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Presence of Archaea in the Indoor Environment and Their Relationships with Housing Characteristics

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Massachusetts Institute of Technology

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April 3, 2016

Subject: Revised Paper Submission to *Microbial Ecology* (paper ID: MECO-D-15-00514R1)

Manuscript Title: Presence of Archaea in the indoor environment and their relationships with housing characteristics

Authors: Sepideh Pakpour, James A. Scott, Stuart E. Turvey, Jeffrey R. Brook, Timothy K. Takaro, Malcolm R. Sears, and John Klironomos

Dear Dr. Nelson,

Following our recent communication and your advice, enclosed please find a copy of our revised manuscript (MECO-D-15-00514R1) that we are submitting for potential reconsideration in *Microbial Ecology*. Below I have also included the authors' responses to each of the respected reviewer's latest comments, which have been accordingly applied in the enclosed text file (yellow highlights). In particular following the reviewer 3 comment we included the new reference [36].

Thank you very much again for giving us the opportunity to submit this revised version.

Sincerely,

Sepideh Pakpour, PhD
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Reviewer 1

-The authors adequately addressed my previous concerns.
> Thank you very much again for your time.

Reviewer 2

-Authors have made all corrections suggested by reviewers. however, reference list is not added in the revised version, therefore reviewer cannot verify the corrections.
> Thank you very much again for your time. This has been merely an unfortunate incident as the

list was deleted during the upload and we added the complete reference list in this revised version.

-On line numbers 110 and 113, duration for denaturation should be 30 Sec not 3 seconds.
> Corrected.

Reviewer 3

-The only type of measurement that was made in this work was quantitative PCR of 16S rRNA genes. Therefore, it is particularly crucial to make sure that the methodology worked well, because otherwise there is nothing left in the report. Here, the reference for the primers in the qPCR method for Archaea is Guo et al. 2013, which is not a methodology paper on qPCR of Archaea. In their paper, Guo et al. 2013 cite Burggraff et al. 1997 and Groβkopf et al. 1998 for the qPCR primers. The paper by Burggraff et al. 1997 deals with Archaea phylogeny, without any qPCR. The paper by Groβkopf et al. 1998 deals with methanogen diversity in soil, here again without any qPCR. In summary, nobody seems to have ever validated the current primers for qPCR of Archaea. In my opinion it is just not possible to base an entire paper on the use of a qPCR method that has not been validated. And sometimes the mere fact of changing the qPCR machine can alter the validity of qPCR data

> Thank you very much again for your time and constructive argument. We also agree that the primer and qPCR workflow validation is a crucial step before running any experiments. Apart from Guo et al. 2013, Kemnitz et al. [cited below and also included in the new revised text (Ref [36])] measured total numbers of archaea by qPCR targeting the 16S rRNA gene using the primer combination A364aF/A934b and A109f/A934b. They specifically noted that A364aF/A934b combination proved to be more specific than the combination A109f/A934b, and it did not result in any unspecific amplification of bacterial 16S rRNA genes.

**Kemnitz D, Kolb S, Conrad R (2005) Phenotypic characterization of Rice Cluster III archaea without prior isolation by applying quantitative polymerase chain reaction to an enrichment culture. Environmental Microbiology 7: 553-565. doi: 10.1111/j.1462-2920.2005.00723.x

It may also be worth noting that this set of primers has been cited in other literatures; some examples have been listed below:

**Zheng YM, Cao P, Fu BJ, Hughes JM, He JZ (2013) Ecological Drivers of Biogeographic Patterns of Soil Archaeal Community. *PLoS One* 8. doi: 10.1371/journal.pone.0063375

**Shen JP, Cao P, Hu HW, He JZ (2013) Differential response of archaeal groups to land use change in an acidic red soil. *Science of the Total Environment* 461: 742-749. doi: 10.1016/j.scitotenv.2013.05.070

**Cao P, Zhang LM, Shen JP, Zheng YM, Di HJ, He JZ (2012) Distribution and diversity of archaeal communities in selected Chinese soils. *FEMS Microbiology Ecology* 80: 146-158. doi: 10.1111/j.1574-6941.2011.01280.x

The authors would also like to share with the respected reviewer that prior to running the tests on dust samples, the same set of primers and the qPCR workflow were widely tested by other students in the UBC lab on the cloned archaeal sequences (positive controls), other samples (e.g., soil) with spiked cloned archaeal sequences (positive controls), and samples (with no internal reference). Result of their sequencing showed no unspecific amplicons.

-The authors speculate that the DNA of the Archaea species may be present in air fresheners, and this could have been tested readily. The authors answer that they do not have access to these air fresheners. Does it mean all these families have moved outside of the area so that it is not possible to contact them again? Have local stores ceased to sell these products?

The use of fresheners was based on a ‘yes/no’ survey question and the authors did not have permission to contact the recruited families as part of the mini-child study privacy agreement to get more specific information. Though, this speculation can be a good step for future studies, yet if you advise we can remove the corresponding sentence from the manuscript.

Presence of Archaea in the indoor environment and their relationships with housing characteristics

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Keywords:

Archaea, Bacteria, Indoor Environment, qPCR, Building Characteristics, Human Activities

Abstract

Archaea are widespread and abundant in soils, oceans, or human and animal gastrointestinal (GI) tracts. However, very little is known about the presence of Archaea in indoor environments and factors that can regulate their abundances. Using a quantitative PCR approach, and targeting the archaeal and bacterial 16S rRNA genes in floor dust samples, we found that Archaea are a common part of the indoor microbiota 5.01±0.14 (log 16S rRNA gene copies / g dust, Mean ±SE) in bedrooms and 5.58±0.13 in common rooms, such as living rooms. Their abundance, however, was lower than for bacteria; 9.20±0.32 and 9.17±0.32 in bedrooms and common rooms, respectively. In addition, by measuring a broad array of environmental factors, we obtained preliminary insights into how the abundance of total archaeal 16S rRNA gene copies in indoor environment would be associated with building characteristics and occupants' activities. Based on our results, Archaea are not equally distributed within houses, and the areas with greater input of outdoor microbiome and higher traffic and material heterogeneity tend to have a higher abundance of Archaea. Nevertheless, more research is needed to better understand causes and consequences of this microbial group in indoor environments.

1. Introduction

The biology and ecology of the third domain of life, Archaea, have been studied far less when compared to the other domains including Bacteria, and Eukarya. Archaea are microorganisms discovered in the late 1970s [1]. For years after their discovery, scientists believed that Archaea were restricted to extreme environments, such as deep-sea

hydrothermal vents, hypersaline waters, or strictly anoxic ecosystems [2]. Development of culture-independent molecular techniques and high-throughput molecular sequencing approaches transformed this belief by illustrating their presence, often with high abundance and diversity, in terrestrial and aquatic environments [3-5], animal care facilities [6-8], deteriorated medieval wall paintings [9], as well as the human and animal microbiome such as gastrointestinal (GI) tracts [10-14] and human oral cavities [15]. However, the presence of Archaea in many other ecosystems has still been investigated scarcely and our understanding of their role in their habitat is limited.

One such overlooked ecosystem is the indoor built environment. There is significant ongoing interest in better understanding the "built environment microbiome" [16], with a focus on characterizing microbial diversity as well as the environmental parameters that would drive its patterns [16-26]. Nevertheless, most of the past studies on the indoor microbiome considered mainly bacteria [16, 17, 27-30], and to a lesser degree fungi [19, 20, 23, 25, 31, 32]. Here, we used culture-independent molecular approaches to study the archaea in indoor dust from homes in the so-called "miniCHILD" study – which is a preliminary cohort of 54 homes in the Vancouver area recruited to assist in the optimization and validation of data collection tools for the larger Canadian Healthy Infant Longitudinal Development (CHILD) study [33, 34]. We sought to answer three general questions: (1) are Archaea regular components of built environment microbiomes? If yes, (2) what would be their magnitude compared to indoor bacteria? And (3) how would building characteristics and occupants' activities relate to the variation of archaeal abundances?

2. Material and Methods

2.1 Sample Collection

Between May 2008 and May 2009, trained research assistants collected dust from the homes of families with new born children using a sterile, depyrogenated custom-designed aluminum collection device attached to the end of a vacuum cleaner (Model S3680, Sanitaire Canister Vac, Charlotte, NC, USA). The collection device held two nylon DUSTREAM filters (Indoor Biotechnologies Inc, Charlottesville, VA). Two dust samples were collected in each house; the first sample was a composite of the mattress and floor in the room where the subject child slept, and the second sample was collected from the floor of the room occupied most often by the family. A standardized floor area was initially sampled (2 m^2) and if insufficient dust was obtained, the sampling area was expanded. Research technicians visually observed the thimbles after vacuuming 2 m^2; if the thimbles were less than half-full, the technician continued vacuuming in a new area of the room until the required amount was met. The exact size of the vacuumed area was recorded for all samples taken. Samples were then fractionated using a sterile depyrogenated 100 Mesh sieve (~150 μm), and the fine fraction transferred to a sterile depyrogenated borosilicate glass vial with a Teflon-lined screw cap (VWR 1 dram glass vial, West Chester, PA) and stored at -80 °C until analysis.

2.2 DNA extraction and quantitative PCR analyses

Total DNA was extracted from 100 mg of collected fine dust samples using a FastDNA® SPIN Kit for Soil (MP Biomedicals, LLC, Solon, OH, USA), which was selected systematically by using the Order Preference by Similarity to Ideal Solution (TOPSIS)

method [35] as the most optimum extraction kit for dust samples in the present case study. Subsequently, extracted DNA samples were checked for integrity by agarose gel electrophoresis with Lambda DNA HindIII Digest standards (New England BioLabs, Ipswich, MA, USA) and their quantities were measured using the QuantiFluor® dsDNA System (Promega, Madison, WI, USA). The purity of extracted DNA samples was evaluated by measuring each sample's ratio of the optical density at 260 nm and 280 nm using the NanoVue Plus™ spectrophotometer (GE Healthcare, Buckinghamshire, UK), before preserving them at –20 °C. Abundances of both archaeal and bacterial 16S rRNA gene copy numbers were measured by quantitative PCR (qPCR); using A364aF (5' CGGGGYGCASCAGGCGCGAA 3') and A934bR (5' GTGCTCCCCCGCCAATTCCT 3') primers for Archaea [36] and BACT1369F (5' CGGTGAATACGTTCYCGG 3') and PROK1492R (5' GGWTACCTTGTTACGACTT 3') for bacteria [37]. Although the abundance of 16S gene sequences is not a surrogate measure of the relative abundance of the archaeal and bacterial cells containing those sequences (because of variations in genomic copy number of the 16S gene in microbial species), in the rest of this manuscript for the sake of brevity, 16S rRNA gene copy numbers will be referred to as archaeal/ bacterial abundances.

All PCR amplifications were carried out in a CFX96 Touch™ Real-Time PCR Detection System (BioRad, Ontario, Canada) and each PCR reaction mixture (20 µL) contained 10 µL of SsoFast™ EvaGreen® Supermix (Biorad, Hercules, CA), 1.5 µL of 1000 µg/ µL T4 gene 32 protein (Biolabs, Ipswich, MA), 0.4 µM of each primer, nuclease-free water (IDT, Coralville, IA, USA), and 2 µL of extracted DNA (5 ng / µL). Thermal-cycling conditions for 16S Archaea were as follows: 95°C for 2 min for the enzyme activation, 40

cycles of 95°C for 30s (denaturation) and 61.5°C for 30s (annealing and extension), followed by 1 cycle of melting analysis (65 – 95°C (0.1°C / 2s)). These conditions for 16S Bacteria included: 95°C for 2 min for the enzyme activation, 40 cycles of 95°C for 30s (denaturation) and 56°C for 30s (annealing and extension), followed by 1 cycle of melting analysis (65 – 95°C (0.1°C / 2s)).

Standard curves were obtained using three replicates of 1:10 serial dilutions of linearized plasmids containing both cloned archaeal and bacterial 16S rRNA sequences, giving a concentration range from 10 to 10^6 copies/ μL. Amplification efficiencies of 92.2 – 94.7 % ($R^2 > 0.985$) and 90.1 – 105.8 ($R^2 > 0.963$) were observed for archaeal and bacteria standards, respectively. Finally, melting curve analyses at the end of all qPCR runs and agarose gel running of qPCR products were performed to check for amplification and specificity of the products.

2.3 Collection of environmental variables and statistical analyses

We monitored and recorded 668 housing characteristics as well as building inhabitant activities by using standardized questionnaires and direct on-site visits for the purpose of statistical analyses. An exhausted list of these factors have been described in recent publications [33, 34] and a subset is shown in **Table 1**. The questionnaire was comprised of questions on the location, history, and characteristics of the unit, such as basic house dimensions, construction details of the building envelope, furniture materials and finishes for interior designs, the occurrence of factors which could influence moisture sources and air change as well as number, type and activities of the occupants.

Statistical analyses were performed in PRIMER 7 and STATICTICA 12 [38, 39]. Regarding the first two questions (listed in section 1), the abundance of archaeal and bacterial genes in bedrooms versus the most used rooms were first plotted (in log scale) to illuminate the indoor archaeal abundance relative to that of bacteria. Subsequently, a Wilcoxon Matched Pairs Test was used to investigate whether or not there is a significant statistical difference between total archaeal abundances in different types of rooms. Then, for the third question of the study (see also section 1), the BEST (Bio-Env) routine, namely BVSTEP, was used to determine which of 668 environmental factors and resident activities 'collectively' best explain the overall variation in archaeal total abundances in both room types. Subsequently, the significance of the BEST analysis result was validated through a permutational null distribution to ensure that the selected combinations of environmental variables were not obtained by chance. Univariate data analyses, namely Mann-Whitney (for two level categorical factors), Kruskal-Wallis (for multi-level categorical variables), and Spearman Correlation tests (for numerical variables) were next employed to explore which individual screened environmental variable would be relatively more associated with the variation of archaeal abundances.

3. Results

Archaeal abundances varied between 5.01±0.14 (log 16S rRNA gene copies / g dust, Mean ±SE) in bedrooms and 5.58±0.13 in the most used rooms. However, these magnitudes were notably lower than for indoor bacteria, which were between 9.20±0.32 in bedrooms and 9.17±0.32 in the most used rooms (**Figure 1**). When we compared sample pairs (bedroom and the most used room of the same houses), a significant difference was detected between their archaeal abundances (Wilcoxon Matched Pairs

Test, p = 0.04), with higher abundance occurring in the most used rooms (**Figure 1A**). However, no similar indication was found for the indoor bacteria (**Figure 1B**). Subsequently by using the BEST procedure, we found that almost 55 % of variation of total magnitudes of indoor Archaea can be explained by 15 and 21 out of 668 environmental factors in bedrooms and the most used rooms, respectively (**Table 2**). When the relative effect size of screened factors by BEST for bedrooms were estimated individually, however, only "use of electric dryer-vented outdoors" (Mann-Whitney U Test, p = 0.005) remained significant and negatively associated with the total abundances of bedrooms' Archaea (**Figure 2A**). Association of this factor was also noted in the most used rooms, albeit to a lesser degree (p = 0.06, **Table 2 and Figure 2B**). Moreover, most used rooms' archaeal abundances were significantly associated with the presence of upgraded plumbing systems (p = 0.029), hanging wet clothes inside the house (p = 0.031), and the use of liquid or solid air fresheners (p = 0.032). In particular, it was found that the presence of an upgraded plumbing system (**Figure 2C**) and hanging wet clothes inside the house (**Figure 2D**) were negatively correlated with the total abundance of Archaea in the most used rooms. In contrast, the use of liquid or solid air fresheners was positively associated with the total abundance of Archaea in the most used rooms (**Figure 2E**).

4. Discussion

We have demonstrated the presence of archaea in the house dust and the influence of selected indoor characteristics on archaeal abundance. These data may add to the existing knowledge that Archaea are not only present in extreme environments with physical limits for biological systems [1, 2], but they are also broadly distributed and abundant in

moderate environments [3, 7, 15, 40-44]. The latter can include Methanomicrobiales and Thermoplasmatales in freshwater and marine habitats [45], Crenarchaeota and Thaumarchaeota in soil [45, 46], and methanogens in the human and animal intestinal tracts [10-14].

Earlier studies have shown that Archaea comprise a significant proportion of microbes in soil and pelagic ocean waters, with a ratio of Archaea:Bacteria around 1:10 [46, 47]. In floor dust, we observed a much smaller archaeal contribution with a ratio of Archaea:Bacteria around 0.02:10 in bedrooms and 0.06:10 in the most used rooms. One of the explanations might be that because we used fine dust particles for sampling while it has been recently suggested that archaeal traces are mostly present in coarse particles [48]. The fact that Archaea were less numerous in indoor dust may also indicate that the indoor archaeal assemblages are mostly allochthonous (passive entrants of archaeal traces such as Halobacteriales, Thermoplasmatales, and the members of Thaumarchaeota [48] brought inside along with the fresh air through windows and ventilation systems or on the shoes and clothing of inhabitants). This is in contrast to the indoor bacterial assemblages, which are a mixture of both allochthonous and autochthonous assemblages (live and active inhabitants of dust). In addition, we found that Archaea, within houses, are not equally distributed and the most used rooms had significantly higher total archaeal abundances than bedrooms (**Figure 1A**). This may be because of the higher human traffic and a greater input of outdoor Archaea propagated indoors through open windows, on footwear or groceries brought inside.

Within each room type, the total abundance of Archaea varied depending on different environmental factors. For example, the use of electric dryer-vented outdoor was

negatively correlated with the total abundance of Archaea (**Figure 2 A and B**). One of the explanations might be that every time a laundry load is dried, some Archaea may be removed from the indoor environment through exhaust fans, and hence the neighboring areas in the house would contain lower amount of these microorganisms. In addition, we found that in houses where wet clothes were hung inside (**Figure 6.3**), the total abundance of Archaea was lower. This could be because when clothes are hung indoors to dry (as opposed to outdoors), the indoor environment may have lower input of outdoor air and thus a lower input of airborne Archaea.

Finally, in addition to outdoor sources, some indoor sources may also contribute to the abundance of indoor Archaea. For example, the use of liquid or solid air fresheners inside houses was positively associated with the total abundance of archaeal sequences (**Figure 2E**). One explanation may be that archaeal traces are embedded in the raw materials and additives of air fresheners, and hence distributed into the indoor environment upon freshener usage. Also houses with old plumbing systems showed higher levels of Archaea (**Figure 2D**), likely because of the accumulation of archaeal biofilm [49, 50] inside the plumbing system where biofilm-forming species can survive, release and disperse into the indoor environment.

In summary, this study provides evidence that Archaea are present in household dust and their abundances may be associated with the physical building characteristics and occupant activities. The results may be further used to form the basis of manipulative studies assessing the causality between factors and total abundance of indoor Archaea, diversity of the indoor archaeal community by using throughput-sequencing methods, as well as studies focusing on determining association of the indoor archaeal community

with human health and disease. Better understanding of indoor microbial diversity can eventually provide more awareness into the role of environment as a determinant of health, particularly in relation to non-infectious diseases in which inflammatory mediators are believed to be important.

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Conflict of Interest

The authors declare no conflict of interest.

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Figure captions:

Figure 1. Total abundance of (A) Archaea and (B) Bacteria in bedrooms and the most used rooms.

Figure 2. (A) The relationship between uses of electric dryer vented outdoors and total archaeal abundance in bedrooms. The relationships between (B) uses of electric dryer vented outdoors, (C) presence of upgraded plumbing system, (D) hanging wet clothes inside house, and (E) use of liquid or solid air fresheners and total archaeal abundance in most used rooms.

Table 1. Sub-sample of 668 collected environmental factors.

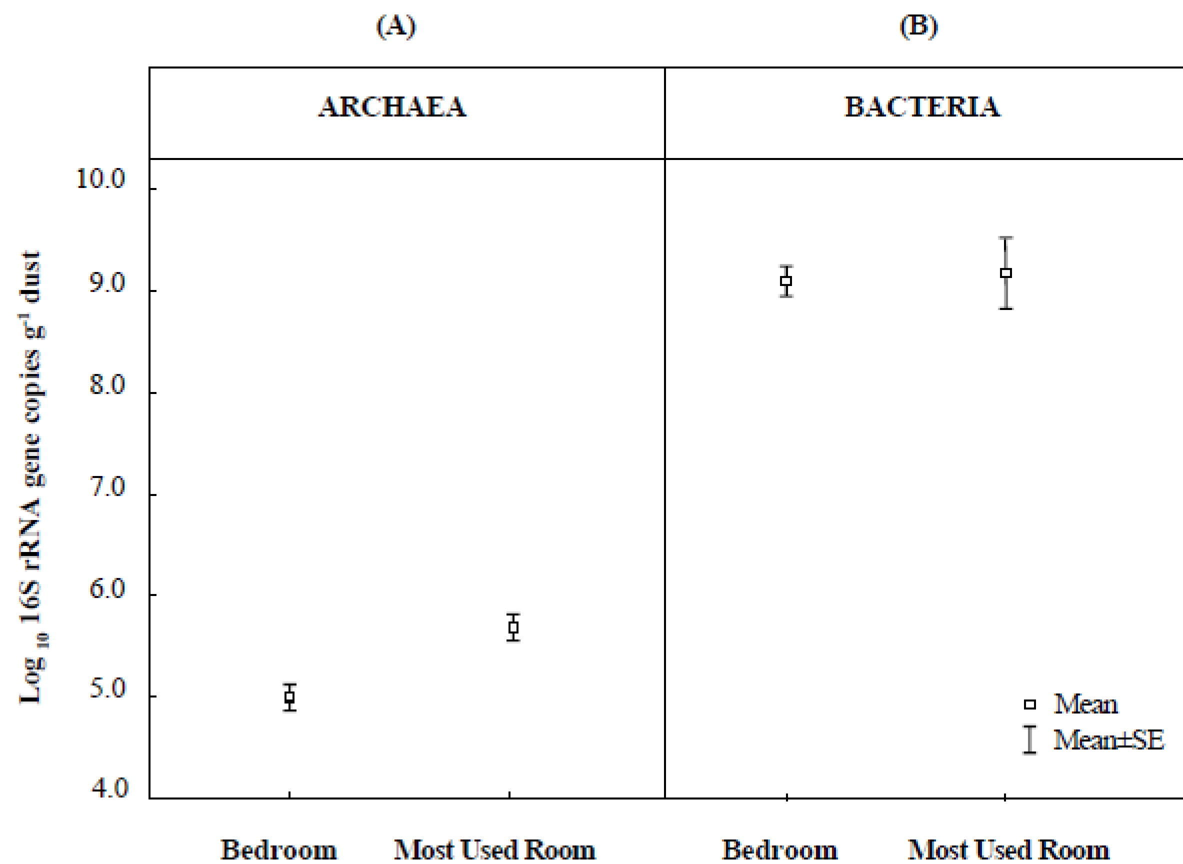
Building Design Characteristics	Type and Density of Occupants	Occupants' Activities
Age of ceiling	Number of adults in the house	Frequency of bathroom fan usage
Age of floor	Number of children in the house	Frequency of house cleaning
Age of house	Number of plants	Frequency of keeping the child bedroom's window open
Basement condition	Number of visitors/day	Frequency of keeping the most used room's window open
Basement dampness	Presence of long-hair cats	Hanging clothes inside the house
Basement foundation	Presence of long-hair dogs	Presence of stuff toys
Child room area (sq. m)	Presence of short-hair cats	Type of vacuum
Child room carpet area (sq. m)	Presence of short-hair dogs	Usage level of gas fireplace
Child room wall cover	Presence of plants	Usage level of radiators
Child room window cover	Presence of moth in house	Use of chemical spray and cloth
Cleanliness of basement	Presence of mouse in house	Use of garden sprays/weed killers
Condensation on bedroom Windows in cooler weather	Presence of pets	Use of mop
Evidence of leak in the house	**Furniture and Equipment**	Use of unscented or scented candles
Finished basement or added insulation	Number of pieces of leather	Use of antibacterial hand cleaner
Floor level	Number of pieces of Metal	Use of broom
Furnace age	Number of pieces of solid woods	Use of chemical sprays for cleaning
Furnace condition	Number of plastic/vinyl furniture	Use of disinfectant in baby's room
Living room area (sq. m)	Number of press wood furniture	Use of disinfectants
Living room carpet area (sq. m)	Presence of air conditioning system	Use of feather duster
Number of rooms in the house	Presence of electronic devices	Use of floor cleaners
Presence of garage	Presence of humidifier	Use of glass cleaners
Type of garage	Presence of stove fan in kitchen	Use of liquid or solid air fresheners
Presence of swimming pool		Use of multi-surface cleaners
Presence of upgraded plumbing system	**Outdoor Related**	

Total volume of the house	Geographic distance	Use of oven cleaners
Type of flooring	Is the house within 100 meters of:	Use of Plug-in deodorizers
Type of foundation	Body of water	Use of plumbing cleaners
Type of fuel in the house	Factory	Use of scented laundry detergents
Type of furnace's filter	Farm	Use of spray air fresheners
Type of garage	Gas station	Use of toilet bowl cleaners
Type of house	Major Highway/Artery	Use of vacuum
Type of insulation	Other source of pollution	Use of wet cloth (water only) for cleaning
Type of lawn	Neighbor currently doing renovations	Use of Swiffer wet jet
Type of wall covering		

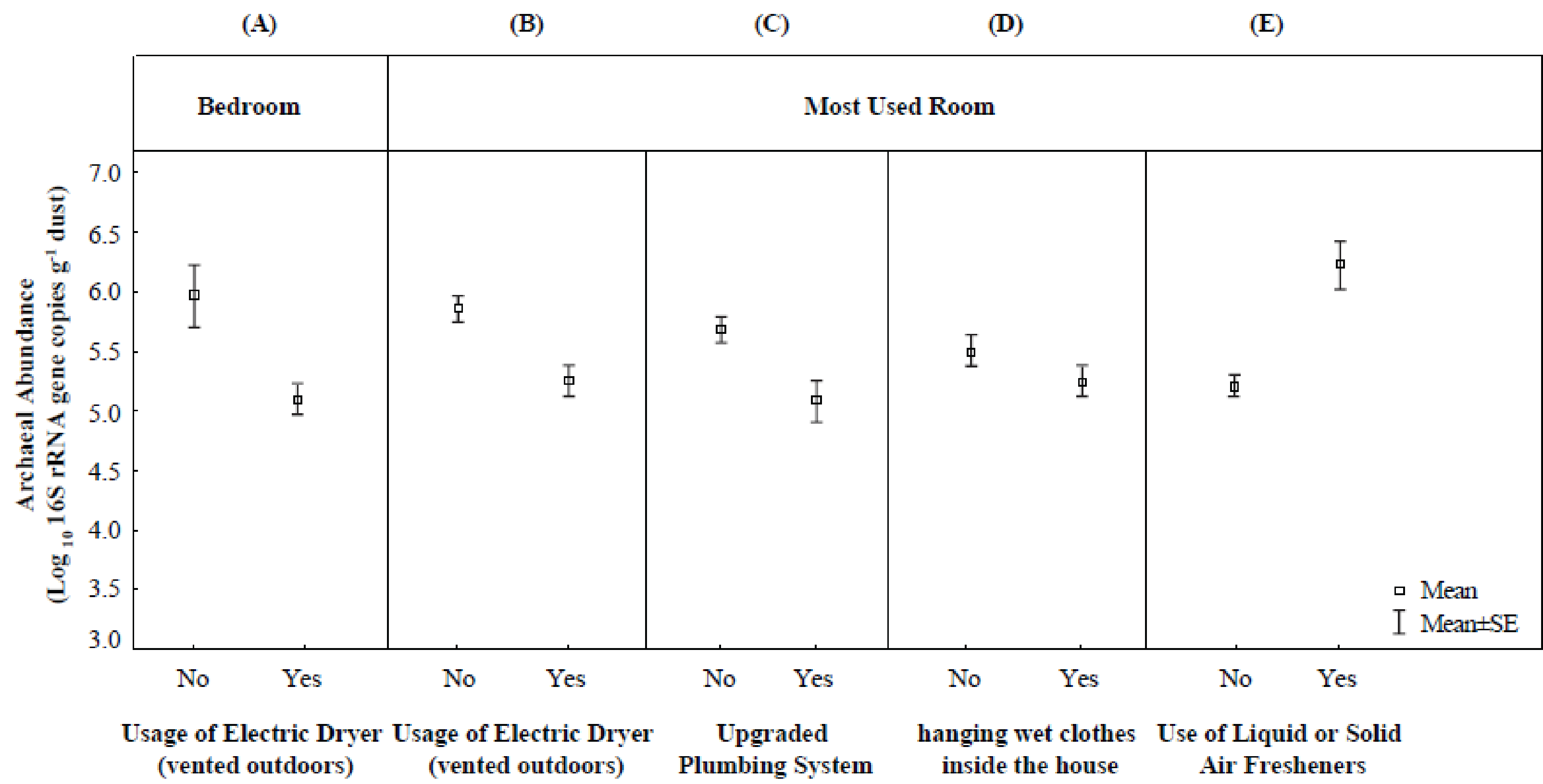
Table 2. Environmental and behavioural factors that best explain variation of the total archaeal abundance in bedrooms and the most used rooms.

Screened Factors by Best [a]	
Bedroom	**The Most Used Room**
Numeric	**Numeric**
Room area (sq. m)+1	Number of plastic or vinyl furniture
Number of plastic or vinyl furniture	
Number of press wood furniture	
Number of leather furniture	
Categorical	**Categorical**
Do they take off shoes when enters the unit	Age of floor
Is there a private child room	Basement condition
Occurrence of condensation on windows in cooler weather	Basement foundation
Presence of humidifier	Hanging wet clothes inside the house
Presence of long hair cat	Presence of air conditioning system
Presence of stuff toys	Presence of Garage
Type of furnace's filter	Presence of plants in home
Type of window's covering	Presence of plastic or vinyl covered furniture
Use of Electric dryer-vented outdoor	Presence of short hair cat
Use of gas fire place	Presence of stove fan in the kitchen
Use of Swiffer wet jet	Presence of swimming pool
	Presence of upgraded plumbing system
	Use of antibacterial hand cleaner
	Use of broom
	Use of floor cleaners
	Use of Electric dryer-vented outdoor
	Use of liquid or solid air fresheners
	Use of oven cleaners
	Use of scented laundry detergents
	Use of vacuum

[a] Multi-factor analyses: All factors are collectively responsible for 55.1% ($p = 0.03$) and 56.3% ($p = 0.02$) variation of total abundance of Archaea in bedrooms and the most used rooms, respectively.



(Figure 1)



(Figure 2)