Characterizing the chemical contaminants diversity and toxic potential of untreated hospital wastewater

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Abstract

Characterizing the chemical contaminants diversity and toxic potential of untreated hospital wastewater

Fras BaasherThis study characterizes 21 wastewater samples collected from Al-Amal hospital between the period of 12 April till 8 July 2020. Al Almal is a hospital that provides drug addiction and psychological treatment to patients. Using solid-phase extraction and liquid chromatography with tandem mass spectrometry (LC-MS/MS), chemical contaminants profiles in these wastewater samples were determined in a non-targeted manner. These chemicals were then individually analyzed in an *in-silico* manner by checking against databases and literature to determine if they were mutagenic. By determining the proportion of mutagenic chemicals against the non-mutagenic ones, we aim to determine if untreated hospital wastewater may potentially negatively impact the downstream municipal biological wastewater treatment process. It was determined that 64% of the identified chemicals were not tested for their mutagenic effect, and hence no prior information is available in the literature and databases. Instead, we further performed *in-vitro* mutagenicity tests using Ames test to determine if the wastewater sample, with all of its chemical constituents, would be mutagenic. Ames test results showed that majority of the samples were non-mutagenic except for 1 sample that imposed a mutagenic effect on *Salmonella enterica* serovar Typhimurium TA98 and 3 samples with mutagenic effect on TA100. In addition, 1 sample showed a toxic effect on TA100. However, in all 5 instances, these samples only imposed a mutagenic and toxic effect at high concentrations of > 10x. The findings in this study suggest that a specialty hospital like Al Amal does not contribute substantially to mutagenic wastewater streams to the municipal sewer, and hence unlikely to significantly perturb the downstream

biological treatment processes. However, there may still be a need to consider ad-hoc contributions of mutagenic and/or toxic wastewater streams from the hospitals.

Keywords: Wastewater, chemical contaminants, Ames test, mutagenicity

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1. Introduction

As demand for water increases due to population growth, wastewater treatment and its subsequent reuse are increasingly becoming an important aspect of integral water resources management to enhance water supply reliability (Jiménez & Asano, 2008). Water reuse is practiced for municipal, industrial, agricultural or ecological purposes. Depending on the reuse purposes, wastewater is cleaned to varying degrees of final quality that would fit the purpose. Among the various reuse purposes, reclaiming water for potable drinking purposes would require the demonstration of a wastewater treatment facility that can achieve reliable pathogen control and attenuation of chemicals present in the wastewater (Trussell et al., 2018). For example, California adopts the "12/10/10 rule", meaning viruses, *Cryptosporidium* and *Giardia* should be reduced by 12-logs, 10-logs, and 10-logs, respectively, from the untreated wastewater (Health, 2014). In addition, source control programs are put in place to control toxic chemicals from entering the wastewater collection system as toxic chemicals may interfere with or pass through the wastewater treatment system to pollute the environment and affect aquatic ecosystems (Neemann et al., 2020).

Municipal wastewater, which is most commonly used as the source to obtain reclaimed potable waters, contains discharges from homes, industries, hospitals and public and private institutions. Among these sources contributing to the municipal wastewaters, hospital wastewaters are particularly of interest as high consumptions of pharmaceutical drugs and disinfectants happen within these facilities. These chemical contaminants may potentially interfere with the downstream wastewater treatment system when hospital wastewaters are mixed within the municipal wastewater. For example, recent studies have shown an increase in horizontal gene transfer rates

caused by non-antibiotic pharmaceuticals such as ibuprofen (Wang et al., 2020). The pharmaceutical compounds were further shown to increase horizontal gene transfer rates for competent bacterial cell hosts by enhancing the stress levels that resulted in reactive oxygen species (ROS) production. In separate studies, it was also shown that chemicals or external stressors that are mutagenic could increase DNA repair rates, in turn triggering natural transformation among competent *Acinetobacter baylyi* ADP1 (Augsburger et al., 2019) (Mantilla-Calderon et al., 2019).

Hence, understanding the level of toxicity imposed by chemicals present in the wastewater would facilitate subsequent assessment on the potential detrimental impact on wastewater treatment processes. To study the toxicity of the wastewater produced by hospitals, it is essential to first have an understanding of its composition and properties. Several studies were conducted on wastewater collected from hospitals in which they target specific compounds and evaluate their toxicity and environmental impact. In one study, single grab samples from three hospitals in Turkey were collected and analyzed for the presence of 55 compounds that include pharmaceuticals, corrosion inhibitors, and pesticides (Yilmaz et al., 2017). Another study targeted 25 pharmaceuticals, quantified them, and assessed their environmental hazards (Mendoza et al., 2015). In general, earlier studies focus on targeting specific compounds and did not provide a full profile of what chemical contaminants may be present in the hospital wastewater. Targeting specific compounds for toxicity analysis does not give a holistic representation of the complex wastewater environment.

In this study, we performed a non-targeted assessment of the chemical composition of wastewater samples collected from a hospital in Jeddah, Saudi Arabia. The mutagenicity of each individual chemical identified after solid-phase extraction followed by liquid chromatography-mass spectrometry was further assessed by

referencing against literature and databases (i.e., *in-silico*). By summing up the overall relative proportion of mutagenic chemicals against non-mutagenic properties, we infer the overall detrimental level imposed by each individual wastewater sample. Mutagenicity was also characterized *in-vitro* for each wastewater sample to verify the observations obtained *in-silico*. As hospital wastewater passes through biological wastewater treatment processes, it is essential to have an understanding on how toxic its constituent chemicals are and what effects they have on the microbial community. Understanding the chemical composition of the wastewater and its toxicity is important to allow us to manage better our wastewater treatment of complex matrixes coming from hospitals.

2. Literature review

2.1. Chemical contaminants in hospital wastewaters

Wastewater generated by hospitals and their facilities contains a plethora of contaminants. Those contaminants include pharmaceuticals such as antibiotics, anti-inflammatories, psychiatric drugs, b-blockers, anesthetics, disinfectants, and others that are not fully metabolized and disposed of along with the wastewater (Zhang et al., 2020). The type of pharmaceuticals and concentration depends on different factors. Those factors are the number of beds and whether it is a specialty or a general hospital (Zhang et al., 2020). Compared to municipal wastewater from an urban area, the pharmaceuticals in hospital wastewater are 4 to 150 times more concentrated (del Álamo et al., 2020). For each pharmaceutical, the concentration was measured to be in the range of ng/L to µg/L (Meza et al., 2020). One study reported the concentration of 38 pharmaceuticals in the wastewater generated by three different hospitals. The highest reported concertation of pharmaceuticals reported were for paracetamol and diclofenac of 7.4-65 µg/L. As for antibiotics, it was ofloxacin at 200 µg/L in one of the samples (Yilmaz et al., 2017). Another study found concentrations as high as 211.9 µg/L of paracetamol and 141 µg/L of ibuprofen (Mayoudom et al., 2018). Meanwhile, for municipal wastewater, the concentration of paracetamol and ibuprofen were between 0.6-49 ug/L and 0.8-21 ug/L, respectively (Kołecka et al., 2020).

To quantify the chemical contaminants in wastewater, different approaches that involve highly sensitive equipment are used due to the low concentrations of the contaminants (Figure 1). A common approach is first to concentrate the samples using Solid Phase Extraction (SPE). The goal of SPE is to concentrate the targeted analyte present in the bulk wastewater into a smaller volume while getting rid of other undesired

compounds (e.g., organics, salts) that may interfere with the downstream analytical characterization. However, this approach is dependent on the physio-chemical properties of the targeted analytes, and hence recovery efficiency depends on the sorbent's affinity to the targeted analytes. Properties such as polarity and ionized state can increase or decrease the recovery (Jiang et al., 2013). The recovery efficiency is the percentage of the analyte retained in the concentrate from the original sample. The recovery efficiency depends on the analyte targeted and the sorbent used. One study compared 12 different sorbent cartridges against 12 pharmaceuticals and reported HPLC Oasis cartridges to be the best at recovering them. The recovery efficiency was between 71-95% for 11 pharmaceuticals, while it was 52% tamoxifen and 9% for thioridazine (Zhang & Zhou, 2007).

After the samples were concentrated using SPE, different approaches can be used to identify and quantify the chemicals present in the samples. The main three approaches are gas chromatography-mass spectrometer (GC-MS), high-performance liquid chromatography (HPLC), and liquid chromatography with tandem mass spectrometer (LC-MS/MS). GC-MS works by injecting the sample and passing it through the capillary column, which will vaporize the sample. The sample passes through a column that will separate compounds and pass them through a detector. Then the GC-MS breaks down the molecules into cations and creates a mass spectrum used to identify the chemicals (Hübschmann, 2015). HPLC runs by passing the analytes using the mobile phase through a column then a detector, converting it into a chromatogram (Kazakevich & Lobrutto, 2007). Finally, LC-MS/MS first ionizes samples using electrospray ionization (ESI). The ionized sample passes through a mass filtering device, and subsequently, a collision chamber breaks down the molecules into anions or

cations. The ions then passed through a second mass filtering device and a detector to build a mass spectrometry (Grebe & Singh, 2011).

Comparing the three analytical instruments, the GC/MS is limited by the derivatization in which only thermolabile, polar, and low volatile compounds can be detected (Díaz-Cruz et al., 2003). The LC-MS/MS can test for those chemicals since it bypasses the derivatization step allowing it to scan more analytes than the GC/MS, which makes it a better replacement to the GC/MS (Tsikas & Zoerner, 2014). Moreover, it has higher specificity than HPLC when it comes to low molecular weight analytes (Grebe & Singh, 2011).

Once a chromatogram is made available, the identification process of each chemical can be done through non-target screening and matching the MS^2 spectra with online databases such as mzCloud, MassBank, and STOFF-IDENT (Itzel et al., 2018). However, such approaches can only provide qualitative characterization of the chemicals present in the wastewater, and additional steps are needed to obtain quantitative measurements of the detected chemicals.

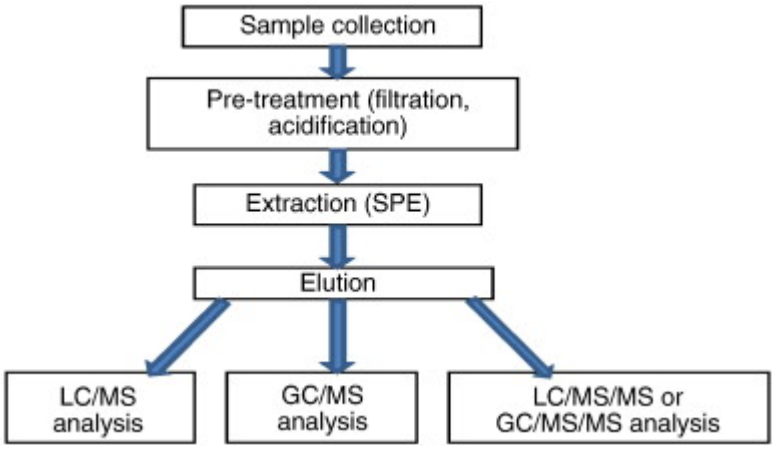

Figure 1. The steps done to concentrate the samples and analyze them using Mass Spectrometry (Jiang et al., 2013)

To achieve quantitative characterization, the method and the parameters in the LC-MS/MS must be built for each chemical. This is done by injecting a pure standard solution in the mass spectrometer directly. The mass spectrometer runs at different parameters to identify the parameters with the strongest peaks. Once the method is built, a selected number of standards are made in HPLC grade solution. Those solutions must have the samples between the minimum and maximum standard solution concentration to ensure an accurate measurement. The solution is run, a linear line of the concentration against the peak intensity can be obtained. Using the same parameters, the concentration is calculated by converting the peak to concentration through the equation generated from the standards.

2.2. Potential toxicity concerns related to the chemical contaminants

From the various studies that characterized the chemical contaminants in hospital wastewater (Diwan et al., 2009), it was known that hospital wastewater contains a plethora of chemicals that are genotoxic and mutagenic. This include ofloxacin and tinidazole along with other pharmaceuticals, which were detected in the hospital wastewater effluent in between 1.4–236.6 µg/L. Oflotoxin is mutagenic for TA98 and TA100, while tinidazole is predicted to be mutagenic by the ADMET score (Isidori et al., 2005; *Tinidazole*).

The effects of pharmaceuticals on bioreactors are not fully understood. One study used a cocktail of organic micropollutants to assess their effect on aerobic and anaerobic membrane bioreactors. That cocktail contains biocides, pharmaceuticals, and flame retardants. The study has shown no change in the removal rate of the bioreactors. However, the microbial community's structure and gene expression changed in terms of

relative abundance and genetic expression levels. Bacterial groups like *Terrimonas lutea* and *Ferruginibacter alkalilentus* increased in relative abundance, and they are linked to biofouling of the bioreactor membrane (Harb et al., 2016). Those phenomena were also reported by another study stating that the micropollutants affected floc formation, the membrane bioreactor performance, and increased the production of polysaccharides, which contributes to membrane biofouling (Besha et al., 2017). An increase in biofouling frequency can lead to higher costs for wastewater treatment. Moreover, the micropollutants posed as an environmental stressor to the microorganisms, hence the increase in the production of polysaccharides.

There have been other studies that showed exposure to mutagenic chemicals could activate the DNA damage response pathway, which in turn activates the *recA* gene and increase the rate of foreign DNA uptake by the process of natural transformation (Mantilla-Calderon et al., 2019). This is a major concern especially in hospital wastewater due to the higher presence of antibiotics and antibiotic resistance genes (ARGs). Different studies have reported that hospital wastewater has concentration of ARGs between 0.4 log to 1.8-fold more than communal wastewater (Paulus et al., 2019; Wang et al., 2018). The presence of ARGs and a mutagen can potentially facilitate the increase in antibiotic resistance in the microbial community through natural transformation events, which poses a significant health hazard.

In addition, sub-lethal mutagenicity associated with chemicals can cause genome mutation and enrich for mutants that show significant resistance to antibiotics or disinfectants. Two studies showed that at a sub-lethal level, heavy metals and nano-metal oxides had a mutagenic effect, which promoted the formation of reactive oxygen species (ROS) that can cause DNA damage. This effect causes cells to start the SOS repair mechanism, which can cause mutations due to its high error rate (Li et al., 2019;

Zhang et al., 2018). Although these studies did not evaluate chemicals found in hospital wastewater, these observations can potentially translate to those mutagenic chemicals that are present in hospital wastewater and persist through the municipal sewers to reach the biological activated sludge processes.

2.3. Methods to evaluate mutagenicity

Considering the abovementioned concerns, it is important to assess the mutagenic properties of hospital wastewater and the compounds present in it. There are different assays to evaluate the mutagenicity of the wastewater samples. The global standard for the mutagenicity assay is the Ames test, which uses different strains of *Salmonella* to test for different types of mutations that could be caused by the mutagen on the histidine operon (Grúz et al., 2020; Mortelmans, 2019). Those strains are TA97 which tests for frameshift hisD6610 mutation, TA98, which tests for frameshift *hisD3052* mutation, TA100, which tests for substitution *hisG64* mutation, and TA102 and TA104, which tests for an ochre mutation in the hisG428 gene (Maron & Ames, 1983). These strains have preexisting mutations that leave the bacteria unable to synthesize the required amino acid histidine and therefore unable to grow and form colonies in its absence. When mutations of these strains occur at the site of these preexisting mutations or nearby in the genes, the gene's function can be restored to allow the cells to synthesize histidine and form colonies on minimal agar media. Ames test was modified throughout the years to enhance the test further and to allow it to test for chemicals such as gases, biological samples such as urine, and highly volatile chemicals (Mortelmans, 2019).

To perform Ames tests, the *Salmonella* strain cells are typically standardized to a certain concentrations (e.g., $2x10^9$ cells/mL) and incubated with the tested chemical for a

defined exposure time at a mesophilic temperature of 37°C. Each sample was then plated onto minimal glucose plates supplemented with Vogel−Bonner (VB) salt solution. In the presence of mutation, the TA strains revert to histidine independence and can form colonies on the agar media. The TA strains have a slow growth rate and require 72 h of incubation at 37°C to denote the number of histidine revertant colonies. The number of colonies can then be counted and normalized against the number of colonies derived from the negative control. Moreover, the standardized cells were grown in a separate plate along with a sterile disk that absorbed crystal violet to ensure that the cells contained *rfa* mutation, which allows higher permeability for larger molecules (Jongen et al., 1978; Maron & Ames, 1983).

Besides the Ames test, there are other tests that can test for mutagenic effects. Those tests include SOS/umu test and *Allium cepa* anaphase-telophase test. SOS/umu test is designed using *Salmonella typhimurium* TA1535/pSK1002 strain that is genetically engineered to assess genotoxicity. The method evaluates mutagenicity based on the SOS signal produced due to a mutagenic effect (Chai et al., 2018). The signal is evaluated by the increase of β-galactosidase activity caused by *Salmonella enterica* serovar Typhimurium TA1535/pSK1002, and that activity was measured by means of spectrophotometer. The activity is calculated based on the absorbances as described by Ye et al., and an SOS is reported when there is a linear dose-effect relationship (Ye et al., 2014). A. cepa anaphase-telophase test is used to screen for the mutagenic effects of chemical compounds by evaluating for chromosomal aberrations using a microscope (Tabet et al., 2015). However, the Ames test remains the golden standard for testing for mutagenicity due to its relatively higher sensitivity towards tested mutagens and its global use for safety testing by different agencies (Grúz et al., 2020).

3. Materials and methods

3.1. Sampling

A total of 22 untreated wastewater samples were collected from Al-Amal Hospital, Jeddah, Saudi Arabia, from 12 April to 8 July 2020. The sample on 22 June 2020 was collected into 2 separate bottles to form independent technical replicates. Al-Amal Hospital is a medical facility that specializes in the treatment of patients with mental illnesses as well as addiction rehabilitation. Grab samples were collected from the equalization tank in the morning on each sampling date and immediately transported to KAUST. Samples were stored at 4 ^{o}C until further analysis.

3.2. Solid-phase extraction (SPE)

200 mL of each sample was individually filtered through Whatman® glass microfiber filters (GF/F grade) and collected for concentration through SPE. SPE was done using Dionex AutoTrace 280 through 500mg Oasis HLB cartridges. The cartridges were pre-conditioned with 5 mL HPLC-grade methanol followed by 5 mL of HPLC water (pH 2.5). The sample was loaded into the cartridge with a flowrate of 3 mL/min, then washed with 10 mL of HPLC-grade water. The sample-loaded cartridge was then dried with nitrogen gas for 60 min, then elution of bound sample was done using 10 mL HPLC-grade methanol. Concentrated samples were collected in a sterile tube and lyophilized in a 40^{o}C water bath under a nitrogen gas blower. The lyophilized sample was then dissolved in 1 mL of HPLC-grade methanol and stored at 4^{o}C prior to analysis as detailed in section 3.3.

3.3. Liquid Chromatography-Mass Spectrometry (LC-MS/MS)

LC-MS/MS was used to provide a non-targeted approach to characterize the chemical constituents that are present in each of the untreated hospital wastewater samples. The system consists of Gemini-NX C18, 4 × 2.0 mm (Phenomenex Inc.) column, Agilent 1260 infinity, and ABSCIEX QTRAP 5500. The system uses two mobile phases: Mobile phase A with 4 mM of ammonium formate and 0.1% formic acid in HPLC-grade methanol and mobile phase B with 4 mM of ammonium formate and 0.1% formic acid in HPLC-grade water. The LC-MS/MS ran on multiple reaction monitoring (MRM) at a flowrate of 200 uL/min for 18 min (Zaouri et al., 2021). The scan range was limited between 150-1000 m/z to improve signal intensity since a preliminary run showed only noise before 150 and after 1000 m/z. The peaks were recorded for data analysis and were compared to the online database mzCloud to find matches. A positive match is defined based on having at least 80% match similarity with a specific compound listed in the database. For each identified chemical, the occurrence frequency was noted and subsequently categorized into thirteen categories, namely (i) medication (i.e., chemicals such as antibiotics, non-steroidal anti-inflammatory drugs NSAIDs, allergy medication, etc., needed for medical treatment), (ii) drugs (i.e., chemicals that were used as illicit or recreational drugs), (iii) pesticide and herbicides (contaminants that are used in agricultural activities), (iv) natural bioactives (chemicals that are secreted naturally by the microorganisms in the wastewater such as toxins), and (v) withdrawn chemicals that have been discontinued from the market. With the exception of the final class, the remaining four classes were further organized into three subclasses. The first subclass was “Parent Compounds” which was defined as chemicals that retained their original chemical structure and did not undergo any reaction. The second subclass was “Analogs” which was defined as chemicals with similar chemical structure and function to the parent compounds but may differ in their pharmacokinetic properties, such as their absorption and toxicity to the human body, amongst others. The third subclass was

"Metabolites" which was defined as parent compounds that have undergone a reaction that resulted in an entirely different chemical compound altogether.

3.4. *In-silico* mutagenicity and ROS characterization

For each chemical, *in-silico* characterization on its mutagenicity and ROS production data was done based on a search conducted on databases collated by US Food and Drug Administration (US FDA), European Chemicals Agency (ECHA). Studies and articles that are within the accessible public domain, written in English, and were published within the duration of 1991 to 2021 were included as an information database to search for the mutagenicity and ROS production capacity of each individual chemical. To search, the name of the chemical and the primary search terms used were *"mutagen"*, *"Ames assay"*, *"Ames test"*, *"Salmonella",* and *"reactive oxygen species"*. These databases were also used to determine if the Ames assay results of each contaminant were available. Apart from the results, the details of such studies, such as the strains of the model organism used and concentration of contaminants tested, amongst others, were also noted to construct a comprehensive catalog. Similarly, these databases were also used to determine if each contaminant had been tested *in vitro* for their capability to induce, suppress or to have no effect on ROS production. Apart from the results, the details of such studies, such as the model organism used and the concentration of contaminants tested amongst others, were also noted to construct a comprehensive catalog. In addition, a computational model, admetSAR accessed through DrugBank (https://go.drugbank.com) was also used to computationally predict the Ames mutagenicity of the contaminant using a probability score so as to further

enhance the comprehensiveness of the in-silico characterization. For chemicals that have no literature data, they were indicated as inconclusive. After analyzing each individual chemical for its mutagenicity and ROS production capacity, the relative percentage of mutagenicity and ROS production capacity for each wastewater sample was further tabulated.

3.5. *In-vitro* characterization of mutagenicity for hospital wastewater samples

The mutagenic effects of the wastewater were evaluated using *Salmonella typhimurium* TA98 and TA100 strains. TA98 strain evaluates for on-point mutation while TA100 tests for base-pair substitution mutation (Maron & Ames, 1983). The positive control was 100 µg of sodium azide/plate for TA100 and 5 µL dichloromethane/plate for TA98, respectively (Jongen et al., 1978). 1x potassium phosphate buffer (PPB) was used as the negative control. Both strains were prepared by growing them overnight in LB broth with ampicillin at 25 mg/mL. The mutagenic effect was evaluated by the Ames test as previously described (Maron & Ames, 1983). The cells were standardized to $2x10^9$ cells/mL and incubated with the SPE-extracted samples (which have an original concentration of 200x) at a final concentration of either 1x, 10x, or 20x for 1 h and 37 °C. Samples of each concentration were done in three technical replicates and plated onto minimal glucose plates supplemented with Vogel−Bonner (VB) salt solution. After 48 h of incubation at 37 °C, the number of histidine revertant colonies was counted and normalized against the number of colonies derived from the negative control. Moreover, the standardized cells were grown in a separate plate along with a sterile disk that

absorbed crystal violet to ensure that the cells contain *rfa* mutation, which allows higher permeability for larger molecules.

4. Results

4.1. Profile of chemical contaminants present in untreated hospital wastewater

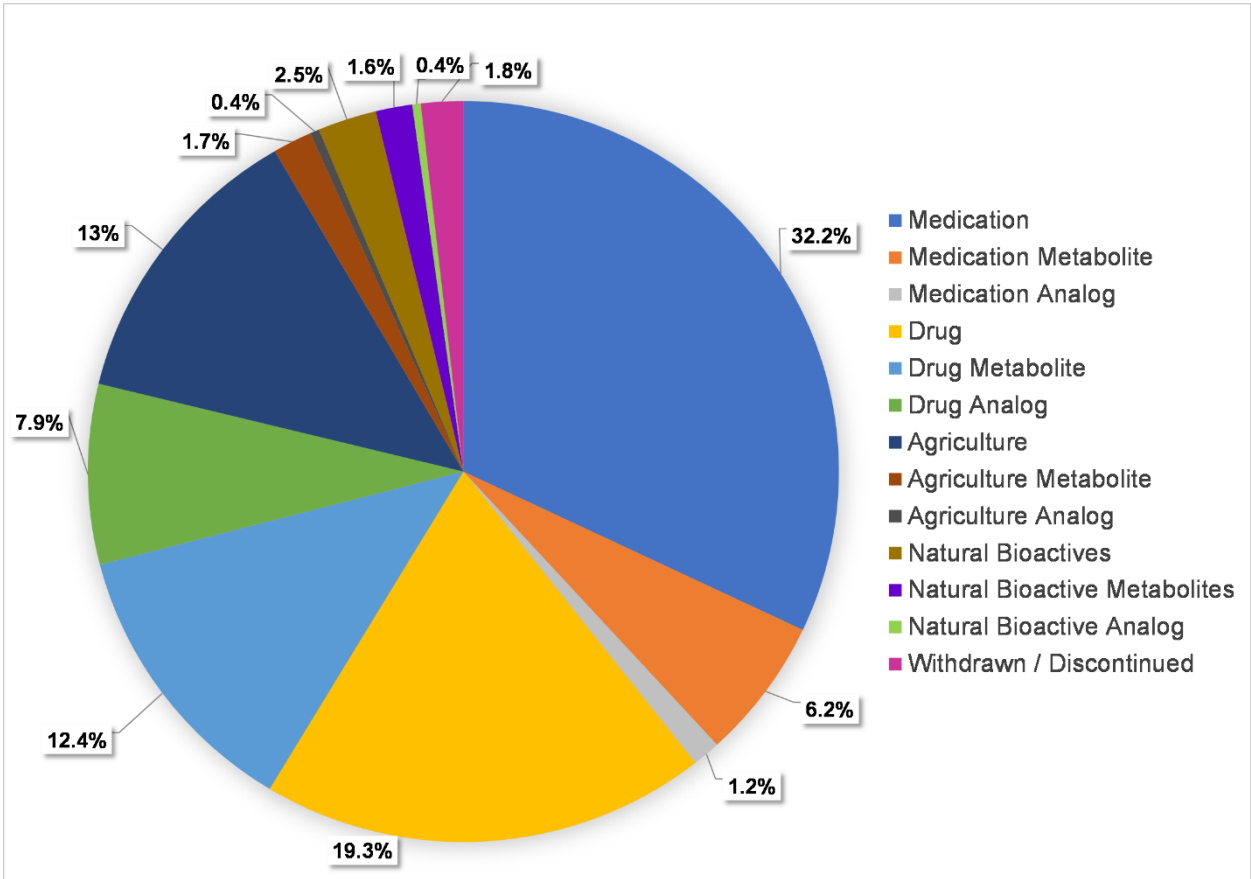

Figure 2. An overall depiction of the chemicals identified in the 22 wastewater samples and their respective categories.

A total of 825 chemical contaminants was identified from all wastewater samples collected from Al-Amal hospital. 32% of the identified chemicals were classified into medications in their parent compound form and include drugs such as antibiotics, antidepressants, and antipsychotics (Figure 2). The percentage of medications that are in its metabolites form account for only 6.3% of the total detected chemicals. In contrast,

illicit drugs (19%) and their metabolites (13%) account for the next two major categories of chemicals detected in the hospital wastewater (Figure 2). Illicit drugs are drugs that were discharged by patients of the hospital in their pure form, while metabolites are the final products after they are metabolized in the patient's body. Those illicit drugs include multiple types of synthetic cannabinoids, heroin, Lysergic Acid Diethylamide (LSD), and others.

Pesticides were the fourth most common chemicals identified in the hospital wastewater, accounting for 12% of the total identified chemicals. This category includes pesticides, herbicides, and fungicides. Both chemicals classified as bioactives or withdrawn chemicals account for only a small percentage of the identified chemicals. Bioactives include chemicals that are secreted naturally by the microorganisms in the wastewater, while withdrawn chemicals include those that were discontinued from the target consumer markets but may be accessed through illegal routes.

4.2. *In-silico* prediction of mutagenicity

For all samples, less than an average of 4% of the chemicals were *in-silico* predicted to be mutagenic (Figure 3). An average of 34% is predicted to be non-mutagenic. However, an average of 62% of the chemicals did not have data available about their mutagenicity, primarily because they are illicit drugs and their metabolites and analogs (i.e., inconclusive).

The sample with a comparatively higher percentage of chemical contaminants classified as mutagens was April 25, 2020 (Figure 3). Moreover, one-way ANOVA was performed on the number of characterized mutagenic, non-mutagenic, and inconclusive chemicals to compare samples collected in April, May, and June 2020. July was excluded due to

the low number of samples. While there was no statistically significant difference in the mutagenic category, there was a statistical difference among the different months for the percentage of inconclusive chemicals ($p < 0.05$). From Tukey's Honest Significant Difference, the different months were April and May for the percentage of inconclusive chemicals. Sample collected on 22 June 2020 was analyzed twice in an independent manner from the SPE to the LC-MS/MS step, and both independently processed samples showed similar *in-silico* profiles, suggesting that the earlier processing protocol is reproducible.

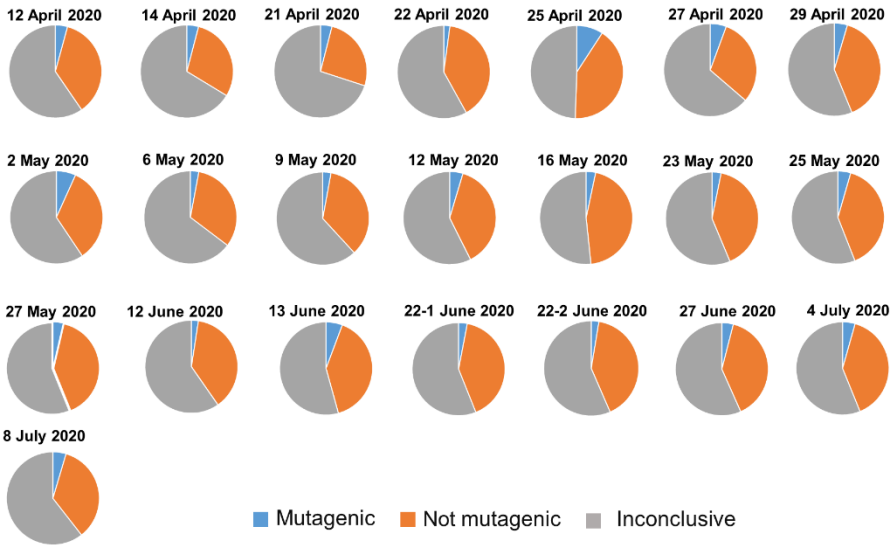

Figure 3. A chart representing the percentages of mutagenic compounds, non-mutagenic compounds, and inconclusive compounds in each of the analyzed Al Amal hospital wastewater samples.

4.3. *In vitro* mutagenicity tests of hospital wastewater samples

Of the 22 samples, most of the samples did not show a mutagenic effect for either TA98 or TA100. The only sample that showed mutagenic response on TA98 was collected on 27 June at a 20x concentration (Figure 4), in which the number of revertants normalized against the NC was 1.4-fold higher.

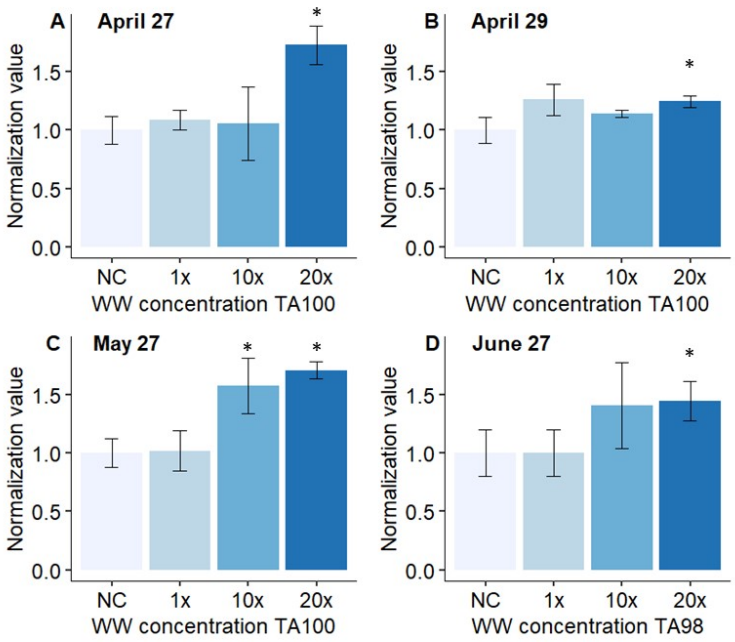

Figure 4. A graph representing the *in-vitro* data of the sample collected on April 27. April 29, May 27, and June 27. A) the mutagenicity of April 27 sample collected at 1x, 10x, and 20x concentrations for TA100. B) the mutagenicity of April 29 sample collected at 1x, 10x, and 20x concentrations for TA100. C) the mutagenicity of May 27 sample collected at 1x, 10x, and 20x concentrations for TA100. D) the mutagenicity of June 27 sample collected at 1x, 10x, and 20x concentrations for TA98.

* denotes statistically significant difference ($p < 0.05$)

Samples that showed mutagenic response on TA100 include those collected on 27 and 29 April and on May 27 (Figure 4). Mutagenic response on TA100 was only observed when April 27 and 29 samples were of 20x concentrate, and that the fold increase in the number of revertants was 1.7 and 1.2, respectively, compared to NC. In contrast, May 27 sample exhibited a mutagenic response on TA100 at both 10x and 20x concentrations. Moreover, a mutagenic effect was detected on TA98 for June 27 sample with a 1.4 fold increase.

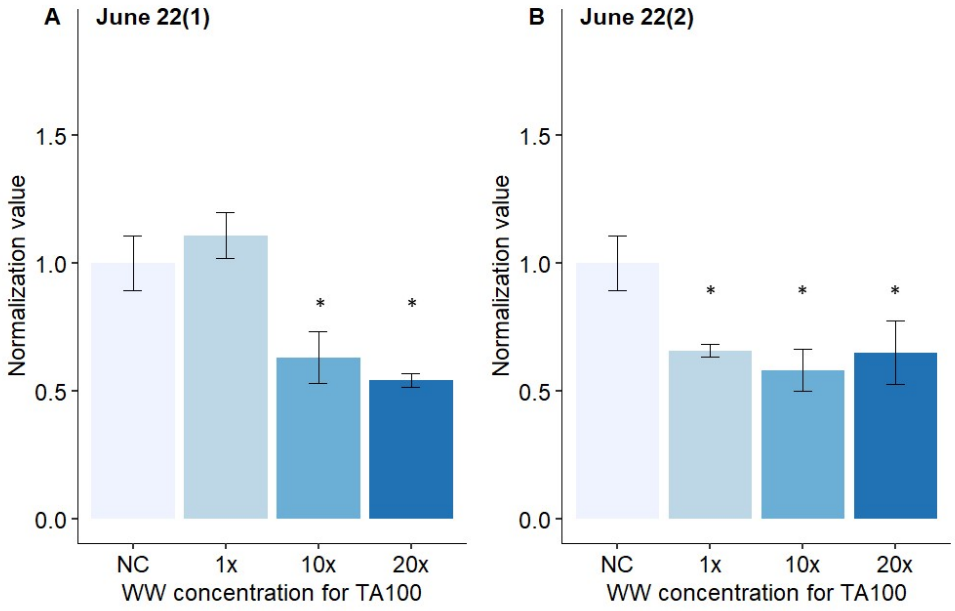

Figure 5. A graph representing the *in-vitro* data of the two samples collected on June 22. A) the mutagenicity of the first sample collected at 1x, 10x, and 20x concentrations for TA100. B) the mutagenicity of the second sample collected at 1x, 10x, and 20x concentrations for TA100.

* denotes statistically significant difference ($p < 0.05$)

Although June 22 sample did not show a mutagenic response on both *Salmonella* strains, both independently processed samples collected on this date exhibited a toxic response on TA100 when tested at 10x and 20x concentrations (Figure 5). The number of revertants counted at both concentrations was 0.5 to 0.7-fold of that in NC.

5. Discussion

Although Al Amal hospital is a rehabilitation hospital for drug addicts, non-targeted characterization of the wastewaters from this hospital showed that the most abundant (32%) chemical constituents are medications, including antibiotics, antidepressants, and antipsychotics. Meza et al. study detected the presence of 52 organic contaminants in Hershey Medical Center wastewater. 43 of the 52 (83%) identified chemicals were pharmaceuticals, while the rest were pharmaceutical metabolites or antifungals (Meza et al., 2020). Another study characterized the wastewater collected from 4 hospitals and detected 31 out of 55 (56%) identified chemicals were pharmaceuticals. The remaining components were pharmaceutical metabolites, corrosion inhibitors, and pesticides (Yilmaz et al., 2017). Those profiles are not entirely similar in terms of chemical composition to our study due to the lack of illicit drugs presence. This could be explained due to the nature of Al-Amal hospital since it is a drug addiction rehabilitation facility. The different chemical profile could also be explained due to the presence of other categories such as bioactives and pesticides.

Majority of the chemicals identified to be present cannot be characterized for its mutagenicity/non-mutagenicity (Figure 2). The highest number of identified inconclusive chemicals are from April 21st. The inconclusive results were due to a lack of information for the mutagenic properties of different pharmaceuticals. For example, quinupramine, finasteride, and robenidine are pharmaceuticals that were identified on April 21st. In addition, Al-Amal hospital is a specialty hospital that uses medications to alleviate the side effects of drug addictions. Patients can discharge illicit drugs and metabolites of the drugs they ingested or injected to the waste stream. Therefore, unless databases improve by expanding characterization effort to a broader suite of chemical

contaminants, particularly that of illicit drugs and drug metabolites, it remains difficult to ascertain the overall mutagenic profile of the hospital wastewater. This is especially true if we consider the extent of complexity in such wastewater streams (in this instance, > 800 different types of chemical constituents are present). Nevertheless, for those that can be *in-silico* characterized, the percentage of chemicals that are mutagenic comprise of a relatively lower percentage than the non-mutagenic ones. This observation is likely because medications detected in the wastewater stream are mostly ingested by patients, and any medications that belong to this category would have to go through approval from Food and Drug Authority to ensure minimal risk of deleterious health impact (Ciociola et al., 2014).

Although *in-silico* characterization assigned the majority of the chemicals to be inconclusive and/or non-mutagenic, *in-vitro* characterization is, in general, well-aligned with the *in-silico* data and showed that almost all, except for four samples, are non-mutagenic. This observation conflicted with studies that reported that hospital wastewater is mutagenic in the majority of their samples. Sharma et al. showed that wastewater from hospitals could be highly mutagenic with a mutagenic rate between 23.13 ± 0.18 and 42.25 ± 0.35 folds increase for TA98 and TA100, respectively. It also demonstrated that proper treatment of hospital wastewater could lower the extent of mutagenicity rate to 3.75 ± 0.35 for TA100 (Sharma et al., 2015). The difference in observation may be due to the earlier studies sampling wastewater from general hospitals that provide treatment for a broader suite of ailments and diseases. The diversity of medications and disinfectants used in general hospitals may be higher than that of a specialty hospital like Al-Amal hospital. As mentioned by Zhang et al., the type of chemicals found in the wastewater depends on what type of hospital (e.g., specialty or general hospital) (Zhang et al., 2020).

A small number of Al Amal hospital wastewater samples, particularly those collected coincidentally at around the same date range (22nd to 29th) of April, May, and June 2020 showed an either mutagenic or toxic effect on *Salmonella* TA strains. The reason behind this trend cannot be easily explained. However, it was observed that the percentage of chemicals that were categorized to mutagenic compounds were higher during those time range of April compared to the earlier days of the same month (Figure 2). Another potential explanation to account for the mutagenicity effect may be associated with ROS production, which was previously shown to be correlated with mutagenicity and number of TA revertants (Mantilla-Calderon et al., 2019). The ROS production capacity associated with each sample was not studied here and future studies should attempt to determine if the level of ROS produced may account for the mutagenic and toxic effect of these samples. In addition, as this study only characterized the presence of chemical constituents in a qualitative non-targeted manner, it cannot exclude the possibility that certain chemical constituents that induce mutagenic effects are present in higher concentrations relative to the non-mutagens.

For those Al Amal wastewater samples that demonstrate mutagenicity, at least 10x concentration is needed. Hence, although the frequency of contributing mutagenicity effect to the bulk water is low, there may be a need to monitor for potential detrimental impact on the activated sludge (AS) processes in the downstream biological wastewater treatment plants. AS systems that are operated with long sludge retention time, SRT, (e.g. 20 to 40 days) may bioaccumulate the chemical contaminants to a level that induces mutagenicity within the sludge blanket. AS systems with longer SRT also have higher mixed liquor suspended solids concentration (i.e., biomass) that can favor horizontal gene transfer frequency. This increase in horizontal gene transfer was noted in a study of the effects of non-antibiotic pharmaceuticals on conjugation rate. The study

demonstrated that the conjugation rate in *E. coli* LE392 increased with the increase in ROS generation caused by the pharmaceuticals. It caused an up to 8-fold increase (at 50 mg ibuprofen/L) in conjugation rate. The paper also noted that the tested pharmaceuticals promoted the increase in ROS generation and that when an ROS scavenger, thiourea, was added, the rate of conjugation declined for all pharmaceuticals (Wang et al., 2021). In addition, although only one sample (e.g. 22 June) was determined to induce toxic effect at 10x and 20x concentration, there may be a potential, albeit low possibility, to detrimentally affect COD, nutrient and organic micropollutants via biodegradation by the AS with longer SRT (Falås et al., 2016; Majewsky et al., 2011).

This study demonstrated a non-targeted approach to characterize the chemical constituents present within wastewater generated by a specialty hospital. The use of databases and literature can serve as a way to *in-silico* assess the overall mutagenicity profile of the wastewater. In general, although *in-silico* characterization resulted in a large number of inconclusive data, the wet-bench experiments also concur with the *in-silico* results that the majority of the samples did not induce mutagenicity unless present at a high concentration of at least 10x. In those instances, there may be a likelihood of detrimentally impacting the downstream biological wastewater treatment processes. Hence, an on-site treatment at the point of generating hospital wastewaters may be an option to minimize such concerns.

6. Conclusion

In this study, the chemical composition of hospital wastewater was identified, and the majority of the chemical components were medications, followed by illicit drugs. As shown in the study, the mutagenic characterization showed that both pharmaceuticals and illicit drugs are not well understood when it comes to mutagenic effects. This mutagenic effect was only observed in 4 samples when they were concentrated by at least 10x. Moreover, there was 0.4-0.6 fold decrease in *Salmonella* cell numbers when June 22 samples were present, in which the sample exhibited toxicity when present in a high concentration of at least 10x. Both effects can be harmful to the downstream wastewater treatment processes. This study's results have demonstrated that a specialty hospital like Al Amal does not contribute substantially to mutagenic wastewater streams to the municipal sewer, and hence unlikely to significantly perturb the downstream biological treatment processes. However, there may still be a need to consider the ad-hoc contributions of mutagenic and/or toxic wastewater streams from the hospitals.

7. Future work

This study is limited by the lack of quantification step. To better understand the mutagenic effects of the identified contaminants, it is essential not only to identify chemicals that were present in wastewater but also to quantify their concentrations. Some mutagens can have a cytotoxic effect at low concentrations. Another critical step is to understand the ROS generation profile of the samples. As mentioned, ROS generation causes mutagenicity by inducing DNA damage and the SOS repair mechanism; hence the understanding of ROS generation can be essential in further understanding the mutagenicity of the hospital wastewater. Finally, it is crucial to understand the effect of that wastewater on the bioreactors in wastewater treatment plants. Although some effects were studied for micropollutants, the effects of hospital wastewater streams that contain a plethora of chemicals are not well understood.

8.References

Augsburger, N., Mantilla-Calderon, D., Daffonchio, D., & Hong, P.-Y. (2019). Acquisition of Extracellular DNA by Acinetobacter baylyi ADP1 in Response to Solar and UV-C254nm Disinfection. *Environmental Science & Technology*, *53*(17), 10312-10319. https://doi.org/10.1021/acs.est.9b01206

Chai, Q., Hu, A., Qian, Y., Ao, X., Liu, W., Yang, H., & Xie, Y. F. (2018). A comparison of genotoxicity change in reclaimed wastewater from different disinfection processes. *Chemosphere*, *191*, 335-341. https://doi.org/https://doi.org/10.1016/j.chemosphere.2017.10.024

Ciociola, A. A., Cohen, L. B., Kulkarni, P., Kefalas, C., Buchman, A., Burke, C., . . . Fang, J. (2014). How drugs are developed and approved by the FDA: current process and future directions. *Official journal of the American College of Gastroenterology| ACG*, *109*(5), 620-623.

del Álamo, A. C., Gonzalez, C., Pariente, M., Molina, R., & Martinez, F. (2020). Fenton-like catalyst based on a reticulated porous perovskite material: activity and stability for the on-site removal of pharmaceutical micropollutans in a hospital wastewater. *Chemical Engineering Journal*, *401*, 126113.

Diwan, V., Tamhankar, A. J., Aggarwal, M., Sen, S., Khandal, R. K., & Lundborg, C. S. (2009). Detection of antibiotics in hospital effluents in India. *Current Science*, 1752-1755.

Díaz-Cruz, M. S., López de Alda, M. J., López, R., & Barceló, D. (2003). Determination of estrogens and progestogens by mass spectrometric techniques (GC/MS, LC/MS and LC/MS/MS). *Journal of Mass Spectrometry*, *38*(9), 917-923.

Falås, P., Wick, A., Castronovo, S., Habermacher, J., Ternes, T. A., & Joss, A. (2016). Tracing the limits of organic micropollutant removal in biological wastewater treatment. *Water research*, *95*, 240-249.

Grebe, S. K., & Singh, R. J. (2011). LC-MS/MS in the clinical laboratory–where to from here? *The Clinical biochemist reviews*, *32*(1), 5.

Grúz, P., Shimizu, M., Sugiyama, K.-i., Yamada, M., & Honma, M. (2020). Effect of episomally encoded DNA polymerases on chemically induced mutagenesis at the hisG46 target in Ames test. *Genes and Environment*, *42*(1), 1-12.

Health, C. D. o. P. (2014). Regulations related to recycled water. In: California Department of Public Health.

Hübschmann, H.-J. (2015). *Handbook of GC-MS: fundamentals and applications*. John Wiley & Sons.

Isidori, M., Lavorgna, M., Nardelli, A., Pascarella, L., & Parrella, A. (2005). Toxic and genotoxic evaluation of six antibiotics on non-target organisms. *Science of the total environment*, *346*(1-3), 87-98.

Itzel, F., Jewell, K. S., Leonhardt, J., Gehrmann, L., Nielsen, U., Ternes, T. A., . . . Tuerk, J. (2018). Comprehensive analysis of antagonistic endocrine activity during ozone treatment of hospital wastewater. *Science of the total environment*, *624*, 1443-1454.

Jiang, J.-Q., Zhou, Z., & Sharma, V. (2013). Occurrence, transportation, monitoring and treatment of emerging micro-pollutants in waste water—a review from global views. *Microchemical Journal*, *110*, 292-300.

Jiménez, B., & Asano, T. (2008). *Water reuse: An international survey of current practice, issues and needs*. IWA London.

Jongen, W., Alink, G., & Koeman, J. (1978). Mutagenic effect of dichloromethane on Salmonella typhimurium. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *56*(3), 245-248.

Kazakevich, Y. V., & Lobrutto, R. (2007). *HPLC for pharmaceutical scientists*. John Wiley & Sons.

Kołecka, K., Gajewska, M., Cytawa, S., Stepnowski, P., & Caban, M. (2020). Is sequential batch reactor an efficient technology to protect recipient against non-steroidal anti-inflammatory drugs and paracetamol in treated wastewater? *Bioresource Technology*, *318*, 124068.

Li, X., Gu, A. Z., Zhang, Y., Xie, B., Li, D., & Chen, J. (2019). Sub-lethal concentrations of heavy metals induce antibiotic resistance via mutagenesis. *Journal of hazardous materials*, *369*, 9-16.

Majewsky, M., Gallé, T., Yargeau, V., & Fischer, K. (2011). Active heterotrophic biomass and sludge retention time (SRT) as determining factors for biodegradation kinetics of pharmaceuticals in activated sludge. *Bioresource technology*, *102*(16), 7415-7421.

Mantilla-Calderon, D., Plewa, M. J., Michoud, G., Fodelianakis, S., Daffonchio, D., & Hong, P.-Y. (2019). Water Disinfection Byproducts Increase Natural Transformation Rates of Environmental DNA in Acinetobacter baylyi ADP1. *Environmental Science & Technology*, *53*(11), 6520-6528. https://doi.org/10.1021/acs.est.9b00692

Maron, D. M., & Ames, B. N. (1983). Revised methods for the Salmonella mutagenicity test. *Mutation Research/Environmental Mutagenesis and Related Subjects*, *113*(3-4), 173-215.

Mayoudom, E. V. T., Nguidjoe, E., Mballa, R. N., Tankoua, O. F., Fokunang, C., Anyakora, C., & Blackett, K. N. (2018). Identification and quantification of 19

pharmaceutical active compounds and metabolites in hospital wastewater in Cameroon using LC/QQQ and LC/Q-TOF. *Environmental monitoring and assessment*, *190*(12), 1-10.

Mendoza, A., Aceña, J., Pérez, S., De Alda, M. L., Barceló, D., Gil, A., & Valcárcel, Y. (2015). Pharmaceuticals and iodinated contrast media in a hospital wastewater: a case study to analyse their presence and characterise their environmental risk and hazard. *Environmental research*, *140*, 225-241.

Meza, L. C., Piotrowski, P., Farnan, J., Tasker, T. L., Xiong, B., Weggler, B., . . . Burgos, W. D. (2020). Detection and removal of biologically active organic micropollutants from hospital wastewater. *Science of the Total Environment*, *700*, 134469.

Mortelmans, K. (2019). A perspective on the development of the Ames Salmonella/mammalian-microsome mutagenicity assay. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *841*, 14-16.

Neemann, J., Colston, J., Krasner, S., Law, I., & Whitson, A. (2020). *Enchanced Source Control Recommendations for Direct Potable Reuse in California*. N. W. R. I. (NWRI). https://www.waterboards.ca.gov/drinking_water/certlic/drinkingwater/docs/dpr-esc-2020.pdf

Paulus, G. K., Hornstra, L. M., Alygizakis, N., Slobodnik, J., Thomaidis, N., & Medema, G. (2019). The impact of on-site hospital wastewater treatment on the downstream communal wastewater system in terms of antibiotics and antibiotic resistance genes. *International journal of hygiene and environmental health*, *222*(4), 635-644.

Sharma, P., Mathur, N., Singh, A., Sogani, M., Bhatnagar, P., Atri, R., & Pareek, S. (2015). Monitoring hospital wastewaters for their probable genotoxicity and mutagenicity. *Environmental monitoring and assessment*, *187*(1), 1-9.

Tabet, M., Abda, A., Benouareth, D. E., Liman, R., Konuk, M., Khallef, M., & Taher, A. (2015). Mutagenic and genotoxic effects of Guelma's urban wastewater, Algeria. *Environmental monitoring and assessment*, *187*(2), 1-13.

Tinidazole. Drugbank. https://go.drugbank.com/drugs/DB00911

Trussell, R. S., Pecson, B. M., Pisarenko, A. N., Idica, E. Y., Howe, E. W., & Trussell, R. R. (2018). *Demonstrating redundancy and monitoring to achieve reliable potable reuse*. T. W. R. Foundation. https://www.waterrf.org/

Tsikas, D., & Zoerner, A. A. (2014). Analysis of eicosanoids by LC-MS/MS and GC-MS/MS: a historical retrospect and a discussion. *Journal of Chromatography B*, *964*, 79-88.

Wang, Q., Wang, P., & Yang, Q. (2018). Occurrence and diversity of antibiotic resistance in untreated hospital wastewater. *Science of the Total Environment*, *621*, 990-999.

Wang, Y., Lu, J., Engelstädter, J., Zhang, S., Ding, P., Mao, L., . . . Guo, J. (2020). Non-antibiotic pharmaceuticals enhance the transmission of exogenous antibiotic resistance genes through bacterial transformation. *The ISME Journal*, *14*(8), 2179-2196. https://doi.org/10.1038/s41396-020-0679-2

Wang, Y., Lu, J., Zhang, S., Li, J., Mao, L., Yuan, Z., . . . Guo, J. (2021). Non-antibiotic pharmaceuticals promote the transmission of multidrug resistance plasmids through intra-and intergenera conjugation. *The ISME Journal*, 1-16.

Ye, Y., Weiwei, J., Na, L., Mei, M., Donghong, W., Zijian, W., & Kaifeng, R. (2014). Assessing of genotoxicity of 16 centralized source-waters in China by means of the SOS/umu assay and the micronucleus test: initial identification of the potential genotoxicants by use of a GC/MS method and the QSAR Toolbox 3.0. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *763*, 36-43.

Yilmaz, G., Kaya, Y., Vergili, I., Gönder, Z. B., Özhan, G., Celik, B. O., . . . Tuerk, J. (2017). Characterization and toxicity of hospital wastewaters in Turkey. *Environmental monitoring and assessment*, *189*(2), 55.

Zaouri, N., Cheng, H., Khairunnisa, F., Alahmed, A., Blilou, I., & Hong, P.-Y. (2021). A type dependent effect of treated wastewater matrix on seed germination and food production. *Science of The Total Environment*, *769*, 144573. https://doi.org/https://doi.org/10.1016/j.scitotenv.2020.144573

Zhang, X., Yan, S., Chen, J., Tyagi, R., & Li, J. (2020). Physical, chemical, and biological impact (hazard) of hospital wastewater on environment: presence of pharmaceuticals, pathogens, and antibiotic-resistance genes. In *Current Developments in Biotechnology and Bioengineering* (pp. 79-102). Elsevier.

Zhang, Y., Gu, A. Z., Xie, S., Li, X., Cen, T., Li, D., & Chen, J. (2018). Nano-metal oxides induce antimicrobial resistance via radical-mediated mutagenesis. *Environment international*, *121*, 1162-1171.

Zhang, Z., & Zhou, J. (2007). Simultaneous determination of various pharmaceutical compounds in water by solid-phase extraction–liquid chromatography–tandem mass spectrometry. *Journal of Chromatography A*, *1154*(1-2), 205-213.

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