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VIABILITY STUDY OF SOME ANTIBIOTIC RESISTANCE BACTERIA PERCENTAGE ISOLATED FROM WASTEWATER EFFLUENT SAMPLES IN MANISA / TURKEY

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ABSTRACT

Antibiotic resistant bacteria and antimicrobial drugs enter the environment via wastewater, especially from hospitals and pharmaceutical plants, and through agricultural runoff, leading to contamination of surface and groundwater. Viability and percentage of antibiotic resistant bacteria (PARB) was investigated in effluent of urban house wastewater of Manisa city wastewater treatment plant system, the investigations done in university of Ege department of industrial microbiology. Samples of wastewater were collected monthly separated in two season (Spring & Summer) 2018, 1 liter in each sampling campaign filtered by aspiration pump with 0.45 µm filters. As a results of samples culturing in this study we noticed that samples for two season contain different levels of bacterial populations including *Pseudomonas, E. coli*, *Salmonella and shigella* that showed different growth rates among the examined samples. High numbers found in summer was 3.39E5, while lowest growth of both groups of bacteria was detected in water samples in June which was about 2.15E2. As results of antibiotics resistance percentage we found that all the isolates were resistant to ampicillin especially in month of Aug. was about 86% resistance of the total number of heterotrophic bacteria followed by the same antibiotic resistance percentage in July was about 67% and 51% for Amoxicillin in July between all of the total number of heterotrophic bacteria. In versa, the lowest antibiotics resistance percentage was with Kanamycin about 2% in the month of June also the resistance to the same antibiotic was not detected-ND in other samples for other months. Finally, we noticed that storing in cold conditions for interval between 10-14 days' lead to reduction of ARB to 90% before effluent of WW to the environment.

INTRODUCTION

Recently the antibiotic resistance (ABR) consider critical problem, and this problem increased day after day because of this issue connected not only with clinical or veterinary, also can connected to our environment system because of increasing in the source of antimicrobial enter the environment via wastewater, especially from hospitals and pharmaceutical plants, and through agricultural runoff, leading to contamination of surface and groundwater (Al-Bahry et al, 2014).

Uncontrolled use of antibiotics as well as Industrialization and the continuous urban development all of this reasons lead us to use new term that is genetically pollution of the environment (Florica Marinescu *et al.*, 2017 and Singer *et al*., 2016).

The issue firstly was widely reported by by Davies in 1973 (Benveniste R and Davies J, 1973), which was elaborated in the following years by several other investigators (Cundliffe E,1989), is that antibiotic resistance is not restricted to pathogenic bacteria, as well as WHO reported this in 2017 issue as major threat to human health (WHO, 2017), Rizzo et al also reported that One of the most significant sources of ARB is the discharge of wastewater treatment plants (WWTPs) (Rizzo et al., 2013).

Rather environmental microbes that are either non-pathogenic such as antibiotic producing bacteria or opportunistic pathogens such as *Pseudomonas aeruginosa* are often drug resistant in comparison to the bacteria typically associated with disease. The role of these organisms as potential reservoirs of resistance genes is only now becoming a focus of researchers (Gerard D Wright, 2010).

The main effect of ABR Increase in the environment may pose a threat to public health. Wastewater is a major source of AR and antibiotics resistance genes ARGs in the environment which acts as a pathway for introduction of antibiotics resistance bacteria ARB and ARGs from anthropogenic sources into natural environmental system (Rizzo *et al*., 2013).

If we think about the vast majority of antibiotic resistance microbes in our environment are not pathogenic, so, from which source is come? These organisms which is nonpathogenic in the origin interact with a multitude of chemicals in the environment produced by other bacteria, fungi, plants, animals, as well as those derived from a biotic process. Calculation of a very rough estimate of the biologically derived chemical diversity in the soil is instructive. One gram of soil holds 10^7–10^9 bacteria comprised of 4000–10,000 species and by the time the nonpathogenic microbes turn to antibiotic resistance then may become extreme pathogen in future (Schloss PD and Handelsman J, 2006).

Over the last decade, studies on the distribution and evolution of AR bacteria and genes ARGs in the environment, particular in wastewater, have gained considerable attention (Berendonk *et al*., 2015). However, the knowledge regarding antibiotic contamination, AR bacteria, and antibiotic resistance genes in ecosystems is still fragmentary, mainly due to methodological bias.

Apart from chemical pollution caused by antibiotics themselves, the use of antibiotics may also accelerate the development of antibiotic resistance genes (ARGs) and bacteria, which shade health risks to humans and animals (Kemper, 2008).

Despite of the Wastewater treatment plant systems in different countries eliminate vast numbers of microbial cells, only it a large number of microbes stay viable in discharge step then distribute in the environment and re increase of the issue, e.g. the viability of coliform bacteria in water distribution systems is an indicator for the presence of pathogenic bacteria including the opportunistic ones like Salmonella and Shigella (Berger et al. 1992). The occurrence of these bacteria does not result from a single factor, instead it is dependent upon complex interactions between various factors (LeChevallier, 2003). These are not well defined but include the quality of the water source, treatment and disinfection effectiveness and physicochemical parameters (Berger et al. 1992).

One of the major problems facing water suppliers is the bacteriological quality of water being distributed (Maggy et al., 1998). It has been reported that water treatment processes and disinfection systems do not inactivate the entire microbial population. This allows some microorganisms to survive the treatment process and adapt themselves to the environment of the distribution system and multiply in the pipelines (Berger et al., 1992). Microorganisms growing in a water distribution network are either indigenous (Ranch et al., 1999), including those growing on the pipe walls e.g. Pseudomonas and Flavobacterium (Ford, 1999), or exogenous e.g. fecal coliform which are introduced and transported by the water in the effluent distribution system (Brion & Lingireddy 1999). Potential pathogenic bacteria may be found among these microorganisms posing a health risks (Simon, 1999). Because of the potential health risk posed by water recovered from sewage treatment systems, it is important to analyze it from the point of view in terms of the microbial populations and the factors influencing their growth.

The viability of coliform bacteria in water distribution systems is an indicator for the presence of pathogenic bacteria including the opportunistic ones e.g. *Salmonella and Shigella* (Berger et al., 1992). The occurrence of these bacteria does not result from a single factor, instead it is dependent upon complex interactions between various factors (LeChevallier, 2003). These are not well defined but include the quality of the water source, treatment and disinfection effectiveness and physicochemical parameters (Berger et al., 1992).

For this reason, this study aimed to investigation the viability percentage of antibiotics resistance bacteria in effluent of wastewater after holding it in cold conditions.

MATERIALS AND METHODS

Describe of sampling sites.

The study is done in Manisa city of Aegean region of turkey which consider is one of the seven regions found in Turkey. It is situated in the western part of Turkey. The Mediterranean region is to the South and Southeast and the Central Anatolia regions are located to the East. The region includes 8 provinces and over nine million people. The Aegean region occupies 11% of the total area of Turkey with its 79.000 square kilometers of land. Most of the population and cities are concentrated on the coast line because of its convenience for sea transportation and tourism. The Aegean region is also both industrialized and agriculturalized. Main products are; textile, leather, carpet weaving, food, machinery and spare parts, marble, tobacco, sugar, olive and olive oil. About half of the total olive trees of Turkey are in this region. There are many important rivers feeding the Aegean Sea.

Sample collected from Waste water treatment plant system (WWTPs) in Manisa city of Aegean region. fig (1).

Sample collection and processing

Treated wastewater effluent samples were taken from the discharge channel of urban WWTPs in *Manisa* municipality in Aegean region by grab sampling method between March 2018 and August 2018 separated into two time, Spring & Summer.

For water samples 1 L collected in each time by using clean Polypropylene PP Bottle from inlet & outlet of WWTPs, Grab samples were always collected in morning time (around 10:00 am) in absence of rainfall before chlorine disinfection.

The samples stored in a portable refrigerator at 4°C and brought to microbiology lab in biology department of Ege university then analyzed immediately within 4 h. about 50 ml of water centrifuged in 2500 rpm, supernatant discarded and 1 ml from bottom portion were serially diluted five time until 10^{-5} dilution.

The samples then stored further for 2 weeks to culturing again.

Total heterotrophic bacteria & ARB Enumeration

To done this step we applied culture-based approach for all samples, Colony forming units (CFU/ml) (# of bacteria / ml = no of colonies X dilution factor / volume) were determined for the total cultivable aerobic bacteria and for the total cultivable antimicrobial resistant aerobic bacteria for all sampling areas. To determine the total number of heterotrophic bacteria THB&ARB 1ml of WW from each entrance and exit part serially diluted 10^{-1}- 10^{-5} by adding 1ml of samples to 9 ml of PBS (pH = 7.4), after that 1 ml from each dilution were directly plated onto R2A agar medium (Difco, Sparks, MD) (Eaton *et al*., 1995 & **Rahim A *et al*, 2014)** in duplicate without antibiotic for counting THB and in the same time 1 ml from each dilution were directly plated onto R2A agar medium dosed with specific antibiotic concentration separately as antibiotic selective media to count ARB The concentrations were determined as the maximum value of the Minimum Inhibition Concentration of bacteria listed in Clinical and Laboratory Standards Institute CLSI 2013 and other sources.

The concentrations of antibiotics were: Amoxicillin(AMX)16mg/L (MAFR, 2014), Erythromycin (Ery) 18mg /ml (MAFR, 2014), Ampicillin (AMP)50mg/ml, Gentamycin (Gen) 10mg/ml, Kanamycin (Kan) 50mg/ml, Tetracycline (TC) 16mg/ml (Munir et al., 2011). Fungal growth was inhibited by adding 200 mg/L cycloheximide to the agar.

These antibiotics subsequently added to the melted R2A agar before pouring then Plates incubated at 37 C for 48h and we count CFU/ml for each ml of WW sample according to the function (# of bacteria / ml = no of colonies X dilution factor / volume), all the samples were carried out in duplicated petri dishes and the mean values were considered by choosing plates with petri plates containing between 30 and 300 colonies. Plates with more than 300 colonies cannot be counted and are designated too many to count (TMTC). Plates with fewer than 30 colonies are designated too few to count (TFTC).

After incubation and enumeration, the bacteria were collected separately for each antibiotic used for backup and colonies were stored in 20-30% glycerol at – 20°C until further use, and at -80°C for long term storage (Ruoting Pei *etal*, 2006 & Rahim A *et al*, 2014).

Statistical analysis

Statistical analysis was performed by the student t-test and Excel program.

RESULTS

Results of total bacterial count

Generally, the total bacteria counted by using the same formula mentioned above which also used for antibiotic resistance bacteria counts, samples connected to Manisa wastewater treatment plant (WWTPS) were investigated for the occurrence of antimicrobial resistant bacteria in the effluent water. The results of total bacteria are showed in table (1).

Total bacteria in July was the **highest** number about 3.39×10^5 CFU/mL in summer season, followed by April month of spring season which was at 3.6×10^4 CFU/ml.

While the **lowest** count was in month of Jun 2.15×10^2 CFU/ml followed by the bacteria number in Aug. with total number about 4.15×10^2 CFU/ml.

After 2 weeks thewe repeated the same method of culturing for the same samples to investigate the viability of total heterotrophic bacteria in WW samples, after 2 weeks we found that about 80% of heterotrophic bacteria still viable even in storage conditions

Table 1: Total bacterial count and antibiotics resistance percentage.

Resistance %							
Season	**Month**	**Total CFU**	**AMX**	**AMP**	**ER**	**TC**	**K**
Spring	March	2.3E4	22	37	13	3	ND
	April	3.6E4	27	35	7	8	ND
	may	2.6E3	33	41	6	4	3
Summer	June	2.15E2	40	60	15	ND	2
	July	3.39E5	47	67	ND	12	ND
	August	4.15E2	51	86	33	19	ND

Result of antibiotic resistance bacteria percentage

For antibiotics resistance percentage we found that the highest resistance percentage was to Ampicillin - Amp in month of Aug. was about 86% resistance of the total number of heterotrophic bacteria followed by the same antibiotic resistance percentage in July was about 67% and 51% for Amoxicillin in July between all of the total number of heterotrophic bacteria.

The lowest antibiotics resistance percentage was with Kanamycin about 2% in the month of June also the resistance to the same antibiotic was not detected-ND in other samples for other months table number (1).

After 2 weeks the we repeated the same method of culturing for the same samples to investigate the viability of antibiotics resistance percentage in WW samples, after 2 weeks we found that only Amx resistance bacteria still viable and other bacteria disappeared.

Results of seasonal change of total bacterial count

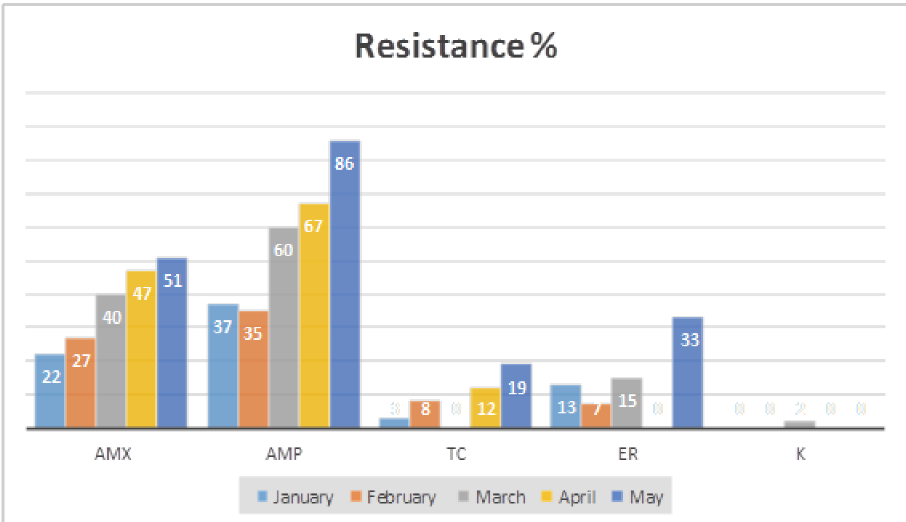
Seasonal change is taken under the consideration because seasonal change absolutely effects on the viability of life cells so we inserted this parameter in our project.

During two season of study we found that the highest number of countable bacterial cells in Summer was in July while the lowest bacterial cell counts was in June.

For spring season, we found that the highest month was in April while the lowest was in May table (1).

Results of seasonal change of antibiotics resistance percentage

By looking to table (1) and graphic (1) we see that the highest antibiotics resistance percentage was in summer season acted by Amp & Amx antibiotic and the lowest antibiotics resistance percentage was in spring season.



Graphic (1) seasonal change of antibiotics resistance percentage.

DISCUSSION

wastewater treatment is generally to allow human and industrial effluents to be disposed of without danger to human health or unacceptable damage to the natural environment. Irrigation with wastewater is both disposal and utilization and indeed is an effective form of wastewater disposal (as in slow-rate land treatment). However, some degree of treatment must normally be provided to raw municipal wastewater before it can be used for agricultural or landscape irrigation or for aquaculture. The quality of treated effluent used in agriculture has a great influence on the operation and performance of the wastewater-soil-plant or aquaculture system. In the case of irrigation, the required quality of effluent will depend on the crop or crops to be irrigated, the soil conditions and the system of effluent distribution adopted. Through crop restriction and selection of irrigation systems which minimize health risk, the degree of pre-application wastewater treatment can be reduced. A similar approach is not feasible in aquaculture systems and more reliance will have to be placed on control through wastewater treatment.

In this study, all the isolates were resistant to ampicillin. In earlier studies the dominant antibiotic bacterial isolates were resistant to ampicillin and were found in human also which studied by Semra Kurutepe *et al* in 2005, they found that most of isolates was resistance to ampicillin. Also Al-Bahry 1999 found the same results in sewage and wild animals including marine turtles. This suggests the overuse use of broad spectrum antibiotics, such as ampicillin.

Samples for two season were found to contain different levels of bacterial populations including *Pseudomonas, E. coli, Salmonella and shigella* that showed different growth rates among the examined samples. High numbers found in summer was 3.39E5, while lowest growth of both groups of bacteria was detected in water samples in June which was about 2.15E2.

In 1999 both of Volk & LeChevallier reporte, higher levels of heterotrophic bacteria were detected in the examined water samples in the absence of chlorine residual. While Berger et al. (1992) supported the fact that the presence of high levels of suspended particles to which bacterial cells adhere provides protection for bacteria from the disinfectant. LeChevallier (2003) found that the bacterial regrowth in distribution systems was stimulated by high water content of organic matter, establishing a linear relationship between the concentration of organic matter and level of bacteria.

These observations also explain the significant inverse relationship between chlorine residual and bacteria, and the latter’s positive correlation with the turbidity and nutrients (Irvine et al. 2002). These factors greatly contributed to the development of biofilm in the distribution system and are for the most part responsible for the high bacterial densities in the analyzed water samples (Berger et al. 1992).

In this study, the cold storage of samples resulted in a greater reduction of microbial population existed in the effluent wastewater. A similar observation was reported by Tree et al. (2003). Berger et al. (1992) The survival of bacteria could be due to protection provided biofilm of these bacteria (LeChevallier 2003).

CONCLUSION

From all the observations above we conclude that strategies must be developed and implemented internationally to avoid dumping residues of various antibiotics and other pharmaceuticals directly into the environment. Stringent sets of rules and regulations will be required to prevent direct sewage drainage into the aquatic and terrestrial habitats. Examination of these effluents for the presence of ARB, heavy metals and other contaminants would reflect the effectiveness of the waste treatment. Since ARB are relatively easily detected, this means that the presence of ARB in wildlife and environmental samples can be used as a biological indicator to monitor the degree of environmental pollution.

As well as we conclude from this study that storing in cold environment for interval between 10-14 days lead to reduction of ARB to 90 % before effluent of WW to the environment.

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