient Gel Electrophoresis) profiles of a) Eubacteria and b) Archeobacteria 16S-rRNA gene sequences from fermented manure

Eubacterial bands 4, 5, 7, 10 and 11 were particularly evident in figure 2a. Sequence 5 showed a 95 % similarity to *Bacillus siralis*, an anaerobic bacterium that hydrolyses casein but not starch or cellulose (Petterson et al. 2000). Sequence 7 had a 98 % similarity to *Clostridium populeti*, which is able to catalyse the highest H_2 production from cellulose (Ren et al 2007). This species produces thermo-resistant endospores (80 °C for 10 min) with an optimal growth temperature of 37 °C. Sequence 10, as well as other three sequences (2, 3, 16) had a 86–95 % identity to *Proteiniphilum acetatigenes*, an acetogenic Bacteroidia, which ferments starch and accelerates the propionate-degradation in anaerobic cultures (Chen and Dong, 2005). Sequence 11 showed a high identity (93 %) to A*cetanaerobacterium elongatum,* a non spore forming bacterium, which grows at 20–42 °C and ferments various mono-, di- and oligo-saccharides to produce acetate, ethanol, H_2 and CO_2 (Chen and Dong, 2004).

Table 2: Uncultured bacterium 16S ribosomal RNA partial sequences isolated by DGGE from buffalo dung batch fermentation at pH 6.0 and 37 °C

OTU	Phylogenetically most closely related Eubacteria and Archeobacteria			
	Description – accession n°	**Sm (%)**	**Phylum**	**Source**
1	Uncultured bacterium - GQ134000.1	83	-	USA: reactor treating swine waste
2, 3, 10, 16	*Proteiniphilum acetatigenes* - AY742226.1	86-95	Bacteroidetes	China: reactor treating brewery wastewater
4	Uncultured bacterium - EU551121.1	91	Bacteroidetes	Finland: anaerobic co-digestion of crops and cow manure
5	*Bacillus siralis strain 171544* (NR_028709.1)	95	Firmicutes	Sweden: silage
6, 7	*Clostridium populeti* strain 743A (NR_026103.1)	96-98	Firmicutes	USA: cellulose culture
8, 15	Unclassified		-	
9	*Ethanoligenens harbinense* YUAN-3 *Clostridium sp.* - CP002400	91	Firmicutes	China: mesophilic digester
11	*Acetanaerobacterium elongatum strain Z7* - NR_042930	93	Firmicutes	China: paper mill waste water
12	Uncultured bacterium – GU607876.1	84	-	Korea: cow dung
13, 14	Uncultured Clostridia – EU887985.1	91-95	Firmicutes	Finland: leach bed reactor
17	*Methanobrevibacter smithii* ATCC 35061 (NR_044786.1)	93	Euryarchaeota	USA: isolated from feces of humans and other animals
18, 19	*Methanocorpusculum labreanum* strain Z (NR_074173.1)	97-98	Euryarchaeota	USA: Tar Pit Lake at the LaBrea tar pits and wastewater treatment, sewage, landfills
20, 21	*Methanobrevibacter millerae* strain ZA-10 (NR_042785.1)	88-91	Euryarchaeota	USA: bovine rumen and faeces

The most evident Archeal bands (19 and 20, 21) in figure 2b belong to *Methanocorpusculum labreanum* (98 % identity) and *Methanobrevibacter millerae* (88-91 % identity), respectively. *Methanocorpusculum labreanum* grows by producing CH_4 from H_2 and CO_2 or formate with a narrow pH range (pH 6.5 to 7.5), with fastest growth near pH 7 (Zhao et al. 1989). *Methanobrevibacter millerae* ZA-10T gains energy for growth by reducing CO_2 to CH_4 using H_2 or formate exclusively as electron donors with an optimal growth temperature of 36-42 °C and pH of 7-8 (Rea et al 2007).

4. Conclusions

The sequences analysis of the bacteria found in the fermented manure revealed that the Eubacterial community during fermentation in batch reactors was much more taxon rich and diversified than the Archeal community, which instead showed the presence of only three main strains very active in methane production. Initially we explained this result by hypothesising the use of antibiotics for the prevention of diseases such as brucellosis, not confirmed by breeders. This treatment could have enabled the survival of a few classes of methanogens, while it could have been more ineffective on the Eubacterial population that is indeed able to produce spores. But a detailed study of the Eubacterial strains present showed that not all of them could produce spores and so this explanation was inconsistent. Actually we think that the most effective bacteria producing methane become largely predominant and they are the only ones found in such biomass. Methane production seems promising both for LB and DB manure and thus it is worth exploring its industrial use for biogas production.

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References

Barnett A., 1978, Biogas technology in the third world: a multidisciplinary Review. International Development Research Centre (IDRC), Ottawa, Canada 51.

Bartocci S., Tripaldi C., Terramoccia S., 2002, Characteristics of foodstuffs and diets, and the quanti-qualitative milk parameters of Mediterranean buffaloes bred in Italy using the intensive system: An estimate of the nutritional requirements of buffalo herds lactating or dry. Livestock Production Science, 77, 45-58.

Borghese A., 2010, Development and perspective of buffalo and buffalo market in Europe and Near East. Proc. 9th World Buffalo Congress, Buenos Aires, 25-28 April. In Revista Veterinaria, 21, 20-31.

Carillo P., Carotenuto C., Di Cristofaro F., Kafantaris I., Lubritto C., Minale M., Morrone B., Papa S., Woodrow P., 2012, DGGE analysis of buffalo manure eubacteria for hydrogen production: effect of pH, temperature and pretreatments. Molecular Biology Reports, 39, 10193-10200.

Chase L., 1981, Energy prediction equations in USA at NY Hydhia Forage Laboratory. Production Agricultural Training School, Ithaca, NY, USA.

Chen S., Dong X., 2004, *Acetanaerobacterium elongatum* gen. nov., sp. nov., from paper mill waste water. International Journal of Systematic and Evolutionary Microbiology, 54, 2257-2262.

Chen S., Dong X., 2005, *Proteiniphilum acetatigenes* gen. nov., sp. nov., from a UASB reactor treating brewery wastewater. International Journal of Systematic and Evolutionary Microbiology, 55, 2257-2261.

Ciarmiello L.F., Pontecorvo G., Piccirillo P., De Luca A., Carillo P., Kafantaris I., Woodrow P., 2013, Use of nuclear and mitochondrial single nucleotide polymorphisms to characterize English walnut (*Juglans regia* L.) genotypes. Plant Molecular Biology Reporter, 31, 1116-1130.

Friis J.C., Holm C., Halling-Sorensen B., 1998, Evaluation of elemental composition of algal biomass as toxical endpoint. Chemosphere, 37, 2665-2676.

Fulford D., 1988, Running a Biogas Programme: A handbook. Intermediate Technology Publications, London, United Kingdom.

Koneswaran G., Nierenberg D., 2008, Global Farm Animal Production and Global Warming: Impacting and Mitigating Climate Change. Environmental Health Perspectives, 116, 578–582.

Kyazze G., Dinsdale R., Guwy A.J., Hawkes F.R., Premier G.C., Hawkes D.L., 2007, Performance characteristics of a two-stage dark fermentative system producing hydrogen and methane continuously. Biotechnology and Bioengineering, 97, 759-770.

Maramba F.D., 1978, Biogas and Waste Recycling: The Philippine Experience. Liberty Flour Mills, Manila, Philippines.

Muyiiya N., Kasisira L., 2009, Assessment of the Effect of Mixing Pig and Cow Dung on Biogas Yield. Agricultural Engineering International: the CIGR Ejournal Manuscript PM 1329, Vol. XI, <www.cigrjournal.org/index.php/Ejounral/article/view/1329>, Accessed 06/04/2014.

Nanda A.S., Nakao T., 2003, Role of buffalo in the socioeconomic development of rural Asia: Current status and future prospectus. Animal Science Journal, 74, 443-455.

Pettersson B., de Silva S.K., Uhle´n M., Priest F.G., 2000, *Bacillus siralis* sp. nov., a novel species from silage with a higher order structural attribute in the 16S rRNA genes. International Journal of Systematic and Evolutionary Microbiology, 50, 2181-2187.

Pietrangeli B., Lauri R., Bragatto P.A., 2013, Safe Operation of Biogas Plants in Italy. Chemical Engineering Transactions, 32, 199-204.

Rea S., Bowman J.P., Popovski S., Pimm C., Wright A.D. 2007, *Methanobrevibacter millerae* sp. nov., and *Methanobrevibacter olleyae* sp. nov., methanogens from the ovine and bovine rumen that can utilize formate for growth. International Journal of Systematic and Evolutionary Microbiology, 57, 450-456.

Ren Z., Ward T.E., Logan B.E., Regan J.M., 2007, Characterization of the cellulolytic and hydrogen-producing activities of six mesophilic Clostridium species. Journal of Applied Microbiology, 103, 2258-2266.

Uzodinma E.O., Ofoefule A.U., 2009, Biogas Production from Blends of Field Grass (*Panicum maximum*) with some animal wastes. International Journal of the Physical Sciences, 4, 91-95.

Yasin M., Wasim M., 2011, Anaerobic digestion of buffalo dung, sheep waste and poultry litter for biogas production. Journal of Agricultural Research, 49, 73-82.

Yeole T., Ranande D., 1992, Alternative feedstock for Biogas. Tropical Animal Production, 9, 10-16.

Zhao Y., Boone D.R., Mah R.A., Boone J.E., Xun L., 1989, Isolation and Characterization of *Methanocorpusculum labreanum* sp. nov. from the LaBrea Tar Pits. International Journal of Systematic and Evolutionary Microbiology, 39, 10-13.