Form 3

Annual Report
LaMer, Ehime University

Date (28, 02, 2018)

To Director of LaMer

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Include the report on the result of the project/meeting in a separate sheet.

1. Project / Meeting title
Environmental risk factors for the development and progression of pulmonary diseases

2. Members of project / meeting

Name	Affiliation	Position	Contribution part
PI Ana Sousa	CNRS-INEE-ECCOREV (Unité FR3098); OHMi Estarreja-OHM Bassin Minier de Provence; Reasercher	Researcher	Sampling, Chemical analysis, Results interpretation; paper writing
M. Ramiro Pastorinho	Faculty of Health Sciences, University of Beira Interior, Portugal	Invited Assistant Professor	Supervisor for the Project in portugal
Rumi Tanoue	CMES, Ehime university	Researcher	Chemical analysis, Results interpretation
LaMer Faculty member in charge	Tatsuya Kunisue	Professor	Supervisor for the project at CMES

3. CONTENTS

3.1. Introduction

Over the last century, a shift from an outdoor to an indoor lifestyle occurred and presently we spend 80-90% of our lives inside buildings [1]. This artificial habitat exhibits unique characteristics (e.g. insulation, humidity) that promote the accumulation of not only biological agents (e.g. bacteria, fungi) but also chemical contaminants and therefore it represents a prime interface between such agents and humans [1]. However, humans are not evolutionary prepared to deal with such an artificial habitat [2] and scientific evidence suggests that the increasing incidence of NCDs in western societies may be a consequence of the ubiquitous exposure to environmental contaminants [3]. Respiratory diseases constitute a paradigmatic case study, as they are deeply responsive to environmental contaminants. Furthermore, the economic burden of respiratory diseases is overwhelming, exceeding 380 billion € in Europe, with asthma and chronic obstructive pulmonary disease (COPD) representing the greatest portion with over 200 billion € [4]. Recent studies have suggested that the indoor environment is an important source of human exposure to not only toxic chemical contaminants but also microbial communities [5-7] and that such exposures deleteriously affect human respiratory health [8, 9]. Furthermore, the microbial community structure can be altered by the use of household products such as antimicrobial agents. In our ongoing microRESPIRA project we have identified the presence of a wide variety of fungi and bacteria including antibiotic resistant bacteria in house dust and indoor air samples [10]. Our preliminary results were the first to disclose the presence of antibiotic resistant bacteria in house dust samples. Given the interest of our dataset and in order to provide for the first compelling evidence of the modulation of indoor microbiome by household products and their joint effect in the respiratory status of COPD patients we focused this LaMer project on the evaluation of antimicrobials in dust samples and matched urine samples from patients with COPD.

3.2. Procedure

3.2.1. Sampling

Dust and urine samples from patients with COPD and respective controls were collected in Estarreja, Portugal between January 2017 and April 2017 under the framework of RESPIRA project. Dust samples were collected from the vacuum cleaner bag that was delivered to the volunteers and that was used for 60 days. The samples were then sieved trough a 63µm sieve and preserved at room temperature in dark conditions until chemical analysis. Urine samples were collected directly into sterile containers and at the laboratory of Beira Interior University, several aliquots of 1.5ml were prepared and immediately frozen. Two aliquots of each sample were transported to CMES in cool conditions and preserved at -20^{o}C until analysis.

3.2.2. Chemical analysis – urine samples

Levels of triclosan, triclorocarban and parabens (Methyl, Ethyl, Propyl, Butyl) were quantified in urine samples. At first, the extraction protocol was optimized. Two different extraction approaches were tested, liquid/liquid extraction and solid phase extraction (SPE).

a) Liquid/liquid extraction: 500 µL of hydrolyzed urine samples (see 3.2.3.) were extracted twice with ethyl acetate, spiked with ultrapure water, and the organic phase concentrated under gentle nitrogen flux. The target compounds were then re-dissolved with methanol/water (1:1, v/v) and the final solution was sonicated, centrifuged and filtered using a 0.2 µm cellulose membrane syringe filter (Satorius stedim biotech, Minisart RC4, 0.2 µm RC). The sample was preserved in amber LC glass vials at 4^0C until injection into the LC-MS/MS.

b) Solid phase extraction: After hydrolysis, cold methanol, ultrapure water and ammonia solution (5% NH_4OH) were added to the urine sample. The sample was then loaded into a pre-conditioned OASIS MAX cartridge (MTBE, Methanol and ultrapure water). Prior to elution with formic acid:MTBE:methanol = 0.2: 3: 7 (v/v/v) the cartridge was washed with 5% NH_4OH and methanol and afterwards dried for 15 min. The eluted target compounds were evaporated to dryness under nitrogen flux and re-dissolved with methanol and ultra-pure water. The sample was preserved in amber LC glass vials at 4^0C until injection into the LC-MS/MS.

3.2.3. Urine Hydrolysis

Urine samples were thawed at room temperature. 500 µL of the thawed urine sample was added to a 10 mL glass vial, to which 50 µL of Internal Standards (ISs) Mixture and 125 µL of 1.0 mol/L ammonium acetate buffer (pH = 4.7) containing β-glucuronidase/aryl-sulfatase was added. The solution was vortexed and incubated at 37°C under gentle agitation for 16hours.

The ISs mixture contained 100 ng/mL of Triclosan-$^{13}C_6$, 20 ng/mL of Triclocarban-13C_6, 20 ng/mL of Methyl paraben-$^{13}C_6$ and 20 ng/mL of Butyl paraben-$^{13}C_6$). The activity of β-glucuronidase/aryl-sulfatase was 290 units per mL of urine. The solution was prepared freshly every week by adding 4.7 ml of 1.0 mol/L ammonium acetate; 5.3 mL of 1.0 mol/L acetic acid and 50 µL of β-glucuronidase/aryl-sulfatase solution (116,000 units/mL). The crude mixture of β-glucuronidase/sulfatase from Helix pomatia (Type HP-2, aqueous solution, 116,000 units/mL glucuronidase and 1020 units/mL sulfatase) was purchased from Sigma-Aldrich (St. Louis, MO, USA); (G7017).

3.2.4. Chemical analysis – dust samples

Levels of triclosan, triclorocarban and parabens (Methyl, Ethyl, Propyl, Butyl) were quantified in dust samples. Since the results obtained for SPE with OASIS MAX Cartridge were better (see Table 1) all the dust analysis were performed using this technique. In brief, ISs mixture was added to

30mg of dust sample. The target compound were extracted twice by ultra-sonication with acetonitrile and methanol. After two centrifugations, the supernatants were combined and afterwards diluted with ultra-pure water. The sample was then loaded into a pre-conditioned OASIS MAX cartridge (MTBE, Methanol and ultrapure water). Prior to elution with formic acid:MTBE:methanol = 0.2: 3: 7 (v/v/v), the cartridge was washed with 5% NH_4OH and methanol and afterwards dried for 15 min. The eluted target compounds were evaporated to dryness under nitrogen flux and re-dissolved with methanol and ultra-pure water. The sample was preserved in amber LC glass vials at 4^0C until injection into the LC-MS/MS.

3.4. Results

3.4.1. Protocol optimization

In order to compare liquid/liquid extraction and solid phase extraction protocols 3 samples of urine plus one procedural blank were tested, furthermore recovery tests with three samples of urine spiked with PPCPs native working solution (100ppb) were also carried out. The obtained results disclose higher absolute recoveries and lower coefficient of variation for antimicrobials when using SPE with Oasis MAX cartridge (Table 1) and thus for the analysis of patients' urine and dust samples this protocol was used.

Table 1. Comparison of the absolute recoveries obtained using different extraction protocols for urine samples and IS corrected recoveries when using solid phase extraction with Oasis Max Cartridge.

Compound	Absolute recovery rate (%, %CV)			IS corrected recovery rate (%, %CV)
	LL extraction	SPE (type of cartridge)		
		Oasis MCX	Oasis MAX	
Methyl paraben	8.2 (0.38)	4.4 (4.2)	28 (4.9)	97 (2.0)
Ethyl paraben	5.9 (1.4)	3.9 (3.1)	25 (4.1)	89 (3.9)
Propyl paraben	28 (1.6)	21 (2.1)	36 (3.3)	79 (1.7)
Butyl paraben	47 (0.88)	34 (1.6)	49 (3.6)	110 (0.58)
Triclocarban	54 (6.7)	3.3 (7.3)	31 (21)	100 (2.5)
Triclosan	9.8 (6.2)	7.8 (4.7)	78 (3.1)	100 (2.4)

3.4.2. Urine and dust samples

Table 2 depicts the levels of parabens (Methyl, Ethyl, Propyl, Butyl) and antimicrobials (triclosan and triclorocarban) in human urine and dust samples. Overall, the concentrations in dust samples are one to two orders of magnitude higher that the concentrations in human urine. Triclosan was detected in 56% of urine samples and in all the dust samples, triclocarban was bellow detection limit (0.25 ng/mL) in all urine samples and was detected in 82% of the dust samples. Amongst parabens, methyl paraben exhibited the highest concentrations in both type of samples. Due to the limited number of dust samples available, it was not possible to find any

association between dust and urine levels. Interestingly, the highest level reported in dust for triclosan (1200 ng/g) corresponded to the house of the patient with the highest triclosan concentration in urine (140 ng/mL). Furthermore in this house, bacteria resistant to ampicillin were also detected in dust, with over 78% of the bacteria detected being resistant to this antibiotic. Such results, although preliminary suggest that the use of antimicrobials might be associated with the presence of resistant bacteria and thus deserve to be further studied.

Table 2. Summary of the concentrations of parabens (methyl, ethyl, propyl, butyl) and antimicrobials (triclosan and triclorocarban) in urine (ng/mL) and dust (ng/g).

		Parabens				Antimicrobials	
		Methyl	Ethyl	Propyl	Butyl	Triclocarban	Triclosan
Urine (n=43)	% detection	72	84	44	23	0.0	56
	min	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	max	1600	160	130	5.1	<MDL	430
	median	6.7	2.3	<MDL	<MDL	<MDL	0.35
	mean	63	12	6.8	0.15	<MDL	15
	stdev	240	28	22	0.77		67
Dust (n=11)	% detection	91	91	91	100	82	100
	min	<MDL	<MDL	<MDL	0.73	<MDL	23
	max	490	420	370	46	160	1200
	median	170	36	77	12	7.2	105.0
	mean	220	85	120	17	29	275.9
	stdev	160	120	130	16	46	362.0

<MDL: below the method detection limits

3.5. Future perspectives

We are planning to include the triclosan and triclocarban dust results in the paper that is being prepared on the house dust samples bacterial community. We expect to have all the data concerning bacterial community characterization by the end of April 2018 and to submit the paper by July 2018.

3.6. Achievements

3.6.1. List of papers published:

The impact factor™ (IF) and quartile values corresponds to Thomson Reuters metric https://jcr.incites.thomsonreuters.com. (*) Corresponding author

- Sousa ACA*. Tanabe S. Pastorinho MR (2017) Organotins: Sources and Impacts on Health and Environment. Reference Module in Earth Systems and Environmental Sciences. Elsevier. http://dx.doi.org/10.1016/B978-0-12-409548-9.09986-3. This paper was also published in the Encyclopedia of the Anthropocene (see 3.6.2).

- Coelho SD. Maricoto T. Pastorinho MR. Itai T. Isobe T. Kunisue T. Tanabe S. Sousa ACA*. Nogueira AJA (2017) Cadmium intake in women from Aveiro University. Portugal – a duplicate diet study. Journal of Geochemical Exploration 183(B): 187-190; http://dx.doi.org/10.1016/j.gexplo.2017.02.003; 5 year IF 2016: 3.024. Q2. 62.5%
- Coelho SD. Maricoto T. Tanabe S. Nogueira AJA. Sousa ACA* (2017) Dietary Habits of a Portuguese Academic Community - A Food Frequency Questionnaire Approach. Journal of Nutrition and Diabetes Research 1 (1)

3.6.2. Book chapters:

- Sousa ACA. Tanabe S. Pastorinho MR (2018) Organotins: Sources and Impacts on Health and Environment *In* Encyclopedia of the Anthropocene. A2 – Dellasala DA. Goldstein MI (Eds). (Oxford: Elsevier). pp. 133-139. https://www.sciencedirect.com/science/article/pii/B9780128096659099869

3.6.3. Oral Communications by Invitation

- Sousa ACA (2018) "Environmental contaminants and endocrine disruption: The story of obesogens". 20th European Congress of Endocrinology. Symposium 4: "Environmental Effects on Endocrine Function". S4.2. Scheduled for 20 May 2018. Barcelona. Spain

3.6.4. Oral Communications

- Sousa ACA. Silva T. Amaro R. Miranda S. Marques A. Valente C. Pastorinho MR. Pereira CC. Teixeira JP. Henriques I. and the RESPIRA Group (2017) microRESPIRA: microenvironmental risk factors for the progression of pulmonary diseases. 5º Colloque de Restitution do OHM- Estarreja. 3 November 2017. Aveiro. Portugal. P.7

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4. European Lung Association. *European Lung White Book*. 2013.
5. Prussin. A.J.. E.B. Garcia. and L.C. Marr. *Total Concentrations of Virus and Bacteria in Indoor and Outdoor Air.* Environmental Science & Technology Letters. 2015.
6. Kettleson. E.M.. et al.. *Key determinants of the fungal and bacterial microbiomes in homes.* Environmental Research. 2015. **138**(0): p. 130-135.
7. Barberán. A.. et al.. *The ecology of microscopic life in household dust.* Proceedings of the Royal Society of London B: Biological Sciences. 2015. **282**(1814).
8. Sousa. A.C.A.. et al.. *Characterization of Fungal Communities in House Dust Samples Collected From Central Portugal—A Preliminary Survey.* Journal of Toxicology and Environmental Health. Part A. 2014. **77**(14-16): p. 972-982.
9. Sharpe. R.A.. et al.. *Indoor fungal diversity and asthma: A meta-analysis and systematic review of risk factors.* Journal of Allergy and Clinical Immunology. 2015. **135**(1): p. 110-122.
10. Silva. T.. *Microbial community in houses from patients with chronic respiratory diseases in Estarreja*. in *Biology Department*. 2017. University of Aveiro: Aveiro.