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Assessing *Methanobrevibacter smithii* and *Clostridium difficile* as not conventional faecal indicators in effluents of a wastewater treatment plant integrated with sludge anaerobic digestion.

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ABBREVIATIONS:

AD: Anaerobic Digestion; **MN**: Methanogens; **TotBact**: Total Bacteria; **WWTP**: Wastewater Treatment Plant; **Clo**: *Clostridium spp.;* **Cdif:** *Clostridium difficile;* **CDI:** *Clostridium difficile* infection; **Cper:** *Clostridium perfringens*

ABSTRACT

Wastewater treatment plants (WWTP) are an important source of surface water contamination by enteric pathogens, affecting the role of environmental water as a microbial reservoir. We describe the release to the environment of certain anaerobes of human and environmental concern. The work was focused on emerging microbial targets. They are tracing, by RT-qPCR, on WWTP effluents, both liquid and solid, when an anaerobic digestion step is included. The focus is placed on *Clostridium spp.* with the specific quantification of *Clostridium perfringens,* as typical bioindicator, and *Clostridium difficile,* as emerging pathogen not only confined into nosocomial infection. Moreover methanogens were quantified for their involvement in the anaerobic digestion, and in particular on *Methanobrevibacter smithii* as major methanogenic component of the human gut microbiome and as not conventional faecal indicator. In the water samples, a reduction, statistically significant, in all microbial targets was observed ($p<0.01$), 2 log for the total bacteria, 1.4 log for the *Clostridium spp*. and *M. smithii*, 1 log for total methanogens, *C. perfringens* and *C. difficile*. The AD process contribute to a significant change in microbial levels into the sludge for total bacteria and total methanogens ($p<0.01$), both when the input sludge are primary and secondary, while for the presence of *Clostridium spp.* and *C. difficile* there was not a significant change. The produced data are innovative showing which is the diffusion of such anaerobic microorganisms throughout the WWTP and opening a discussion on the implementation of possible techniques for a more efficient microbial removal from effluents, particularly bio-solids, to reduce the potential release of pathogens into the environment.

1. Introduction

Wastewater treatment is primarily intended to reduce the organic and pathogen load of sewage through a sequential series of physical, biological and chemical treatments. Maintenance of the microbiological quality and safety of water resources is paramount, especially for water systems used for drinking, recreation, and harvesting seafood, where a public health hazard could occur without adequate treatment (WHO, 2011). Over the last 15 years, a large number of drinking water outbreaks were reported by surveillance systems in the US (Craun, 1991; Wallender et al., 2013) and in Europe (Divizia et al., 2004; Kourenti et al., 2007)(WHO Europe, 2009).

The assessment of contamination often relies on monitoring a variety of sewage tracers (Hunt et al., 2010), because the direct monitoring and enumeration of all pathogens associated with

environmental human and animal contamination is impractical. For this purpose, microbiological indicators are utilized.

Water contamination events often result from WWTP discarge (Sercu et al., 2011). While robust evidence on various common pathogens and bio-indicators has been collected, there is little knowledge of anaerobe presence and persistence in the WWTP, such as methanogens or *C. difficile*. These latter became more relevant in consequence of the introduction of anaerobic digestion to WWTP, which can represent a selective factor for methanogens and other anaerobes (Wang et al., 2010). Moreover, their role in human diseases seems to be indirect but not negligible, if nothing else for their role in microbe interactions and human gut microbiome equilibrium (Dridi et al., 2011; Scanlan et al., 2008).

Among the methanogens – which are normal inhabitants of marine and terrestrial environments (Miller and Wolin, 1986)– Methanobacteriales inhabit only the intestines and oral cavities of animals (Belay et al., 1990; Conway de Macario and Macario, 2009), where they enhance the digestion of cellulose (Lin and Miller, 1998). In particular, *Methanobrevibacter smithii* is the main species among the Archaea known to specifically colonise the human large intestine and vaginal tract (Belay et al., 1990; Miller and Wolin, 1986). Given its host specificity and high abundance in the human intestine, *M. smithii* may be useful to track sewage and human faecal pollution in the environment (Ufnar et al., 2006).

Among the resistant microorganisms are Clostridia members. *Clostridium* is a common genera found in the WWTPs (Ye and Zhang, 2011), especially in effluent, and is associated with biosolids, contributing to the environmental burden of this microorganism. Studies suggest that *Clostridium* spp. are probably able to grow in multi-species biofilms; moreover, *Clostridium difficile* biofilms are able to produce toxins that may participate in virulence and in the production of spores that may promote new infections (Pantaléon et al., 2014). Clostridia inhabit the intestinal tract in 1% to 3% of healthy humans and are responsible for *Clostridium difficile*-infections (CDI), a common cause of nosocomial diarrhoea associated with substantial morbidity and mortality (Ricciardi et al., 2007).

In WWTP, the anaerobic digestion of sludge from wastewater depuration represents a step aimed at the reduction of bacterial load; however, the robustness of spore-forming microorganisms could ensure their survival from such treatment. Anaerobic digestion (AD) has been used for more than a century to stabilize the sewage sludge produced by WWTP and potentially to eliminate pathogens (Kjerstadius et al., 2013). Thus, accurately determining the presence of human gut

indicators in water is imperative for assessing public health and safety. There are numerous strategies for the detection of specific bacteria from environmental samples. Biomolecular techniques are useful for anaerobe studies avoiding the difficulties into the application of cultural methods and increasing the sensitivity. These are commonly applied in microbial ecology studies as tools to analyse the structure of microbial communities or to detect specific microorganisms and pathogens in complex samples (Dridi, 2012). The objective of this study was to monitor the prevalence, fate and disposal of certain anaerobe communities (*Clostridium spp*. and methanogens) including species with a human and environmental concern (*C. difficile*, *C. perfringens* and *M. smithii*), along with the principal steps of wastewater treatment: from the incoming wastewater to the depurated water, including the anaerobic digestion treatment of the sludge.

2. Materials and methods

2.1 WWTP description

The plant studied in this work is a WWTP in Castiglione Torinese (Italy) belonging to the SMAT (Società Metropolitana Acque Torino) S.p.A. group. This plant treats wastewater from the Torino metropolitan area. This plant is the largest WWTP in Italy, serving over 2 million population equivalents in the city of Torino and treating over 620.000 cubic metres of wastewater daily. The treatment plant operates on two treatment lines: the first treats water (*Water Line*) through parallel modules, where water is purified by a chemical-physical-biological process; the second (*Sludge Line*) is able to receive and treat the particulate waste material produced by water sedimentation. In the Sludge Line, sludge is collected, thickened, biologically stabilized, partially converting its organic content to biogas, and then dewatered before disposal. Briefly, during the primary wastewater treatments, particles with settling rates of 0.3-0.7 mm/s are removed. At the end of the process, the degraded primary sludge is pumped to the plant's sludge-handling facilities for further processing, and the partially treated wastewater from the primary settling tanks flows to the secondary treatment system. This treatment includes pumping air into the sludge to facilitate further settlement of particles. Some of this settled sludge is circulated back to the aeration tanks to stimulate an activated sludge process. The remaining secondary sludge is removed from the settling tanks and added to the primary sludge for further processing in anaerobic reactors (mixed sludge). There are 6 anaerobic digesters (12000 m^3 each) configured to operate continuously. Sludge is stored with a mean hydraulic retention time of approximately 20

days under mesophilic conditions. After anaerobic digestion, the sludge is heat dried, evaporating most of the water from the sludge, and then disposed.

2.2 Sample collection

We sampled influent wastewater and effluent water from the Water Line and anaerobically digested sludge from the Sludge Line over a period of one year. Water samples were designated as W-IN and W-OUT for influent wastewater and effluent water samples, respectively. Sludge samples were designated as P-IN, P-OUT, S-IN, S-OUT, M-IN and M-OUT, to distinguish the sludge origin (sludge from primary-P, secondary-S and mixed-M treatments) and the sampling point (inlet-IN or outlet-OUT to the anaerobic bio-digester) (Table 1).

SMAT's WWTP employs anaerobic digestion to minimize the volume of wastewater and to produce raw biogas to be used industrially. The chemical characteristics of sludge from primary treatment make them less interesting for anaerobic digestion because in practice the digesters were generally fed with mixed sludge. For this reason, the data from these samples (P-IN and P-OUT) are referred to as spot-sampling activity when the digester was fed only with P sludge. The main physical-chemical characteristics of the secondary and mixed sludge are showed on articles published using the same WWTP digester (Traversi et al., 2015). Each sample consisted of 500 ml of water or sludge collected in sterile PET bottles intended for microbiological analysis and freshly undergone to DNA extraction procedure.

2.3 Total DNA extraction

Water samples were freshly processed as previously described by Lemarchand and co-authors in the description of "Procedure 4" (Lemarchand et al., 2005). To prevent filter clogging, 30 ml and 450 ml were tested as the appropriate volumes from W-IN and W-OUT samples, respectively. Then, the filters underwent DNA extraction using a commercial kit (PowerSoil DNA Isolation Kit, MoBio Laboratories Inc., Carlsbad, CA), following the manufacturer's instructions with appropriate modifications for very wet samples. Moreover a manual step of bead-based homogenization was performed to increase the extraction efficiency for the spores. On the other hand we have to consider a probable less efficiency of our extraction for such microorganisms. Fluorimetric quantification of each DNA sample was performed using a Qubit™ fluorometer and the Qubit™ dsDNA HS Assay from Invitrogen (distributed by Life Technology Ltd. – Paisley, UK) according to the manufacturer's instructions. The mean values of total extracted DNA concentrations are

reported in Table 1. DNA quality and integrity were controlled and the samples were stored at -20°C until RT-qPCR analysis.

2.4 RT-qPCR

Water and sludge samples were all tested in triplicate. The samples were quantified for the following targets: total bacteria (TotBact) with a ribosomal 16S subunit target codified by *16S rDNA* (Dridi et al., 2009), total methanogens (MN) with a functional gene target *mcrA* codifying for α subunit of the methyl-coenzyme M (Steinberg and Regan, 2009), *Clostridium spp.* with a ribosomal RNA 16S subunit target *16sRNA* (Xia et al., 2012a), *Clostridium difficile* evaluating a specific 16S rRNA gene (Nakayama and Oishi, 2013) ending *Clostridium perfringens* using *plc* gene codifying for the α-toxin (Shannon 2007); *Methanobrevibacter smithii* evaluation both ribosomal 16S subunit codified by 16S rDNA (Dridi et al., 2009) and the functional *nifH* gene, encoding a critical component of the nitrogenase enzyme complex (Johnston et al., 2010). Real-Time Quantitative PCR (RT-qPCR) was performed using a Chromo4 thermal cycler (Bio-Rad – Hercules, CA) and Opticon Monitor 3 Software.

The determination of MN, *Clostridium spp.* and *C. difficile,* is performed starting from 2 µl of extracted DNA (pure DNA from water samples, ten-fold diluted DNA for sludge samples).It was added to reaction mixtures consisting of 10 µl SsoFast EvaGreen® Supermix (Bio-Rad – Hercules, CA), 0.5 µl of the forward and reverse primers (10 µM final concentration, Thermo Fisher Scientific, Waltham – MA) and 7 µl of ultrapure water in a 20 µl final reaction volume. The reaction conditions were 95 °C for 3 min (1X), 95 °C for 3 sec, 55 °C for 45 sec, 72 °C for 30 sec and 83 °C for 5 sec (40X). A final melting curve analysis was performed to verify the specificity of the PCR products. The melting curve program was as follows: denaturation for 1 min at 95 °C, cooling for 1 min at 65 °C and then heating to 95 °C at a rate of 0.5 °C per cycle.

For TotBact, *M. smithii* (both using *16SrRNA* and *nifH* genes) and *C. perfringens*, 2 µl of extracted DNA (pure DNA from water samples, ten-fold diluted DNA for sludge samples) was added to a reaction mixture consisting of 8 µl IQ™ Multiplex PowerMix (Bio-Rad – Hercules, CA), 0.2 µl of molecular probe (10 µM concentration), 0.5 µl each of the forward and reverse primers (10 µM final concentration, Thermo Fisher Scientific, Waltham – MA) and 8.8 µl of ultrapure water in a 20 µl final reaction volume. The reaction conditions were 95 °C for 3 min (1X), then 95 °C for 30 sec and 55 °C for 1 min (39X); the subsequent amplifications were performed for 30 sec at 55 °C for 1 min (40X).

To obtain an absolute quantification of all targets in sludge samples, the genomic DNA of each microorganism, provided by the American Type Culture Collection - LGC (ATCC – Manassas, VA), was used as standards for the most of the targets. Serial ten-fold dilutions of each ATCC standard were assayed and are expressed as gene copy number/100 ml for water samples and as gene copy number/ml for sludge samples, assuming four 16S RNA gene copies *per* bacterium during TotBact quantification, and one gene copy number *per* bacterium for the other gene targets (Merlino et al., 2013). Total MN was quantified using a standard curve as previously described (Traversi et al., 2015). *C. perfringens* was quantified starting from a pure culture of a standard (ATCC® 13124™) following the propagation instruction and then extracting the DNA from the concentrated suspended cells in a physiologic solution (PowerSoil DNA Isolation Kit, MoBio Laboratories Inc., Carlsbad, CA).

To confirm the amplification of each target, gel electrophoresis was performed on 2% agarose gels, and the size of each fragment was compared with the literature data. Finally, triplicate averages were accepted only when the coefficient of variation was below 20%. Supplementary material 1 provides detailed information regarding the sequences and standard genomic DNA used in our PCR analyses.

2.5 Statistical analysis

The statistical analyses were performed using the SPSS Package, version 21.0. In particular, we applied (1) a log transformation of non-normally distributed data, (2) the Spearman rank-order correlation coefficient to assess relationships between variables, (3) Mann-Whitney and ANOVA tests to compare two or more means, respectively, (4) a paired T-test when the same target was assessed on each sample by different methods.

3 Results and Discussion

3.1 Characterization of microbial communities in the Water Operating Line

The extraction were successfully performed in all the collected samples. The amount of extracted DNA is comparable to other study on waste water and sludge samples. Table 1 shows a significant decrease in the mean values of total DNA concentration comparing W-IN and W-OUT samples (approximately -95%; $p<0.0001$), demonstrating an abatement of biological contamination load, as expected.

Figure 1 reports mean values of the microbial target concentrations in all types of samples. For all the targets, the abatement was approximately 2 log comparing W-IN and W-OUT samples, with

highly significant differences (p<0.0001). The treatments applied to such water samples are intended to reduce not only the growth of microbial groups but also the presence of viable microorganisms. At the same time, there is evidence of valuable microbial contamination of the W-OUT samples (the lowest is meanly 10^2 gene copies into 100 ml of water for *C. difficile*); this is an important indication of their potential dissemination in the environment, because they are reduced but still present in effluent samples at the 2, 3.5 and 4 magnitude load respectively for *C. difficile*, *M.smithii* and *C. perfringens* (Figure 1).

The TotBact concentrations were similar to those described for the WWTP in the Milwaukee metropolitan area, expressed as total culture count (McLellan et al., 2010), and at the same order of magnitude compared with other studies (Hata et al., 2013; Samie et al., 2009). No literature data were available on the presence of MN in inlet and effluent water, although a significant decrease close to 2 log is shown, in line with the other targets. The most interesting literature data are referred to *M. smithii* – one of the most investigated methanogens - detected in water samples, by the amplification of the *nifH* marker, as a human faecal tracer. Sercu et al. detected *nifH* in all influent samples, while other authors highlighted the higher positive percentage of such markers in faecal contaminated water samples when compared to environmental water. Our results showed the presence of *M. smithii* at a detectable level in both all influent and all effluent water samples, showing an expected higher prevalence in wastewater influent compared to effluent (T-test p<0.01). Such evidence is valid both for *nifH* and *16SrRNA* gene quantification. Moreover when we consider the water samples we observed a high and highly significant correlation between the results obtained with the two detection design and the paired quantifications can be considered not different (paired T-test rho=0.932; p>0.05). Our quantifications of *M. smithii* in the sewage samples are quite similar at other published (meanly 1 gene copy for each ng of DNA) (Layton et al., 2013).

Among the microbial communities assessed in this study, *Clostridium spp.* were well represented in our samples, especially in influent raw water. *Clostridium spp.* have been previously reported as one of the most abundant phylotypes in different aquatic environments within operational drinking water networks of drinking water distribution systems. *Clostridium spp*. was one of the most abundant genera, with an average relative abundance ranging from 4.37% and 3.69%, with the variability depending on the analysed flushing steps, so their presence at a detectable level is widely in line with our findings (Douterelo et al., 2014).

C. perfringens is a common parameter used in water quality evaluation, moreover the use is generally base on cultural method, the contamination can reach 10^5 cells for 100 ml of wastewater and the abatement of the WWTP is generally about 98% (Gantzer et al., 2001; Lisle et al., 2004). Our data are comparable to such levels (Figure 1), on the other hand it is probable an overestimation of the biomolecular methods, including not vital bacteria DNA. Moreover the detected concentration of *C. perfringens* is quite similar as magnitude as other published articles, also including biomolecular technique, that analyse the influent and effluent water in WWTP in Europe (Mayer et al., 2016).

In our samples, *C. difficile* is not constantly present in the samples and less abundant if compared to the other quantified targets (figure 1). In previous studies, *C. difficile* has been recovered from water samples supposing a faecal source (Kelly and LaMont, 2008; Romano et al., 2012; Xu et al., 2014). *C. difficile* was under the LOD into the 50% of the water samples. In the influent and effluent water were detected 3.78 log copies/100 ml and 2.28 log copies/100 ml, respectively. Because treated water goes back to the environment, the presence of microbial communities in W-OUT samples is an important hygienic issue, especially for microorganisms such as *C. difficile*, whose pathways of transmission are not entirely explained. The ability to produce spores explains how spore-forming bacteria such as Clostridia - anaerobic organisms in their vegetative state - can be easily acquired from the environment (Longo et al., 2015a, 2015b). *C. difficile* human infections (e.g., CDI) are supported by endogenous sources; however, there is circumstantial evidence to suggest that this pathogen could be transmissible and acquired from external sources, such as food and water (Gupta and Khanna, 2014; Otten et al., 2010). For example, Al Safi and co-authors found a high percentage of river (81.2%) and lake water (40%) samples positive for *C. difficile* in South Wales, demonstrating the potential of water supplies as a source of infection (Al Saif and Brazier, 1996). On the other hand, a few very early studies evaluated the presence of different *Clostridium spp*. in water supplies, but *C. difficile* was not found (Pantaléon et al., 2014). From a wider point of view, the presence of *C. difficile* in the wastewater effluent should be considered a potential opportunity for the further dissemination of the pathogen in the environment. To confirm this possibility, the literature contains several studies in which 50% or more of hospital patients colonized by *C. difficile* are symptomless carriers, suggesting the possibility of natural immunity (Bauer et al., 2011). Recently the presence of *C. difficile* is monitored into households where recurrent CDI was observed, suggestion a crucial role of environment in the infection persistence (Shaughnessy et al., 2016).

3.2 Characterization of microbial communities in the sludge operating line

Different microbial trends are evident in the Sludge Line, in which the anaerobic digestion bio-chemical conditions strongly affect the selection of specific microbial communities. The DNA data reflect differences among the treatments. In particular, the secondary treatment contains sludge coming from an aerobic treatment, with microbial communities reflecting a richer situation in terms of DNA quantification (table 1). Conversely, the anaerobic conditions established in the mixed treatment digestion result in a general closer selection for microbial communities that are preferably or obligated anaerobe. The TotBact community remains at high and constant concentrations along the Sludge Line (~ 7 log for all three types of sludge treatments). These results were lower than the findings from untreated sewage collected at the Duluth-Superior harbour site, in which total Bacteroides were at a concentration of 8.8 ± 0.6 log/100 ml gene copies (Eichmiller et al., 2013). On the other hand such parameter is very variable in relation to the WWTP characteristics and also in relation to the design of the primers involved into the determinations (Wang et al., 2010)(Araùjo dos Santos et al., 2015).

MN are numerically well represented in these sludge samples, at approximately 5-6 log. Digesters are one of the typical habitats of MN. In fact, their positive selection is evident for each anaerobic sludge treatment system.

No significant differences were found for *M. smithii*, comparing IN and OUT samples from the anaerobic reactors, this is a constant evidence independent from the primers and probe set used. Moreover the quantifications performed using the different target gene for *M. smithii* are significantly correlated considering sludge samples (rho = 0.503 $p<0.01$). Such evidence is previously discussed also for the water samples, and it is showed for all the samples on table 2. The concentrations of MN and *M. smithii* were not correlated (Spearman's Rho=0.076, p=ns) using *16SrRNA* target highlighting the unselective conditions recognized for *M. smithii* in the anaerobic reactors. While *M. smithii* levels, using *nifH* target, were weakly but significantly correlated to MN (Spearman's Rho=0.446, $p<0.01$). Such incongruity highlight a possible over-detection with such functional gene. It could cover also other methylotrophs as well other methanogens. In fact, microorganisms catalyse biological nitrogen fixation with the enzyme nitrogenase, which has been highly conserved through evolution. Only a few alternative nitrogenase sequences have yet been obtained from the environment (Gentry-Shields et al., 2012). In particular methanogens such as *Methanosarcina* and *Methanococcus* closing to the cluster I (2B sub-cluster) and IV (4, 4A add 4D

sub-cluster) where *nifH* sequences of environmental strain are very similar (Zehr et al., 2003). This is reinforced by the significant paired T-test ($p<0.01$) observed, considering all the sludge samples, between the mean level calculated for the *16SrRNA* gene target (2.76±1.29 gene copies/ml sludge) and *nifH* gene (4.25±0.55 gene copies/ml sludge). Such results is not overlapping to the water samples where the two kind of determinations are strictly closed and of course the MN presence is limited to few species (Figure 2) and it is masked considering all the samples (table 2).

The *Clostridium* spp. community is well represented in sludge samples as well, at values of more than 5 log. This is confirmed by the literature where the Clostridia quantification in terms of relative abundance and richness is positively correlate with substrate availability and biogas generation (Westerholm et al., 2016).

C. perfringens in the sludge is present at a 10^3 gene copies level, independently by the sludge characteristics: both mixed or secondary and both in input and output respect the digester. Such constant value is not a surprise considering the anaerobic capability of such microorganism, on the other hand also a growth into the digester seems to be not probable for the competition with other microorganisms in not favourable environment. Recent data showed a stability of such parameter into the sludge in the ingestate and digestate from agro-zoothecnic biogas plants in Italy (Orzi et al., 2015).

C. difficile is present in all the sludge samples (ranging between 2 and 3 log). A recent study has illustrated that *C. difficile* is highly prevalent in sewage, although lower than our results (concentrations ranging from 1 and 2 log/ml) in both primary and digested sludge, and the levels are not significantly affected by the wastewater treatment process (Xu et al., 2014). On the other hand, a US survey of wastewater biosolids showed, for 1 dried sludge gram, a mesophilic anaerobic sludge concentration of ~ 6 log for *Clostridia spp.* and of ~ 4 log for *C. difficile* (Viau and Peccia, 2009). They are higher but not so different, considering the transformation in mass, from our results (figure 1). Moreover a statistically significant difference of *C. difficile* level is achieved only in the mixed treatment system. The average abatement was equal only to one order of magnitude. A recent Canadian study showed as *C. difficile* is not reduced by mesophilic digestion while the endospore level is reduced of more than 1 log CFU/day by thermophilic digestion, moreover acidification inhibit the spore germination (Xu, 2016). The mixed sludge digestion are generally characterized by a higher acidity respect the secondary sludge (Traversi et al., 2015), such data can supply a possible explanation of the *C. difficile* reduction observed only when mixed sludge are involved in input.

The comparison of M-IN and S-IN samples using the Mann-Whitney Test revealed no statistically significant differences in the microbial populations between the two types of sludge, with the exception of *M. smithii* and *C. difficile* (p<0.0001). While the decreased concentration of *C. difficile* seems to be attributable to the lack of tolerance towards oxidation conditions established in the secondary treatment tanks, the increased concentration of *M. smithii* in secondary treatment sludge has no plausible explanation. We hypothesize that the objective thickening of the secondary sludge could have a role in the formation of flocks that border the anaerobic bacteria populations that are particularly oxygen-sensitive, such as *M. smithii*.

Finally table 2 showed the correlation among the various parameters considering all the samples collected. It highlights a not correlation between methanogens and *Clostridium spp.* moreover significant correlation between the genera and its species.

4. Conclusion

Even if mesophilic AD is the most commonly used sludge stabilization process in WWTPs, the hygienic performance of this treatment is very low, achieving the removal of approximately 1-2 log units of common bacterial indicators, as observed also in this study (Astals et al., 2012; Mocé-Llivina et al., 2003; Sahlström et al., 2004). Attention should be placed on the possible reuse of dry biosolids that results from the mesophilic anaerobic fermentation. Today daily sludge production ranges from 60 to 90 g dry solids *per* population equivalent (Bodík et al., 2011) and a disposal without further treatment to land is of interest especially if a new restrictive EU legislation will be introduced (Tony, 2010). Moreover any infections propagation arising from the disposal of dry sludge as fertilizer could be amplified through the soil and food chain (Levantesi et al., 2014).

In this study an efficient reduction in the water (influent versus effluent) was observed, but currently such reduction can't be judged acceptable for pathogenic microorganisms such as *C. difficile*. The water effluents generally impact on a surface water receptor, in Italy such same receptor are rivers used as water supply for drinking water production. All potential sources are of significant interest as identification of risk factors for CDI and may help in the early diagnosis and subsequent management of the infection. Without knowing the infectious dose such environmental results may still be of relevant significance.

To our knowledge, this study is one of the very few to report the presence of *C. difficile* in water samples coming from a wastewater treatment plant. For this reason, it could represent a significant contribution to the knowledge of the ecology and the diffusion of such organism.

For digested sludge, the situation is more complex, because biochemical conditions established in the reactors control the microbial population equilibrium. The use of AD in WWTP is currently related to the use of sludge for biogas production, rather than for its hygienic ability, as demonstrated by the concentrations of methanogen communities growing in the effluent digested sludge samples. Moreover an improve of the thermophilic digestion could be auspicial under an hygienic-sanitary point of view.

The determination of *M. smithii* was suggested as a bio-indicator of faecal contamination in food such as water through biomolecular methods based on the analysis of specific constitutional or functional genes (Dridi et al., 2011; Ufnar et al., 2006). The quantification in wastewater and digested sludge produces reliable data regarding the excretion rate from the human gut and diffusion among the population. *M. smithii* faecal concentration is associated to host susceptibility towards certain intestinal diseases, as well as in drug metabolism, absorption of nutrients, and the metabolism of toxic compounds. Moreover recently it was observed a higher concentrations of *M. smithii* in intestinal microbiota in children living near landfill, suggesting a relevant role of the environment quality into the *M. smithii* persistence (Bezerra De Araujo Filho et al., 2014). Of course such link has to be further investigated.

Considering the conformity to the reuse of sludge in agriculture, this study confirms that microbiological aspects are currently not fully understood, with an abatement capacity of the depuration process that could be not adequate for water but above all bio-solid effluents. Moreover, an accurate assessment of the abatement level required for specific groups of microorganisms of pathogenic concern cannot be separate from the minimum infective dose assessment, as well as the consideration of the protective effect induced by herd immunity, due to the circulation of the microorganisms among the population.

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Table 1: Mean values of total extracted DNA concentrations in water and sludge samples

Sample Type - Acronym	***N***	**mean ± SD (µg/ml)**
Raw Water Inlet – W-IN	*10*	$(7.18 \pm 1.50)\ 10^{-2}$*
Primary Sludge Inlet – P-IN	*Spot sample*	7.88
Primary Sludge Outlet – P-OUT	*Spot sample*	10.3
Mixed Sludge Inlet – M-IN	*25*	30.53 ± 18.34
Mixed Sludge Outlet – M-OUT	*25*	33.43 ± 20.72
Secondary Sludge Inlet – S-IN	*25*	78.23 ± 42.68
Secondary Sludge Outlet – S-OUT	*25*	50.42 ± 22.12
Effluent Water Outlet – W-OUT	*10*	$(0.36 \pm 0.19)\ 10^{-2}$*

* Mann-Whitney test for W-IN and W-OUT extracted DNA comparison: $p < 0.0001$

Table 2: Correlation among the microbial communities considering all the data collected

	TotBact	***MN***	***M. smithii (16SrRNA)***	***M. smithii (nifH)***	***Clo spp.***	***C. difficile***	***C. perfringens***
Extracted DNA	0.424**	ns	0.208*	0.195*	ns	-0.198*	-0.310**
TotBact	-	0.577*	0.469**	0.678**	0.411**	0.302*	ns
MN	0.577**	-	0.263**	0.527**	ns	ns	ns
M. smithii (16SrRNA)	0.469**	0.263**	-	0.535**	0.415**	ns	ns
M. smithii (nifH)	0.678**	0.527**	0.535**	-	0.329**	0.206*	ns
Clostidium spp.	0.411**	ns	0.415**	0.329**	-	0.449**	0.436**
C. difficile	0.302**	ns	ns	0.206*	0.449**	-	0.398**
C. perfringens	ns	ns	ns	ns	0.436**	0.398**	-

* significant Spearman rho correlation

**high significant Spearman rho correlation.

Supplementary material 1: Oligonucleotide primers, probes and genomic standards used in RT-qPCR analyses.

TARGET		SEQUENCES	Standard genomic DNA ATCC number if present LOQ (gene copies number/µL)	SIZE FRAGMENT (base-pairs)	REFERENCES
Total Bacteria (***TotBact***)	F R Probe	5'-AGAGTTTGATCMTGGCTCAG-3' 5'-TTACCGCGGCKGCTGGCAC-3' 5'-CCAKACTCCTACGGGAGGCAGCAG-3' (FAM-BQ1)	*Desulfovibrio vulgaris* ATCC 29579D-5 85.2	327	(Dridi et al., 2009)
Methanogens (***MN***)	mlas f mcrA	5’-GGTGGTGTMGGDTTCACMCARTA-3’ 5’-CGTTCATBGCGTAGTTVGGRTAGT-3’	*mcrA plasmid* 25.2	470	(Steinberg and Regan, 2009)
Methanobrevibacter smithii (***M. smithii 16S***)	Smit.16S-740f Smit.16S-862r Smit.16S FAM	5’-CCGGGTATCTAATCCGGTTC-3’ 5’-CTCCCAGGGTAGAGGTGAAA-3’ 5’-CCGTCAGAATCGTTCCAGTCAG-3’ (FAM-BQ1)	*Methanobrevibacter smithii* ATCC 35061 16.4	123	(Dridi et al., 2009)
Methanobrevibacter smithii (***M. smithii nifH***)	Mnif 202 Mnif 353 Mnif Prob	5'-GAAAGCGGAGGTCCTGAA-3' 5'-ACTGAAAAACCTCCGCAAAC-3' 5'-CCGGACGTGGTGTAACAGTAGCTA-3' (FAM-BQ1)	*Methanobrevibacter smithii* ATCC 35061 16.4	151	(Johnston et al., 2010)
Clostridium spp (***Clo***)	F R	5’-AAAGGAAGATTAATACCGCATAA-3’ 5’-TGGACCGTGTCTCAGTTCC-3’	*Clostridium acetobutylicum* ATCC 824D-5 73.6	179	(Nakayama and Oishi, 2013)
Clostridium difficile (***C. diff***)	Cdif 706f Cdif994r	5’-ATTAGGAGGAACACCAGTTG-3’ 5’-AGGAGATGTCATTGGGATGT-3’	*Clostriudium difficile* ATCC 9689D-5 14.5	307	(Xia et al., 2012b)
Clostridium perfringens (***C. perf***)	Clper-F Clper-R Clper	5' - GCATGAGTCATAGTTGGGATGATT - 3' 5' - CCTGCTGTTCCTTTTTGAGAGTTAG - 3' 5' - TGCAGCAAAGGTAACTT - 3' (FAM-BQ1)	*Pure culture isolation followed by DNA extraction* *31.4*	283	(Shannon 2007)

Figure 1: Descriptive analysis of the microbial communities for different samples; means (bars) and standard deviations (SD) are given to characterize the sample distributions with respect to the different types of water (per 100 ml of water) and sludge (per ml of sludge) samples. Square brackets show ANOVA p values from influent and effluent comparisons: * indicates a significant difference, **indicates a high significant difference.

Figure 2: Mean log gene copies and standard deviations observed for the qRT-PCR quantification using different primers and probe in water (per 100 ml of water) and sludge (per ml of sludge). The paired T-test comparing the two quantification designs is not significant for water samples while it is highly significant for sludge samples (T-test $p<0.01$).

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