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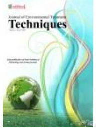

Effects of Different Wastewater Treatment Processes on Occurrence and Prevalence of Antibiotic Resistant Bacteria and Their Resistance Genes

Mahdi Asadi-Ghalhari[1], Rahim Aali[2]*, Mohammad Aghanejad[3], Reza Fouladi Fard[4], Hassan Izanloo[5], Ali Shahryari[6], Hamed Mirhossaini[7], Mohsen Mehdipour Rabori[8], Reza Ghanbari[9]

[1]Research Center for Environmental pollutants, Department of Environmental Health Engineering, Faculty of Health, Qom University of Medical Sciences, Qom, Iran
[2]Research Center for Environmental pollutants, Department of Environmental Health Engineering, Faculty of Health, Qom University of Medical Sciences, Qom, Iran
[3]Department of Environmental Health Engineering, Khoy University of Medical Sciences, Khoy, Iran. Email: mamamaxy01@yahoo.com
[4]Research Center for Environmental pollutants, Department of Environmental Health Engineering, Faculty of Health, Qom University of Medical Sciences, Qom, Iran
[5]Research Center for Environmental pollutants, Department of Environmental Health Engineering, Faculty of Health, Qom University of Medical Sciences, Qom, Iran
[6]Department of Environmental Health Engineering, Gorgan University of Medical Sciences, Gorgan, Iran
[7]Department of Environmental Health Engineering, Arak University of Medical Sciences, Arak, Iran
[8]Department of Environmental Health Engineering, Kerman University of Medical Sciences. Kerman, Iran
[9]Department of Environmental Health Engineering, Qazvin University of Medical Sciences, Qazvin, Iran

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Abstract

This study aimed to explore the difference between hospital and municipal wastewater treatment processes regarding the reduction of antibiotic-resistant bacteria (ARB) and antibiotic resistant genes (ARGs). Samples were collected from raw and final effluent of four different wastewater treatment plants (WWTPs). ARB were evaluated by modified HPC method. Extraction and purification of DNA from the samples were conducted by Freeze-Thaw and DNA extraction kit. Real-time PCR (qPCR) was utilized to obtain the quantity of *Sul*1 and *Erm*B genes in the samples. For standard control in qPCR, was used plasmid containing each gene sequence. The average ARB concentration in the raw wastewater and effluent was 1.03×10^{7}-6.63×10^{7} CFU/100mL. Quantitative range of the *Sul*1 and *Erm*B genes were obtained as 0-8.3×10^{10} Copies/100 mL and 9.29×10^{5}- 9.64×10^{9} Copies/100 mL, respectively. The results show that urban wastewaters play a more significant role than hospital wastewaters in the emission of sulfonamides and erythromycin-resistant bacteria and genes to the environment. Findings revealed that conventional wastewater treatment plants cannot be regarded as reliable barriers for the control of these agents.

Keywords: Antibiotic-resistant bacteria, ARGs,Hospital wastewater,Urban wastewater, Real-time PCR, *Sul*1, *Erm*B

1 Introduction

Increasing concerns have been reported about the negative impacts of antibiotic residuals on the environment (1-3). The major representation of this problem is the development of antibiotic resistance (4-6). Antibiotic resistance has been reported all around the world. The WHO has mentioned antibiotic resistance as one of the three major problems of the 21st century (4, 7). This resistance has been observed across a wide variety of environments such as water, soil, air, and wastewater (8, 9). Antibiotic resistance can be developed by different ways, including the direct entrance of resistant bacteria from therapeutic settings or the antibiotic residual pressure in environmental resources (10). The developed resistance can cause changes in the natural ecosystems. Urban and hospital wastewaters are the most important sources that release these contaminants to the environment (4). Wastewater treatment plants are one of the most important and recent obstacles in the emission of resistant bacteria and genetic elements to the environment (11). Researchers have not reached a consensus regarding the effects of WWTPs yet. Some have reported the reducing effect of these plants. Others, however, have mentioned the increasing effects of treatment plants on the emission of agents that can develop antibiotic resistance. A number of studies have regarded the effect of urban WWTPs, and other researchers believe the hospital WWTPs to be more efficient. Research shows that the destiny of various antibiotic resistance factors in the environment is contingent upon different factors, including the type of treatment processes, procedure of operation, wastewater

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Corresponding author: Rahim Aali, Research Center for Environmental pollutants, Department of Environmental Health Engineering, Faculty of Health, Qom University of Medical Sciences, Qom, Iran. E mail: r.aali@qum.ac.ir.

organic load, the variety of wastewater microorganisms, the amount of discharged antibiotics, conditions of hospitals, consumption patterns of antibiotics and the economic and cultural situation of a society. In Iran, the consumption of antibiotics has not become standardized in therapeutic settings and the society. Iran is among countries with the highest antibiotic consumption. Antibiotic resistance has been reported in almost all clinical, agricultural, and animal husbandry studies and against all antibiotic groups (12-16). Limited research has been conducted on antibiotic resistance in the Iranian environment (17). In this research, two antibiotic groups, i.e. sulfonamides (STX) and erythromycins (ER), were investigated because of their wide application in therapeutic, agricultural, and animal husbandry environments, as well as their informal use by the people. The common coding genes (*Sul*1, *Erm*B) in relation to these two antibiotic groups were also studied quantitatively.

2 Materials and Methods

1.2 Sampling and enumeration of antibiotic resistant bacteria

Samples were collected from wastewater treatment plants in Isfahan province, Iran. Four sampling sites were selected to study antibiotic-resistant bacteria (ARB) and antibiotic resistant genes (ARGs). The first (MW_1), second (MW_2), and third (MW_3) sampling sites were the influent and effluent of municipal WWTPs with different processes, conventional activated sludge (two-step), conventional activated sludge, and stabilization pond, respectively. The capacity of these WWTPs were 25×10^4, 13×10^4 and 9×10^4 m^3d^{-1}, respectively. The disinfection process of all WWTPs were chlorination. The fourth sampling site (HW_1) was the influent and effluent of extended aeration supported with high-speed sand filter. Its capacity was 890 m^3d^{-1}, and its disinfection process was chlorination. To determine ARB concentration, samples were diluted and 0.1 mL of each dilution was spread on R_2A (Difco), amended individually with erythromycin (15µg mL^{-1}), sulfamethoxazole (50 µg mL^{-1}), and additional antifungal nystatin (18, 19). Plates were incubated for 48h at 37˚C. ARB results were derived by comparing heterotrophic and ARB cultivable concentrations (20, 21). All assays were performed in duplicate. Positive samples were rechecked.

2.2 DNA extraction and qualified PCR

DNA was extracted from original samples. Fifty mL of the original samples was prepared (centrifuged at 6000 rpm for 15 min) and the pellet was resuspended in 300 µL of distilled water. The pellets were frozen in liquid nitrogen and boiling water for three times (22). The DNA was extracted and purified by DNA extraction kit (promega wizard genomic DNA purification kit, Madison, WI) according to the manufacturer's manual. Primer pairs were used to amplify *sul*1 and *erm*B genes, as taken from Munir et al (19). The total volume of the reaction mixture (25µL) contained 0.5 µL of each primer, 1.5 µL $MgCl_2$, 0.5 mM dNTP, 2.5 µL PCR buffer, 1 µL of template DNA and 5 units of Taq DNA polymerase (22). All PCR assays contained a positive and a negative control. PCR amplification was performed using a thermal cycler (Corbett, Australia). The PCR profile included initial denaturation at 94˚C for 10 min, denaturation at 94 ˚C for 45 s, annealing (varied) for 30 s, and extension at 72˚C for 45s for 30 cycles, followed by a final extension at 72˚C for 10 min. PCR products (6 µL) were mixed with 2 µL of DNA safe stain and loaded on 1.5% agarose gel. Gels were viewed on a UV trans illuminator, and DNA fragment sizes were compared with the 100-bp ladder (14).

2.3 Quantitate PCR

Plasmid DNA was used as the standard control in real-time PCR (qPCR). Fresh PCR products of *erm*B and *sul*1 were separated and excised from the agarose gel. The gel fragments were purified with a gel extraction kit (Gel purification kit, Cat NO: K-3035-1, Bioneer, USA) and ligated into the PTZ 57R vector. DNA was transformed into Escherichia coli Top 10 using $CaCl_2$ and heat-shock. Clones containing the correct insert were confirmed by PCR amplification with m13 universal primer [F-m_{13} (5-ttgtaaaacgacggccagt-3) and R-m_{13} (5-acaggaaacagctatgaccatg-3)] and sequencing (14). Plasmids were purified and plasmid concentration was then determined by spectroscopy (Nano drop® ND1000). The copy number of each plasmid was calculated using the molecular weight of nucleic acid and length (in base pairs) of the cloned plasmid. To generate the standard curve, the threshold cycle (Ct) value was used for each concentration. The qPCR was used to quantify the concentrations of *erm*B and *sul*1 genes. Prior work had shown that *erm*B and *sul*1 genes were present in substantial concentrations in wastewater. The qPCR was carried out on the applied bio systems (Applied Bio systems, USA) using the SYBER green method. All qPCR reactions were performed in 20 µL of reaction mixture (23). T-tests were run to compare quantity averages in influents and effluents. McNamara statistical test was used to compare the examination results from just before and after the WWTPs. ANOVA was employed to compare the variation results from different sites. Finally, Pearson's correlation coefficients were used to determine the correlations between HPC (heterotrophic plate count), incidence of ARB, and ARG genes (SPSS 16 for Windows, SPSS Inc., Chicago, IL).

3 3 Results and Discussion

3.1 ARB and ARGs in wastewater sources

The average concentrations of ARB and ARGs in influent and effluent were obtained to be 5.39×10^6-4.22×10^7 and 1.38×10^9-9.29×10^9 Copies/100 mL, respectively (Fig 1, 2 and 3). Results in all of figures were shown by standard deviation.

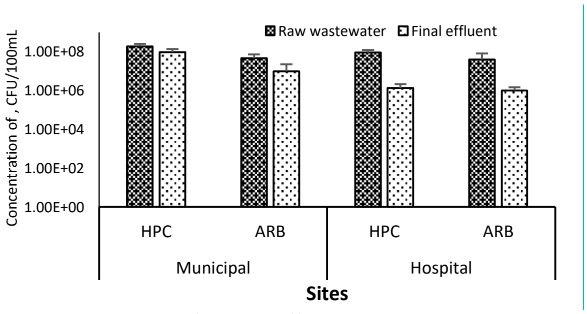
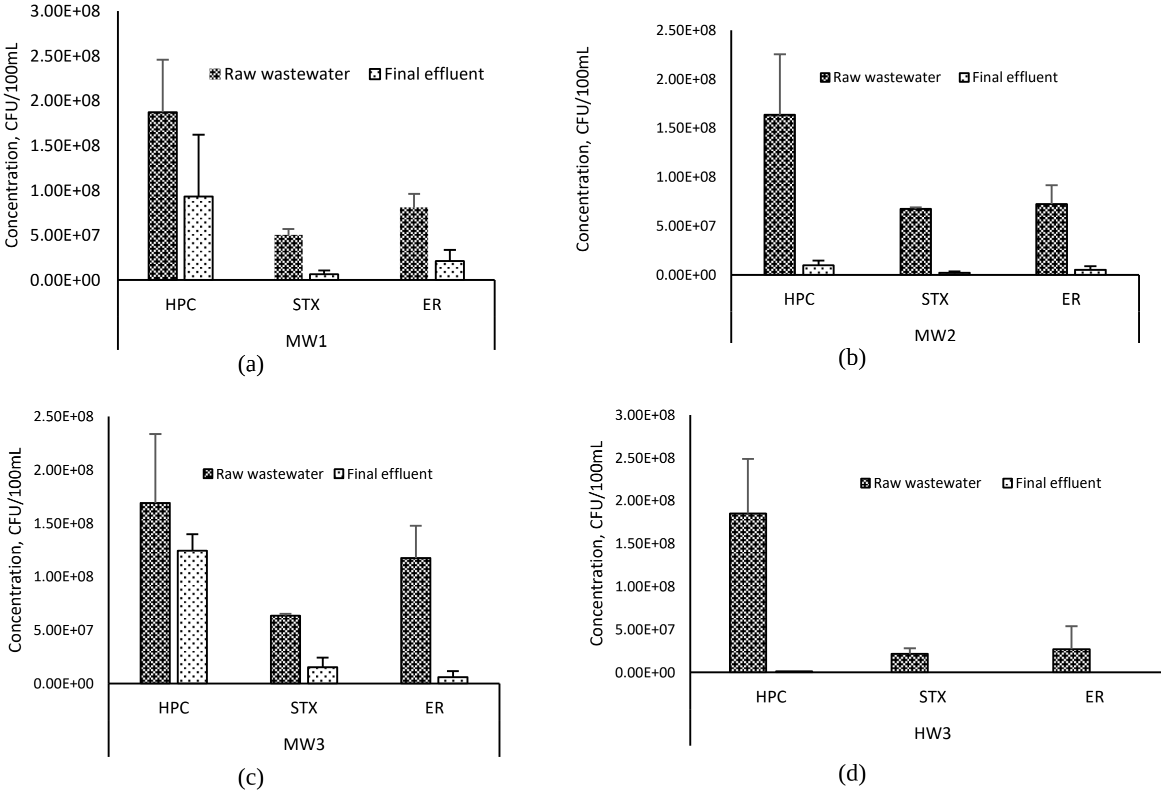


Figure 1: Compare of raw and effluent in municipal and hospital wastewater

These values are larger than the means of ARGs and ARB achieved in other studies (19, 24, 25). This can be due to insufficient management of WWTPs in Iran and the nature of the produced wastewater. The generated wastewater in Iran has a larger organic load compared with Western countries because of different nutritional patterns. For example, Kim et al (2006a) reported a strong relationship between organic load and the ARB growth rate. Previous studies have reported a high organic load in the studied urban WWTPs in Isfahan province, Iran (26). In addition, high quantitative values of resistant genes in wastewater can be due to high population and horizontal gene transfer (19, 27).

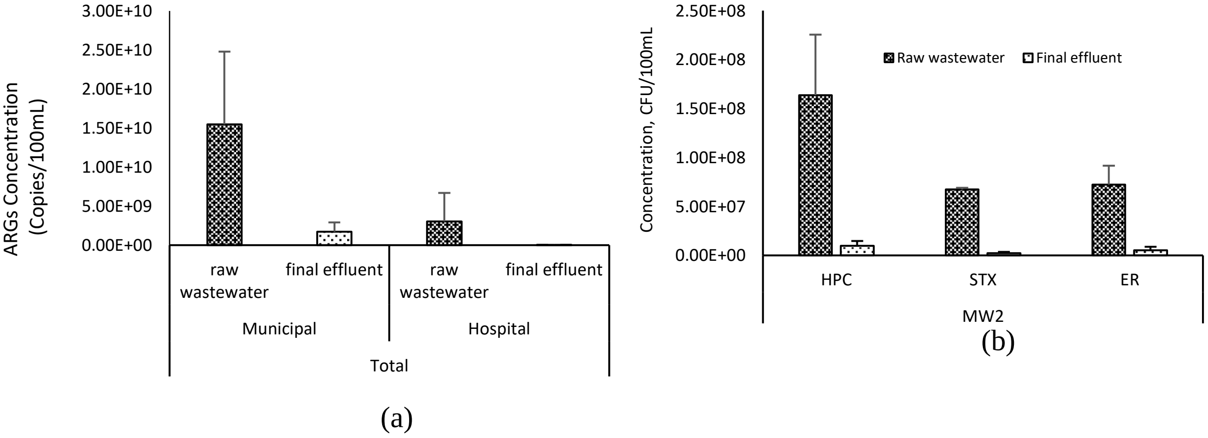


(a) (b) (c) (d)

Figure 2: Compare of ABR and HPC in municipal (a,b and c) and hospital (d) wastewater treatment plants

The results of this study revealed a moderate reduction in ARB and ARGs in the WWTPs. The findings of other studies are in agreement with the results of this study (19, 28). The rate of ARB and ARGs discharge to the environment through wastewaters was very high (the output range of ND-7.7×10^7 CFU/100mL and ND-8.29×10^9 Copies/100 mL). The findings of this research generally show that the identified values are much larger than the values of ARB and ARGs available in the receiver sources such as water resources (19, 29). Therefore, abundant release of these agents causes a high pressure on water resources and natural ecosystems (30). The results of the effluent indicated that although ARB and ARGs were slightly affected during the treatment stages, conventional wastewater treatment facilities could not have a significant effect on the removal of ARB and ARGs. Thus, the findings of this study do not agree with the reports of Huang et al. (2012) and Rodriquez-Mozaz et al. (2015) regarding the great effect of treatment on the reduction of antibiotic resistance (31). The investigation of the resistance developed by individual ARB and ARGs revealed that the resistance patterns in the raw wastewater and effluent were not necessarily similar (Fig 2 and 4).

The pattern obtained based on quantitative results of genes in raw wastewater and effluent indicated that the *Sul*1 gene had the highest value in the input and output (Fig 4). In a study conducted by Munir et al. (2011), the quantitative value of *sul*1 gene identified in the WWTPs was the highest (19). These results are also in accordance with those of the investigation of Rodriquez-Mozaz et al. (2015). The high frequency of this gene can be due to the high frequency of genetic elements in the wastewater.



(a) (b)

Figure 3: Compare of ARGs in hospital and municipal wastewater treatment plants

3.2 ARB and ARGs in municipal and hospital wastewater

The results of this study indicate that the average ARB and ARGs are larger in urban wastewaters (UWW) than in hospital wastewaters (HWW) (Fig 1 and 3). This can be due to the higher microbial load of UWW, presence of organic and nutrient materials to bacterial growth, and easy transmission of resistance among bacterial population because of receiving wastewater from a wider spectrum of sources (32, 33). Excessive use of antibiotics, especially oral antibiotics by people and the subsequent release of antibiotics from body to the UWW are not negligible. Geli et al. (2012) and Udekwe et al. (2009) have suggested that the increased resistance to vancomycin in the US in 1980s could have been a result of the over-consumption of oral vancomycin to control C. difficile (34). This trend can also be applied to other antibiotics. Overall, many researchers consider UWW as a key source of ARB and ARGs’ emission into the environment (19) The lower levels of ARB and ARGs by hospitals in comparison with the UWW may be attributed to the vast application of disinfection and acidic compounds, as well as the transmission of these compounds into the wastewater. This is not in congruence with the findings suggesting that HWW are richer in ARB and ARGs (24, 35). These studies attribute higher ARB and ARG levels to the high concentration of antibiotics in the HWW and in turn, the increased chance of contact between bacteria and antibiotics and resistant strains (24). Some studies consider the wide application of antibiotics in hospital as a selective advantage for ARB. In an investigation of the biofilm of urban and hospital wastewaters, Shwarz et al. (2003) demonstrated that ARB was higher in UWW and ARGs in HWW. In addition, some researchers argue that the mechanisms of resistance development in the HWW and UWW are different (36). The incidence of antibiotic resistance in the treatment plants effluent is not necessarily similar in different antibiotics and microbial groups.

3.3 The effect of WWTPs on ARB and ARGs

As can be seen in Fig 2, the reduction of ARB in the HW_3 treatment plant had the highest percentage (over 2 logs) among the studied treatment plants. This hospital treatment plant possesses an extended aeration process supported by a dual disinfection system (CL+UV) and rapid sand filter. Although STX and ER were not detected in the effluent, the results of quantitative ARGs indicated that *erm*B was identified and measured in the effluent (9.64×10^5 copies/100 mL). The lack of identification of resistant bacteria in the effluent is possibly related to the dual system of disinfection and filtration. On the other hand, positive results of molecular experiments indicated that genetic parts (genome DNA, plasmid, etc.) related to ARB existed in the wastewater. This shows that cultivation-based tests on their own are not reliable for investigation of the status of ARB and ARGs in environmental sources. Thus, the application of cultivation-based and molecular tests can present reliable results for the estimation of the level of activity and the genetic makeup of resistant agents (37). Our results demonstrated that the reduction of ARB and ARGs had taken place desirably. Rizzo et al. (2013) believe that rapid passage over surfaces of infiltration systems is not suitable for the process of conjugation of microorganisms and can cause sexual cell damage in them (32). It seems that more studies are required based on the applied advanced systems for the removal of contributing factors to the development of resistance in WWTPs (36). According to the results of the study, after HW_3, the MW_2 plant had the second highest reduction of ARB with 1-1.5 log. This plant has a strong management system. From among the studied treatment plants, MW_1 had the lowest reduction percentage, possessing a dual active sludge process. This plant has no significant effect on ARB removal, despite benefiting from a dual process. It also suffers from a poor management system. The pattern of ARB reduction indicates that in addition to the effect of the process type, the operation procedure also affects the efficiency of ARB removal. The MW_3 process is a stabilization pond. Some researchers attribute the reduction of ARB and ARGs in this process to solar radiation. The present study showed that the level of log reduction for STX and ER was equal to 1.43. This reduction is 1.44 for ARGs (*erm*B and *sul*1).

Investigating the effects of sunlight on resistant bacteria, Mezrioui and Echab (1995) observed that these ponds can cause an increase in population of these bacteria. Rizzo et al. (2013) attribute the increased number of resistant strains of *E.coli* in aeration lagoons to the support of the process type from the mentioned strains (32).

4 Conclusions

Municipal wastewater plays a more significant role in discharging ARB and ARGs into the environment. This study proves that although the processes of UWW treatment can be effective to some extent in removing genes, they are not successful to sustainable and reliable controlling of the bacterial resistant genes to the environment. Performance of treatment plants based on their process demonstrated that the process of active sludge with a widespread aeration mechanism supported by a dual disinfection system together with sand filter is the most efficient method. It seems that multiple disinfection systems complemented by a sand filter are effective and efficient in the removal of bacteria and genetic-resistant elements. Furthermore, the poor performance of MW_1 plant benefiting from a dual system of active sludge highlights the fact that in addition to the process type, management can play a major role in this regard

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Ethical issue

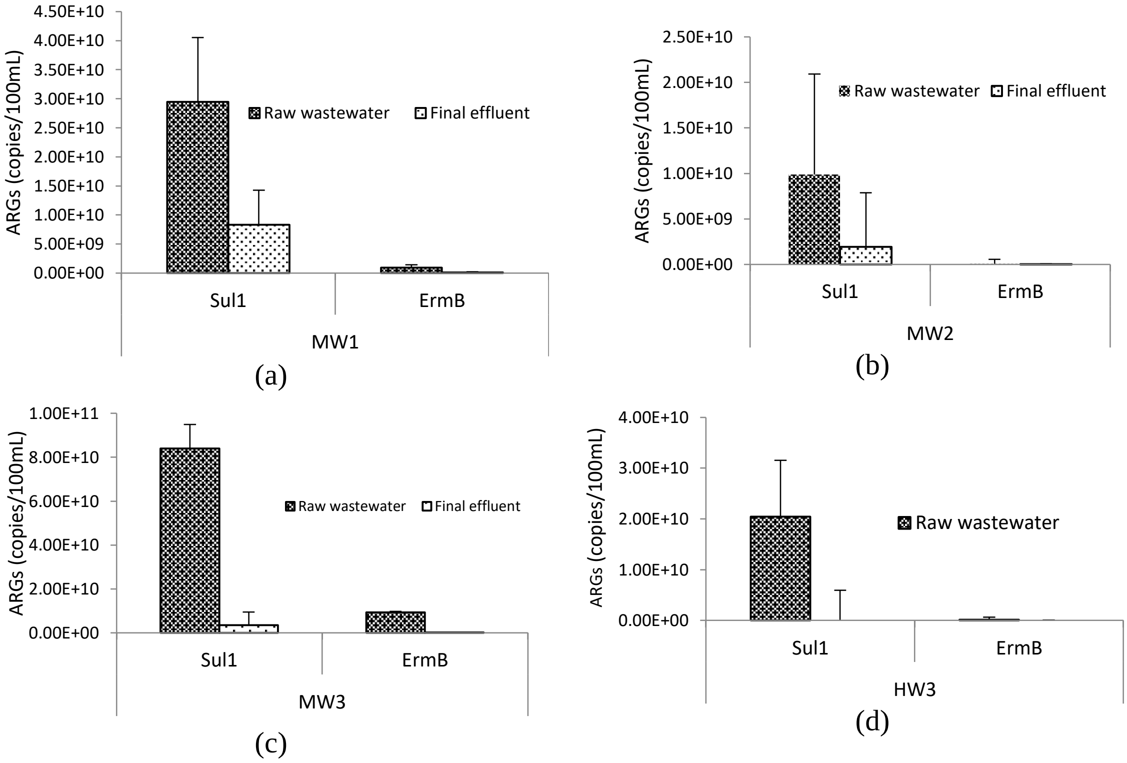
Authors are aware of, and comply with, best practice in publication ethics specifically with regard to authorship (avoidance of guest authorship), dual submission, manipulation of figures, competing interests and compliance with policies on research ethics. Authors adhere to publication requirements that submitted work is original and has not been published elsewhere in any language.

Competing interests

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Authors’ contribution

All authors of this study have a complete contribution for data collection, data analyses and manuscript writing.



(a) (b)

(c) (d)

Figure 4: Quantities average of *Sul*1 and *erm*B genes in different WWTPs

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