Sewage reclamation process as multifactorial public health risk concern: a longitudinal study.

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Abstract

This year-long research analysed emerging risks in influent, effluent wastewaters and biosolids from six wastewater treatment plants in Spain's Valencian Region. Specifically, it focused on human enteric and respiratory viruses, bacterial and viral faecal contamination indicators, extended spectrum beta-lactamases-producing *Escherichia coli* and antibiotic resistance genes. Additionally, particles and microplastics in biosolid and wastewater samples were assessed. Human enteric viruses were prevalent in influent wastewater, with limited post-treatment reduction. Wastewater treatment effectively eliminated respiratory viruses, except for low levels of SARS-CoV-2 in effluent and biosolid samples, suggesting minimal public health risk. Antibiotic resistance genes and microplastics were persistently found in effluent and biosolids, thus indicating treatment inefficiencies and potential environmental dissemination. This multifaced research sheds light on diverse contaminants present after water reclamation, emphasizing the interconnectedness of human, animal, and environmental health in wastewater management. It underscores the need for a One Health approach to address the United Nations Sustainable Development Goals.

1. Introduction

Water is a fundamental resource for human life, being also essential for crops and livestock production. However, the increasing global population and limited freshwater resources pose significant challenges to meeting the demands of various sectors, including agriculture. Water reuse has emerged as a sustainable solution to preserve freshwater resources and reduce environmental pressure. Reclaimed water, also known as recycled water or effluent from wastewater treatment plants (WWTPs), refers to the treated wastewater that undergoes a series of physical, chemical, and biological processes to remove contaminants and pathogens. The reclaimed water is then suitable for non-potable uses, such as irrigation, industrial processes and groundwater recharge according to national regulations[1].

Water reuse has become increasingly important in agriculture due to the limited freshwater resources and the growing demand for food production. Agriculture accounts for approximately 70% of global freshwater withdrawals and the water demand for crops and livestock is projected to increase in the coming decades[2]. Reclaimed water offers a sustainable solution to reduce the demand for freshwater resources and ensure the availability of water for irrigation, while reducing the discharge of treated wastewater into the environment and the cost of water supply. However, water reuse also poses several challenges, particularly in terms of microbiological and chemical safety. Reclaimed water may contain a variety of contaminants, including bacteria, viruses, protozoa, and emerging pollutants, such as microplastics (MPs), antibiotic resistant genes (ARGs) and pharmaceuticals[3].

In particular, human enteric viruses are responsible for causing viral gastroenteritis, hepatitis, and various illnesses primarily transmitted through the faecal-oral route[4]. The spread of these viruses is primarily linked to person-to-person contact and the consumption of contaminated food and water. Enteric viruses are excreted in substantial quantities, up to 10^{13} particles per gram of stool, by both symptomatic and

asymptomatic individuals[5,6]. Major causative agents of waterborne viral gastroenteritis and hepatitis outbreaks worldwide include rotaviruses (RVs), norovirus genogroups I (HuNoV GI) and II (HuNoV GII), hepatitis A and E viruses (HAV and HEV), and human astroviruses[5] (HAstVs). In this context, and related to microbiological risks dissemination, a new European regulation (EC, 2020/741) on minimum quality criteria (MQR) for water reuse is in place since June 2023, outlining the guidelines for the use of reclaimed water for agricultural irrigation[7]. However, questions have arisen concerning potential non-compliance scenarios in European water reuse systems[8–12]. According to EC 2020/741 regulation, validation monitoring needs to assess whether the performance targets reductions are met. Monitoring of pathogen elimination in the water reclamation process is necessary to assess the suitability of reclaimed water in its secondary uses. In this respect, the WHO has suggested that another problem to be tackled in the framework of "One Health" is the rise of antibiotic resistance (AR)[13]. AR is frequent in places where antibiotics are employed, but antibiotic resistant bacteria (ARB) and ARGs are also widely prevalent in water environments[14,15]. According to several reports, surface water and reclaimed wastewater used for irrigation are significant sources of ARBs and ARGs[16]. Due to inadequate removal of ARGs, which are crucial in the growth of extremely unfavourable drug-resistant superbugs, reuse of WWTP effluents may be harmful to human health[17].

On the other hand, plastic pollution is currently one of the most important environmental problems that humanity must face. The exponential growth of plastic production since 1950s (up to 368 million of tons were produced in 2019) and the massive use of plastics, together with insufficient/inadequate waste management/disposal strategies, are the main causes of the global presence of plastics in every environmental compartment[18]. The European Commission has recently published an amending Annex to Regulation (EC) No 1907/2006 concerning the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) as regards synthetic polymer microparticles, where the intentional use of microplastics in commercial products is prohibited[19].

Current research is showing that one of the main concerns about plastics, apart from the fact that they persist in the environment for an extremely long time, is their constant fragmentation into even smaller particles called microplastics (MPs, 1 μm – 5 mm) or nanoplastics (< 1 μm), depending on their final dimensions, though they are also released as such[20].

MPs are emerging global threats as they can end up in our body through water and food ingestion or by air inhalation[21]. The larger MPs can cause mechanical damage to the intestinal epithelium, while the smaller particles can cross the epithelial barrier[22] and end up in the lung[23], colon[24], placenta[25], and even blood[26].

MPs can transport pathogens over long distances, due to their ability to harbor biofilms on the surface, which can lead to the spread of pathogenic viruses and bacteria to new areas where they were not previously found[27]. Another of the main risks associated with MPs is that plastic materials include approximately 4% by weight of additives[28], some of them declared as possible human carcinogens and

most of them considered endocrine disruptors[29]. In addition, MPs also contain traces of persistent organic pollutants (COPs), such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and organochlorine pesticides[22].

It is important to highlight that depending on the performance of WWTPs high amounts of pathogens, MPs and ARGs can be released on a daily basis into rivers, lakes, and oceans[9,14,30]. On the other hand, the sludge generated as well as the effluent water from the WWTPs are generally used in agriculture as a fertilizer and for irrigation respectively, and, therefore, the presence of emerging contaminants in these biosolids and reclaimed waters can favour the propagation of plastic particles, emerging pathogens, and ARGs through agricultural soils which could reach cultivated vegetables and ultimately the human body through the trophic chain.

In overall terms, understanding the distinct risk factors involved in the water reclamation process is critical to ensuring the safety of water reuse in agriculture and other sectors, and the analysis of the water reclamation process can serve as an important risk assessment tool. Moreover, by analysing wastewater, we gain valuable insights into the collective health of a community, as it contains traces of chemical pollutants, pathogens, and biomarkers from human and animal sources. Thus, monitoring wastewater helps identifying trends in the prevalence of diseases, antibiotic resistance patterns, zoonotic pathogens, and exposure to environmental pollutants as microplastics, providing early warning and valuable data for public health interventions. This integration of environmental, human, and animal health data underscores the significance of wastewater analysis in promoting a comprehensive and proactive “One Health” approach to public health and the well-being of both the planet and its inhabitants.

2. Results

Incidence of human enteric viruses, respiratory viruses and viral faecal indicators in influent and effluent wastewater samples.

The presence of human enteric viruses, including HuNoV GI, HuNoV GII, HAstV, HAV, HEV, and RV, was analysed, along with novel viral faecal contamination indicators pepper mild mottle virus (PMMoV), crAssphage and somatic coliphages in influent, effluent and biosolid samples from six different WWTPs in the Valencian region of Spain (Figs. 1 and 2).

In influent wastewater samples, the mean highest levels of viruses were observed for RV (8.55 log GC/L), followed by HuNoV GII (7.80 log GC/L) and HAstV (7.72 log GC/L). The lowest concentration levels were detected for HuNoV GI (4.46 log GC/L), HEV (4.13 log GC/L), and HAV (3.47 log GC/L) (Fig. 2). HAV was only detected in 4 out of 72 influent wastewater samples (Fig. 1). PMMoV and crAssphage were detected in all influent samples, with mean levels of 5.95 log GC/L and 8.44 log GC/L, respectively.

In the effluent wastewater samples, the titres of all viruses decreased after the water reclamation process. HuNoV GI, HuNoV GII, HAstV, and RV showed mean concentrations titers of 3.51, 6.25, 6.35, and 7.69 Log GC/L when detected, respectively (Fig. 2). On the contrary, HEV was not detected in any of the effluent

samples. In the case of faecal viral indicators, PMMoV (4.72 Log GC/L) and crAssphage (6.23 Log GC/L) were present in all effluent samples. The highest reduction in virus levels were observed for HEV, with a reduction of 4 Log GC/L, even though the vast majority of viruses' reduction levels were below 2 Logs GC/L (Figure S1). Interestingly, viable coliphages were found at levels of 4.73 Log plaque forming units (PFU)/100 mL in effluent waters, with a mean reduction of 1.83 Log PFU/100 mL compared to the influent waters (6.54 Log PFU/100 mL) when testing positive.

As for biosolid samples, HuNoV GI, HuNoV GII, HAstV, and RV showed the highest mean concentrations, with titers ranging from 5.37 (HuNoV GI) to 7.27 Log GC/L (RV) when detected (Fig. 2). HAV and HEV rendered lower mean concentrations of 3.24 and 3.91 Log GC/L, respectively. Besides, proposed viral faecal indicators yielded mean concentrations levels of 7.06 Log GC/L for crAssphage, 4.85 Log GC/L for PMMoV. and 5.63 Log PFU/100 ml for somatic coliphages.

Regarding respiratory viruses, respiratory syncytial virus (RSV) showed a remarkable seasonality, with almost all positive samples being collected on November and December 2022 (Fig. 3). Influenza A virus (IAV) was intermittently detected over the year, with the most noteworthy peaks taking place in spring and winter (Fig. 3). Finally, SARS-CoV-2 was present in 99% and 32% of the influent and effluent samples, respectively. When testing positive, mean concentration values for RSV, IAV, and SARS-CoV-2 were 4.57, 6.20, and 5.27 Log GC/L, respectively. Notably, any of the analysed effluent wastewater samples tested positive for either RSV or IAV.

Regarding biosolid samples, SARS-CoV-2 was found positive in the 71% of the samples at mean concentration of 4.44 Log GC/L, while RSV and IAV only tested positive in three biosolid samples.

In general, no significant differences were found among the six different WWTPs analysed neither for enteric nor respiratory viruses.

Quantification of Escherichia coli, Extended Spectrum Beta-Lactamases-producing E. coli and ARGs in wastewater and biosolids samples.

In influent wastewater samples, the mean concentration of *E. coli* and ESBL-*E. coli* were 7.08 Log CFU/100 mL and 6.19 Log CFU/100 mL, respectively (Fig. 4). After the wastewater treatment process, the mean concentrations of *E. coli*, and ESBL-*E. coli* in the effluent wastewater samples were significantly reduced, with mean concentrations of 5.43 Log CFU/100 mL, and 4.76 Log CFU/100 mL, respectively.

Regarding biosolid samples, the mean concentration of *E. coli* was 5.64 Log PFU/100 mL, while ESBL-*E. coli* yielded a mean concentration of 4.89 Log CFU/100 mL.

Furthermore, a deeper analysis on the ARGs present in effluent and biosolids samples was performed due to the high levels of ESBL-*E. coli* in biosolids and the observed low performance of the water reclamation process (less than 2 log reduction; Figs. 2 and 4). ARGs including tetPB_3, tetA_1, and qacA_1 were not detected in effluent wastewater and biosolids. ARG sul1_1, sul2_1, pbp2b, bla_{CTX-M}, cmlA_2, nimE, and

ermB were detected in effluent samples at mean concentrations of 9.20, 8.78, 8.57, 8.42, 8.31, 8,24, and 8.39 Log GC/100 mL, respectively (Fig. 5).

ARGs were identified in biosolids, with the following values: 9.87, 9.25, 8.58, 8.42, 8.50, 8.64, 8.28 Log GC/100 mL for sul1_1, sul2_1, pbp2b, bla_{CTX-M}, cmlA_2, ermB, and ermA, respectively. Notably, nimE was not found in any of analysed biosolids.

Quantification of particles and microplastics present in biosolids and reclaimed water samples.

The presence of solid particles and microplastics was bi-monthly analysed in both influent and effluent wastewater samples. In general, a great reduction in both the number of particles between 1 µm and 5 mm or (T)-P and particles larger than 300 µm or (S)-P was observed after the wastewater treatment process (Fig. 6). Although there was not a clear effect derived from seasonality, WWTPs were slightly less efficient in removing (T)-P in January and March.

The efficiency of each WWTPs regarding the reduction of (T)-P and (S)-P particles was determined considering the average number of particles in the influent and effluent wastewater samples (Fig. 7). At the WWTP level, the calculated efficiency in (T)-P reduction was approximately 84, 68, 69, 46, 80 and 71%, for the different WWTPs (P1-P6) samples analysed. Notably, the efficiency in removing (S)-P was higher than in removing (T)-P, with the most noteworthy reduction taking place for P2 and P6 wastewater samples (91 and 93% approximately and respectively), while the lowest efficiency in (T)-P reduction was approximately 40% for P5.

Once (T)-P and (S)-P particles were quantified, all samples were spectroscopically characterized in order to identify the presence of MPs derived from synthetic polymer particles, fibres, and films. In general terms, the highest reduction was observed in (S)-MPs as compared to (T)-MPs, thus suggesting the lower efficiency of wastewater treatments in removing microplastics smaller than 300 µm (Fig. 7). It should be highlighted that the efficiency of WWTPs for removing MPs of smaller particle size or (T)-MPs was lower than for removing all solid particles or (T)-P, being 59% the highest (T)-MPs efficiency (sample P6). In general, a higher efficiency in reducing (S)-MPs was observed (around 98–100%) in all samples, except in P2 (77%) (Fig. 7).

Considering the pre-treatment (T), the annual average MPs concentration in influent samples was around 1816 MPs/L which was slightly reduced in effluent samples (1724 MPs/L). In contrast, the annual average concentration of (S)-MPs (larger than 300µm) in influent samples was 198 MPs/L and it was significantly reduced in effluent wastewater samples until 11 MPs/L in average (Fig. 8).

The annual average percentage of MPs respect to all solid particles in influent and effluent wastewater samples and biosolids was also determined and the results (Figure S2). It is worth mentioning that, regarding the particles larger than 300 µm, the MPs/all solid particles ratio in biosolid samples was similar to the MPs/all solid particles ratio in influent wastewater samples, reaching values up to 35 in some of the WWTPs (Figure S2).

In all the analysed biosolid samples a significant number of (S)-P was also detected, and no significant effects due to seasonality were found (Fig. 9). The average highest concentration of (S)-MPs was 122 MPs/g and 99 MPs/g for P1 and P2, respectively. In contrast, the lowest level of MPs was detected for P3 (23 MPs/g) (Figure S3).

Analysing the morphology and type of MPs identified in the WWTPs samples may help to understand the origin of water pollution. As depicted in Fig. 10, the majority of MPs existing in influent wastewater samples had the shape of fragments (86%), percentage that was further increased in effluent wastewater samples. The percentage of particles identified as films was negligible both in influent or effluent samples. Most of the MPs found in influent samples were between 0-100 µm (61%) in size, percentage that was increased in effluents (up to 73%), and a small fraction of MPs (3–5%) were larger than 300 µm in size, in agreement with the results commented above (Fig. 8). The composition of the MPs was dominated by common polymers, whereas the PS, PA, PVC, and PET were greatly decreased in effluent samples (Fig. 10). It is worth mentioning that the distribution of polymer type was quite different when comparing wastewater and biosolids samples. PE was dominant in all samples, accounting for 56, 46 and 57% of the total MPs, for wastewater (T)-MPs and (S)-MPs, and for biosolids (S)-MPs, respectively (Figure S4). The amount of PA was more than two-fold higher in (T)-MPs samples from wastewater than in (S)-MPs from biosolids (31% vs. 12%, respectively). PET represented around 21–28% of the (S)-MPs in wastewater and biosolid samples. Other polymers such as PS, polytetrafluoroethylene PTFE, PVC and PS were detected in lower amounts.

3. Discussion

Reuse of effluent wastewater and biosolids in agriculture is essential to face the increasing demand of water and agricultural products in combination with global warming and water scarcity[31]. Effluent wastewater and biosolids, however, are sources of emerging contaminants of concern such as viral pathogens, antibiotic resistance genes and microplastics. The reuse of water and the release of reclaimed water into the environment may compromise public health due to the combination of several risk factors. In recent years, several publications have pointed out the low efficiency of WWTPs in removing viral pathogens[9]. While decay rates of human enteric viruses in effluents wastewater samples are frequently studied, very few studies have reported the incidence of respiratory viruses, MPs and ARGs in effluent wastewaters and biosolids, with potential of being used in agriculture.

The present study investigated the presence of human enteric viruses, including HuNoV GI and GII, HAstV, HEV, and RV, as well as ARBs, ARGs, MPs and two novel viral faecal contamination indicators (PMMoV and crAssphage) in influent, effluent and biosolids samples. Consistent with findings from earlier research, influent wastewater samples exhibited elevated concentrations of human enteric viruses, MPs and ARBs[14,32] (Figs. 1, 2, 4, 6, and 8).

Following the water reclamation process, the concentrations of all analysed viruses decreased in the effluent samples. However, it is worth noting that the reductions for HuNoV GI, HuNoV GII, HAstV, and RV

(when detected in effluent) were below 2 Logs, suggesting the persistence of these viruses to a relevant extent after being exposed to either UV or chlorination treatments. Only HEV was not detected in any of the analysed effluent samples thus resulting in higher reductions (> 4 Log GC). The reductions observed for human enteric viruses along the year substantially differ from current European legislation (Regulation (EU) 2020/741, 2020) on water reuse, which indicates the need for ≥ 6 Log decreases on the presence of these pathogens[7]. Even though enteric viruses' presence detected by RT-qPCR in this study might not correspond with infectious particles, several publications have pointed out the presence of infectious enteric viruses in reclaimed waters by capsid-integrity or cell culture approaches[8–11,33].

Owing to the microbiological risk that the presence of enteric viruses in these waters could entail, this study also aimed to assess the levels of somatic coliphages and *E. coli* in influent and effluent wastewater samples, as well as biosolid samples. Coliphages have been found in locations where faecal contamination is present[34,35], and numerous studies have suggested utilizing coliphages as markers for enteric viruses' presence[34–39]. Following the water treatment process, reductions of 1.83 Log PFU and 1.65 Log CFU were observed for somatic coliphages and *E. coli*, respectively. These reductions, which are far from those stipulated by the legislation EU 2020/741, 2020, highlight the low performance of the WWTPs in decreasing the microbial load and mitigating the potential risks associated with these pathogens (pathogenicity and antibiotic resistance transmission)[7]. For somatic coliphages and *E. coli*, obtained counts in biosolids were similar to those obtained in effluent wastewater samples, pointing out the risk of using biosolids without any further treatment in agriculture. Besides, in recent years, both crAssphage and PMMoV have been proposed as viral indicators of faecal contamination in water bodies and as a virus model to assess the performance of WWTPs[40–46]. Regarding effluent samples, the mean concentration of crAssphage detected in reclaimed waters was 6.25 Log GC/L, which consistently matches the reported mean concentrations of 6.5 Log GC/L in high income countries as reviewed by Adnan et al. (2022)[47]. PMMoV concentrations in effluent wastewater samples are in line with existing bibliography, which reports mean concentration values of ~ 4 Log GC/L[48–50]. Notably, obtained mean concentrations of PMMoV in untreated wastewaters (5.95 Log GC/L) are slightly under-average when compared with previously reported data, as the common concentration values of PMMoV published in influent wastewater samples range from 6 to 10 Log GC/L[48–54]. Interestingly, to our knowledge, this study includes the first report on PMMoV levels in biosolid samples which may also pose a risk for the dissemination of this plant pathogen.

As for respiratory viruses, SARS-CoV-2 and IAV were detected at mean titres similar to those reported in the US, Canada, Australia, and other regions in Spain covering the same time period, while RSV levels were at least one Log GC/L over the reported in the aforementioned studies[55–60]. In recent years, the possibility of transmission of various respiratory viruses through food and water consumption has been discussed[61]. The absence of RSV and IAV in all effluent samples analysed in this study indicates an almost non-existent risk of transmissibility caused by ineffective water treatment. Nevertheless, the high presence of SARS-CoV-2 in effluent samples, together with the presence of these respiratory viruses in

several of the analysed biosolids samples and the lack of studies regarding non-respiratory routes of transmission, warrant the need for further studies to assess public health risks.

Recently, a new proposal by The Urban Wastewater Treatment Directive (UWWTD), requested that member states should monitor antibiotic resistance at WWTPs serving over 100,000 individuals[62]. As this monitoring has been proposed to be performed for both influent and effluent wastewater samples, it should tackle both environmental transmission risks arising from WWTPs and provide insights into resistance patterns within specific regional areas.

In this study, ESBL-*E. coli* levels in influent samples were very high, with 6.63 Log CFU /100 mL on average, with no statistical differences among the different WWTPs and along the year. When analysing the reclamation treatment applied by the WWTPs, only mean reductions of 1.43 Log were observed for ESBL-*E. coli*, with 4.30 Log counts on average in effluent samples, which surpass by 3 Logs the levels reported in other studies, suggesting the important role of effluent water in the dissemination of ARB in the food chain if used for irrigation and the need to improve water reclamation processes[14,63,64]. Similarly, the high levels of ESBL-*E. coli* in biosolids, suggest the need for further treatments before application in agriculture.

As well as resistant bacteria, the spread of ARGs needs to be addressed worldwide[13]. Thus, it is important to understand and mitigate their occurrence in different ecological systems. This study has shown the prevalence of 11 different ARGs belonging to 7 of the most widely used antibiotic groups in effluent water and biosolids[65]. Our study revealed that sulfonamide ARGs (sul1 and sul2) were the genes with higher concentrations in effluents and biosolid samples. In line with previous studies, levels of sulfonamide resistance genes in effluent samples were higher than macrolide, tetracycline, and quinolone resistance genes[65,66]. Furthermore, sulfonamide gene levels were higher in biosolids than effluents (Fig. 5) as in the Mao et al. 2015 aforementioned study, highlighting the risk of biosolids as carriers of ARGs[65]. Levels of bla_{CTX-M}, ARG that confer resistance to beta-lactamase, were 4 Log higher than levels of viable ESBL-*E. coli*, which could be explained by the longer persistence of DNA[67], the presence of extracellular genetic material with bacterial surfaces, colloids, and bacteriophages, which shields it from nucleases[68–71]. This fact supports the idea that the dissemination of ARGs is not only carried out by viable bacteria but also by being found free in the environment or carried by other microorganisms such as bacteriophages[72].

ARGs profiles were comparable in effluents and biosolids despite gene concentration differences except for cmlA_2 and ermB_1. The cmlA_2 gene, which confer resistance to phenicol, was not found in any effluent samples indicating that environmental conditions, microbial populations, or the presence of contaminants in water treatment facilities may have impacted effluents but not biosolids. In March–May 2022, the ermB_1 gene was only detected in effluent samples, whereas the ermA gene, conferring resistance to macrolide-lincosamide-streptogramin B group antibiotic, was only detected in biosolid samples collected in January, consistent with previously reported data, whereas erm genes were only detected in biosolids[73]. Cold stress, which is linked with low temperatures, may increase horizontal gene

transfer of ARGs, explaining this fluctuation along the year[74]. The significant presence of the ARGs and ESBL-*E. coli* supports assertions that land application of biosolids may disseminate ARGs to soil bacteria and demonstrate their potential introduction to food products via both irrigation and amendment[75].

The wide distribution of MPs present in wastewater sources undoubtedly brings about environmental pollution and risk. Therefore, removing MPs before they reach environmental water courses is highly recommended. In this sense, WWTPs play an important role in hindering MPs from entering water environments[76]. As observed in this work, the concentration of MPs in wastewater decreased in effluent samples as compared to influent samples, being the water treatment more efficient in removing higher size particles. The number of MPs found in the different samples agreed with those reported in the literature. Previous works investigated the abundance of MPs in urban WWTPs, with ranges of 0.28 to 3.14×10^4 particles/L in the influent, which significant differed from 0.01 to 2.97×10^2 particles/L in the effluent[77]. However, they did not refer to the removal efficiency depending on the particle size. In this work, a higher efficiency in reducing MPs (between 77–100%) of higher particle size (S)-MPs has been observed, which was similar to the 88–94% efficiency of municipal WWTPs previously reported[78]. However, this value was significantly reduced for MPs with smaller particle size (S)-MPs and presented a great variability depending on the WWTP studied (4–59%). Deng et al. (2023) reported that the removal efficiency of MPs in a petrochemical WWTPs reached 92% and highlighted that the primary treatment removed most of the MPs[79] (87.5%). Talvitie et al. (2015) also stated that the primary treatment could remove most of the MPs, although they did not refer to their particle size[80]. They reported that the major part of the fibers can be removed already in primary sedimentation process, which agreed with the lower proportion of fibers (as compared to fragments) found in these samples.

Concerning the type of polymers detected, there is a higher prevalence of PE, PET, PS and PA, as it has been previously reported for drinking water and petrochemical and urban WWTPs[79,81–83]. Furthermore, WWTPs were more efficient in removing polymers with higher density such as PA and PET, probably during the density separation step, favouring a significant reduction of these polymers in the effluent wastewater. Furthermore, the size of more than 90% of microplastic particles detected in WWTPs ranged between 1 and 300 μm and fragments were found to be the most prevalent shape of microplastics, in agreement with other works[84].

Within this context, MPs release into the environment through sludge and effluent wastewater can also pose another risk, since MPs can accumulate/transport harmful pollutants, posing concerns about their role in treatment resistance and disease spread[85]. Bacteria and viruses have been reported to adsorb onto MPs, forming plastispheres[86]. Pathogenic bacteria, including those harmful to humans and fish, have also been found in communities of MPs[87–89]. Regarding viruses, the primary interaction with MPs involves electrostatic adhesion, increasing the risk of waterborne viral transmission. These viral or bacterial plastispheres not only resist UV treatment but can also promote infections, as shown for polystyrene MPs, which have been observed to facilitate IAV infection of host cells[89,90]. Additionally, the persistence of pathogen-carrying MPs in aquatic environments raises concerns about reverse zoonosis,

where these plastispheres might be ingested by aquatic organisms, potentially endangering human populations through the food chain[100]. In summary, MPs can act as carriers for pathogenic bacteria and viruses in municipal sewage, intensifying concerns about public health and the environment.

Overall, the findings of this research underscore the potential threats to public health associated with the reuse and release of reclaimed water, particularly concerning microbiological pathogens and environmental pollutants like microplastics, as well as the release of emerging contaminants into the environment and food chain through the use of biosolids in agriculture. These risk factors, including the persistence of enteric viruses, the inadequate reduction of microbial load and antibiotic resistance genes, and the prevalent presence of microplastics, emphasize the need for a holistic approach in addressing health concerns. Integrating these insights from wastewater analysis as well as human epidemic respiratory viruses monitoring into the broader One Health framework is crucial for devising effective policies, improving water treatment processes, and safeguarding both human and ecosystem health in a sustainable manner.

4. Materials and methods

Water concentration method and nucleic acid extraction for viruses and ARGs

Grab influent (n = 72) and effluent (n = 72) wastewater samples were collected along with dehydrated biosolid samples (n = 72) from 6 different WWTPs over a one-year period (January 2022 – December 2022). Samples were grabbed early in the morning (8 am) by collecting ~ 500 mL of wastewater in sterile HDPE plastic containers (Labbox Labware, Spain). Collected samples were transferred on ice to the laboratory, kept refrigerated at 4°C, and concentrated within 24 h. Samples were artificially contaminated with 10^6 PCR units (PCRU) of porcine epidemic diarrhea virus (PEDV) strain CV777, serving as a coronavirus model. Additionally, 10^6 PCRU of mengovirus (MgV) vMC_0 (CECT 100000) were used as a non-enveloped counterpart for recovery efficiency assessment. Effluent wastewater samples were concentrated through a previously validated aluminium-based adsorption-precipitation method[11,91]. Alternatively, 40 mL of influent wastewater samples were processed with the Enviro Wastewater TNA Kit (Promega Corp., Spain) vacuum concentration system following the manufacturer's instructions[92]. For biosolid samples, 0.1g of biosolid were resuspended in 900 µL PBS for nucleic acid extraction prior to PCR analyses.

Nucleic acid extraction from influent and effluent wastewater concentrates and biosolid suspensions was performed by using the Maxwell® RSC Instrument (Promega, Spain) with the Maxwell RSC Pure Food GMO for viral and ARG extraction. Specific programs, namely 'Maxwell RSC Viral Total Nucleic Acid' and 'PureFood GMO and Authentication,' were employed for viral and ARG extractions, respectively.

Virus detection and quantification

The detection of process control viruses, PEDV and MgV, was carried out through RT-qPCR using the One Step PrimeScript™ RT-PCR Kit (Perfect Real Time) (Takara Bio Inc., USA) as detailed elsewhere[93]. Levels of HuNoV GI and GII, HAstV, RV, HAV and HEV were determined using the RNA UltraSense One-Step kit (Invitrogen, USA), following previously described procedures[9,11]. The occurrence of crAssphage was established using the qPCR Premix Ex Taq™ kit (Takara Bio Inc)[94]. PMMoV detection was determined using the PMMoV Fecal Indicator RT-qPCR Kit (Promega, Spain) following the manufacturer's instructions. SARS-CoV-2 detection was performed by targeting the N1 region of the nucleocapsid gene. The One Step PrimeScript™ RT-PCR Kit (Perfect Real Time) was used with N1 primers and conditions described by CDC[95]. IAV detection followed the protocol described by CDC (2009) using primers from CDC (2020) and the One Step PrimeScript™ RT-PCR Kit (Perfect Real Time)[96].

Different controls were used in all assays: negative process control consisting of PBS; whole process control to monitor the process efficiency of each sample (spiked with PEDV and MgV); and positive (targeted gene reference material) and negative (RNase-free water) RT-qPCR controls. The recoveries of PEDV and MgV, spiked as enveloped and non-enveloped viral process controls, respectively, ranged between 6.31 and 59.65% (data not included). The validation of results for targeted viruses adhered the criteria specified in ISO 15216-1:2017, where a recovery of the process control of ≥ 1% is required[97].

Commercially available gBlock synthetic gene fragments (Integrated DNA Technologies, Inc., USA) of HuNoVs GI and GII, HAstV, RV, HAV, HEV, and crAssphage were used to prepare standard curves for quantification. For IAV and RSV quantification, Twist Synthetic InfluenzaV H1N1 RNA control (Twist BioScience, South San Francisco, CA, USA), and purified RNA of RSV (Vircell, S.L., Spain) were used. The PMMoV Fecal Indicator RT-qPCR Kit (Promega) provided PMMoV RNA for generating a standard curve. A table, featuring primers, probes, PCR conditions, limit of quantification (LOQ/L), and limit of detection (LOD/L) for all targeted viruses in this work is available in the Supplementary materials (Table S1).

Quantification of viable somatic coliphages, E. coli, and Extended Spectrum Beta-Lactamases producing E. coli.

Somatic coliphages were determined from wastewater samples filtered through sterile filters (0.45 µm pore) by using a commercial Bluephage Easy Kit for Enumeration of Somatic Coliphages (Bluephage S.L., Spain), following manufacturer's instructions. For biosolid samples, 1g of biosolid was resuspended in 100 mL PBS for both somatic coliphages and *E. coli* enumeration.

For all water and biosolid samples, *E. coli* and Extended Spectrum Beta-Lactamases producing *E. coli* (ESBL-*E. coli*) enumeration was assessed by using selective culture media Chromocult coliform agar (Merck, Darmstadt, Germany) and CHROMagar ESBL (CHROMagar, Paris, France), respectively. Spread plating (0.1 mL) or membrane filtration (200 mL) was used depending on the anticipated bacterial concentration. Influent wastewater samples were diluted serially, and 0.1 mL aliquots were spread-plated. Effluent samples were filtered through a 0.45 µm cellulose nitrate membrane filter (Sartorius, Madrid, Spain). Following incubation at 37 ºC for 24 hours, results were interpreted, with. dark blue-violet colonies

considered positive for *E. coli* and dark pink-reddish colonies considered positive for ESBL-*E. coli*. The analysis was performed in duplicate, and the results were expressed as CFU/100 mL. The detection limit (LOD) for *E. coli* and ESBL-*E. coli* counts in the influent and biosolid samples was 2.0 Log CFU/100 mL (100 CFU/100 mL), while in the effluents, the LOD was 0 Log CFU/100 mL (1 CFU/100 mL).

Detection and quantification of antimicrobial resistance genes in effluent waters and biosolids

In this study, 11 ARGs that confer resistance to Sulfonamides (sul1, sul2_1), beta-lactamase (pbp2b, bla_{CTX-M}), phenicols (cmlA_2), nitroimidazoles (nimE), MLSB (ermB_1, ermA), tetracyclines (tetPB_3, tetA_1) and fluoroquinolones (qacA_1), were only detected in effluent waters and biosolids. The 16S rRNA gene was used as positive control for qPCR measurement. Quantification of the 12 selected genes was performed by high-throughput quantitative PCR (HT-qPCR) using the SmartChip™ Real-Time PCR system (TakaraBio, CA, USA) by Resistomap Oy (Helsinki, Finland). qPCR cycling conditions and processing of raw data were described elsewhere[98–100]. Each DNA sample was analysed in duplicate. Data processing and analysis were performed by using a python-based script by Resistomap Oy (Helsinki, Finland)[101,102].

Digestion of organic material and isolation of MPs

Initial steps consisted on optimizing the protocol for the removal of organic material and the isolation of the maximum number of MPs from wastewater and biosolid samples. Different volumes of water, amounts of biosolids and digestion strategies for organic biomass removal were tested to remove the greatest amount of organic material without compromising the integrity of the MPs. Avoiding filter clogging was a requirement during the methodology development, to facilitate further identification of MPs. To reduce the risk of external contamination by MPs, laboratory consumables made of glass were used, the reagents were purified by filtering through a 0.2 µm pore size nitrocellulose filter (Whatman, Maidstone, UK), 100% cotton lab aprons were used, samples were processed in a laminar flow cabinet, the beakers were covered with a watch glass, disposable nitrile gloves were used and, before and after using the material, all used materials were rinsed thoroughly with deionized water. In order to assure that the isolation of MPs was effective and external contamination did not occur, a negative control (NC) was included every month and a positive control (PC) was carried out every 3 months. The positive control was made with fluorescent polystyrene microspheres (Invitrogen, Waltham, USA) of 1 µm in diameter. Specifically, a solution of 1000 beads/20 µL was prepared and 20 µL of this solution was incorporated before the pre-treatment and, the number of remaining microbeads after the digestion protocol was determined to calculate the percentage of recovery. The average value of particle recovery was 93.9%.

Two different pre-treatment protocols were finally defined:

1) Sieved > 300 µm or (S): With this pre-treatment, all solid particles (including MPs) larger than 300 µm were isolated from 2 L of wastewater or 5 g of biosolid samples after sieving, oxidative digestion, and filtration steps.

2) Total Particles or (T): With this pre-treatment all solid particles (including MPs) with a size between 1 µm and 5 mm were isolated from a 10 mL aliquot of wastewater after oxidative digestion, density separation, and filtration steps.

Through protocol (S), a larger and more representative amount of wastewater was treated, but particles smaller than 300 µm were lost. In the other hand, protocol (T) allowed the analysis of particles down to 1 µm in size, but the amount of analysed wastewater was much smaller to avoid filter clogging.

In both protocols (S) and (T), oxidative digestion was performed to remove organic material, adapting the method described by the National Oceanic and Atmospheric Administration (NOAA)[103].

In the case of the Sieved 300 µm or (S) protocol (Fig. 11), 2L of wastewater or 5 g of biosolids were treated. The 5 g of biosolids were previously dispersed in 100 mL of ultrapure MilliQ water by applying stirring and heat during 30 minutes at 30 ºC. The wastewater or biosolid dispersion were subsequently poured through a 300 µm mesh stainless steel sieve. The retained particles were collected by washing with MilliQ water into a beaker and digested by adding an equivalent volume of NaClO (14%, VWR chemical, USA). After heating at 75 ºC for 3 h under stirring, the sample was sieved again to remove the disaggregated smallest particles. The particles retained on the sieve were collected by washing with MilliQ water on a 0.8 µm pore size nitrocellulose filter (Whatman, USA). The filter was protected from external contamination between a microscope glass slide and a glass cover, and finally dried at 40ºC for 24 h in a convection oven.

In the case of the Total Particles or (T) protocol, an oxidative digestion (Fenton reaction) was performed on a 10 mL wastewater sample by adding 20 mL of a H_2O_2 (30%, Sigma- Aldrich, USA) solution and 20 mL of a 0.05 M Fe (II) solution prepared by mixing $FeSO_4$ (Sigma- Aldrich, USA), H_2SO_4 (96%, PanReac AppliChem, ITW Reagents, USA) and deionized water. The sample was then heated at 75°C for 30 min under stirring. The digestion step was repeated if any remaining organic material was visually. Thereafter, a density separation was performed after adding NaCl (99.5%, Sigma- Aldrich, USA) until saturation. Subsequently, the sample was left to sediment for 30 min in a separatory funnel and the supernatant was filtered through a 0.8 µm pore size nitrocellulose filter (Whatman, USA) under vacuum. The filter was also protected between glass slide and coverslip and dried at 40ºC for 24 hours.

Characterization of particles present in biosolid and wastewater samples.

Filters obtained after pre-treatment protocols (S) and (T) were photographed using an EVOCAM II macrophotography equipment (Vision engineering, Woking, UK) and the ViPlus software (2018, Vision Engineering). Two partially overlapping 2MPx color photos were taken for each filter, always at 20x magnification, with half of the filter appearing in each photo. These images were fused by digital stitching techniques using the mosaic J command of the FIJI software (ImageJ 1.49q Software, National Institutes of Health, USA). Each image showed a 25*15mm field of view. The pixel size was 13.3 microns, obtaining an image to calibrate in each photo session to have a precise external calibration data. A rough quantification was performed, and all particles, including MPs, were characterized using the Nis Elements

BR 3.2 software (Nikon corporation, Japan). To achieve this, a macro of programmed actions was designed in which, firstly, the pixel size was calibrated in the complete image of the filter, then a matrix-iterative detection tool for particles less bright than the filter was applied, which facilitated a binary segmentation by brightness levels and achieve the selection of the particles of each filter in an automated way, only in the filtration zone. Finally, the data of all the particles were exported to obtain the count and the different morphological values of numerous parameters and perform the statistical calculations.

For the characterization, the particles were classified into 3 size ranges of 1-100 µm, 100–300 µm and 300–5000 µm. The particles were also classified according to their circularity, calculated from the measured perimeter and area of each particle according to Eq. 1, in 3 ranges: 0-0.4, 0.4–0.8 and 0.8-1. A circularity value of 1.0 indicates a perfect circle. As the value approaches 0.0, it indicates an increasingly elongated polygon. Particles with a circularity less than 0.4 were considered as fibers.

$$Circularity = 4\pi(\frac{area}{perimeter^2}) \quad (1)$$

In addition, the efficiency of WWTPs in removing particles was calculated according to the following equation:

$$Efficiency = \frac{\text{influent} - \text{effluent}}{\text{influent}} \times 100$$

2

Where: Efficiency = particle removal efficiency (%); influent = number of particles detected at the WWTP influent; effluent = number of particles detected at the WWTP effluent.

Quantification of microplastics present in biosolid and wastewater samples.

Quantification, identification and characterization of MPs was carried out only on samples from the odd months. The analysis was performed using an automated Raman microscope Alpha300 apyron (Witec, Ulm, Germany). First, each filter was mapped by acquiring a total of 1089 images, which after reconstruction represented a 27% of the filter area or 1 cm^2. The present particles were detected and selected by performing image analysis using the ParticleScout 6.0 software in automatic mode.

After particle selection, analysis on each particle by Raman spectroscopy and subsequent identification were carried out. The optimal conditions for Raman spectra acquisition were as follows: 785 nm laser which facilitates to identify fluorescent particles, 300 lines/mm diffraction grating opening, spectral range between 0 and 3000 cm^{-1}, 10 accumulations, 0.2 second acquisition time, and 40 mW laser power. The spectrum of each particle was registered and compared with an in-house build spectral library of polymers. The reference polymer materials included in the spectral library were polyethylene (PE), polyethylene terephthalate (PET), polyamide (PA), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), polytetrafluoroethylene (PTFE), polyacrylamide (PAM), Polyarylsulfones (PSU),

Polymethylmethacrylate (PMMA), nitrile rubber (NBR), Cellophane and Melamine. Particles that had a 75% or better match (HQI) between the sample and reference spectra were identified as composed of the same material or of a similar chemical nature. In addition, a visual inspection was carried out and the spectrum acquisition was repeated on the particles where a clear identification was not initially possible. Three rules were considered to discriminate between plastics and non-plastics and to prioritize the particles to be analysed: i) the object must not show cellular or natural organic structures; ii) the fibre thickness must be uniform along the entire length; iii) the colour of the particles must be clear and homogeneous[104]. The MPs already identified were classified based on material type, size, morphology, and area.

Statistical analysis

Results were statistically analysed and significance of differences was determined on the ranks with a one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests. In all cases, a value of $p < 0.05$ (confidence interval 95%) was deemed significant.

Declarations

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Declaration of competing interest

All authors declare no financial or non-financial competing interests.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used ChatGPT (https://chat.openai.com/) in order to improve readability and language. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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Figures

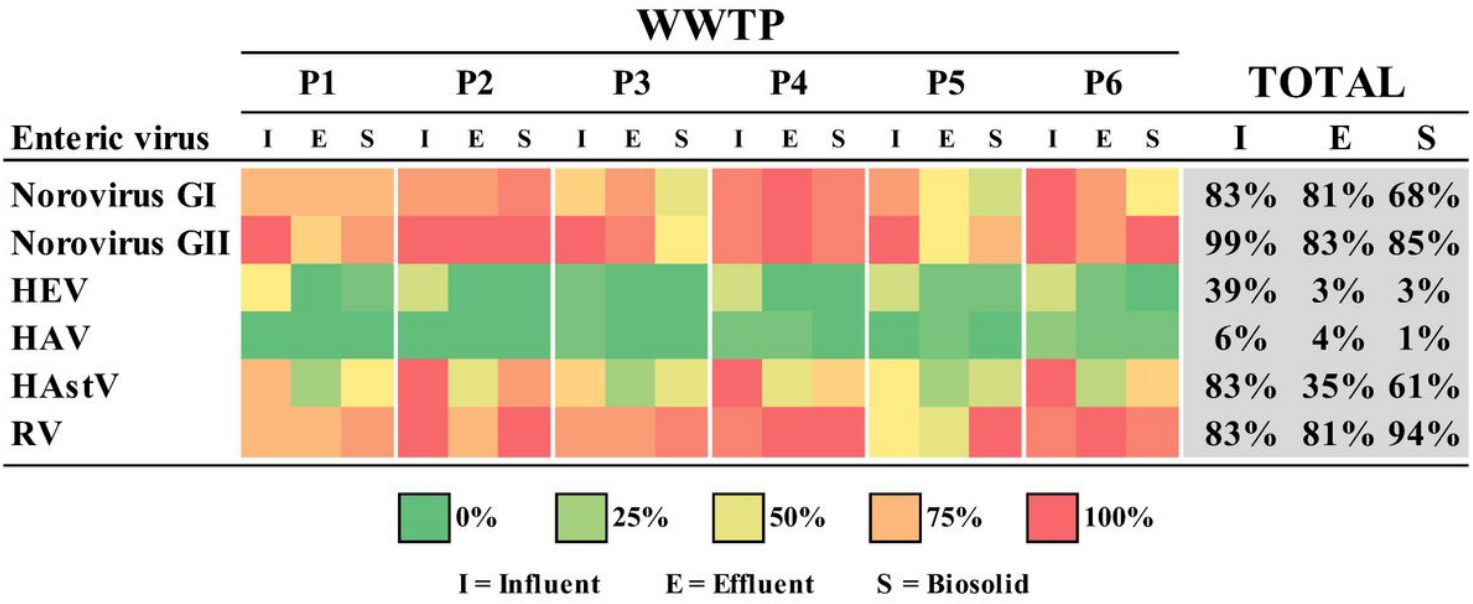

Figure 1

Prevalence of human enteric viruses (%) in influent (I), effluent (E), and biosolid (S) samples collected from six different WWTPs (P1-P6).

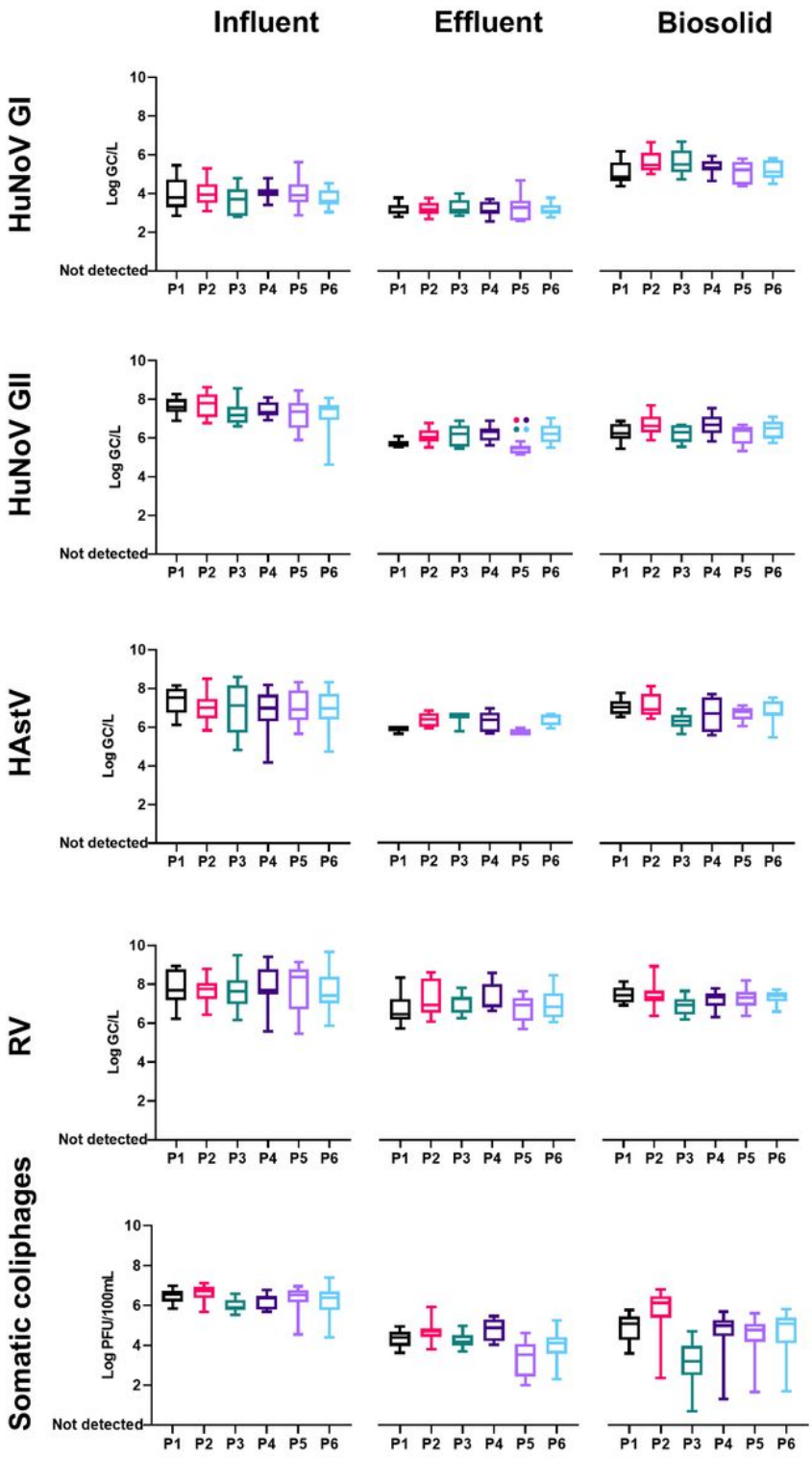

Figure 2

Mean concentrations of human enteric viruses (when detected) and somatic coliphages in influent wastewater, effluent wastewater, and biosolid samples in each of the six WWTPs analysed (P1 - P6). Coloured circles above a box indicate significant differences between that box and the box with that same colour ($p < 0.05$). GC: genome copies; PFU: plate forming units, RV: rotavirus; HuNoV: human norovirus, HAstV: human astrovirus.

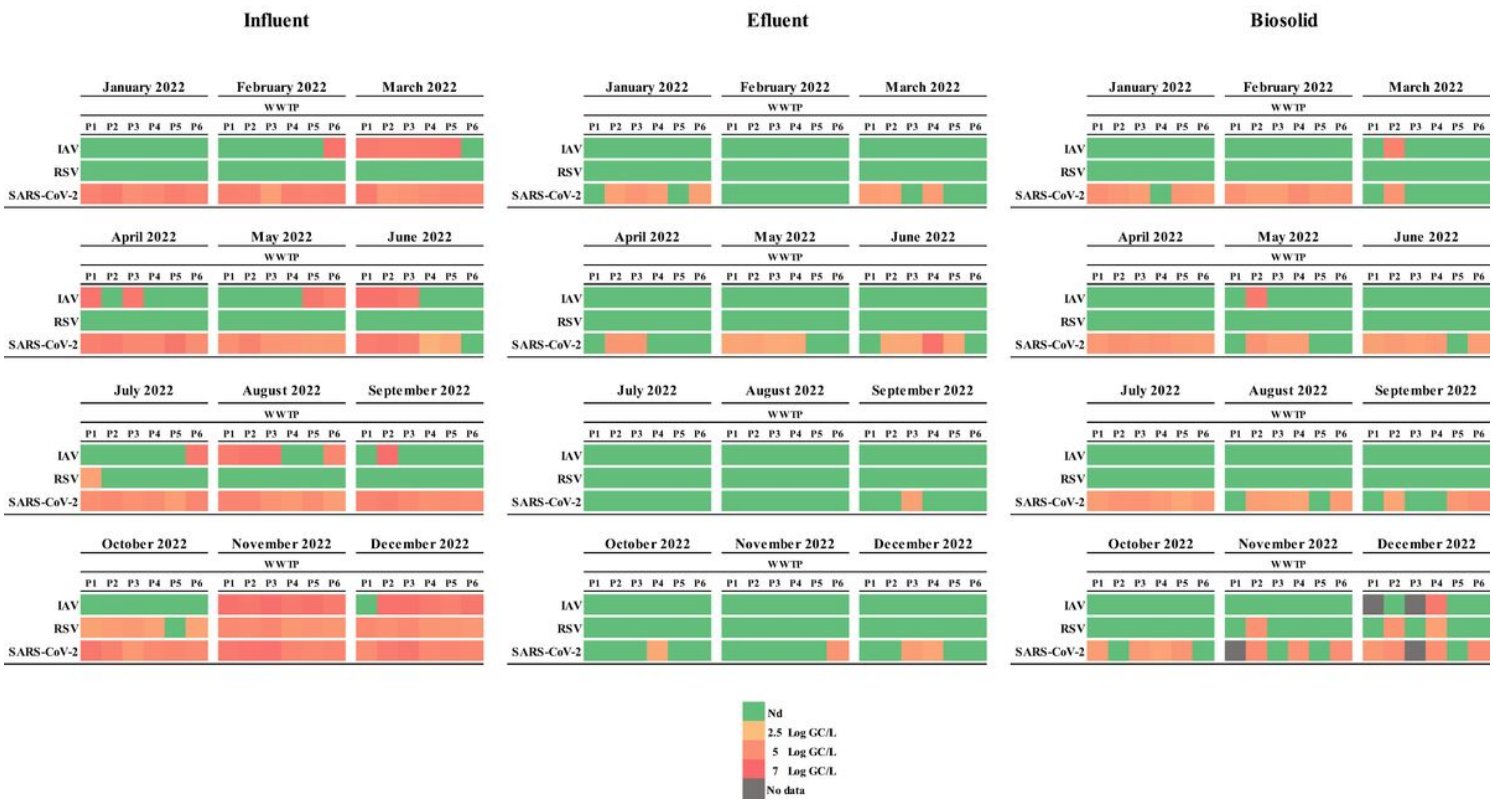

Figure 3

Concentration (in Log GC/L) of RSV, IAV, and SARS-CoV-2 in influent, effluent, and biosolid samples collected over a one-year period in six different WWTPs (P1-P6). Nd: not detected. GC: genome copies; RSV: respiratory syncytial virus; IA: Influenza A virus

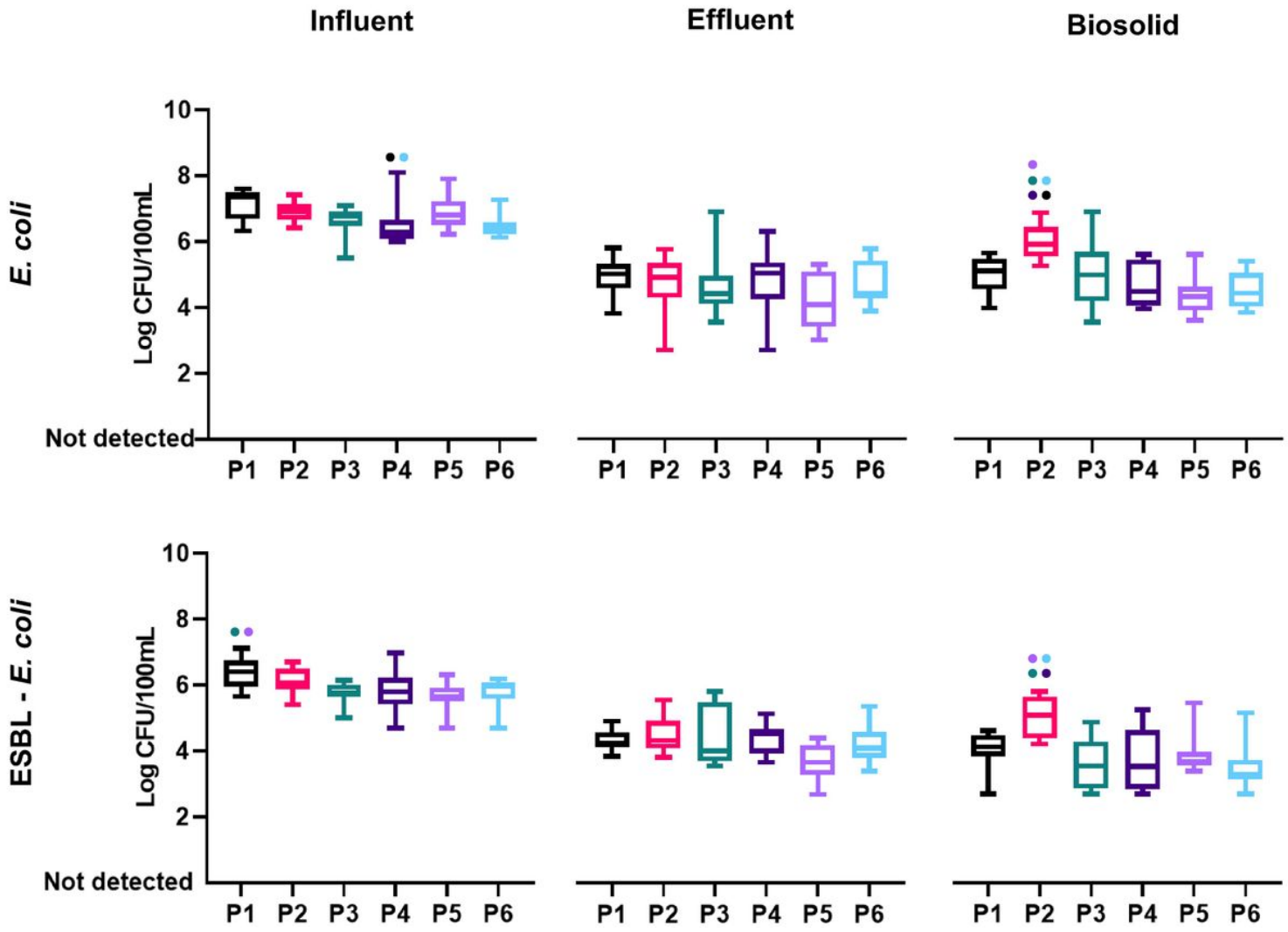

Figure 4

Levels of *E. coli* and ESBL-*E. coli* in influent, effluent, and biosolid samples in each of the six WWTPs analysed (P1-P6). Coloured circles above a box indicate significant differences between that box and the box with that same colour ($p < 0.05$). CFU: colony forming unit.

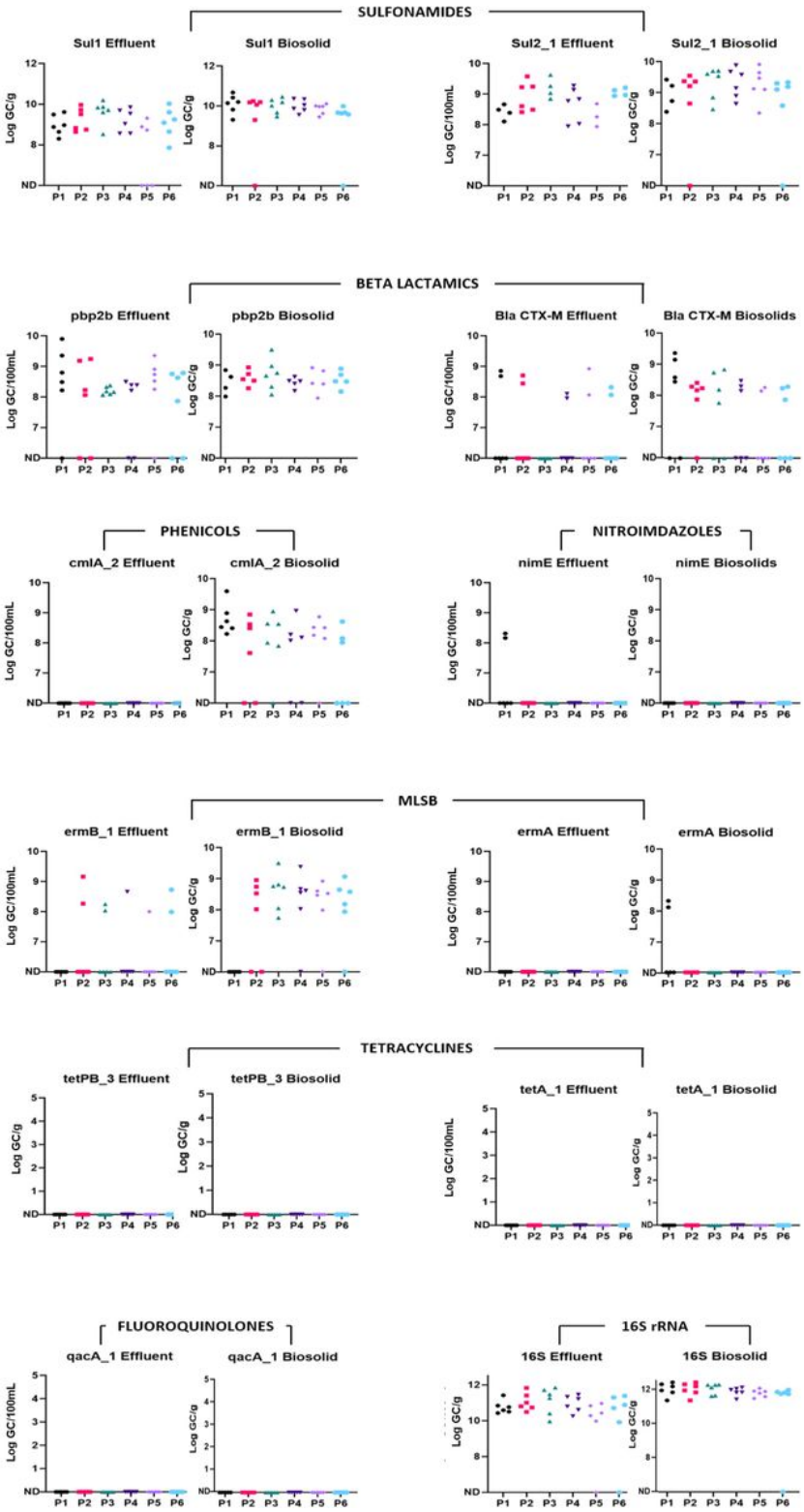

Figure 5

Levels of different ARGs in effluent wastewaters (in Log GC/100 mL) and biosolids (in Log GC/g) samples for each of the six WWTPs analysed (P1-P6). ND: Not detected. MLSB: Macrolide-lincosamide-streptogramin B group antibiotics; GC: genome copies.

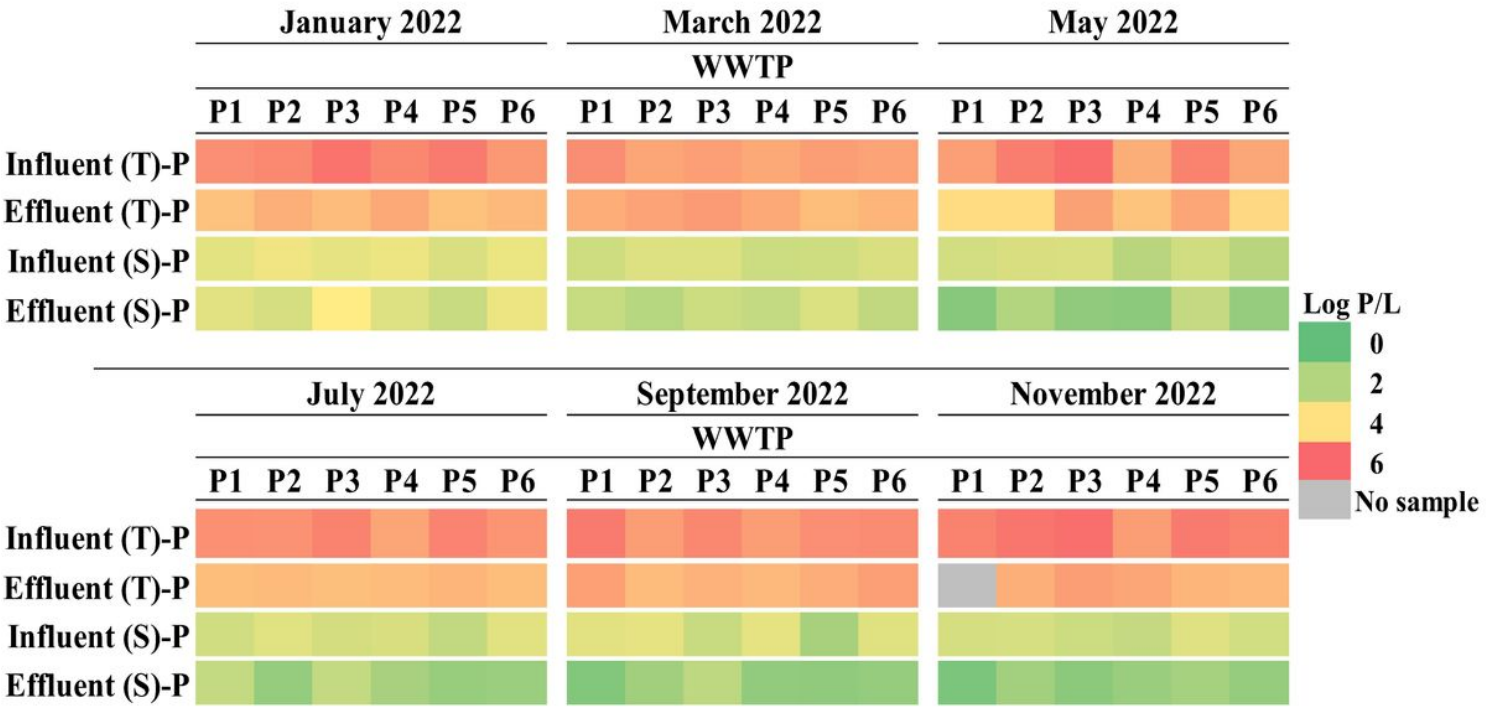

Figure 6

Concentration (log P/L) of total particles (T)-P and sieved particles (> 300 mm, (S)-P) in influent and effluent wastewater samples in even months over a one-year period in six different WWTPs (P1-P6).

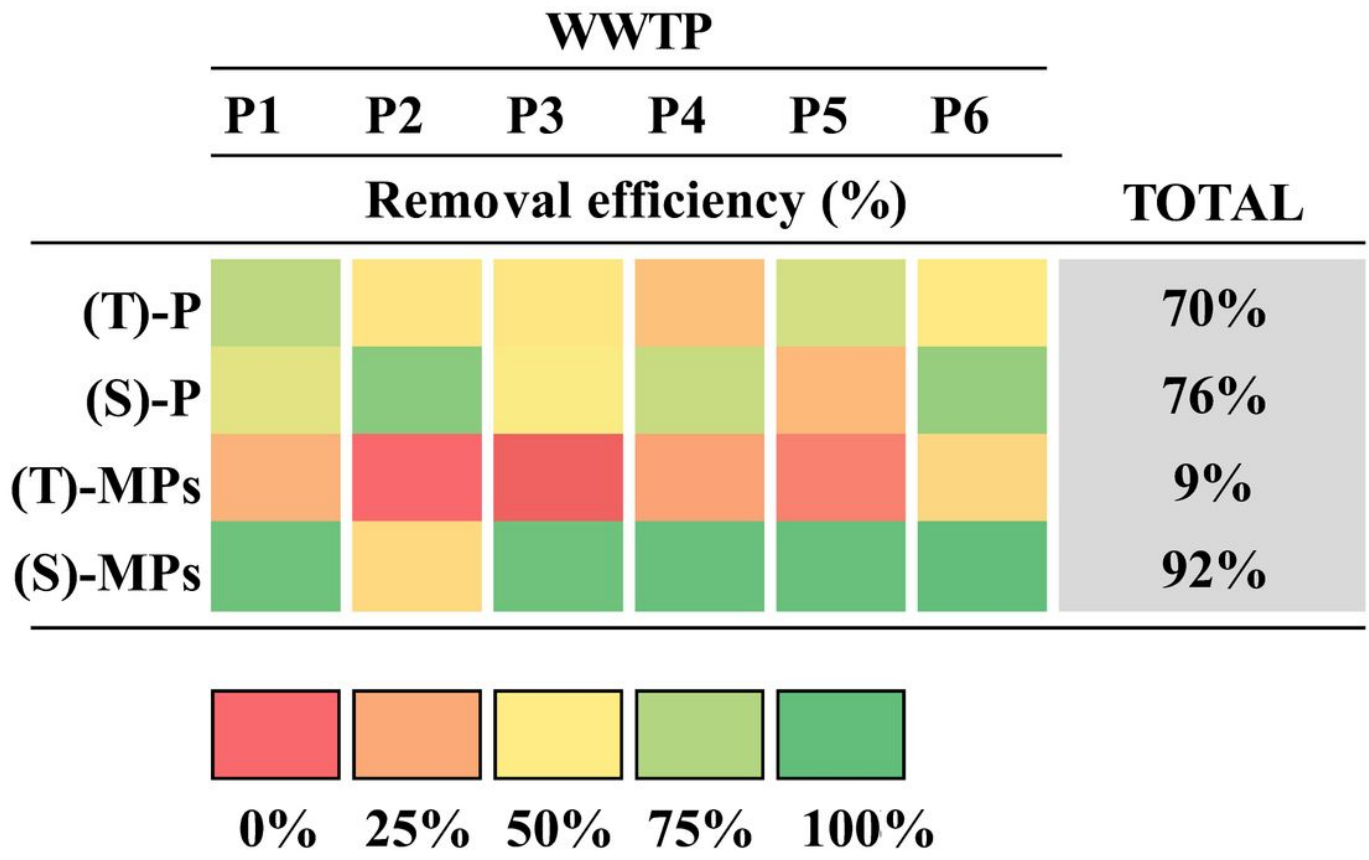

Figure 7

Removal efficiency (%) of all solid particles (P) and microplastics (MPs) between influent and effluent samples collected from six different WWTPs (P1-P6) after both pre-treatment protocols (T) and (S).

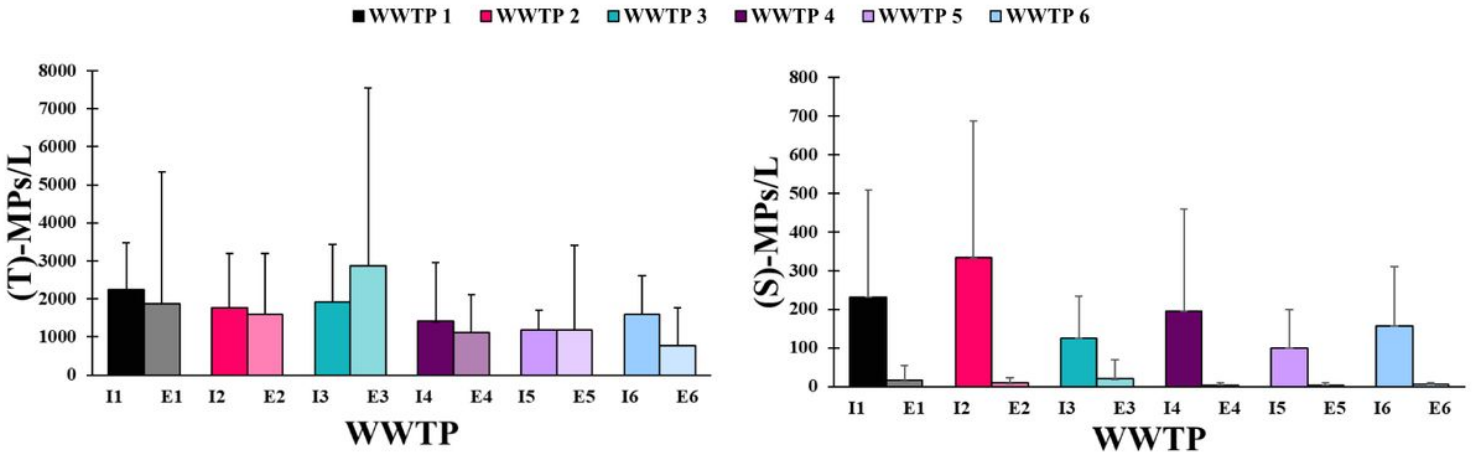

Figure 8

Annual average concentration of microplastics (MPs) in influent (I) and effluent (E) after (T) (Panel A and (S) (Panel B) protocols collected from six different WWTPs.

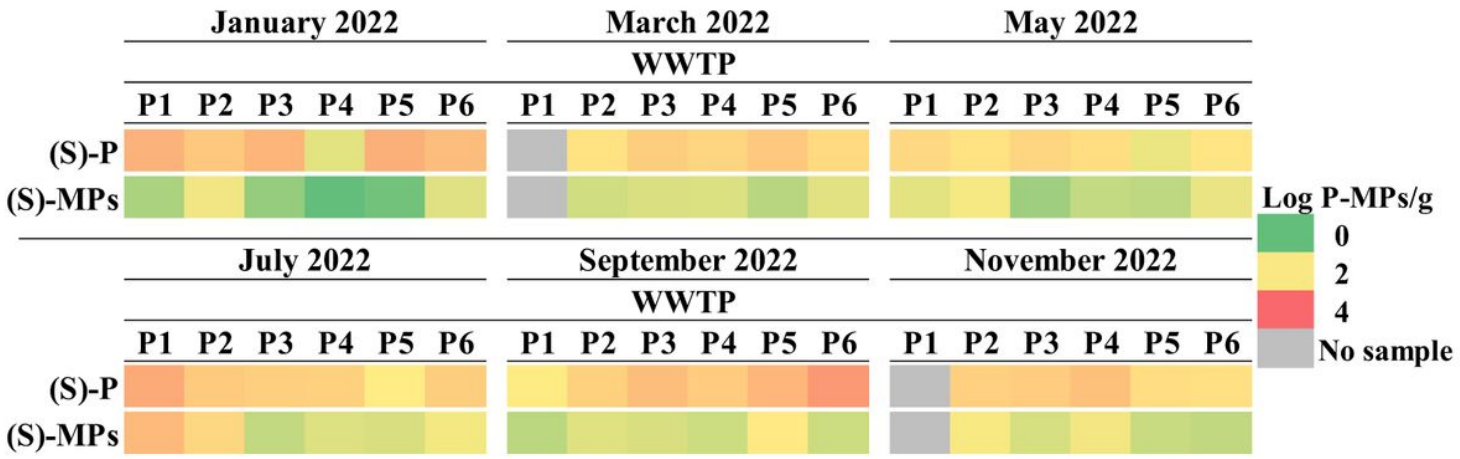

Figure 9

Concentration (in log/g) of (S)-P and (S)-MPs in biosolids in even months over a one-year period in six different WWTPs (P1-P6).

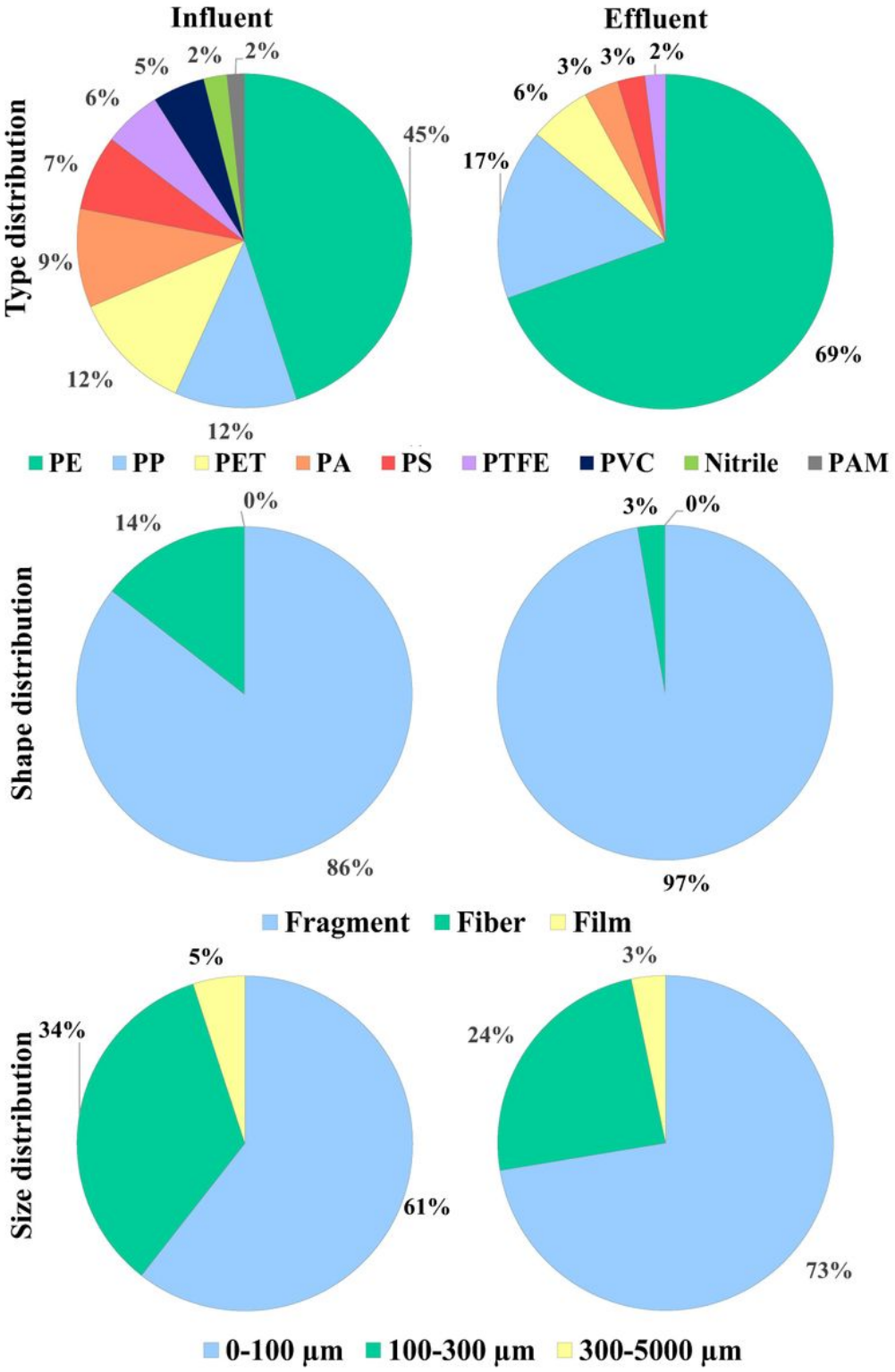

Figure 10

Morphological distribution of type, size, and shape of (T)-MPs in influent and effluent wastewater samples from six different WWTPs (P1-P6). PE: polyethylene; PET: polyethylene terephthalate; PA: polyamide; PP: polypropylene; PS: polystyrene; PVC: polyvinyl chloride; PTFE: polytetrafluoroethylene; PAM: polyacrylamide.

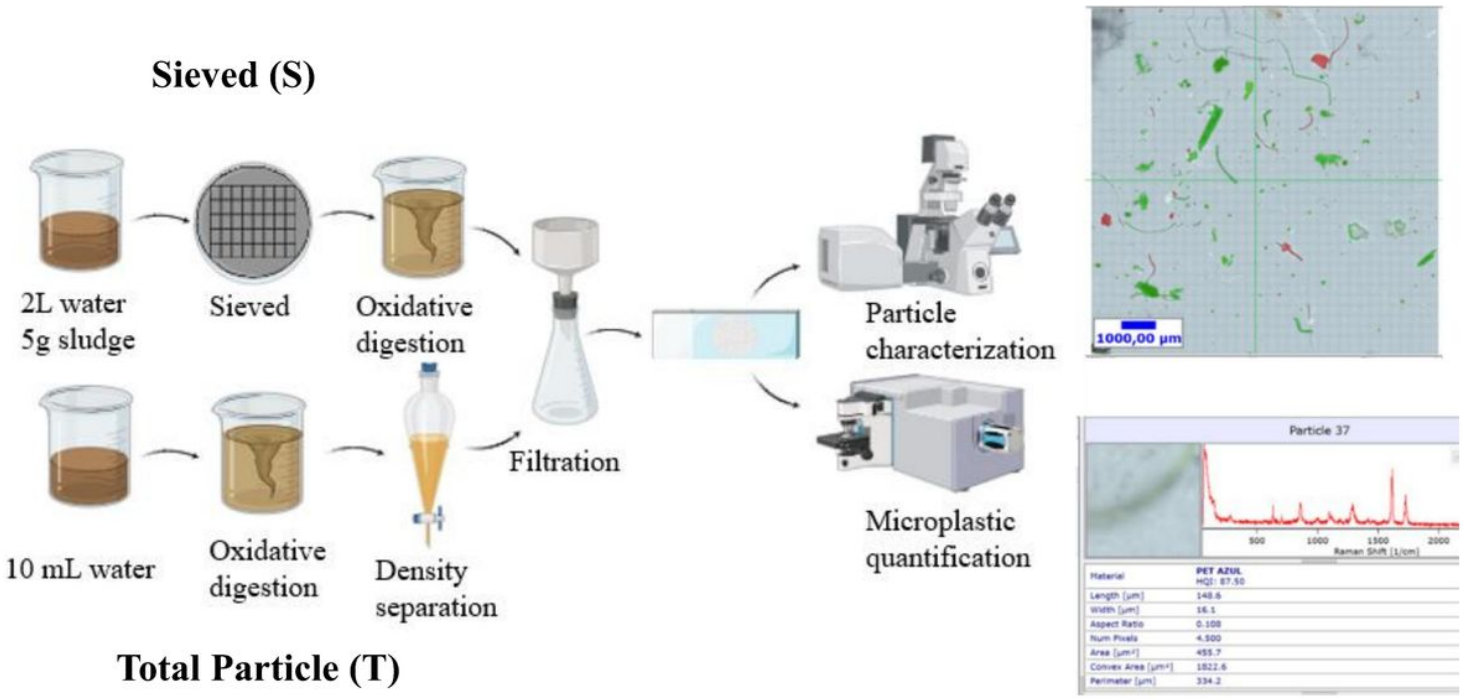

Figure 11

Scheme summary of the methodology used for the isolation, quantification and identification of MPs.

Supplementary Files

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