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Insights into the antibiotic resistance dissemination in a wastewater effluent microbiome: bacteria, viruses and vesicles matter

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Running Title

Antibiotic resistance genes in wastewater

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Originality-Significance Statement

Presence of antibiotics in wastewater might induce selection of bacterial populations and antibiotic resistance genes (ARGs) that can be spread from wastewater treatment plants. Here, to provide a comprehensive view, in addition to the bacterial resistome (ARG collection in a metagenome), we studied the viral resistome and the potential role of vesicles -usually ignored when studying natural communities- in antibiotic resistances in a representative wastewater effluent microbiome in natural conditions and amended with polymyxin, which is used as last resort to treat infections by multidrug-resistant bacteria.

Summary

Wastewater treatment plants (WWTPs) effluents are considered as hotspots for the dispersion of antibiotic resistance genes (ARGS) into natural ecosystems. The bacterial resistome (ARG collection in a metagenome) analyses have provided clues on antibacterial resistance dynamics. However, viruses and vesicles are frequently ignored. Here, we addressed the bacterial, viral and vesicle resistomes from a representative wastewater effluent in natural conditions and amended with polymyxin, which is used as a last resort antibiotic. Metagenomics showed that the natural prokaryotic resistome was vast (≈9,000 ARG hits/Gb metagenome) and diverse, while viral resistome was 2 orders of magnitude lower (≈50 ARG hits/Gb metagenome) suggesting that viruses

rarely encoded ARGs. After polymyxin amendment, data showed no ARG enrichment - including to polymyxin- in the microbiome. Remarkably, microbiomes responded to polymyxin with a vast release of putative vesicles (3-fold increase compared to the control), which might be used as `traps´ to decrease the antibiotic concentration. Intriguingly, although polymyxin resistance genes (PRGs) were rare in the microbiome (0.018% of total ARG found), in the viral and vesicle fractions, PRGs were more abundant (0.5-0.8% of total ARG found). Our data suggest that vesicles could have a more active role in the context of transmission of antibiotic resistances.

Introduction

The discovery of antibiotics has been a major breaktrough in the human history. However, antibiotic resistance is an urgent and growing global health concern. By 2050, predictions estimate that over 10 million of deaths and ≈100 trillion USD total cost derived from antibiotic resistance worldwide (Brogan and Mossialos, 2016; O'Neill, 2016). Globally, significant amounts of antibiotics used in domestic treatments, hospitals and cattle farming end up, directly or indirectly, in wastewater (Priyanka and Nandan, 2014). In municipal wastewater and even in natural streams and wells receiving water from agricultural areas dominated by animal-feeding operations, concentrations of different antibiotics remain very high (Loper *et al.*, 2007; Hong *et al.*, 2013; Ju *et al.*, 2018). Wastewater and wastewater treatment plants (WWTPs) are thus considered to be hotspots for dissemination of antibacterial resistance into the environment. In a recent prokaryotic metagenomic study of several WWTPs analyzing 20 different antibiotics, authors found that the pool of antibiotic resistance genes

(ARGs) in bacteria is persistent and shared among different WWTP compartments (Ju *et al.*, 2018). Thus, these findings clearly underline the paramount role of WWTPs regarding disease control in humans and ARG spreading.

Between the different antibiotics that end up in wastewater and WWTPs, polymyxins -that destabilize the cell membrane- are most likely to have the lowest concentration compared with other antibiotics. This is because polymyxins are used mainly as last resort antibiotics to treat infections caused by multidrug-resistant gram-negative bacteria, which are resistant to carbapenem, and thus considered to be highly-drug resistant bacteria and of the most difficult pathogens to treat (Magiorakos *et al.*, 2017). Therefore, when comparing with other more commonly used antibiotics, polymyxins only account for a minor fraction of total antibiotic consumption (e.g. 0.068% in the European Union). Recent research has demonstrated that selection for antibiotic resistance occurs even at very low antibiotic concentrations in single-species experiments (Murray *et al.*, 2018). Thus, sub-inhibitory concentrations can induce contrasting biological responses in prokaryotes, such as selection of some specific microbial populations and positive selection of ARGs (Grenni *et al.*, 2018), but the relevance of these results in natural complex communities is unclear (Murray *et al.*, 2018).

The increase in polymyxin resistance is a serious global health concern due to the low number of alternative and effective antibiotics. Most polymyxin resistance mechanisms have been described in the context of bacterial isolates from human samples (Mammina *et al.*, 2012; Lean *et al.*, 2014; Olaitan *et al.*, 2014). In the case of

known pathogens, some clinical isolates of *Klebsiella pneumonia* or *Acinetobacter baumannii* have acquired resistance to polymyxin, while others, such as, *Burkholderia* spp. are naturally resistant to this drug (Olaitan *et al.*, 2014). Gram-negative bacterial isolates use several contrasting polymyxin resistance strategies that act at different cellular levels, such as the modification of the outer membrane, the use of efflux pumps or the formation of capsules (Manning and Kuehn, 2011; Yu *et al.*, 2015), but whether these polymyxin resistance mechanisms apply to uncultured wastewater bacteria is mostly unexplored, with only a few reports, such as one PCR-based study in wastewater that detected the gene *mcr-1* involved in the modification of lipid A of outer membrane (Hembach *et al.*, 2017), or a very recent metagenomic survey which pointed that polymyxin resistance genes were upregulated and enriched in the effluent of WWTPs (Ju *et al.*, 2018).

In the context of ARG dispersion from WWTP effluents, most studies have focused on the analyses of the bacterial resistomes (Ju *et al.*, 2018), while the viral and vesicle fractions have been frequently ignored, in spite of being important factors involved in gene transmission in bacteria. To the best of our knowledge there is no metagenomic report addressing the ARG pool in mixed fractions of WWTP effluent containing viruses and vesicles. It is well known that viruses usually outnumber bacteria in all aquatic ecosystems, including wastewater (Ma *et al.*, 2013). In addition, recently, vesicles have been found to be naturally abundant in seawater and data suggest that they could be ubiquitous in all aquatic environments (Biller *et al.*, 2014, 2017). In wastewater biofilms, vesicles have been observed by electron microscopy (Schooling

and Beveridge, 2006), although their abundance remains to be investigated in wastewater. Viruses, via generalized transduction, can spread ARGs (Balcazar, 2014). In addition, over the last years, different viral metagenomic studies have reported high levels of ARGs in viruses from feces samples of antibiotic-treated mice (Modi *et al.*, 2013) or human pulmonary samples (Rolain *et al.*, 2011). However, a recent study on 1,181 viral isolates suggests that *bona fide* ARGs attributed to viruses in viromes were previously overestimated (Enault *et al.*, 2017). Finally, recent evidence from pure bacterial cultures indicate that vesicles could also mediate resistance to antibiotics (Chattopadhyay and Jaganandham, 2015).

Thus, in the context of the polymyxin resistance health concern, here, in addition to the effluent wastewater bacterial resistome, we aim to get some insights into the viral resistome integrating as well the study of vesicle in the context of polymyxin resistance. For that, we analyzed uncultured microbial communities (cells and viruses) from untreated and polymyxin-treated samples in order to 1) monitor changes and responses of the microbiome, virome and vesicle fraction, 2) unveil the natural prokaryotic and viral resistomes, 3) assess the potential effect of polymyxin concentrations on the ARGs enrichment and their potential spreading through the bacterial, viral and vesicle fractions into nature.

Results

The effect of polymyxin B on wastewater prokaryotic, viral and vesicle abundance

The collected effluent wastewater samples were amended with sub-inhibitory polymyxin concentrations (30 μg/mL final antibiotic concentration; Fig. 1 and S1) that

allowed the survival of ≥95% of cells (see methods for details) following a previously reported method with culture reference models to study the effect of polymyxin sub-inhibitory concentrations (Manning and Kuehn, 2011). The effect of polymyxin on the abundance of prokaryotes, viruses and vesicles was monitored at times 0, 24 and 48 h (Figs. 1b and S1).

Natural bacterial concentration in the wastewater effluent was $4.4x10^7 \pm 1.8x10^7$ cells/ml (mean±SE). A day after the antibiotic treatment (24 h), changes in mean prokaryotic abundance in both the untreated controls and the treated samples behaved nearly identical, with a slight decrease to $\approx 3.4x10^7 \pm 2.7\ x10^6$ cells/mL (mean±SE; Fig. 2a). Two days afterwards treatment (48 h), the number of cells increased in the control and treated samples reaching similar abundances to those observed at time 0 h when sample was collected, which indicated that nevertheless, the used polymyxin concentrations were sub-inhibitory and did not affect overall bacterial abundance. Furthermore, the addition of polymyxin did not increase the number of dead cells detected by LIVE/DEAD® BacLight bacterial viability kit compared with the untreated control (Fig. S2). However, polymyxin concentrations did change the bacterial population patterns observed in the flow cytometric biplot (Fig. S1). Regarding the viral fraction, the natural wastewater showed an abundance of $1.6x10^9 \pm 7.2\ x10^8$ viruses/ml (mean±SE), and as observed for cells, virus-like particle (VLP) abundance followed a similar trend, albeit the decrease of VLP/mL in both control and treated samples was more evident throughout the experiment (Fig. 2a).

The abundance of putative vesicle-like particles (vesicle-LPs) in the control and the treated wastewater was determined using nanoparticle tracking analyses with NanoSight. For this, vesicle-LPs present in the 0.2 μm-filtered fractions free of cells were stained with the lipophilic FM4-64 dye. Previously, in these samples, we demonstrated the effectiveness of this dye at staining cell membranes from which vesicles are originated (Fig. S3). The natural concentration of vesicle-LPs in the wastewater effluent was $2x10^6$ per mL (Fig. 2b) and most vesicle-LPs had ≈125 nm size (Fig. S4). After 48 h, control samples showed very similar vesicle-LP abundance ($3.3x10^6$ vesicle-LP/mL) and size distribution to that of the natural samples (Fig. S4). However, data for antibiotic-treated samples showed a greater amount of vesicle-LP in the polymyxin B-treated samples ($1.0x10^7$ vesicle-LP/mL, ≈3-fold increase compared to controls, Fig. 2b). Furthermore, results indicated that released vesicles could have larger sizes (around 175 nm; Fig. S4), and a more homogenous size distribution. In addition, when we measured the total bulk fluorescence of these stained putative vesicles by high-resolution fluorometry, data showed larger fluorescence values for the polymyxin B treated samples in line with the absolute counting of vesicle-LP (Fig. S5). The presence of putative vesicle-LPs in the studied samples was proved with TEM (Fig. 2c) since spherical particles within <200 nm size were observed in the analysed samples.

Microbial community structure and diversity of wastewater microbes

Variation in microbial diversity from control and treated samples was assessed by 16S rRNA gene amplicon sequencing. A total of 5,376,908 reads were obtained and analysed with Qiime (Caporaso *et al.*, 2010; Kuczynski *et al.*, 2011) (Table S1). As shown in the Principal Component Analysis (PCA) of the 16S rRNA gene (Fig. 3a) performed at different taxonomic levels, control samples gathered together but separate from antibiotic-treated samples, which indicated differences in microbial diversity structure. The natural effluent water sample (named "nw"), processed as soon as it was obtained, was separated from both antibiotic and control samples in the PCA probably due to the effect of incubation conditions that differ from the natural ones. It is worth noting that these observed differences in PCA plot among samples (control vs antibiotic treated samples) is only due to significant variations in proportion of taxa, and not because of the disappearance or appearance of new taxa in the control or treated samples (Fig. 3b). Data showed that the predominant phyla in control and treated samples were *Proteobacteria*, *Saccharibacteria* and *Bacteroidetes*. Furthermore, candidate phyla usually found in wastewater, such as WCHB1-60 and TM6 (Tian *et al.*, 2015; Yeoh *et al.*, 2016; Allievi *et al.*, 2018), were also detected in samples. At the genus level, *Aquicella*, uncultured *Saccharibacteria* and *Flavobacterium* dominated the community in both control and treated antibiotic samples (Fig. 3b). Among the 20 different detected phyla, 45% of them increased their relative abundance in the antibiotic treated sample compared to the control, while the remaining 55% of phyla decreased their abundance. Variation in relative proportions between controls and samples was statistically significant (p-value <0.05) in 70% of the analyzed phyla

(Table S2). At the genus level, some bacteria, among the most prevalent, such as uncultured WCHB1-60 bacteria, *Flectobacillus*, and *Flavobacterium* -more abundant in the treated samples- showed significant differences between the control and antibiotic treated samples (p-value <0.02; Fig. 3b). Some strains of the former genus have been described as polymyxin B resistant (Bernardet and Bowman, 2006). Furthermore, the Shannon–Weaver diversity index and one-way ANOVA analysis confirmed that the control's diversity was significantly higher than the antibiotic treated samples (p-value<0.01) (Fig. 3b).

Metagenomics of wastewater prokaryotic and viral fractions

Since 16S rRNA gene Illumina tagging data showed no differences in bacterial diversity among replicates of each type (control and antibiotic-treated replicates; Fig. 3a), two of them were selected at random from the control (C1 and C2) and treated sample (Ab1 and Ab3) for shot-gun metagenomic sequencing (a total of ≈7.2 Gb, Table S3). Furthermore, all replicates (control and treated samples) from the 0.2 µm-filtered fractions free of cells containing viruses and vesicles were sequenced (a total of ≈1.6 Gb per replicate, Table S4). From each replicate of the 0.2 µm-filtered fractions free of cells, ≈15% of all assembled contigs were classified as viral contigs by employing VIRSorter and VirFinder programs (Fig. S6) (Roux *et al.*, 2015; Ren *et al.*, 2017). When more relaxed parameters were used in the detection of viral contigs, that value increased up to >35%. However, in this study, we used very strict and conservative thresholds (p-value of 0.01, Fig. S6). The origin of those other contigs not detected *in silico* as viral contigs could be free recalcitrant cellular DNA to DNase digestion and/or

DNA fragments putatively packaged in the detected putative vesicles and even unknown viruses not detected by the used programs. Control and antibiotic treated samples showed strong correlation when plotting VLP counts and DNA extracted (Fig. S7). In addition, low 16S rRNA gene contamination was found in all 0.2 µm-filtered fraction samples (≤0.0425‰; Table S5). The contigs for the 0.2 µm-filtered fraction mapped 26.86%-39.10% of total reads depending on the analyzed sample (Table S6). A metagenomic comparison of the unassembled and assembled data from the prokaryotic metagenomes confirmed these differences observed by 16S rRNA gene analyses since control and antibiotic-treated samples did not cluster together (40-60% of difference in genetic content between the control and treated samples, Fig. 3c and S8). Within control and treated samples from the 0.2 µm-filtered fractions, no differences in genetic content were obtained, which suggested a very similar viral composition (>96%) among replicates from control and treated-antibiotic samples (Fig. S8b).

The effluent wastewater resistome of prokaryotes, viruses and putative vesicles

To assess whether resistance to polymyxin is naturally present in the studied wastewater samples and how sub-inhibitory antibiotic concentrations might affect the overall repertoire and relative abundance of ARGs leading to a potential positive selection for ARGs, a metagenomic analysis of the resistome was performed for the prokaryotic and the 0.2 µm-filtered fractions. The characterization of the resistome was performed by employing two independent approaches: a machine learning algorithm that predict ARGs from the unassembled metagenomic data (Arango-Argoty *et al.*, 2018) and a complementary ARG search from the assembled and unassembled metagenomic data by

using a best hit approach with BLAST program against the ARG databases CARD (Jia *et al.*, 2017), ARG-ANNOT (Gupta *et al.*, 2014) and RESFAMS (Gibson *et al.*, 2015).

First, in the analyzed prokaryotic wastewater metagenomes, nearly all 30 ARG categories recently described by machine learning models (Arango-Argoty *et al.*, 2018) were found in the wastewater metagenomes (26-28 detected antibiotic categories, Supplementary File 1) indicating a vast diversity of major ARG categories both in the control and antibiotic treated sample. No significant differences in the proportion of the ARG categories were found in the prokaryotic resistomes of the control and antibiotic treated samples (Fig. 4a) and indeed, they clustered together in a PCA analysis (Fig. 4b). In the case of ARG sub-categories or groups, we found 412 out of a total of 2149 described groups (Arango-Argoty *et al.*, 2018). The most abundant ARG category in the control and antibiotic-treated samples (50%) were multidrug efflux pumps (e.g. *sav1866* gene) (Fig. 4a). Other abundant ARG categories were macrolide-lincosamide-streptogramin (MLS, ≈10%), tetracycline, beta-lactamic, mupirocine (e.g. *ileS* gene) and dyaminopyrimidine (Fig. 4a). The resistome composition detected in the 0.2 µm-filtered fractions was more heterogeneous among the replicates than that found for wastewater prokaryotes (Fig. 4b). The multidrug resistance category, as observed in prokaryotes, was the most abundant one. This category was also highly predominant in bacterial resistomes from different WWTPs (Ju *et al.*, 2018). In the 0.2 µm-filtered fraction, the dyaminopyrimidine category was also abundant, while it was undetectable in the bacterial resistomes (Fig. 4a).

Both independent ARG searching approaches used in this study showed that the prokaryotic resistome of controls was more enriched in ARGs (Figs. 5a, 5b). According to the machine learning method, a total of ≈4,000 ARG hits per Gb of metagenome were detected in the treated antibiotic samples (Supplementary File 1), while 2-fold more ARG hits were found in the control samples (Fig. 5a). Similar trend of ARG enrichment was obtained using BLAST search approach from the unassembled data (≈7,000 and ≈13,000 ARGs hits in antibiotic treated and controls, respectively; Fig. 5a) and assembled prokaryotic contigs (4.3 and 5.4 ARGs/Mb in antibiotic treated and control samples, respectively; Fig. 5b). However, these observed differences between control and antibiotic treated samples based on one-way ANOVA were not significant. Indeed, when the data was normalized per number of detected 16S rRNA gene hits, as recently recommended for resistome analyses (Arango-Argoty *et al.*, 2018), nearly identical ARG abundance was obtained in the control and antibiotic-treated prokaryotic fractions (≈10 ARGs/16S rRNA hit, Fig. S9). Therefore, regardless of the *in silico* method employed to detected ARGs and the normalization data approach, no ARG enrichment was found in the antibiotic-treated samples. The resistome data indicated that wastewater effluent microbes of the studied samples have a mean of ≈10-32 ARGs per genome, upon normalization method (number of 16S rRNA genes or assembled Mb; assuming a mean genome size of 4 Mb).

Regarding the 0.2 µm-filtered fractions comprised mainly by viruses and vesicles as well, the overall ARG abundance in unassembled metagenomes was significantly lower, with only 40-356 ARG hits per Gb of sequenced data

(Supplementary File 2), meaning up to 2 orders of magnitude below prokaryotes, and around ≈0.005 ARG per viral genome (assuming a mean viral genome size of 34 kb; data extracted from 9,700 viruses deposited in IMG-JGI database). Similarly to bacterial resistomes, differences in ARG abundance between the antibiotic-treated and control samples were not statistically significant since variance among replicates was very high (One-way ANOVA; Figs. 5c andd). Corroborating the low ARG abundance frequency in assembled contigs annotated as putative viruses by VIRSorter and VirFinder from the 0.2 μm-filtered fraction resistome, only 16 ARGs from the control and antibiotic-treated samples were detected (Table S7). From the viral contigs detected in the prokaryotic metagenomes corresponding to viruses actively replicating or prophages, 91 ARGs were found (1.40 hits/Mb sequenced in the antibiotic treated samples and 1.56 hits/Mb in control samples) out of 3,027 ARGs found in the prokaryotic metagenome. This supports the conclusion of ARG presence in wastewater effluent viral genomes being a rare phenomenon and not significantly affected nor enriched by sub-inhibitory presence of polymyxin.

In both the 0.2 μm-filtered fraction and prokaryotic wastewater metagenome, among the different ARG databases used (CARD (Jia *et al.*, 2017), RESFAMS (Gibson *et al.*, 2015) and ARG-ANNOT (Arango-Argoty *et al.*, 2018)) CARD was that that retrieved more hits (Jia *et al.*, 2017). The prokaryotic metagenome showed for instance 99 hits shared and detected by all three ARG databases (Fig. S10), while in the 0.2 μm-filtered fraction and the viruses found in the prokaryotic metagenome, ≤1 hit were detected and shared by using all three ARG databases.

Regarding specific polymyxin resistance genes (PRGs), in the prokaryotic assembled metagenomes, *arn*A, *pmrC* and *pmrE* genes were detected, although no difference (One-way ANOVA) was found between the control (0.06 PRGs/Mb) and antibiotic-treated (0.05 PRGs/Mb) samples (Table S8). From the unassembled data provided by learning machine models, PRGs (mainly *arnA*) represented only ≈0.018% of the prokaryotic resistome in both control and antibiotic-treated samples (Supplementary File 1). These genes are involved in the modification of lipopolysaccharides of outer membrane in gram-negative bacteria (Olaitan *et al.*, 2014). Intriguingly, although PRGs were extremely rare in the prokaryotic resistome, in the 0.2 µm-filtered fractions, PRGs (mainly *arnA* gene) were indeed detected at a higher frequency (0.5-0.8%) and only in the antibiotic-treated samples (Supplementary File 2). In the assembled data from this 0.2 µm-fraction, almost all detected ARGs (44 out of 60; mostly involved in diaminopyrimidine and multiple-drug resistance; Table S9) were not found in circular plasmids (according to VIRSorter) nor in *bona fide* viral annotated contigs, but in contigs that according to the gene annotation (e.g. lack of phage terminases, capsid proteins or other viral hallmark genes) and their presence also in cellular metagenomes, resembled to have a prokaryotic origin (Table S10). For instance, in several of these contigs, genes encoding common prokaryotic proteins such as *RadD, RepB*, DNA ligase, DNA polymerases, or *spoJ* involved in bacterial chromosome replication and segregation were detected (Table S10). Furthermore, in these putative non-viral contigs, we were able to detect PRGs (*arnA*, *pmrE* and *pmrF* genes, Table S11) when we applied a more relaxed bit-score threshold (<70) for the ARG search (see

Methods for details). Thus, nearly all detected ARGs were more abundant in non-viral contigs of the 0.2 μm-fraction, which precisely were more prevalent in the antibiotic-treated sample than in controls (Fig. S11). Since vesicles commonly package fragments of genomic DNA including ARGs (Bitto *et al.*, 2017), our data suggest the hypothesis of these above mentioned non-viral contigs containing ARGs and PRGs being packaged in the putative detected vesicles.

Finally, in the antibiotic treated samples from the bacterial fraction, we explored the enrichment of genes not annotated as ARGs and thus involved in other metabolic functions or with unknown function. Data showed a total of 65 different genes statistically enriched in the antibiotic-treated samples (Two-proportion Z-Test with pooled variance; Table S12). One of these genes was a glycosyl hidrolase, most likely involved in lipopolysaccharide modification in outer membrane and cell wall, which is frequently required for polymyxin's resistance. Although speculative, some of these genes may have potential to confer polymyxin resistance.

Discussion

In the effluent wastewater, commonly antibiotic concentrations are in the order of hundreds or dozens of ng/L (Ju *et al.*, 2018). Given that polymyxin is typically used as a last resort, concentrations in wastewater is among the lowest of all antibiotics and below ng/L (Ju *et al.*, 2018). Sub-inhibitory antibiotic concentrations or minimal selective concentration, which is the lowest concentration of antibiotic at which resistance is positively selected, are typically obtained from pure cultures (Murray *et al.*, 2018). When studying a complex uncultured microbial community, such as in this

study, these sub-inhibitory concentrations are provided for the bulk of the community and can be significantly different from pure culture data because of the cross-protection and rapid extracellular degradation by resistant populations of the community (Murray *et al.*, 2018). To the best of our knowledge, there are no environmental based studies estimating sub-inhibitory polymyxin concentrations for uncultured microbiomes. However, for reference bacterial isolates (Manning and Kuehn, 2011; Sato *et al.*, 2018), the criterion applied to estimate the sub-inhibitory polymyxin concentration was to use the maximum concentration of antibiotic that resulted in the lowest amount of killing, with ≥ 95% survival (Manning and Kuehn, 2011; Sato *et al.*, 2018). For instance, for *E. coli* and *Acinetobacter baumannii* (Manning and Kuehn, 2011; Sato *et al.*, 2018), authors used 0.15 and 2 µg/mL, respectively. In our study, we used the same criterion and methodology, and the concentration, among the different doses tested, which met the criterion of ≥95% survival was 30 µg/mL (Figs. S1 and S12). With this concentration, total cell abundances remained very similar after 48h, although it was noticed by flow cytometry that cell population patterns began to change (Figs. S1 and S12) indicating that microbes "sensed" the antibiotic. Indeed, it did trigger changes in the proportion of bacterial taxa, mainly related to gram-negative bacteria and an overall diversity loss (Figs. 3b and S8).

In many cases, antibiotics can induce an SOS response in microbes that promote the dissemination of antibiotic resistance and activation of mobile genetic elements, such as viruses (Beaber *et al.*, 2004). Here, we have not detected a general activation nor a viral release, but rather an effect of amelioration of viral replication. In contrast, in

all three treated antibiotic replicates of the 0.2 µm-filtered fractions free of cells, after 48 h of incubation, a significant enrichment and release of putative vesicles was detected by two independent methods: high-resolution fluorimetry and absolute putative vesicle-LP counting. The nanoparticle tracking analysis (NTA) has been proved to be an accurate method for estimating vesicle abundance in natural aquatic samples (Dragovic *et al.*, 2011; Biller *et al.*, 2017). The styryl dye FM 4-64 employed in our experiments to stain vesicles, is a lipophilic compound for general lipid staining of cell membranes, vacuoles, and vesicles. However, although not reported yet, this dye could stain lipid enveloped viruses as well. Distinguishing actual vesicle-LPs from lipid enveloped viruses, in practice, remains unfeasible and very complex with the NTA method. Nonetheless, as enveloped viruses typically infect eukaryotic cells, which, compared to bacteria, are supposed to be a minor component in wastewater microbial communities, we assume that its potential contribution to the nanosight vesicle count data is not significant. As shown in Figs. 2 and S2, an increase in the number of total and dead cells was not found in the antibiotic treated samples after 48 h of polymyxin treatment. Thus, it is very unlikely that the fluorescence signal from lipids came from cell debris after lysis which passed through the 0.2 µm-filter. In contrast, TEM and nanotracking particle-based methods along with a high-resolution fluorometry approach demonstrated the presence of vesicle-LPs and suggested a specific vesiculation response of the wastewater effluent microbiome, with an apparent change in the vesicle distribution size (Fig. S4). The differences between the estimated vesicle size by nanosight and TEM images are due to the different fractions used to obtain them. While TEM images were

recovered from a sub-fraction of the Optiprep density gradient from the 0.2 µm-filter fraction (Pérez-Cruz *et al.*, 2013), the Nanosight data was obtained for the whole 0.2 µm-filter fraction without any previous sample partition or purification, being the latter more accurate and complete to estimate the size of the total pool of vesicles in samples. It is worth noting that with Optiprep gradient, each purified fraction usually might contain different types and density of particles, including vesicles (Pérez-Cruz *et al.*, 2013).

Recently, vesicles have been acknowledged as essential factors in the interaction between cells, with being involved in host-virus interactions, quorum sensing, adaptation to lack of nutrients and horizontal gene transference, including ARGs (Schatz and Vardi, 2018). The use of vesicles released by bacteria as a defense against antibiotics, such as polymyxin B, has been observed only in *E. coli* and *Vibrio tasmaniensis* culture isolates, but has never been studied in the context of uncultured natural wastewater microbial communities. In the case of *E. coli*, in which authors used sub-inhibitory polymyxin concentrations, externally added isolated vesicles incremented the survival of the bacteria population against polymyxin B, proving vesicles to reduce the effective antibiotic concentration (Manning and Kuehn, 2011). In *V. tasmaniensis*, vesicles increased the minimal inhibitory concentration of polymyxin B by 16x (Vanhove *et al.*, 2015). Thus, as demonstrated with these reference isolates, it is reasonable to think that the effluent wastewater microbes use vesiculation as a general defense mechanism against polymyxin, and vesicles could behave as `traps´ to effectively decrease the antibiotic concentration in the water. Nevertheless, we cannot

rule out the option that the increase of vesicles could be produced by the dose of polymyxin B employed that could have affected the membranes.

Even though DNA packaging and dissemination of ARG through vesicles has been proved in pure cultures (Pérez-Cruz *et al.*, 2015; Bitto *et al.*, 2017), here in our samples, we could not confirm that the ARGs and PRGs detected in the 0.2 µm-filtered fraction -containing viruses and vesicles- were actually packaged in vesicle particles. Although the presence of polymyxin ARG category was rare in the prokaryotic resistomes, using a machine learning approach, it was only detected in the antibiotic treated samples of the 0.2 µm-filtered fraction (Supplementary File 2). Given the very low frequency of polymyxin ARG in our bacterial resistome and the low number of total ARGs detected in the 0.2 µm-filtered fraction, it is surprising that some of these ARGs were precisely for polymyxin. Therefore, we speculate that some of these ARGs could be packaged in the detected vesicles, although we cannot rule out that they might come also from free cellular DNA recalcitrant to DNase digestion, GTAs, or/and unknown viruses not detected by VirFinder or VIRSorter. An obvious methodological approach to answer this question would be to physically separate vesicles from viruses. In this study, although we comprehensively applied and tried different existing methodologies to purify vesicles (Cantin *et al.*, 2008; Biller *et al.*, 2014) from the 0.2 µm-filtered fraction, such as ultracentrifugation in density gradients (either in Optiprep - Sigma-Aldrich, D1556-250ML as recommended (Chutkan *et al.*, 2013)- or in cesium-chloride(Pietila *et al.*, 2010) or antibody-lipid purification (data not shown) using a novel methodology (Nakai *et al.*, 2016), we and another independent group at the

University of Helsinki failed to do so, and in all cases, viruses co-purified as well. These vesicle purification techniques have been successfully applied in simple models, such as bacterial cultures in presence of one or a few phage and vesicle types, or reasonably well in marine samples (Biller *et al.*, 2014), which is far less complex than wastewater samples. But even in these marine samples, authors noticed viral contamination after vesicle purification. The enormous complexity and diversity of wastewater viruses and vesicles with overlapping density rates makes it in practice, so far, very unfeasible to separate them. Nevertheless, according to our data, vesicles should be more thoroughly considered in further studies since they could play an important but underappreciated role in ARG dissemination.

Regarding the resistome of the effluent´s viral fraction, which has been overlooked by recent metagenomic studies (Forsberg *et al.*, 2012; Yang *et al.*, 2013; Zhu *et al.*, 2013; Li *et al.*, 2015; Pehrsson *et al.*, 2016; Ju *et al.*, 2018), our data indicated that in contrast to bacteria, effluent wastewater viruses rarely carry ARGs. Remarkably, when comparing our data to those from untreated sewage viromes from different cities and hospitals (Cantalupo *et al.*, 2011; Subirats *et al.*, 2016; Lekunberri *et al.*, 2017), ARG abundance was significantly higher in raw sewage, indicating that wastewater treatment is effective in reducing numbers of viruses carrying ARGs. At the JGI-IMG database, there is only one viral wastewater metagenome (IMG-ID 121188; from sludge, as date of 11/6/2018) and had an enrichment of ARGs per Mb of metagenome of 0.5; thus in the same order as our obtained values for the viral metagenomes (0.29-0.30). Thereby, our findings regarding the low ARG prevalence in

viruses are consistent with data obtained for the analyses of 1,181 phage isolate genomes (Enault *et al.*, 2017). The amount of ARG per bacterial genome estimated in our samples (10-32 ARG/genome) is lower than the average calculated (≈51 ARG/genome) for 26 strains of *E. coli* isolated from wastewater from different geographic points (Zhi *et al.*, 2019).

In our experiments of polymyxin amendments at sub-inhibitory concentrations, it was not observed a positive ARG selection neither in prokaryotes nor viruses when employing two independent metagenomic ARG search approaches based on machine learning and best hit score from unassembled and assembled resistomes (Forsberg *et al.*, 2012; Yang *et al.*, 2013; Zhu *et al.*, 2013; Li *et al.*, 2015; Pehrsson *et al.*, 2016; Ju *et al.*, 2018). Recently, a metagenomic study indicated that precisely polymyxin resistance, compared to other antibiotics, does not seem to be ‘‘highly mobilized’’ among different WWTP compartments with low potential for horizontal dissemination (Ju *et al.*, 2018). Our study indicates that after spiking the microbiome with polymyxin sub-inhibitory doses well above the natural concentration in effluent wastewater, ARGs and PRGs were not apparently selected in the microbiome, which indicates that the chance of dissemination from WWTPs to other ecosystems, in natural original conditions, is very low or insignificant. Indeed, to get deeper into that potential polymyxin resistance dispersion into the ecosystem, we also collected seawater from a sampling site that continuously receives the discharging treated wastewater of the studied WWTP (Fig. S13 top panel). If marine microbes from that location have acquired polymyxin resistances from effluent wastewater microbiomes, the amount of sub-inhibitory

polymyxin concentrations that allowed >95% of cell survival should be somehow very similar to that obtained for the effluent wastewater (30 µg/mL). However, only 0.1 µg/mL of polymyxin was enough (Fig. S13 bottom panel), indicating that the studied effluent wastewater microbes are naturally more resistant (≈300-fold) to polymyxin than those marine microbes. This phenomenon could be explained by a low probability of them being horizontally transferred or the little selective pressure to maintain those genes that could exist in seawater.

Conclusions

This study performed with natural samples from the effluent of a representative WWTP shows that, in addition to bacteria, viruses carry ARGs, albeit at a very low frequency. Since viruses are host-specific, data suggest that effluent viruses are unlikely to spread out those ARGs into nature. Furthermore, resistome data indicate that the presence of polymyxin antibiotic, critical for treating fastidious infections, does not seem to trigger the selection of ARGs and PRGs in effluent wastewater prokaryotes and viruses. In contrast, data demonstrate that wastewater microbes released vesicles when polymyxin was added, as it has been described only for clinical isolates. Given that, as previously reported, vesicles may be a general mechanism to exchange genetic cargo between bacterial species and that transfer gene rate did not correlate with the relatedness of the donor and recipient species (Tran and Boedicker, 2017), our data open the question whether vesicles might have a more active role on the ARG spreading in effluent wastewater microbiomes as well as in other natural environments.

Experimental procedures

Sample collection, antibiotic treatment and experimental design

A water sample (7 L) was collected from the effluent of the WWTP "Edar Alicanti Norte" (11/05/16; 38°25'35.8"N 0°25'03.5"W, Alicante, Spain; Fig. 1a) and immediately transported to the laboratory at ambient temperature within the next 15 min. The collected sample was divided into two groups: controls (3 replicates of 1 L each; named C1, C2 and C3) and polymyxin B treated samples (3 replicates of 1 L each; named Ab1, Ab2, and Ab3). Polymyxin B powder stock (Sigma, polymyxin B sulfate salt powder, St. Louis, MO, USA, Ref. 4932-1MU) was suspended in mQ water (150 mg/mL). Initially, different polymyxin concentration doses were tested and added to the samples (0.2-100 µg/mL final polymyxin concentration in samples) to assess and select that sub-inhibitory concentration that, as previously reported (Manning and Kuehn, 2011), allowed the survival of the 95% of the cell population of the microbiome after 48 h of incubation. Finally, a single antibiotic dose was added (0.2 mL of antibiotic stock solution) to each 1 L replicate sample (30 µg/mL final polymyxin concentration; 95% of survival). Controls and antibiotic treated samples were incubated for 48 h at 20ºC (same temperature as the water collected at the moment of sampling, Fig. 1b) and then processed for bacterial, viral and vesicle abundances through electron microscopy, 16S rRNA sequencing and metagenomic sequencing. In addition, 1 L of the natural wastewater without treatment or incubation (called "nw") was immediately processed after collection as explained above.

To test the potential polymyxin resistance dispersion, we spiked with different amounts of polymyxin B a marine sample that continuously receives the treated wastewater of

the studied WWTPs. Using cytometry, we measured which concentration allowed >95% of population survival and we compared it with the one found at the studied WWTP.

Sample processing, DNA extraction and sequencing of prokaryotic and viral fractions

Samples were processed for prokaryotic and viral DNA extractions after 48 h of incubation as follows. For prokaryotes, a total of 10 mL water sample from each replicate after 48 h of incubation was filtered through a 0.2 µm-filter (Isopore Membrane Filters, Cork, Ireland, Ref. GTTP02500) and DNA from this filter was extracted with MasterPure Complete DNA and RNA Purification Kit according to the manufacturer's protocol (Epicentre, Madison, WI, USA, Ref. MC85200). For purifying the viral fraction, from each replicate, 980 mL of the water sample were filtered through 0.22 µm-Polycap 150TC filters (Whatman, Amersham, UK, Ref. 6717-9502) in order to remove the cells. The filtered samples were ultraconcentrated to a final volume of 19 mL using tangential flow filtration with a polyethersulfon membrane (Sartorius, 100 kDa cut-off, Stonehouse, UK, Ref. VF20P4). The concentrated water samples were filtered again using 0.22 µm PES syringe filters (Millipore, Cork, Ireland, Ref. SLGP033RS) to remove any remaining cell, and the sample was inspected under the microscope with DAPI staining to ensure that no cells were in the concentrated fraction. This concentrated fraction, which contained viruses and vesicles, is hereinafter called "0.2 µm-filtered fraction". Prior to the DNA extraction, potentially free DNA present in this fraction was digested with 5 µL of Turbo DNase I 2U/µL (Invitrogen, Graciurno,

Lithuania, Ref. AM107) per 1 mL for 1 h at 37ºC, followed by incubation with proteinase K (Epicentre, Madison, WI, USA, Ref. MPRK092) for 1 h at 65ºC (0.5 mg/mL final concentration). DNA extraction from that fraction was performed using QIAamp Ultrasense Virus Kit (QIAgen, Hilden, Germany, Ref. 53704) according to the manufacturer's instructions. DNA was then stored at -80ºC until use.

Metagenomes from prokaryotic and 0.2 μm-filtered fractions from replicates were shot-gun Illumina sequenced by employing the Nextera XT DNA library Prep (Illumina, San Diego, CA, USA, Ref. FC-131-1024) in a MiSeq sequencer (2 × 300, pair-end) according to the manufacturer's protocol. Illumina sequencing was carried out in FISABIO Genomics Centre (Valencia, Spain).

Viral and prokaryote abundance

From each replicate, 4 mL of water was taken at different times (0, 24 and 48 h) in order to analyse its abundance of prokaryotes and viruses by flow cytometry. Prokaryote and viral abundances were monitored by using the reference flow cytometry protocols (Gasol and Del Giorgio, 2000; Brussaard *et al.*, 2010) except that SYBR Gold dye was used. Stained samples for prokaryotes and viruses were measured with a FACS Canto II cytometer (BD Biosciences). Triplicate measurements were performed for each replicate. The cytometer settings were as follows: the threshold was set in green fluorescence (200 units), FITC voltage=525, SSC voltage=640, FSC voltage=250, and the flow rate was established as low. The polymyxin B effect on microorganism's death after 48h of incubation was compared for control and treated samples using

LIVE/DEAD® BacLight Bacterial Viability Kit L7012 (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions.

Vesicle abundance and lipid-bulk fluorescence measurements

NanoSight NS300 (NanoSight Ltd., UK) was used to measure the abundance and size of vesicles present in the concentrated 0.2 μm-filtered fraction samples. Samples were divided in two aliquots (0.5 mL each) and frozen at -80ºC. Each aliquot was stained with 2 μL of FM *4*-64 dye (final concentration 0.5 μg/mL; Molecular Probes Life Technologies, Eugene, OR, USA, Ref. T13320) and incubated at room temperature in dark conditions for 10 minutes. The styryl dye FM *4*-64 has been reported to selectively stain membranes with red fluorescence. Previous to Nanosight, we demonstrated in a doble stained sample (DAPI-FM4-64) by confocal microcopy and epifluorescence microscopy that FM *4*-64 dye effectively stained membranes of microbes present in the collected samples (Fig. S3). The Nanosight instrument was equipped with a 488 nm laser, and a sCMOS camera. The data was analysed with NTA 3.1 software (Build 3.1.45), with detection threshold established at 8. Blur Size and Max Jump Distance were set to auto and camera levels to manual. The analyses were performed at room temperature (between 23.6 and 27.3 °C). Triplicate measurements were taken for each sample. In addition, lipid content of the fraction containing vesicles and viruses was measured with high resolution fluorescence in a CLARIOStar fluorimeter (BMG). These fractions were stained with the red fluorescent dye FM *4*-64 dye. Conditions of FM *4*-64 bulk staining were identical as above for Nanosight. Triplicate measurements

were taken for each sample. The vesicle-LP measures were normalized calculating the concentration of vesicles per mL in concentrated samples and then multiplying it by the dilution factor.

Transmission electron microscopy

Vesicles and viruses from the 0.2 μm-filtered fraction were purified by ultracentrifugation in Optiprep (Sigma, St. Louis, MO, USA, Ref, D.1556) density gradients following a previously reported methodology (Pérez-Cruz *et al.*, 2013). The different density Optiprep gradients were then visualized using a transmission electron microscope JEM-2010 (JEOL, Japan) operating at 120 kV. A total of 5 μL of sample was settled on formvar-coated carbon grids (Electron Microscopy Sciences, Hatfield, PA, USA, Ref. FCF300-CU) and stained with 1 μL of uranyl acetate (concentration 2%; Electron Microscopy Sciences, Ref. 22400) for 1 minute. The excess dye was removed with filter paper soaked in mQ water (0.02μm-filtered), and then grids were air-dried. For comparison, vesicles from *Shewanella vesiculosa* were also processed in parallel as described (Pérez-Cruz *et al.*, 2013). As discussed, purification and separation of vesicles from viruses was not achieved.

16S rRNA gene amplicon sequencing and analyses

Microbial diversity from samples was conducted by 16S rRNA gene PCR amplicon sequencing using the primer pair Illumina Pro341F-805R (Pro_Illumina_341F: 5´-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNBGCASCAG-3´;

Pro_Illumina_805R:5´GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACT

ACNVGGGTATCTAATC-3´), with the following PCR conditions: Initial denaturation at 94 °C for 3 minutes, followed by 25 cycles of 94 °C for 45 seconds, 51 °C for 1 minute, and 72 °C for 2 minutes, with a final extension for 10 minutes at 72 °C .The purification and indexation was performed as detailed in the Illumina´s MiSeq 16S Sequencing Library Protocol .The product was sequenced using Miseq sequencer with pair-end reads (2 x 300). Next, the obtained 16S rRNA gene reads were processed with Qiime as described (Caporaso *et al.*, 2010; Kuczynski *et al.*, 2011). Vegan package (Oksanen *et al.*, 2018) was used to calculate the Shannon–Weaver Index (α-diversity) and the β diversity (betadisper function); both were individually compared with one-way ANOVA performed with the statistical program R (version 3.3.0) (R Core Team, 2014). In addition, ANOVA analyses were performed separately for each phylum and species.

Metagenomics and antibiotic resistance gene (ARG) analyses

Illumina reads from prokaryotic and 0.2 µm-filtered fraction metagenomes were quality filtered by Trimmomatic 0.36 (Bolger *et al.*, 2014) with the following parameters: ILLUMINACLIP:NexteraPE- -PE.fa:2:30:10 (sliding window 4:15, Phred 33 and MINLEN as 36) (Magoc and Salzberg, 2011; Schmieder and Edwards, 2011; R Core Team, 2014). 16S reads contamination in reads from 0.2 µm-filtered fractions was detected using RNAmmer (Lagesen *et al.*, 2007). 16S RNA reads returned by the program were checked against NR database (09/05/19, NCBI). Metagenome assembly was carried out with METASpades version 3.10 applying the following option "-k 33,55,77,99,127". Contigs below 500 pb were removed from the analysis and gene

annotation was performed at the JGI-IMG platform (Markowitz *et al.*, 2012) for the prokaryotic and viral metagenomes. Metagenome comparison of trimmed reads from control and antibiotic treated metagenomes was done with MetaFast (Default settings; Ulyantsev *et al.*, 2016). To analyze the amount of reads that were kept for the analysis, both paired and unpaired trimmed reads were aligned with the contigs in each sample using blast. Only the reads with 97% identity and query coverage of 95% were considered.

To distinguish those *bona fide* viral DNA assembled fragments (hereinafter "viral contigs") from non-viral contigs in the sequenced 0.2 µm-filtered fraction, such as putative DNA fragments packaged in vesicles, the programs VIRsorter (version 1.0.3)(Roux *et al.*, 2015) which identifies viral hallmark genes and circular sequences, and VirFinder (Ren *et al.*, 2017) , which detect viruses based on their k-mer signatures, were used. Both non-viral contigs and viral contigs were used in further analyses for ARG detection. The open reading frames (ORFs) from these contigs were predicted with Prodigal version 2.6.3 (Hyatt *et al.*, 2010). Then, predicted proteins from contigs were compared with the four different antibiotic resistance protein reference databases: CARD (Jia *et al.*, 2017), ARG-ANNOT (Gupta *et al.*, 2014) and RESFAMS (Resfams AR Proteins, Gibson *et al.*, 2015), by using BLASTp. The taxonomic affiliation of non-viral contigs was performed using BLASTp 2.2.31+ against the NR database (28/10/2015, NCBI). For both analyses the e-value threshold was set to 1e-5, the sequence subjects with greater bit-score were identified using Best hit from Enveomics bioinformatics package (Rodriguez-R and Konstantinidis, 2016) and only those proteins

with an identity ≥50% and a bit-score greater than or equal to 70 were finally considered. Previously, a bit-score above 70 has been proven to be a conservative parameter to avoid false positives (Enault *et al.*, 2017). However, here, more strict parameters, including the identity cut-off have been also considered. In order to compare ARG abundances between different samples, number of ARGs was normalized against metagenome size. BLASTx (version 2.5.0+) analysis was performed to confirm the ARG results obtained, comparing the unassembled data with the different ARG databases (e-value 1e-5; bit-score 70). The comparison between ARG and PRG genes normalized by metagenome size between control and treated samples for all the fractions were performed with one-way ANOVA, as indicated above. Unassembled metagenomic data was also analyzed for ARG presence following the recently described machine learning algorithm developed for metagenomic data (Arango-Argoty *et al.*, 2018). For this, cleaned filtered metagenomes were analyzed and submitted to the comprehensive DeepARG platform that contains 14,933 reference ARG sequences. Detection of ARGs in the analyzed metagenomes was done by applying the default parameters (e-value 1e-10, identity ≥50%, minimum probability 0.8 and gene coverage (Arango-Argoty *et al.*, 2018).

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Conflict of interest

The authors declare that they have no conflict of interests.

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Availability of data and materials

The metagenomic data is available under the NCBI BioProject PRJNA505772. The 16S rRNA amplicon sequences are available under the NCBI Bioproject number PRJNA505907. The annotated metagenomes are available at the IMG-JGI database. For prokaryotes: IMG-IDs 3300027927 (C1), 3300027913 (C2), 3300027920 (Ab1), 3300027923(Ab3). For viral metagenomes, IMGs: 3300010940 (C0), 3300010946 (C1), 3300010945 (C2), 3300010942 (C3), 3300010944 (Ab1), 3300010943 (Ab2), 33000109401 (Ab3).

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Figure legends

Figure 1. Sampling and experimental design. Location of the studied WWTP in Alicante city (Spain). This municipal plant treats 5,933 m^3/day from a population of

≈72,000 people and it has a tertiary treatment based on coagulation /flocculation and UV light. The sampling point of the WWTP effluent is indicated in the middle and right panels with a black arrow **(a)**. Experimental design **(b)**. Triplicate samples (1L each) from the control (named as C1, C2, and C3) and polymyxin-antibiotic treated (30 µg/mL; named as Ab1, Ab2, and Ab3) water were incubated for 48 h and monitored for resistome analyses and microbiome and virome changes. Natural effluent wastewater (named as nw) was also analysed.

Figure 2. Microbiome, virome and vesicle abundances in the effluent wastewater samples and antibiotic-treated samples. Abundance of virus-like particles (Virus-LP) and bacteria were monitored for 48 h with flow cytometry **(a)**. Abundance in time 0 h corresponds to that of the natural effluent wastewater. Vesicle abundance measured by nanoparticle tracking analyses in the 0.2 µm-filtered effluent wastewater fraction. Vesicle-like particles were stained with a lipidic dye FM4-64. Standard deviation is shown in graphic Triplicate measurements were taken from each replicate. **(b)**. Transmission electron microscopy of a sub-fraction from the 0.2 µm-filtered effluent wastewater fraction of the antibiotic treated samples. **(c)**. Vesicles from *Shewanella vesiculosa* for cross comparison in shape and size in left panel. Middle and right panels are from vesicles detected in the antibiotic-treated sample.

Figure 3. 16S rRNA gene Illumina sequencing data and metagenomics of effluent wastewater microbiome. Microbiome composition and structure of natural wastewater effluent, control and antibiotic-treated samples depicted in a principal component analyses showing the differences at the phylum level **(a)** and changes in proportion of most abundant genera including the Shannon diversity Index that was significantly higher in the control compared with the antibiotic treated samples **(b)**. Metagenomic comparison depicting the overall distance of genetic content performed by Metafast program with unassembled data **(c)**. A distance of 1 means two metagenomes completely different.

Figure 4. Resistome analyses of the microbiome and the 0.2 µm-filtered fraction comprised by viruses and vesicles. Detection of ARGs was performed by a machine learning algorithm from unassembled metagenomic data. Frequency of ARG categories found in each one of the resistomes. MLS stands for Macrolide-Lincosamide-Streptogramin **(a)**. Often, after assembly, a large fraction of reads remain unassembled. Resistome analyses of unassembled quality filtered reads likely provided a more complete picture of ARGs in resistomes. Assembled metagenomes were also screened for ARGs (Figure 5) and compared with machine learning approach. Principal component analyses of the relative proportion of genes assigned to each ARG categories from the studied resistomes **(b)**.

Figure 5. Frequency and enrichment of ARGs in microbiomes and the 0.2 µm-filtered fraction comprised by viruses and vesicles. ARG abundance was performed by independent approaches: Unassembled resistomes from prokaryotes **(a)** and 0.2 µm-

filtered fraction **(c)** were scrutinized for ARG with learning machine approach and best BLAST hit against the antibiotic resistance gene databases CARD, ARG-ANNOT and RESFAMS. For assembled data, the best BLAST hit was used for both prokaryotes **(b)** and 0.2 μm-filtered fractions **(d)**. Open reading frames (amino acids) from assembled contigs were compared by employing the thresholds of e-value 1e-5, score>70 and and identity of 50%; more conserved and strict than previous publications on ARG detection. Standard error is shown in bar chart. The color legend applies for all the panels (blue indicates control samples and red, antibiotic treated samples).

a

b