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ABSTRACT

Anaerobic digestion is a consolidated biotechnology able to produce renewable energy from biomasses. In the European countries, quick growth of biogas production from different organic matrices including wastes has been observed. In relation to the characteristics and quantity of the anaerobic digestion of feedstock, there are different technologies, advantages and criticisms. An accurate occupational risk assessment and development of management tools for green jobs involved in the anaerobic digestion plants are due. The aim of this work is to assess the aerosol exposure for such workers, focusing on the bioaerosol risk. Full scale plants for the treatment of organic municipal waste, waste water treatment sludge, agro zootechnical and food producing byproducts were involved for this purpose. The bioaerosol levels were monitored during activities through culturing and biomolecular methods; moreover, the sub-fractionated PM10 and carried endotoxins were measured in different plant areas. Global microbial contamination is higher (>5,000 UFC/m^3) in the area where organic wastes are handled and pretreated, both for organic municipal waste plants - with a bacterial prevalence - and agro zootechnical plants - with a fungi

prevalence. Moreover, the microbial contamination is higher where organic municipal waste is present in respect to other biomasses (ANOVA $p<0.01$). Numerous pathogens are carried by the aerosol. HAdV-4 presence is lower than LOQ (50 gene copies/m^3) in all the samples. Environmental PM10 reached the 280 μg/m^3 level including PM3 for 78%. Endotoxin pollution overtakes the 90 EU/m^3 limit sporadically. Personal PM4.5 reached 10 mg/m^3 only for maintenance technicians in the pretreatment area for organic municipal waste. The risk can be evaluated under a quantitative and qualitative point of view highlighting risk management improvement for anaerobic digestion plants.

KEYWORDS

Anaerobic digestion; bioaerosol; fractioned PM; endotoxin; occupational exposure

ABBREVIATIONS

WWTP:	WasteWater Treatment Plant
AD:	Anaerobic Digestion
OFMSW:	Organic Fraction Municipal Solid Waste
ALB:	Agricultural and Livestock Biomasses
FFbP:	Food and Feed producing by-Products
COPD	Chronic Obstructive Pulmonary Disease
GIMC	Global Index of Microbial Contamination
IMC	Index of Mesophilic Bacteria Contamination
PNOC	Particulate Not Otherwise Classified

1. INTRODUCTION

Green Jobs refers to all occupational employment - from agriculture to administration and services - that sharply contributes to preserve or to restore environmental quality in terms of pollution impact removal, reduction or mitigation. The green jobs diffusion is in line with the stimulus to adopt more ecological production methods to limit global warming and to avoid irreversible climate change (WHO, 2014). Moreover, both increasing energy needs and the price of raw materials have been factors in the diffusion of such jobs. It has been estimated that in 2016, approximately 8 million people worldwide worked in renewable bioenergy (IRENA, 2017), and by 2020, 120,000 new net jobs are expected in the whole-EU bio-anaerobic sector (IEA, 2017).

Biomethanization consists of the production of methane from biomasses through a biological anaerobic process. The environmental advantage of such biotechnology includes the wide spread of biomethanization, particularly in the agro-zootechnic and waste treatment sectors. Anaerobic digestion is a process in which microorganisms breakdown organic biomasses in anaerobic or micro-aerophilic conditions (Díaz et al., 2011). This process leads to the production of biogas, a mixture mainly composed of methane and carbon dioxide with other trace compounds (Ali Shah et al., 2014). According to qualitative standards of the European certification, it has recently become possible (after an upgrading process and a cleaning phase) to turn biogas into biomethane; this can be used for vehicle fuels, natural gas net introduction, residential heating and so on (Scholz et al., 2013). Moreover, in many countries, including Italy, a subsidy is recognized for specific end uses and for specific biomasses such as wastes and production by-products (Wall et al., 2017). There are more than 6,000 active biogas plants in modern Europe (European Bioplastics, 2015), within which various occupational and environmental risks can be identified; these include explosive, chemical and biological risks (ECORYS, 2012). In general, there are not new risks, but risks with different magnitudes in relation to the source and to the quantity of the input biomasses. Such peculiarity is relevant, especially for biological agents and bioaerosols, which are generally underestimated in the occupational settings (Douglas et al., 2017). Bioaerosols include particles with a biological origin such as microorganisms, microorganism fragments such as endotoxins and biological-derived particles such as animal fur (ACGIH, 2006). They can produce adverse human health effects, such as transmissible diseases, decline of lung functionality and respiratory symptoms (De Vizcaya-Ruiz et al., 2006). On the other hand, bioaerosol compositions are not well-known, and surveillance data are weak to describe the real human health impact on groups generally composed of few individuals. (Douglas et al., 2016; Walser et al., 2015; Wéry, 2014). Furthermore, biomass treatments produce much fine particulate matter, which is also

correlated to relevant effects on human health. Some of these effects – such as, primarily, COPD and cardiovascular diseases - are also correlated to bioaerosol exposure.

The increment of the air temperature rise recorded in the last years - 0.2°C per decade (World Meteorological Organization, 2017) - worsens the potential bioaerosol burden in relation to human health effects.

The aim of this study was to evaluate the biological risk of biogas plants by identifying the green jobs involved, measuring the bioaerosol generated, assessing the worker exposure and finally proposing a semi-quantitative risk assessment method.

2. MATERIAL AND METHODS

2.1. Anaerobic digestion plants

In Italy, the number of anaerobic digestion plants has overtaken 1550 units. Moreover, the volume of biogas produced in 2015 is 5-fold higher in respect to 2007. The increment is more marked in the last 5 years and for the production of the final product biomethane, including the cleaning and up-grading steps (Sun et al., 2015). There are 167 biogas plants local to Piedmont (ARPA Piemonte, 2017), and these involve approximately 800 green jobs exposed directly to biological risks (3.2 persons/100 km^2); moreover, in Italy, the future perspective accounts for a total 25.000 green jobs involved in AD processes. This is double the green jobs measured to be involved in such sectors during 2015 (Green Report, 2015). Biogas plants can use different kinds of biomasses, including agricultural and livestock biomasses (ALB), food and feed producing by-products (FFbP), waste water treatment sludge (WWTS) and the organic fraction of municipal solid waste (OFMSW). In this study, we considered 3 types of plants in relation to the origin of the biomasses introduced into the digester: 3 plants that use ALB (1 in thermophilic and 2 in mesophilic conditions), 1 plant that mainly uses WWTS (mesophilic) and 1 plant that mainly uses OFMSW and FFbP (thermophilic conditions). The process flowcharts of the three types of plant are reported in Supplementary data 1 (a-b-c).

In ALB plants, the input matrix (solid vegetal origin biomasses and liquid cattle manure) is located in a storage tank and loaded into the hopper, directly or with the tractor aid, where the biomasses can directly reach the digester. In this type of plant, the greatest part of the workplace is outdoors, though these conditions are partly influenced by the different distances of plants from highly populated areas. Generally, in ALB plants, few workers are involved in anaerobic digestion management: on average, 4 workers, ranging from 1 to 7 workers in relation to different plants. When there is only 1 worker in the workforce, a large number of operations are allocated to

outsourcing. The ALB plants have a nominal installed electricity of 1 MW. All the ALB sampling sessions were conducted during the summer of 2016, involving 9 full sampling days. During each sampling day, we conducted samplings both in the morning and in the afternoon (Table 1).

In the WWTS plant, the AD input biomasses are sludge from primary and secondary sedimentation. In OFMSW treatment plants, solid biomasses are disposed of indoors in open tanks and then, mechanically pre-treated, from the unpacking to the grinding, mixing and heating. They reach the digester through pipes. Mechanical pre-treatment is generally only introduced for OFMSW, while for WWTS only thickening is included.

The number of AD workers in WWTS and OFMSW plants is on average 13, ranging from 7 to 20. The installed electrical capacity is on average 3 MW. The sampling session are conducted from August 2016 (OFMSW) to February 2017 (WWTS), involving 6 full sampling days. During each sampling day, we conducted samplings in the morning and in the afternoon, they are detailed on Table 1.

In all the involved plants, the produced biogas is recovered and piped in a co-generation system to obtain heat and electricity. System upgrades to produce biomethane were being implemented, even if they were not yet operative, during the sampling period.

The digested sludge can be used differently in relation to its hygienic characteristics (EU directive 1535/2015 and regulation EU 142/2011). In Italy, the latest law is the D.M. 5046 and the update of the D. Lgs. 75, both published on 2016. In the no-waste AD plants, sludges - often dewatered - can generally be used directly as fertilizer, taking into account the nitrate directives (European Commission, 1991) and the common agricultural policy (European Commission, 2010). On the other hand, when wastes are used, the output biosolids can be employed for composting or for incineration in relation to its toxicity.

2.2. Sub-fractionated PM environmental sampling

Sub-fractionated PM was performed in the 5 plants between August 2016 and February 2017. In each plant at least 2 different sites were chosen as sampling sites (“storage and loading” and “digestate output”) where the occupational exposure could be relevant.

The sampler was positioned near the site’s tank or the hopper, called "Storage and loading", not far from biomass storage area. Meanwhile, the sampler was positioned near the digestate collection emitted from the digester, where solid-liquid separation took place for the site; this was called "digestate output".

An additional sampling point was included in OFMSW in pretreatment, because both activities in the hangar and in a control room were present, and there additional protective systems are applied (filters for the air entering to the room). Each sampling was performed in the morning and repeated in the afternoon, for 4 hours each. PM were collected using a high-volume cascade impactor (the AirFlow PM10-HVS sampler is a multi-stage cascade impactor, with pre-selectors complying with the UNI EN-12341 norm, by Analitica Strumenti, Pesaro Italy) at an electronically controlled flow of 1.27 m^3/min. First, PM10 was selected by a pre-selector, and then the multistage impactor determined the division of different sampled particle sizes according to the aerodynamic diameter. The particle size fractions were as follows: 10.0–7.2, 7.2–3.0, 3.0– 1.5, 1.5–0.95, 0.95–0.49, and <0.49 μm. Glass microfibre filters with splits (Type A/E; 8'' x 10''; Gelman Sciences, MI, USA) were used to collect particles on each impactor plate. Glass microfibre filters (203 x 254 mm; Pall Corporation, NY, USA) were also used as back-up filters to collect the finest particles (<0.49 μm).

2.3. PM4.5 personal sampling

Personal sampling was conducted to evaluate worker PM4.5 exposure. It was performed on November 2016 and February 2017, in two plants (OFMSW and WWTP) where some workers are effectively dedicated only to the AD process. Personal exposure assessments were conducted over 2 or 3 days and the sampling periods covered a complete work shift (6 hours). PM4.5 samples were collected using the SKC AirChek XR5000 Sample Pump, a constant flow air sampler, at 2.2 L/min with a Casella Cyclone (Aquaria, Milan Italy, cod B.2030), glass microfibre filters (Nupore Filtration System Pvt. Ltd., India, cod NV20), and 25 mm diameter on filter support (Aquaria cod B.2031). A portable air floating flowmeter (ranging from 0.4 to 5 L/min) was used for air flow calibration after the filter assembly. The samplers also had an internal flow maintenance control system. The workers who received the personal sampler were 8 maintenance technicians and 10 workmen involved in both ordinary and extraordinary AD operations (cleaners).

2.4. Gravimetric and endotoxin analyses

Each filter regardless of sampling source (environmental or personal) was treated individually. First, they were conditioned before and after the sampling by placement in a dry and dark environment for 48 h, then weighed in a room with controlled temperature and humidity. The PM concentration in the sampled air volume was calculated based on the mass of the particulate

sampled and the volume of the air passed through the filters, as previously described (Traversi et al., 2010); then, it was expressed as µg per 1 m^3 of sampled air.

For the endotoxin extractions, different portions of the filters were used: one-half (51.75 cm^2) of the impactor plate filters and one-fourth (140 cm^2) of the back-up filters, with the full filter used for personal exposure. Each portion was cut into single strips and placed into a 50-ml sterile polypropylene pyrogen-free tube with 15 ml (for the personal filters, 5 ml) of RPMI-1640 medium (Biowest, France) and then supplemented with 0.025% Tween-20 (Sigma Aldrich, USA). The tubes with the filter strips were placed in an ultrasonic water bath for 10 min and then vortexed for 30 s. This procedure was repeated three times. The samples were then centrifuged at 5,000 rpm for 10 min to remove the glass fibre, and the supernatant was collected in 15 ml clean tubes. The resulting clear supernatant was assayed for endotoxin evaluation. The determination was performed as previously described (Duquenne et al., 2013; Paba et al., 2013).

Endotoxin was assayed using the endpoint chromogenic Limulus Amebocyte Lysate (LAL) method (QCL-1000™ 50-648U, Lonza, Walkersville, MD, USA) at 37°C with a microplate reader (TECAN Infinite® 200 Pro, Switzerland) following the kit instructions. The limit of detection (LOD) of the kit was 0.1 EU/ml, so considering the lower volume sampled and that the protocol applied the LOQ for the personal sampling, which was 1 EU/m^3, for the environmental sampling, the LOQ used was the lower 0.01 EU/m^3, because we can concentrate the sample, using more air in less extraction volume. Endotoxin concentration is expressed as EU per 1 m^3 of sampled air for each PM sub-fraction.

2.5. Bioaerosol sampling and analyses

Bioaerosol sampling was performed using a DUO SAS Super 360 sampler (PBI International, Milan, Italy), which allows microbial monitoring through air contact on apposite Petri plates (RODAC™ ContactPlates, VWR, USA). Twelve microbiological parameters were selected as described in Table 2. In particular: total bacteria at 22°C is an environmental contamination indicator; total bacteria at 37°C is an animal/human contamination indicator; and total Gram-negative bacteria - including such pathogens as *Salmonella spp.*, *Shigella spp.*, and Enterobacteriaceae in general, as faecal contamination indicators - linked to endotoxin presence, while Actinomycetes is another class linked to involved biomasses and *Staphylococcus spp.* and Clostridia are members resistant to and sometimes with an affinity for anaerobic conditions. Moreover, *Pseudomonas spp.* and *Bacillus spp.* are included as genera associated with biofilm formation (Sadiq et al., 2017; Wang et al., 2017). In particular, *Pseudomonas* is an indicator for contaminated water, and *Bacillus* for

contamination with plant origins. In the plants, biofilm could be present simultaneously in the storage tank, in the hopper and in the pipelines for sludge and recycled water. Moreover, biofilm formation is possible for all surfaces where biomasses are present. The plates were prepared in the laboratory few days before the samplings, following the medium instructions (Table 2). Different volumes were assigned for the various indicators (from 50 L to 2000 L). For all determinations, three plates were used, as a technical reproducibility guarantee. At the end of sampling plates were transported to the laboratory and placed in a thermostat-controlled environment set at the opportune temperature (Table 2). Microbiological contamination levels were also expressed as Global Index of Microbial Contamination (GIMC) and Index of Mesophilic bacteria Contamination (IMC) (Dacarro et al., 2005, 2000; Grisoli et al., 2012) (Table 4).

2.6. Viral analyses

Viral detection was conducted starting both from filters (finest fraction: PM0.49) and from specific plates. After sampling the filters were cut in half and stored at -80°C prior to DNA extraction. For the other samples, plate preparation was performed as previously described (Zhao et al., 2014; Ziros et al., 2011) and on each plate 2,000 L of air were sampled. Low Melting Agarose (LMA) (Ultra Pure™ LMP Agarose 16520-050, Invitrogen, USA) was prepared with deionized water following the product instructions. 4 ml of medium were transferred on each Petri plate. After sampling the LMA was transferred in sterile Falcon with a sterile spatula, then stored at -80°C prior to extraction. The LMA was used as the means of capture, not as cultural substrate for aero dispersed virus. Such method is able to limit the high dehydration observable for filtration on solid membrane and to produce, in theory, less degradation of the microbiological component.

DNA extraction protocol was the same for filters and for LMA and was performed using PowerViral Environmental RNA/DNA kit (#28000-50 Qiagen, USA):

- For filter extraction (20 total samples) 1/16 of glass microfibre filter, which corresponds to 19 m^3 of sampled air, was used. Filters are made of porous material and in the first step of extraction, more lyses buffer was used (2 ml);
- For LMA extraction (26 total samples, 20 corresponding to PM0.49 filters and 6 sampled in the control room, Table 1, where the environmental PM sampling was not possible) the agarized medium was depolymerized by a microwave for a few seconds; the extraction was performed starting from 200 µl, corresponding to 0.1 m^3 of sampled air.

After viral extraction, the nucleic acids were quantified using a NanoQuanto Plate (Tecan Trading AG, Switzerland) which allows the quantification through a spectrophotometer read at 260 nm.

The spectrophotometer used was Tecan Infinite® 200 PRO and the software was i-control™ (version 1.11.10). The protein and lipid contamination indices were calculated through two ratios A260/A280 and A260/A230 respectively to certify the purity of the extract. The average DNA extracted concentration from filters was 4.27 ± 1.29 ng/ml and from LMA, 1.89 ± 0.76 ng/ml.

Real time PCR was performed, with the CFX Touch System (Bio-Rad, USA), to identify and quantify the HAdV-4 (human adenovirus 4; strain RI-67, ATCC® VR-1572). The primers used were: AdF-1 (5'–CWTACATGCACATCKCSGG–3'), AdR-1 (5'–CRGGGGCRAAYTGCACCAG–3') and probe AdP1-1 (5'–CCGGGCTCAGGTACTCCGAGGCGTCCT–3') (ThermoFisher Scientific, U.S.A.) at 10 µM (Ziros et al., 2011). Standard curves were created with serial seven-fold dilutions of purified viral DNA starting from a concentration of $5.07*10^7$ gene copies. The reaction was set as follows: 50°C (2 minutes), 95°C (2 minutes), 45 cycles of 95°C (15 seconds), 55°C (30 seconds) and 72°C (15 seconds) and final step at 72°C for 5 minutes. The reaction was performed using iQ™ Multiplex Powermix (BioRad, USA).

2.7. Statistics

Statistical analyses were performed using the SPSS Package, version 24.0 (IBM Corp.). We applied: (1) a log transformation of non-normally distributed data, (2) the Spearman's correlation to assess relationships between variables; (3) T-test to compare means, (4) an ANOVA for multivariate analysis, in which we assumed an equal variance, followed by a Tukey post hoc test for multiple comparisons. The mean differences and correlations were considered significant if $p<0.05$ and highly significant if $p<0.01$.

3. RESULTS AND DISCUSSION

3.1. Environmental sub-fractionated PM10 and personal PM4.5

The particulate matter was sampled in all 5 plants both during storage and loading activities and during digested sludge outing. In Figure 1 the concentration of the particles are shown in relation to the sampling plant considered. The PM10 sub-fractions contribution are illustrated in Figure 1A, while the total summarized PM10 is illustrated in Figure 1B.

Six PM10 fractions (10.0–7.2, 7.2–3.0, 3.0–1.5, 1.5–0.95, 0.95–0.49, and <0.49 µm) were evaluated, and the particles with an aerodynamic diameter smaller than 0.49 µm were the most represented compared to the other fractions for ALB and WWTS plants (ANOVA, $p<0,01$). In fact, in such conditions, the contributions of the finest fraction (<0.49 µm) to the PM10 were respectively 48% and 59%. On the other hand, OFMSW plants showed a more homogeneous

distribution of the single fractions, with a contribution of 15% of each sub-fraction to the total PM10. This evidence was consistent with pollution of indoor environments needed for the OFMSW plants. In such conditions, particulates were scarcely scattered compared to in the outdoor and semi-outdoor environments typical of ALB and WWTS plants, which showed greater particulate dispersion.

Regarding the breathable fraction of PM10, PM3 (the sum of the 4 finest fractions) was estimated. Values were approximately 128 μg/m^3 for OFMSW plants, 55 μg/m^3 for agro-livestock treatment plants and 70 μg/m^3 for WWTS. Moreover, in the OFMSW plants the finest fraction was more abundant in the storage and loading biomasses areas respective to the digested output area (T-test, $p<0.01$). Both ALB and WWTS plants did not show significant differences between the two sites. This is congruent with the two plants' features, in which there are not clear separation between the areas generally outdoor or only partially confined.

Environmental PM3 concentrations were lower compared to the occupational limit of dust exposure as a PNOC breathable fraction, 3 mg/m^3 (ACGIH, 2014), though such a limit is referred to as personal exposure assessment. On the other hand, a characterization of the quality of the dispersed particles is beneficial in order to exclude or to estimate the proportion of particular dust such as, for example, wood or flour for which more stringent limits are imposed (ACGIH, 2014). Moreover, the observed fine PM pollution is widely higher than the guideline values, introduced for general population and continuous exposure, by the Environmental Air Quality Guideline values proposed by both the EU Regulation (25 μg/m^3 year mean, 20 within the 2020) and WHO (10 μg/m^3 year mean) (Shneider et al., 2014).

The PM10 levels were higher in OFMSW plants (mean 204.88 μg/m^3), followed by agro ALB plants (mean 69.05 μg/m^3) and WWTS plant (mean 57.77 μg/m^3) (ANOVA, $p<0.01$) (Figure 1B). The higher concentration of the PM10 was justified by the indoor characteristics of the OFMSW plants, in which there is less dispersion of the airborne particles. Even if the PM10 levels were far from reaching the occupational limit of dust exposure (PNOC inhalable fraction, 10 mg/m^3)(ACGIH, 2014), they were markedly higher than both the EU (50 μg/m^3 day mean) and WHO (20 μg/m^3 year mean) Environmental Air Quality Guideline values (Shneider et al., 2014) proposed for general population and continuous exposure in life not occupational environment.

Personal sampling was assessed only in solid and liquid waste treatment plants where AD dedicated workers were present. In the ALB plant the workers have more heterogenic activity. The OFMSW plant showed a higher concentration in storage and loading areas (>4,000 μg/m^3)

compared to WWTS plant (~300 µg/m^3) (Table 3). Meanwhile, in WWTS plants there was a higher, even if moderate, contamination in the digested sludge output area (Table 3).

3.2. Endotoxin pollution

The endotoxin contamination was generally limited and the levels ranged from 0 to 137.83 EU/m^3, with a mean value of 4.68 $\pm$ 15.52 EU/m^3. Comparing the three plants, endotoxin concentrations were higher in the OFMSW plant, which is consistent with the indoor characteristics of sampling points in such plants (Figure 2A and 2B). However, the endotoxin contamination in the three anaerobic digestion plants studied was generally comparable to the data observed in the literature (Duquenne et al., 2013). Figure 2A shows the PM10 sub-fractioned distribution analysis of airborne endotoxins in the two sampling sites within the three different anaerobic digestion plants. In the storage and loading areas of the OFMSW plants, the endotoxin presence was higher in the finest particles (<0.49 µm) and in the 1.5-3.0 and 7.2-10 µm fractions. Moreover, the difference (not shown in figure 2) between input biomass area and output area at OFMSW is significant (for total PM10: 119.75 *vs* 5.12 EU/m^3; T- test, p <0,05).

Moreover, no significant difference was detected between the sampling sites in the ALB plant, where endotoxin levels were limited, showing a limit contribution of gram-negative microorganism contamination.

Finally, in the WWTS plants, the most contaminated sites were the digested output areas, in which endotoxin concentrations were more abundant than in the storage and loading sites, specifically in the finest fraction (<0.49 µm) and in the total PM10.

The determination of personal PM4.5 samples showed the same circumstances of contamination, but endotoxin levels are not relevant in such samples (Table 3).

At present, there is no occupational exposure limit for endotoxins at the international level. However, there is currently a health-based occupational exposure limit of 90 EU/m^3 established by the Dutch Expert Committee on Occupational Safety (DECOS, 2010). In this study, the endotoxin content is largely below the Dutch level with the exception of the pretreatment area of the OFMSW plant, where the endotoxins carried by PM10 reach level above 100 EU/m^3.

Finally, a significant correlation (p=0.594; p<0.01) was observable to highlight an influence of the aero dispersed particle mass on the endotoxin pollution. The maximum endotoxin contribution calculated, in mass (considering 1EU ~ 0.1 ng endotoxin), to the total particulate is 1/50,000.

3.3. Bioaerosol results

The results of the cultural microbiological analysis were summarized in Table 4. The microbiological indicators with a higher concentration were the total bacteria population at 22°C, 37°C and 55°C, and fungi and yeast, followed by Actinomycetes for all the ALB and OFMSW plants (Figure 3). In decreasing order, we found Staphylococci, Bacilli, Enterococci and Clostridia. Moreover, the levels of gram-negative Pseudomonadaceae, *Salmonella spp.* and *Shigella spp.* were very limited. Only sporadically typical and metabolically confirmed colony are identified. Our results are consistent with other, previous studies, produced for other production activities that include biomasses (Dacarro et al., 2005, 2000; Eduard et al., 2012; Fracchia et al., 2006; Gladding and Gwyther, 2017; Grisoli et al., 2012).

Comparing the three types of anaerobic digestion plants studied, we observed a greater contamination in the OFMSW plant for the total bacteria counts at 22°C (ANOVA $p<0.01$) and 37°C, following by ALB plants and then WWTS plants. On the other hand, in the agro-livestock treatment plants there was a large amount of fungi and yeast contamination (ANOVA $p<0.01$).

The comparison between the first steps of the operation during the biomass storage and loading and the final steps during the digestate output in the three types of plants (Figure 3) showed that in the OFMSW plant, the total bacteria at 22°C and 37°C were higher in the input sites. In the agro-livestock treating plants, only the total bacteria at 22°C were greater in the input operations, but such differences are limited, especially for plants where pig sludge was treated. In the WWTP, the higher microbiological parameter was the total bacteria count at 55°C, with no particular differences between the two sampling areas. Moreover, such data are very low dispersed near the median (Figure 3, last box). In the ALB plants no difference between the two areas was present. Considering all the data, the GIMC is higher in the input area (T-test $p<0.05$).

Bacillus spp. and Pseudomonadaceae were higher in OFMSW plants compared to the other plants and significantly correlated (Spearman's correlation =0.894 $p<0.01$). Notably, we observed greater contamination in the storage and loading areas. *Bacillus spp*. and Pseudomonadaceae were generally associated with the biofilm maturation, followed by biofilm dispersion. The first was found in the presence of vegetal biomasses, the second in the presence of water (Flemming and Wingender, 2010)(Sadiq et al., 2017). The contamination is described in Table 5 and the two biofilm indicators are significantly correlated (Spearman's rho =0.539 $p<0.01$), showing an effective problem with respect to biofilm formation in the presence of great amounts of biomasses. Moreover, Bacillus spp. correlated significantly to the GIMC (Spearman's rho =0.434 $p<0.05$) and to the bacterial counts (Spearman's rho =0.720 $p<0.01$) and can be considered a

simple bioindicator of bacterial contamination in such plants as discussed in the literature (Tasaki et al., 2017).

On the other hand, no significant differences between input and output operations were found for Clostridia levels in the three types of plants (T-test $p>0.05$). The same evidence was observed generally in the literature (Romanazzi et al., 2016), even if the problem of spore-forming and anaerobic pathogens is discussed (Neuhaus et al., 2015).

Table 6 shows the results of qualitative analysis, starting from the CFU on plates sampled in the studied workplaces. Those analysis revealed the presence of biological agents that may cause disease in humans and are classified in the Risk Group 2 and opportunistic pathogens (Advisory Committee on Dangerous Pathogens, 2013). In Table 6, other microorganisms generally present in the three biomasses are also included (for example *B. subtilis*), as reported in the literature (Haagsma et al., 2012).

There is still a lack of implementation of standardized procedures for microorganism characterization in the various environments, even if guide lines and technique procedures are published (UNI EN 13098:2002; UNI CEN/TS 16115-1/2:2016). Moreover, since the human response to bioaerosol exposure is variable, there are no internationally accepted threshold limit values or occupational exposure limits for biological agents. Nevertheless, a few countries proposed acceptable values. For example, Germany, where a limit of 50,000 CFU/m^3 of mesophilic fungi (includes *Aspergillus* sp.) was set for breathable air in the workplace (Douglas et al., 2016). In Russia, the proposed limits for some fungi species and actinomycetes ranged from 10^3 to 10^4 cells/m^3 (Rao et al., 1996) on the basis of a LOEL (Lowest Observed Effect Level) of 10^5 spores/m^3 of no pathogenic and no mycotoxins-producer fungi species, considering the inflammatory effects in the respiratory tract (Eduard, 2006).

Recently, in England, provisional guidelines for composting operators was proposed; this established 10^3 CFU/m^3 for total bacteria, 300 CFU/m^3 for Gram negative bacteria and 500 CFU/m^3 for *Aspergillus fumigatus,* as acceptable levels of bioaerosols. Such levels must be kept at 250 m away from the source to protect public health (Pearson et al., 2015).

3.4. Virus results

The Real-Time PCR was performed and all samples showed a concentration under the LOQ. The results showed presence of HAdV-4 <50 gene copies/m^3 sampled air for each sample. The simple PCR, without the use of photoreactive DNA-binding dye, is not able to distinguish between genome units coming from infective or not infective biological agent. The presence of other

airborne viruses cannot be excluded, in particular the bioaerosol could contain both other DNA virus and RNA viruses such as NoV (Norovirus) and HEV (Hepatitis E Virus) (Masclaux et al., 2014). The presence of harmful viruses in the air emission was previously examined, especially NoV, in a hospital WWTP. NoV genomes were detected in the air inside the WWTP and in the exhaust air, even if in low concentrations (Uhrbrand et al., 2017). Furthermore, airborne virus analysis could need more quantitative assessments with new viral targets or with universal sets of primer that could identify family of viruses, as already possible for bacteria and archaea (Muyzer et al., 1993; Nicol et al., 2003). An additional step is represented by metagenomic methods and sequencing of the viral genome. This approach could be applied to the viral component of bioaerosol; currently, the most used platforms are Illumina/Solexa and Roche 454. Despite the high resolution, the next generation sequencing techniques show some disadvantages in relation to this matrix (Behzad et al., 2015): (a) high viral DNA or RNA concentrations in the environment; (b) inefficient sampling for airborne virus extraction; (c) no standardized methods; and (d) requirement of specific bioinformatic abilities to process the reads.

3.5. Occupational risk evaluation

For reason of the results that we obtained, the occupational risk due to PM10 or endotoxin was no negligible; thus the risk's control could be obtained by applying good work practices and using protective measures, provided by specific equipment such as cabin and collective air extractors with filters and as final resource individual protective devices. Moreover, the high microbiological risk could be managed using occupational safety and control measures, including limited and protected access to the areas and use of vehicles with cabin filters and systems of air's filtering. In Italy, the management of the biological risk refers to the law introduced during 2008 (D. Lgs. 81/2008), which follows the EU directive 2000/54/CE. An accurate and exhaustive risk assessment is due for all the occupational tasks, evaluating both the probability and the gravity of the effects (using the regulation group - from 2 to 4) (2000/54/CE). When calculating the probability (P) of bioaerosol-related effects, a wide list of occupational factors have to be considered with the estimation of bioaerosol levels in terms of quantity and quality. On Figure 4 the conceptual construction of a quantitative risk evaluation for AD plant is proposed. Moreover, the risk (R) for two specific work tasks are calculated in each of the plant kinds (grey bottom box Figure 4). The risk could be considered high and additional specific control activities due above 8, so in the example shown in Figure 4, it can be deduced that based on the monitoring activities discussed,

such levels frequently being reached for the OFMSW plant specifically in the input and pretreatment area (ARPA and INAIL, 2013).

Of course, the quantitative microbial risk assessment is also an alternative in the occupational environment (Carducci et al., 2016), but it is actually an expensive method that includes specific evaluation for each pathogen. Moreover, there are not clear safety limits due to both heterogeneity of the microbial mixture, also conformed in this work and to the variable inter individual susceptibility.

3.6 Study limitation and future perspective

Selection bias could be present in this work. The enterprises needed to accept the invitation to participate in the study on a voluntary basis even if without cost, so it could be supposed that such enterprises are more careful of occupational risk management. For example, an AD plant sample cannot be assumed as really representative of occupational settings, and results from this study could probably could be considered a final underestimation of the real risk for the AD green jobs.

An in-depth analysis for fungi was not performed in the work, for lack of a mycologist, and this could be auspicious for especially the ALB plants. The bioaerosol was analysed using culturing methods on an air contact plate; however, there are also other methodologies useful for such purposes, such as impinge samplings. Moreover, the literature showed the presence of viable but non-cultural (VBNC) microorganisms that could be relevant in such samples. Recently, the application of methods based on loop-mediated isothermal amplification seems to also be useful for VBNC estimation (Li et al., 2017).

The viral fraction of the bioaerosol in particular needs technical and scientific improvement of samplings methods, following extraction and detection. The methods actually used are not able to produce exhaustive results.

The epidemiologic evidence with respect to dose-effect estimation in occupational settings for bioaerosol and infective material, especially data on chronic effects, are not well known, so a real risk characterization is difficult. This is the reason behind the lack of regulatory limit definitions. However, such problems have to be overcome, for example by analysing early effect biomarkers on population exposed to various bioaerosol levels.

4. CONCLUSION

The management of biological risk deserves specific attention, especially in indoor areas, where organic wastes are treated. These settings are where bacterial and mould contamination are quite

relevant, and where the presence of various pathogens is shown dispersed into the air also, as part of the bioaerosol. In such working areas, various green workers conduct their tasks. Among the various jobs, the maintenance technicians and workmen involved in the cleaning procedure, near the biomass, showed respirable PM exposure.

The estimated occupational risk is relevant for bioaerosol, endotoxin and particulate exposure. On the other hand, such problems cannot be an obstacle to the diffusion of the AD treatment for organic waste and biomasses. International reports discussed the role of waste-to-energy in the circular economy COM(2017)34, highlighting waste-to-energy processes among which anaerobic digestion of biodegradable waste is expressly included. Advanced management methodologies, such as the application of collective protection devices such as air extractors and filters, are beneficial where the risk is high. Human health concerns cover not only infective disease and eventually outbreaks but also as much as chronic disease, such as COPD, or other chronic effects observed in populations with occupational exposure to bioaerosol. Further close examination might concern the characterization of the viral presence, as well as bacteria such as *Legionella spp.,* moulds such as *Aspergillus spp.* and the impact on the resident population in the surrounding areas of the plants.

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FIGURE CAPTIONS

Figure 1. (A) Box-plot of the PM10 sub-fraction levels for each AD feedstock (B) Total PM10 levels in the three types of plants. Circles indicate outliers, while asterisks indicate extreme values.

Figure 2. (A) Box-plot of the endotoxin carried by PM10 sub-fraction for each AD feedstock (B) Endotoxin level carried by total PM10 in the three types of plants. Circles indicate outliers, while asterisks indicate extreme values.

Figure 3. Box-plot of the main bioaerosol components for the different feedstock plants in the two main sampling sites (INPUT: delivery, storage and pretreatment area; OUTPUT: digested sludge disposal area). Circles indicate outliers, while asterisks indicate extreme values.

Figure 4. Risk assessment scheme suggested for the AD plants.

TABLE CAPTIONS

Table 1. Description of the collected samples: sampling site, work shift, number of samples both in PM filters and microbiological plates

Table 2. Microbiological parameters adopted for bioaerosol pollution assessment and technical information on the cultural method adopted.

Table 3. Mean concentration of PM4.5 and of the carried endotoxins for the personal samplings in the three types of plants, divided by sampling sites.

Table 4. Descriptive analysis of the microbiological contamination of the air observed during the samplings in all the plants and reference value previously published in the literature (grey column). For each parameter, we have 25 different samples; moreover, all the samples were the result of at least a technical triplicate for bacteria counts; fungi and *Bacillus spp.* were sampled different air at different volumes to observe a readable plate.

Table 5. Pseudomonadaceae, *Bacilli spp.* and Clostridia mean concentrations (CFU/m^3) in the sampling sites of the three types of plants. Number of collected samples: 6 in OFMSW, 7 in ALB, 4 WWTP; each as results of plate technical triplicate

Table 6. Microorganisms metabolically identified in the three types of plants from the bioaerosol cultured plates.

Table 1

Where		Work shift	N of filters*	N of plates [(1)]
OFMSW	Pre-treatment control room (INPUT)	morning	1 C	42
		afternoon	1 C	/
	Storage and loading, including pretreatment (INPUT)	morning	5 A 1 B 3 C	40
		afternoon	5 A 1 B 2 C	40
	Digestate OUTPUT	morning	5A 1 B 3 C	40
		afternoon	5 A 1 B	40
ALB	Storage and loading (INPUT)	morning	30 A 6 B	211
		afternoon	15 A 3 B	85
	Digestate OUTPUT	morning	25 A 5 B	248
		afternoon	15 A 3 B	206
WWTP	Storage and loading (INPUT)	morning	5 A 1 B 4 C	42
		afternoon	5 A 1 B	42
	Digestate OUTPUT	morning	5 A 1 B 2 C	42
		afternoon	5 A 1 B	42

* A = PM subfractions; B= PM <0.49; C= PM 4.5. Each C filter correspond to a worker.
[(1)] included LMA plates

Table 2

Parameters	Incubation period	Incubation temperature (°C)	Cultural medium
Bacterial environmental total count	48 h	22	Plate Count Agar
Bacterial Total count 37°C	48 h	37	Plate Count Agar
Thermophilic total count	48 h	55	Plate Count Agar
Yeasts/fungi	5-7 days	25	Sabouraud glucose 4% chloramphenicol agar
Pseudomonadaceae	18-48 h	37	Cetrimide
Bacillus spp.	24-48 h	30	HiCrome Bacillus Agar
Clostridia	18-24 h	44	m-CP
Negative Gram bacteria	24 h	37	MacConkey Agar
Salmonella spp. Shigella spp.	18-24 h	37	Xylose lysine deoxycholate agar (XLD)
Actinomycetes	5-10 days	55	Starchcasein agar
Enterobacteriaceae	48 h	37	Slanetz+ Triphenyl tetrazolium chloride (TTC)
Staphylococcus spp.	48 h	37	Baird-Parker Agar

Table 3

	OFMSW			**WWTP**		
	Input area	Output area	Control room	Input area	Output area	Control room
PM4.5 µg/m^3	4065±2140	446±179	214±21	310±162	613±541	n.d.
PM4.5 EU/m^3	6.03±2.86	4.04±1.61	3.67±4.85	1.49±0.68	12.52±7.56	n.d.

Table 4

Parameters	**Mean (UFC/m^3)**	**Standard Deviation (UFC/m^3)**	**MAX (UFC/m^3)**	**References** (Gladding, 2017; Dacarro 2010; Fracchia 2006; Eduard 2012)
Environmental count 22°C	546	573	26,303	10^2-10^5
Mesophilic count 37°C	1,420	1041	13,183	10^3-10^6
Thermophilic count 55°C	934	1456	2,630	10-10^4
Yeasts/Fungi	4,820	7071	26,303	10^3-10^5
Pseudomonadaceae	3	3	1,318	-
Clostridia	25	26	479	-
Bacillus spp.	187	47	13,183	-
Gram-negative	4	2	339	0-10^3
Salmonella spp. Shigella spp.	2	1	372	-
Actinomyces	738	692	2,630	10^3-10^4
Enterococcus spp.	80	124	1,318	-
Staphylococcus spp.	291	364	1,445	-
GIMC	5,969	7,197	38,904	>5,000
IMC	2.6	1.8		>3

Table 5

Sampling site	Microbiological parameters	OFMSW (UFC/m^3)	ALB (UFC/m^3)	WWTP (UFC/m^3)
Storage and loading	Pseudomonadaceae	729 ± 78	4 ± 1	1 ± 1
	Bacillus spp.	7,175 ± 697	320 ± 35	135 ± 28
	Clostridia	118 ± 28	27 ± 14	5 ± 2
Digestate output	Pseudomonadaceae	1 ± 1	6 ± 1	1 ± 1
	Bacillus spp.	225 ± 95	202 ± 14	135 ± 54
	Clostridia	17 ± 3	54 ± 4	5 ± 1

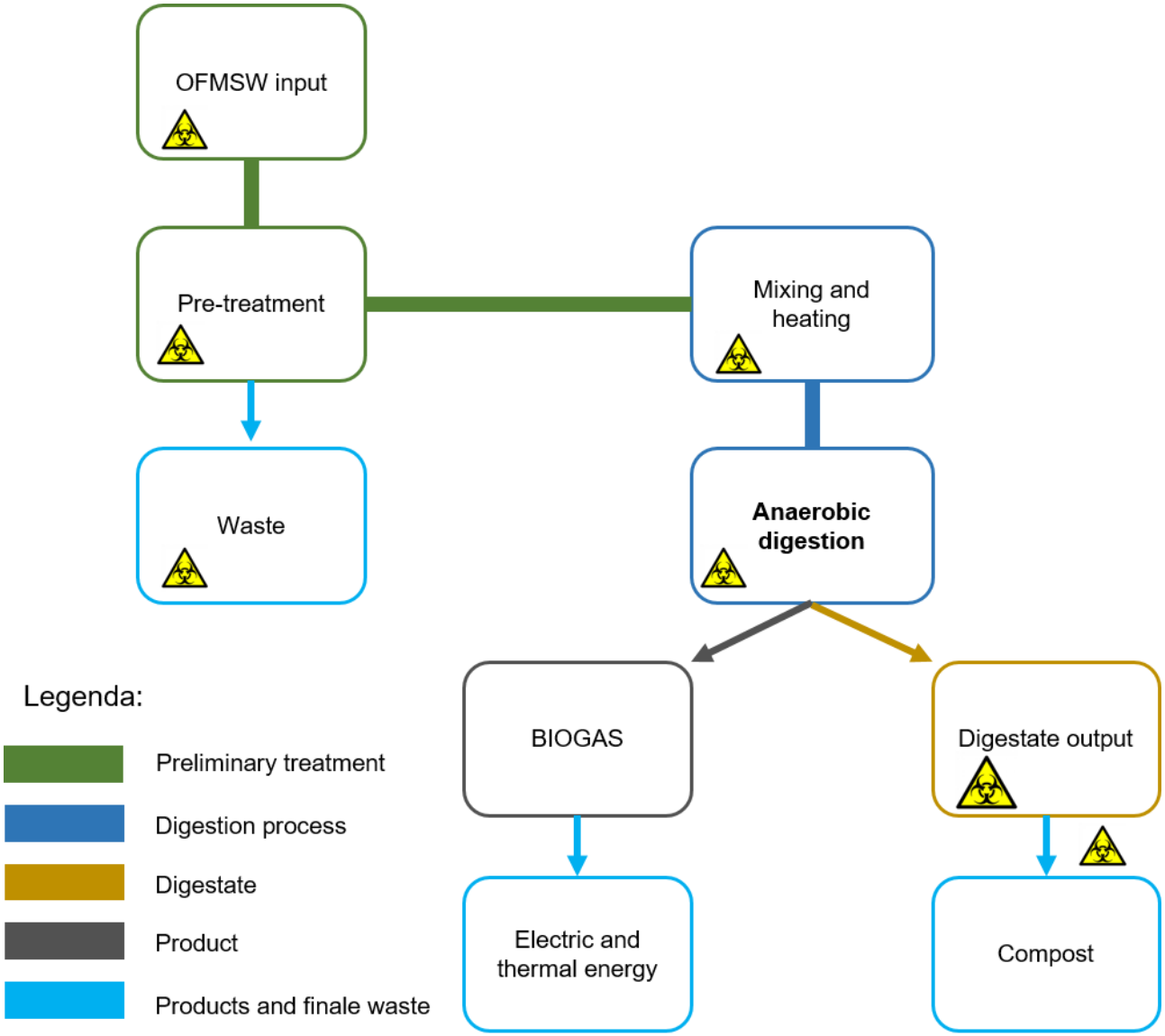
Table 6

		CFU confirmed from plates		
Microorganisms	Group (ACDP, 2013)	OFMSW	Agro-livestock	WWTP
Bacillus cereus	2	-	11	-
Bacillus coagulans	-	-	1	-
Bacillus lentus	-	-	1	-
Bacillus megaterium	-	6	50	23
Bacillus subtilis	-	103	172	23
Bacillus thuringiensis	-	-	117	10
Clostridium perfringens	2	-	5	-
*Escherichia coli**	2	3	4	-
Enterobacter cloacae	2	-	2	-
*Enterococcus avium**	2	-	2	-
*Enterococcus faecalis**	2	92	16	-
*Enterococcus faecium**	2	101	3	-
Hafnia alvei	-	6	-	-
Klebsiella oxytoca	2	2	3	-
*Klebsiella pneumoniae**	2	5	10	-
Proteus mirabilis	2	111	7	-
Serratia liquefaciens or Serratia marcenscens	-	8	62	-
Salmonella spp.	2	1	-	-
*Staphylococcus aureus**	2	-	16	1
Staphylococcus xylosus	2	-	214	-
Streptococcus uberis	2	298	-	-

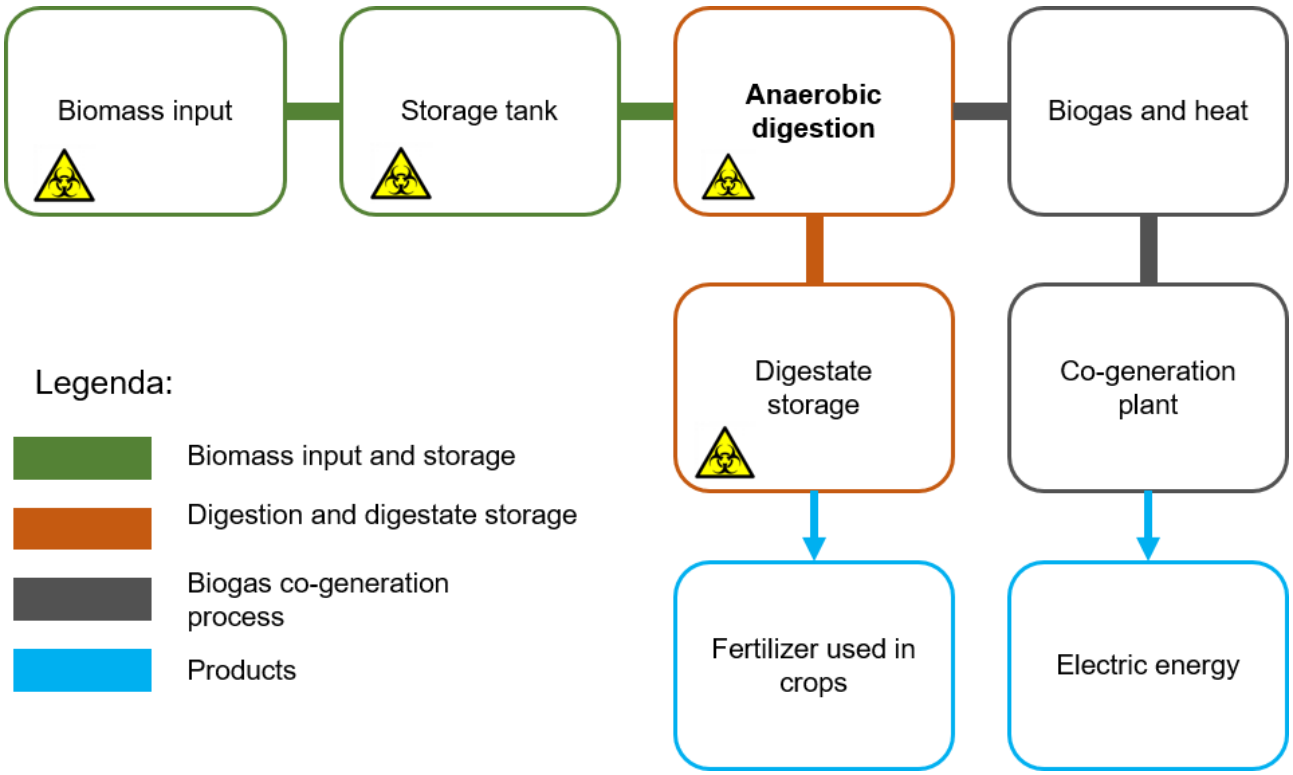
* species frequently involved in resistome diffusion

Supplementary data 1.

a. OFMSW plant process flowchart; the biologic risk sign is included for the phase where a non-negligible risk can be individuated.



b. Agro-livestock plant process flowchart; the biologic risk sign is included for the phase where a non-negligible risk can be individuated.



c. WWTS process flowchart; the biologic risk sign is included for the phase where a non-negligible risk can be individuated.