Microbial community structure of activated sludge in treatment plants with different seasons

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Abstract: Biological treatment is based on activated sludge that shows filamentous bulking with change in temperature, which has effect on wastewater treatment process. The aim of study focuses on variation microbial (Bacteria- Fungi) in the activated sludge in two seasons and affects them on treatment of wastewater. The wastewater samples were collected in October-November of 2018 and March–April in 2019 during (autumn - spring) from (old project - second expansion - third expansion, Al-Rustmiya). The results showed 418 isolates representing 9 fungal species screened from wastewater samples during two consecutive monthly surveys seasons (for October-November, 2018 and (March-April, 2019). Results showed that fungal number varied through the survey months. The highest number was 112 isolates. Also, presence of more fungus was there in the old Al-Rustmiya plant, second expansion Al-Rustmiya plant, especially in April, despite the lack of dominance, where Aspergillus flavus species predominated by 40%. For all projects the highest isolation count found was in April (143 isolates). All projects (old Al-Rustmiya plant and second, third expansion Al-Rustmiya plant) show increase in sludge volume index due to the high fungi, especially the filaments fungus. In addition, four types of bacteria were isolated; Escherichia coli, Pseudomonas aeruginosa, shigella sp. and Klebsiella sp. Also observed during statistical analyses program was correlation between phosphate and Nitrate, Aspergillus tamarri, as well as the relationship between phosphate and Biochemical Oxygen Demand Fusarium solani Aspergillus Tamari, Nitrate relationship with Total Dissolve Solid. Although, the sludge volume index is high but it is close to specification and does not indicate about sludge bulking.

Keyword
Al-Rustmiya station, Biological treatment, Fungal taxa colonization, Properties of wastewater, Seasons.

1. Introduction
The complex microbial community is the basis for the treatment of pollutants in Wastewater treatment plants (WWTPs), and is widely applied for the removal of contaminants from wastewater, which is disposed to the natural environment (Jaranowska, 2013). Activated sludge (AS) consists of microorganisms, whether aerobic or anaerobic, such as protists, archaea, fungi, and bacteria, which can degrade organic matter such as benzopyrene, toluene and petroleum products (Seviour and Nielsen, 2010). In the case of suitable and effective conditions for activated sludge, that removes 85-95% of the solids and that reduces biochemical oxygen demand (BOD), and the removal efficiency depends on the climate and on the characteristics of wastewater, the entry of toxic waste into this system caused the disruption of biological activity. This toxicity resulting from the collection of
industrial waste in the sanitary wastes caused aesthetic problems and therefore, the industrial waste must be treated from toxic chemical waste before it enters the biological treatment (Dan, 2014). There are five major groups of microorganisms generally found in the aeration basin of the activated sludge process: 1. Bacteria-Aerobic bacteria removes organic nutrients: 2. Protozoa-Removes dispersed bacteria and suspended particles: 3. Metazoa-Dominate longer age systems including lagoons: 4. Filamentous bacteria-bulking sludge (poor settling and turbid effluent): 5. Algae and fungi-Fungi is present with pH changes and older sludge (Dan, 2014). During the chemical composition of nutrients, the final choice is made for the dominant bacterial families, where feces are the main source of bacteria in wastewater (Arumugam et al., 2011; Nam et al., 2011; Wu et al., 2011; Yatsunenko et al. 2012). The biomass produced during fungal wastewater treatment has, potentially, a much higher value than that from the bacterial activated sludge process. (Sindhuja, et al. 2010). Fungi are able to colonize, multiply and survive in diversified habitat that makes them ubiquitous and cosmopolitan. Geographic location, climatic conditions, microhabitat, substrate type, distribution of fauna and flora are the significant factors contributing to fungal distribution and diversity (Manoharachary et al., 2005). Various studies also previously demonstrated the presence of fungi in both groundwater and surface water (Hageskal et al., 2006- and Hageskal et al.2007- Pereira et al. 2009).

Filamentous fungi from genus *Aspergillus* were previously detected in wastewater treatment plants (WWTP) as being *Aspergillus flavus* (*A. flavus*), an important toxigenic fungus producing aflatoxins (Viegas et al., 2014), but their excessive growth leads to an increase in the level of foam and foaming. Specific filamentous fungi (FF) have been recognized for sludge treatment and possibly these strains can be utilized for simultaneous bioflocculation, solids and pathogens reduction and, removal and degradation of toxic compounds. The fungi have greater rates than bacteria to perform denitrification (More et al., 2010). Two of the most common technical problems in running a bioreactor in WWTP. Nematode bacteria primarily consume carbohydrates and excrete many external enzymes, for example, ketinase, glucuronides, and galactosidase (Kragelund et al., 2007). The value of sludge volume index (SVI) during 2017 in inlet of Al Rastamiya WWTPs treat domestic wastewater were 150 ml/g, but SVI for outlet old Al-Rustmiya plant (R0)= 152 ml/g, second extension Al-Rustmiya plant (R2) = 147 ml/g, and third extension Al-Rustmiya plant (R3) = 131ml/g. The average SVI= 141 ml/g (Al-Hussainy et al., 2017) is considered as normal sludge [Al-Hussainy et al., 2017]. The purpose of this study was to analyze the presence and diversity of bacteria and fungi from activated sludge samples in three sewage treatment plants in southeastern Baghdad in the winter and spring seasons. Dilution technique was used for isolating and studying the fungal populations. In addition, study the properties of wastewater and its impact on bacteria and fungi. This study facilitates the understanding of the macrobiotic-performance of the Fungal -activated sludge in the Rastamiya plants.

2. Materials and Methods

2.1 Description of WWTPs and Sample Collection

Al-Rustamiya Project is the largest domestic and industrial waste treatment plant in south east of Baghdad city, with suspended growth process and high capacity but not sufficient to treat all waste water coming to this project, this project has three parts, old project (R0), second extension (R2), and third extension (R3). The discharge of R0 project is 175m3/day. The discharged of R2 is 375m3/d but, a 25% of these discharges exceed the capacity of the project and was thrown directly to Diyala River without treatment, in addition, the discharge of R3 is 450m3/day but a 50% of these discharges exceed the capacity of the project and was thrown directly to Diyala River without treatment.

Activated sludge samples were collected from the aeration tanks from R0, R2, R3 stations, located southeast of Baghdad in central Iraq in same manner that was used in study of Al-Hussainy, 2017, that is shown in Figure 1. In two seasons (Autumn, Spring) (4 months) starting from October- November, 2018 and March- April, 2019. Samples for microbial analysis were stored in the laboratory at -4 °C and were sent to a microbial laboratory. Wastewater characteristics were measured according to
standard method (APHA, 1998) are also illustrated in Table 1.

| parameter | Standard | parameter | Standard | parameter | Standard |
|-----------|----------|-----------|----------|-----------|----------|
| BOD       | APHA 5210B | TDS       | 2540-D   | Temperature | 2550    |
| DO        | APHA 4500 –O G | NO3       | APHA 4500 NO₃⁻ | pH   | APHA 4500- HB |
| EC        | 2540-E   | PO4       | ASTM D 511 –03 B | SVI | 2710 D |

Figure (1): location of old, second and third expansion of Al-Rustamiya WWTPs

2.2 Screening of fungal population from wastewater samples:
In order to study the Microbial population in wastewater, fungi were isolated from different samples. In this study, the direct plating technique was used for isolating and studying the fungal and bacterial populations. One milliliter of each samples were distributed in a series of sterile Petri-dishes. A volume of 50 ml of potato-dextrose agar medium was melted and cooled down to 45°C, poured into each plate followed by gentle brisk rotation of the plate to secure homogenous distribution of the sample. These plates were then incubated at 28 °C± 2 for 5-7 days. The growing colonies were counted, sub cultured on Dox's medium and identified. Total counts of isolates (colony forming units, CFU /cm²) relative density of each species to the total count (%) were calculated. The fungal species were identified using morphological and microscopic features.

2.3 Identification of fungi:
Identification of the genera and species isolated from the leaf, stem, root and rhizosphere of straw berry were carried out using the following reference methods: 1- Barnett and Hunter, (1972) for the genera of imperfect fungi:- 2- Barron, (1968) for the genera of Hypomycetes:- 3- Booth, (1971) for Fusarium species:- 4- Kendrick, (1971) for imperfect fungi:- 5- Laurence, (1989) for Aspergillus and Penicillium:- 6- Raper and Fennell, (1965) for the genus Aspergillus.

2.4 Maintenance of fungal cultures
Fungal cultures were maintained on Dox's agar composed in (g/ l) (Pitt and Hocking, 1985) as shown in Table 2.
Table 2: the Dox’s agar composed

| Composed of Dox’s agar | Quantity (g/l) |
|------------------------|----------------|
| Sucrose                | 30.0           |
| NaNO3                  | 3.0            |
| KH2PO4                 | 1.0            |
| MgSO4.7H2O             | 0.5            |
| KCL                    | 0.5            |
| FeSO4.7H2O             | 0.01           |
| Agar                   | 20.0 (For solid medium) |
| Distilled H2O          | 1 Liter        |

2.5 Statistical analysis

Analysis of experiments were set up with three replicates. Means were compared using the least significant difference (LSD) at the 5% probability level; standard deviation (SD) was calculated and represented in Tables 3 and 4.

3. Results and Dissociation

Total fungal count of 418 isolates constituting 9 species were screened from different wastewater samples during four monthly surveys (October, November, March and April 2019). Results showed that the total fungal count varied among the four survey months. The highest count (143 isolates) was obtained at the Forth isolation month (April) while the lowest count (74 isolates) was at the first isolation month (October) (Table 3). Furthermore, samples showed different percentage of fungal taxa colonization. R2 was colonized by the highest fungal taxa (205 isolates) representing 48.9% of the total count, followed by R0 and R3 that were colonized by 131 and 87 isolates representing 31.3%, and 20.8% of the total counts, respectively.

Table (3): Fungal count, diversity and relative density of species isolated from wastewater samples

|          | R0     | R2     | R3     | All Samples |
|----------|--------|--------|--------|-------------|
| Tc / 4 surveys | 13.9  | 20.6   | 31.3   | 31.3        |
| Oct      | 21     | 7      | 43     | 43          |
| Nov      | 35     | 7      | 54     | 54          |
| Mar      | 27     | 9      | 39     | 39          |
| Apr      | 43     | 9      | 69     | 69          |
| Tc        | 43     | 9      | 39     | 39          |

Tc: Total fungal count
RD: Relative density (%)

Considering the fungal species isolated from different water samples, results showed that there were no fungal species detected with high dominance in the screened samples. Consequently, the relative density ranged from 40 % to 100 % remain blank. A niger was the most frequent fungus observed which had been recorded in all surveys with relative density of 34.8 at the R0, followed by Rhizoctonia solani (with 20 % relative density). The other species were arranged, descendingly, according to their relative densities as: A. flavus (17.6 %), Aspergillus tamarii (11.5 %), A. sulphorius (9.6 %), P. cyclopium (9 %), Penicillium notatum (7.6 %), Rhizopus nigricans (8.2 %) and Fusarium solani (5.1 %), as shown in Table 4.
Table 4: Identification and relative density of isolated fungi species

| Relative density (%) | R0 Fungal species | R.D (%) | R2 Fungal species | R.D (%) | R3 Fungal species | R.D (%) |
|----------------------|-------------------|---------|-------------------|---------|-------------------|---------|
| 50 – 100             | -                 | -       | -                 | -       | -                 | -       |
| 40 – 50              | -                 | -       | -                 | -       | -                 | -       |
| 30 – 40              | Aspergillus niger | 34.8    | -                 | -       | -                 | -       |
| 20 – 30              | Rhizoctonia solani| 20.0    | Aspergillus niger | 26.9    | -                 | -       |
| 10 – 20              | A. flavus         | 17.6    | A. tamarii        | 11.5    | A. flavus         | 10.3    |
|                      |                   |         |                   |         |                   |         |
| 5 – 10               | As. sulphorius    | 9.6     | As. flavus        | 8.0     | A. sulphorius     | 6.1     |
|                      | As. tamarii       | 8.9     | A. sulphorius     | 9.0     | A. flavus         | 5.1     |
|                      | F. solani         | 5.0     | F. solani         | 5.0     | F. solani         | 5.1     |
|                      | P. cyclopium      | 6.6     | P. cyclopium      | 9.0     | P. cyclopium      | 7.1     |
|                      |                   |         |                   |         |                   |         |
| 5%                   | -                 | -       | Pe. notatum       | 4.0     | R. solani         | 4.1     |

Fungal population was affected by the survey month, low temperature in October was associated with lower fungal isolation than in hot temperature in April. The genus Aspergillus was more predominant and the genus was observed in a broad range of habitats principally in soils and decaying vegetation. Species of Aspergillus are important both medically and commercially (Goldman and Osmani, 2008). The density and diversity of the fungal communities in waterbodies are governed by physicochemical factors like temperature, hydrogen-ion concentration, oxygen content, dissolved organic and inorganic matter, concentration of ions like phosphate, sulphate etc. (Jan et al., 2014) which reflects local environmental conditions. It can be concluded that the seasonal differences in water chemistry is the reason for the differences in colony count and number of isolates (Thomas and Thengave, 2017). The fungi colony is shown in Figure 2: deep of sample showed different percentage of fungal taxa colonization. The most frequent species, which had been recorded in all surveys, was Aspergillus niger with a total count of 58 isolates, representing, relative density of 34.8 %, followed by Rhizoctonia solani (34 isolates with 20.0 % relative density). The other species were arranged, descendingly, according to their relative densities as: Aspergillus tamarii (12.6 %), Penicillium notatum (12.0 %), A. sulphorius (9.8 %), A. flavus (9.5 %) P. cyclopium (9.0 %), Fusarium solani (5.0 %) and Rhizopus nigricans (1.84 %) in addition to four genus of bacteria that were isolated are Escherichia coli, Pseudomonas aeruginosa, shigella sp. and Klebsiella sp.

Figure 2: fungi colony:  a) first isolation with different colony  b) Fusarium solani  c) P. notatum
The fungi are significantly affected by temperature, where there is more presence of fungus in April with higher temperature but, lowest in low temperature in March, as shown in Figure 3. The highest values of the TC are in April for all stations, the R2 project have higher fungus value than R0 and R3, respectively, and R3 project have higher RD values than R0 and R2, respectively. In addition, the degree of interaction is affected where more values of fungus are recorded in April as shown in Figure 4 where more fungi exist.

![Figure 3. Seasonal variation of wastewater temperature.](image1)

![Figure 4. Seasonal variation of wastewater pH.](image2)

The presence of fungi is affected by the increased concentration of phosphate, which is affected by temperature. This corresponds to Akpor, et al. 2013 and found the highest concentration of phosphates in March and April as shown in Figure 5. *Aspergillus niger* was found in R0, R2 projects as the presence of fungi while *A. flavus R. solani* presented in the R3 project. Nitrates decreased with temperature rise, as shown in Figure 6, where it was found that the relationship is inverse with the biochemical oxygen demand while the temperature of the three projects was consistent with that of Al-Qusai, 2012. The highest value of R0 2.0 mg / 2.1 mg / l was for the R2 plant, 1.5 mg / L for the R3 plant.

![Figure 5. Seasonal variation of wastewater Phosphate.](image3)

![Figure 6. Seasonal variation of wastewater Nitrate.](image4)

The results show Filamentous fungi in wastewater treatment plants that have higher R.D% in R0 project then R3 and finally R2. The Filamentous fungi is used to treat the COD, BOD and Lignin concentration of effluent of wastewater (Viegas et al. 2014: Dhanushree and Hina, 2017), and shown in Figure 7, less handling of the BOD in R2 project is due to lack in Filamentous fungi. In Figure 8, the dissolved oxygen was found to be the highest in April due to drifting torrents from Iran, the Rustamiya plant flooded by the Diyala River, mixing the waste with surface water, also affected on the total dissolved solids shown in Figure 9 and on the electrical conductivity shown in Figure 10.
The standard of SVI in activated sludge is 150 and this study showed that SVI have higher value in R0 reach to 158.5 than R3 reach to 155, while the SVI in R2 plant was within the determinants. This indicates the presence of Filamentous in abundance, whether bacteria or fungus, and the conclusion of these results are similar to that found in the study (Al-Hussainy, 2017).

From the SPSS program version 22 correlation matrix shown in Table 4, indicated strong relationships between temperature and A. flavas, P. cyclopium and between SVI and TDS. The strong relationship between PO4 and NO3, A. tamarri, and also the relationship between PO4 and BOD F. solani A. tamarri, NO3 relationship with TDS, Rhizoctonia solani Aspergillus niger, and A. sulphorius, with temperatures, having a strong BOD relationship with PO4 and F. Solani.
Table 5. correlation matrix

|     | T    | TDS | PH | PO₄ | NO₃ | SVL | BOD | Aspergillus niger | Rhizoctonia solani |
|-----|------|-----|----|-----|-----|-----|-----|------------------|------------------|
| T   | Pearson Correlation | Sig. (2-tailed) | .451 | .557 | - .781 | .950 | - .557 | .950 |
| TDS | Pearson Correlation | Sig. (2-tailed) | .702 | .624 | .503 | .429 | .201 | .482 | .624 | .203 |
| PH  | Pearson Correlation | Sig. (2-tailed) | .557 | -.491 | - .909 | .151 | - .992 | .708 |
| PO₄ | Pearson Correlation | Sig. (2-tailed) | .624 | .673 | .873 | .946 | .423 | .894 | .752 | .827 |
| SVL | Pearson Correlation | Sig. (2-tailed) | .704 | .350 | .951 | .941 | .364 | .915 |
| NO₃ | Pearson Correlation | Sig. (2-tailed) | .781 | .909 | -.084 | 1 | .548 | 1 | .954 | .937 |
| BOD | Pearson Correlation | Sig. (2-tailed) | .727 | .096 | .999 | .997 | .954 | .975 |
| Aspergillus niger | Pearson Correlation | Sig. (2-tailed) | .624 | .079 | .752 | .121 | .482 | .203 | .421 |
| Rhizoctonia solani | Pearson Correlation | Sig. (2-tailed) | .950 | .708 | .269 | .937 | .805 | .789 | 1 |
| A. flavus | Pearson Correlation | Sig. (2-tailed) | .997 | .377 | .622 | .728 | .972 | .488 | .921 |
| A. sulphurias | Pearson Correlation | Sig. (2-tailed) | .051 | .754 | .573 | .554 | .481 | .150 | .533 | .675 | .254 |
| A. tamari | Pearson Correlation | Sig. (2-tailed) | .476 | 1.000 | -.466 | -.921 | .179 | .995 | .727 |
| F. solani | Pearson Correlation | Sig. (2-tailed) | .684 | .018 | .691 | .182 | .255 | .885 | .202 | .061 | .482 |
| P. cyclopium | Pearson Correlation | Sig. (2-tailed) | .155 | .952 | -.735 | -.738 | - | .907 | .456 |
| P. notatum | Pearson Correlation | Sig. (2-tailed) | .016 | .819 | .508 | .619 | .546 | .085 | .598 | .740 | .319 |

* Correlation is significant at the 0.05 level (2-tailed).
4. Conclusions
The conclusion of this study about wastewater treatment plants (R0, R2, R3 projects):

a) The SVI in WWTP increases in the value due to the high fungi, especially the filamentous fungus, which helps to treat the organic matter in activated sludge, but harmful effects are there if it increases, in addition to the presence of more fungus in the R0 and R3, especially in April, despite the lack of dominance, where A. flavus species predominated by 40%, and found a direct relationship with phosphates and reverse with BOD.

b) Good relationship between PO4 and NO3, A. tamarri, as well as the relationship between PO4

|       | A. flavus | A. sulphurias | A. tamarri | F. solani | P. cyclopium | P. notatum |
|-------|-----------|---------------|------------|-----------|--------------|-----------|
| T     | Pearson Correlation | .997 | .476 | .155 | -.364 | -.983 | -.624 |
|       | Sig. (2-tailed) | .051 | .684 | .901 | .763 | .116 | .571 |
| N     | 3 | 3 | 3 | 3 | 3 | 3 |
| TDS   | Pearson Correlation | .377 | 1.000 | .952 | -.996 | -.281 | .416 |
|       | Sig. (2-tailed) | .754 | .018 | .199 | .060 | .819 | .727 |
| pH    | Pearson Correlation | .622 | -.466 | -.735 | .571 | -.698 | -.996 |
|       | Sig. (2-tailed) | .573 | .691 | .475 | .613 | .508 | .054 |
| PO4   | Pearson Correlation | -6.45 | -.960 | -.810 | .918 | -.564 | -.115 |
|       | Sig. (2-tailed) | .554 | .182 | .398 | .260 | .619 | .927 |
| NO3   | Pearson Correlation | .728 | .921 | .738 | -.866 | -.655 | .000 |
|       | Sig. (2-tailed) | .481 | .255 | .472 | .333 | .546 | 1.000 |
| SVL   | Pearson Correlation | .972 | .179 | -.160 | -.057 | -.991 | -.836 |
|       | Sig. (2-tailed) | .150 | .885 | .898 | .964 | .085 | .369 |
| BOD   | Pearson Correlation | -.669 | -.950 | -.791 | .904 | .590 | -.083 |
|       | Sig. (2-tailed) | .533 | .202 | .419 | .281 | .598 | .947 |
| Aspergillus niger | Pearson Correlation | .488 | .995 | .907 | -.976 | -.397 | .301 |
|       | Sig. (2-tailed) | .675 | .061 | .277 | .139 | .740 | .806 |
| Rhizoctonia solani | Pearson Correlation | .921 | .727 | .456 | -.637 | -.877 | -.349 |
|       | Sig. (2-tailed) | .254 | .482 | .698 | .560 | .319 | .773 |
| A. flavus | Pearson Correlation | 1 | .403 | .075 | -.288 | -.995 | -.685 |
|       | Sig. (2-tailed) | .736 | .736 | .952 | .814 | .065 | .519 |
| A. sulphurias | Pearson Correlation | .403 | 1 | .943 | -.992 | -.308 | .390 |
|       | Sig. (2-tailed) | .736 | .217 | .078 | .801 | .745 |
| A. tamarri | Pearson Correlation | .075 | .943 | 1 | -.976 | .027 | .675 |
|       | Sig. (2-tailed) | .952 | .217 | .138 | .983 | .528 |
| F. solani | Pearson Correlation | -.288 | -.992 | -.976 | 1 | .189 | -.500 |
|       | Sig. (2-tailed) | .814 | .078 | .138 | .879 | .667 |
| P. cyclopium | Pearson Correlation | -.995 | -.308 | .027 | .189 | .1 | .756 |
|       | Sig. (2-tailed) | .065 | .801 | .983 | .879 | .454 |
| P. notatum | Pearson Correlation | -.685 | .390 | .675 | -.500 | .756 | 1 |
|       | Sig. (2-tailed) | .519 | .745 | .528 | .667 | .454 |
and BOD F. solani A. tamari, NO3 relationship with TDS.

c) The relative density is of 25.5%, and found that the higher isolated Aspergillus niger is 61.7%, but the minimum P. notatum is 7.1%.

d) Finally, the treatment in activated sludge is good and has diversity in bacteria and fungus and is effected with temperature although the value of SVI is high but close to specification and does not indicate about sludge bulking.

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