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Characterisation of indigenous microbial community isolated from wastewater treatment phases Baghdad/Iraq

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Abstract: Biodegradation processes could be efficient for such organic contaminants like *o*-xylene within sewage. Since the biodegradation processes is mainly controlled by microbial communities therefore, this research paper intended that the bioaugmentation process application might speed up or improve biodegradation process in Rustumihia plant. It delivers an initial knowledge of the effects of one of the most complicated organic contaminants at Rustumihia plant. In addition to that, it suggested the using of indigenous microbial communities that is isolated from the treatment plant within the application of bioaugmentation. It reveals findings on the ecology of *o*-xylene degradation via using bacterial communities that were already enriched and isolated from the four important treatment phases of Iraq's Rustumihia plant

Molecular biology analyses were used to show how bacterial diversity changed under various concentrations of *o*-xylene 0.5, 5 and 50 mM, pH value of 7.0 and incubation temperatures of 25°C, 35°C, 45°C and 55°C. The *o*-xylene degradation efficacy was measured after 14-day incubations of 10 ml triplicate microcosms. Chemical analysis demonstrated that Rustumihia microbial community from the four phases was tolerant towards the higher concentrations of *o*-xylene and temperatures at the same time. According to the Hill number, diversity and richness should be considered the increased that is occurred in common and most dominant taxa values. According to the Hill number the evenness values at 50 mM and 55°C, as ${}^{1}D/{}^{0}D$ for combining phase S1=63.3%, activated sludge S2=69%, settlement tanks S3=82% and river discharge S4=58%. These results indicated that Rustumihia microbial community were able to tolerate and degrade *o*-xylene at 55°C, results are supported by GC-FID. The degradation efficiencies were recorded for different *o*-xylene concentrations with the help of the Rustumihia indigenous microbial community. At 35°C, degradation efficacy was the highest amongst the tested temperatures for four phases with recorded results of 64.75%, 82.1%, 60% and 64.2% for S1, S2, S3 and S4 respectively of Rustumihia WWTP. In summary, biodegradation process that is occurred by Rustumihia microbial community proposing that the cooperation between process engineering and microbial ecology may be appropriate and viable for efficient biological wastewater treatment processes in Iraq. For a thorough assessment of the wastewater microbial community's total and functional structure, it is important to apply high-throughput approaches such as next-generation sequencing. Identifying gene regulatory patterns and their functional populations at the translational and transcriptional levels is another important step forward.

1.Introduction: The removal of hydrocarbon from wastewater during the treatment process often focuses on the microbial biodegradation abilities [1; 2; 3; 4 & 5]. Despite the fact that wastewater treatment is a well-established technique, there are still substantial knowledge gaps regarding the composition and structure of the indigenous microbial community in WWTP and their metabolism efficiency of the different organic pollutants [6 & 7]. This chapter provides an overview of the findings concerning microbial ecology and *o*-xylene degradation inside the Rustumihia WWTP in Iraq. The study employed the molecular techniques of DNA extraction, 16S rRNA gene PCR, AGE and DGGE to analyse the dynamics of the microbial community profiles associated with the different temperatures and carbon source concentrations [8 & 9].

A microcosm in an artificial and simplified ecosystem that is used to study ecological processes in a well-controlled environment. The study-microcosm is an established and robust procedure that has been used widely with significant impacts for wastewater research to, for example, control contaminants/organic loads and apply different bioremediation and biodegradation techniques in laboratory and/or pilot scales [1; 10; 11 &12]. Parallel to its economic importance, the transformation of some quantitatively crucial and organically hazardous substances such as petroleum hydrocarbons by microbial community activity, especially *o*-xylene in wastewater treatment systems, is considered as one of the ecosystem services reflected in the ecological indices. However, the

Limitations and key controls of the biological processes (biodegradation) within wastewater treatment plants are still poorly understood [13; 14; 15 & 16], suggested that the dominant microbial community, and its key determining factors, is an important factor that needs to be closely understood especially for heterotrophic microorganisms that might ultimately have an influence on the bioremediation processes used to manage petroleum contaminated environments. Therefore, meeting these requirements make laboratory investigations, using controlled and replicated microcosm, an essential step for knowledge development. In domestic wastewater, the major components of organic matter are proteins, oils, carbohydrates, and fats. Both proteins and carbohydrates are made up of easily biodegradable molecules, in addition to containing small fractions of phenolic compounds, synthetic detergents, herbicides, and pesticides. Based on their concentration, these compounds, in particular, the volatile organic ones, are not biodegradable [17; 18; 19; 20 & 21]. The origin of hydrocarbons comes about as a result of some faulty practices by Iraqi local residents, which have resulted in an increase of hydrocarbon ratios in domestic wastewater. For example, because of the deteriorating economic situation in Iraq, many Iraqi families clean inner car parts, inside houses, with oil products such as benzene in order to save money, and the residual benzene may be poured away via the houses' main sewage systems, which increases the ratio of hydrocarbon contamination, thus resulting in carcinogenicity or foaming. Another source of hydrocarbon compound is the improper disposal of industrial waste directly into river streams [22; 23; 7 & 24]. While in hospital sewage water the situation is different.

From an ecological point of view, Rustumihia WWTP is one of the most important areas of sewage treatment in Baghdad [25; 6 & 7]. The ecological investigations in *situ* are considered as crucial evaluation evidence regarding ecological indices [26; 27 & 28]. Sewage wastewater was subjected to the physical, chemical and biological treatments [24 & 21] since microbial degradation are considered as part of the biological treatments [29; 4 & 30]**.** Therefore, there is a need for further evaluation of the ecological and microbial analysis inside the biological process in Rustumihia WWTP. As a result, the focus is on the ecological indices which were not recognised before as an essential need. Nevertheless, numerous factors including contaminant types, their concentrations, the variations in temperature and pH, determine the indigenous microbial structure for each treatment phase, which might subsequently, affect the heterogeneity of the treatment phase [31; 21 & 32]. Hence, the phenotypic, physiological

and phylogenetic diversities of the real microbial communities are important factors that assess the *in situ* stability because, in the contexts of other microbial ecology, a fully comprehensive analysis of the roles of the microbial community indicator shifts through the biodegradation process is critical for the following applications in real WWTP.

Recently, with the aid of molecular techniques, it is possible to predict the outcomes for an ecosystem as a result of disturbances to its microbial communities [33; 34 & 16]. Hydrocarbon degradation is influenced by both abiotic and biotic factors, which vary according to the treatment phase and the type of pollutant [35 & 4]. Numerous studies compared the types of pollutants and the phases of treatment by using various microbial ecology techniques to categorise more likely the microbial taxa that are related to and/or affected by hydrocarbon degradation [36] and to correlating microbial community composition shifts with wastewater treatment stages [37]. In this study, each combining phase activated sludge, settlement tanks, and river discharge is highlighted, and the use of microbial community composition shifts is proposed to estimate the quality of the microbial community and its activity in *o*-xylene degradation. The molecular techniques are used to evaluate the microbial community structure, dynamics and function in the environment [38]. The shifts in the efficiency of the degradative community in a common effluent treatment plant correlated directly with the shifts in the microbial community [39]. Therefore, the analysis of the environmental parameters that affect the microbial community is important to evaluate the treatment efficiency, particularly in the activated sludge. In addition, [40 & 41] support the idea of using the microbial community as a predictable clock for measuring the efficiency of the microbial quality inside the *situ* in order to investigate the changes in the taxa microbial communities because of the changes in the 16S rRNA gene for the wastewater microbial inhabitants at family and phylum taxonomic levels. They suggested that the changes might be used to evaluate the unique type that participates in the hydrocarbon degradation process inside the biological treatment process in the Rustumihia WWTP.

The current paper research was conducted to test the ability of the Rustumihia model microbial community to degrade *o*-xylene through an assessment of ecological indices and to correlate them with chemical analysis via the overarching research programme in addition to that, the composition and structure of an indigenous microbial community will shift under stressful growth conditions, such as variations in temperature, and *o*-xylene concentration as a carbon source (three different concentrations were added to BMS).

This paper aimed to:
Investigate the ecological indices of indigenous WWTPs microbial community and their *o*-xylene degradation capabilities and role of temperature on the response.

2.Experimental design: The four samples in triplicate for each phase of the Rustumihia WWTP subjected to different *o*-xylene concentrations 0.5, 5, 50 mM, and the chemical analysis that include (Gram staining, Catalase activity, oxidase activity, spore forming test, lipase and protease expression) according to [42] were applied to all samples. The assessment of *o*-xylene concentrations changes was carried out on two steps. First, the liquid phase extraction was done on all samples with volume (10 mL). Samples extracted from the culture of each microbial community or bacterial strain to monitor degradation of *o*-xylene by gas chromatography-flame ionisation detector GC-FID. For each experiment, triplicated samples (diluted with 5 mL of hexane) were run, 5 mL of hexane were added to *o*-xylene feed twice, and the mixture was shaken by hand for 2 min to ensure that phase equilibrium

was reached. Each sample extraction was performed for 15 min with the two phases allowed to settle for 20 minutes. The top aqueous phases of each extraction were transferred into sterile 2.5 mL GC-FID vials and stored at -80°C until needed [43]. And second the chemical analysis includes volatile aromatic GC-FID was used to measure *o*-xylene concentration. The analysis was achieved via split injection with a 2 µL sample volume and 30:1 split ratio. The GC-FID interface was held at 260°C and the injector at 280°C. Initially, the oven temperature was held at 50°C for 1.5 min, then programmed to 290°C at a ramp rate of 7°C/min alongside at 290°C and held for 15 min. In the selected ion mode, the mass selective detector was operated at *m/z* values of 91.1 for xylene. Quantitation of aromatic hydrocarbons was performed by running a series of *o*-xylene standards as prepared from 0.114 mL of *o*-xylene in 100 mL of hexane (v/v). A standard calibration curve for *o*-xylene was then used to estimate the amount of biodegraded hydrocarbon [44] by the 45 individual bacterial isolates and one consortium developed from the four phases, combining (S1), activated sludge tanks (S2), settlement tanks (S3) and discharge to Tigris river (S4) of the Rustumihia wastewater treatment plant. By using a standard curve, calculations are made to measure the degradation of the *o*-xylene was constructed by plotting the concentration on the *x*-axis and related peak area value on the *y*-axis intercept to determine the unknown concentration (*x*-value) from the peak area reading corresponding to the standard curve, the following steps were followed. Using a linear trend-line equation $y = mx+c$, the *x*-coordinate corresponds to the unknown concentration of *o*-xylene to be determined, and y = 1E+07x-59192 was used to calculate the unknown concentrations of *o*-xylene produced from different treatments.

DNA extractions from wastewater samples were performed using FastDNA™ Spin Kit for Soil, according to the manufacturer's instructions. All DNA extracts were stored at -20°C until needed for concentration and purity measurements, agarose gel electrophoresis and PCR amplification.

1. The concentration and purity of DNA were determined by UV spectrophotometry. The ratio of absorbance of A260:A280 was used to assess DNA and RNA purity. A ratio of ~1.8 is generally accepted as a signature of pure DNA; a ratio of ~2.0 is accepted for RNA [45].
2. All the ecological indices were calculated for the four Rustumihia WWTP phases. After conducting the DGGE analysis as follows PCR amplicons (20 µL) were separated on 10% (w/v) polyacrylamide gels (acrylamide/bis-acrylamide gel 37.5:1) with a 30% to 70% and 40% to 70% denaturing gradient [46]. Gels were cast and run on a Biorad DCODE System at 60°C and 110V for 16 hours in a 5X TAE buffer. The gels were stained with SYBR Gold and viewed under UV light by Alpha Imager HP . DGGE data out of community profiles were analysed by Phoretix ^{1}D software. This analysis assumed the number of bands and their volumes equated to bacterial taxa (operational taxonomic units; OTUs) and their relative abundance respectively. This assumption permitted the determination of bacterial diversity, using Hill numbers (^{0}D; ^{1}D; ^{2}D), and evenness, using the Hill ratio ($^{1}D/^{0}D$). Both [47] and[48] suggested Hill numbers are better indices for measures of alpha diversity, since, they observe the replication principal and the determined values carry ecological meaning such that ^{0}D, ^{1}D *and* ^{2}D equate to species richness, the number of common species and the number of dominant species. Similarly, Hill ratios can be used as a measure of evenness and determine the proportion of common ($^{1}D/^{0}D$) or dominant species ($^{2}D/^{0}D$) associated with a community (Equations 1-4).

$$^{q}D = {}^{S}(\sum_{i=1} pi^{q})^{1/[1-q]} \quad \textbf{Equation 1}$$

$$^{0}D = {}^{s}\sum_{i=1} pi^{0} \quad \textbf{Equation 2}$$

$$^{1}D = e^{H} = e\text{-}\,{}^{s}\sum_{i=1} pi^{Lnpi} \quad \textbf{Equation 3}$$

$$^{2}D = 1/\,{}^{s}\sum_{i=1} pi^{2} \quad \textbf{Equation 4}$$

3.Results:

3.1 O-xylene degradation by indigenous microbial community from the WWTP

To assess the potential ability of the indigenous microbial community to degrade *o*-xylene, the wastewater samples from four phases of Rustumihia WWTP were subjected to three different

concentrations of *o*-xylene 0.5, 5 and 50 mM in BMS; the *o*-xylene concentrations were measured using gas chromatography flame ionization detector GC-FID.

The bar charts in Figure 1a, 1b, 1c and 1d show observed changes in *o*-xylene degradation trends by the indigenous microbial community from the four phases of the WWTP with respect to the different temperatures after 14 days of incubation.

In the present study the achieved degradation efficacy of the added 0.5 mM *o*-xylene at 25°C were 22.2%, 88.8%, 50.1% and 66.6% for the combining phase S1, activated sludge S2, settlement tanks S3 and river discharge S4 phases, respectively. At 35°C, degradation efficacy were the highest amongst the tested temperatures as they were 64.75%, 82.1%, 60% and 64.2% for combining phase S1, activated sludge S2, settlement tanks S3, and river discharge S4, respectively. At 45°C degradation efficacy were 21.3%, 33.2%, 33.8% and 33.81% for combining phase S1, activated sludge S2, settlement tanks S3 and river discharge S4, respectively. At 55°C, the degradation efficacy were 62.5%, 25%, 25.2% and 12.5% for combining phase S1, activated sludge S2, settlement tanks S3 and river discharge S4, respectively.

The Figure 2a, 2b, 2c and 2d described a noticeable change in *o*-xylene degradation trends by the indigenous microbial community from the four phases of the WWTP with respect to the different temperatures after 14 days of incubation time. The degradation efficacy of *o*-xylene at 25°C, 35°C, 45°C and 55°C were found to be variable at 5 mM of *o*-xylene for all four different phases.

The degradation efficacy of the added 5 mM *o*-xylene at 25°C were 60%, 20%, 40% and 58.4% for each of combining phase S1, activated sludge S2, settlement tanks S3, and river discharge S4, respectively. At 35°C, 98%, 97.8%, 98.2% and 98.5% efficacy of *o*-xylene degradation were recorded for each of combining phase S1, activated sludge S2, settlement tanks S3, and river discharge S4, respectively. At 45°C, the degradation efficacy were 98.3%, 98.4%, 97.8% and 97.7% for each of combining phase S1, activated sludge S2, settlement tanks S3 and river discharge S4, respectively. At 55°C the degradation efficacies were 95.5%, 96.3%, 98.18% and 97.2% for each of combining phase S1, activated sludge S2, settlement tanks S3 and river discharge S4, respectively.

In Figure 3a, 3b, 3c and 3d, the bar chart demonstrates an interesting change in *o*-xylene degradation trends by the indigenous microbial community from the four phases of the WWTP with respect to the different temperatures after 14 days of incubation time.

The degradation efficacy at the highest *o*-xylene concentration (50 mM) at 25°C were 68.3%, 76.6%, 60% and 33.3% for combining phase S1, activated sludge S2, settlement tanks S3 and river discharge S4, respectively. At 35°C the degradation efficacy were 92.85%, 96.19%, 92.4% and 47.61% for combining phase S1, activated sludge S2, settlement tanks S3 and river discharge S4, respectively. At 45°C the degradation efficacy were 95.12%, 95.6%, 94.9% and 26.8% for combining phase S1, activated sludge S2, settlement tanks S3 and river discharge S4, respectively. At 55°C the degradation efficacy were 57.9%, 47.3%, 52.6% and 36.8% for combining phase S1, activated sludge S2, settlement tanks S3 and river discharge S4, respectively.

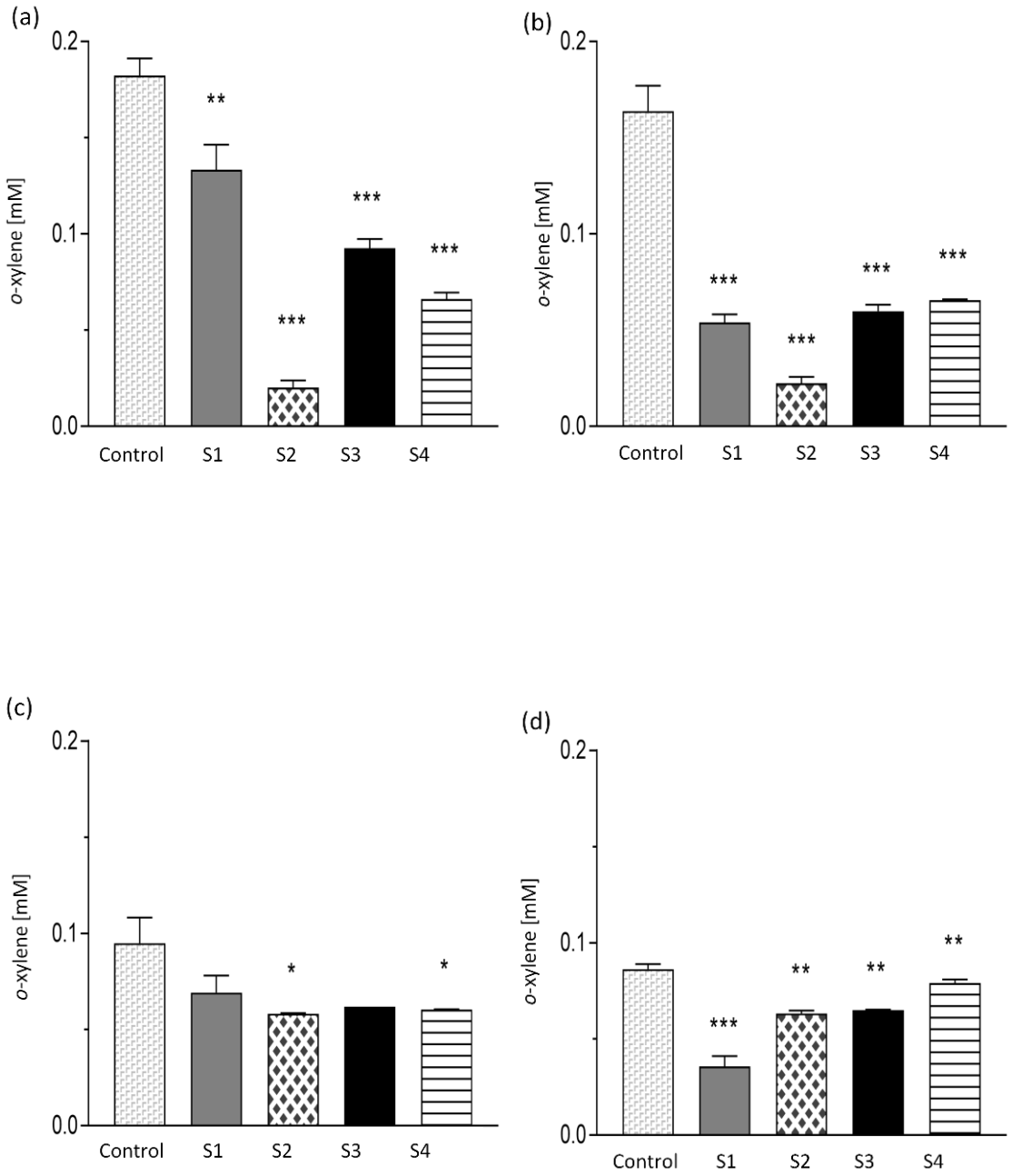

Figure 1: Efficacy of *o*-xylene degradation by indigenous microbial community for each treatment phase and control with (n=3), (abiotic control / without any microbial community, combining phase / S1, activated sludge / S2, settlement tanks / S3 and river discharge / S4). All samples subjected to 0.5 mM of *o*-xylene and different temperatures ((a) 25°C, (b) 35°C, (c) 45°C and (d) 55°C), incubated at 150 rpm for 14 days.

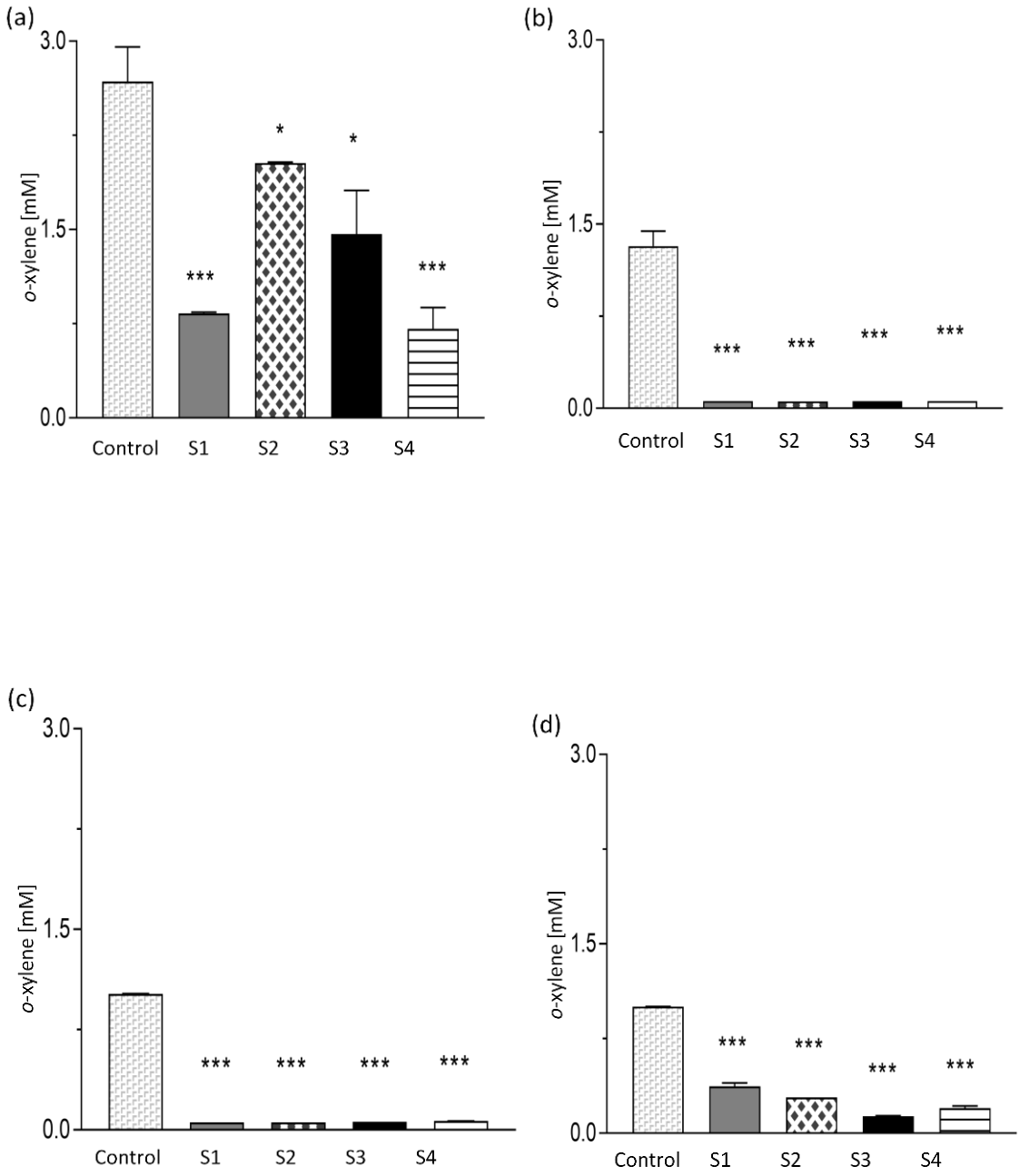

Figure 2: Efficacy of *o*-xylene degradation by indigenous microbial community for each treatment phase and control with (n=3), (abiotic control / without any microbial community, combining phase / S1, activated sludge / S2, settlement tanks / S3 and river discharge / S4). All samples subjected to 5 mM of *o*-xylene and different temperatures ((a) 25°C, (b) 35°C, (c) 45°C and (d) 55°C), incubated at 150 rpm for 14 days.

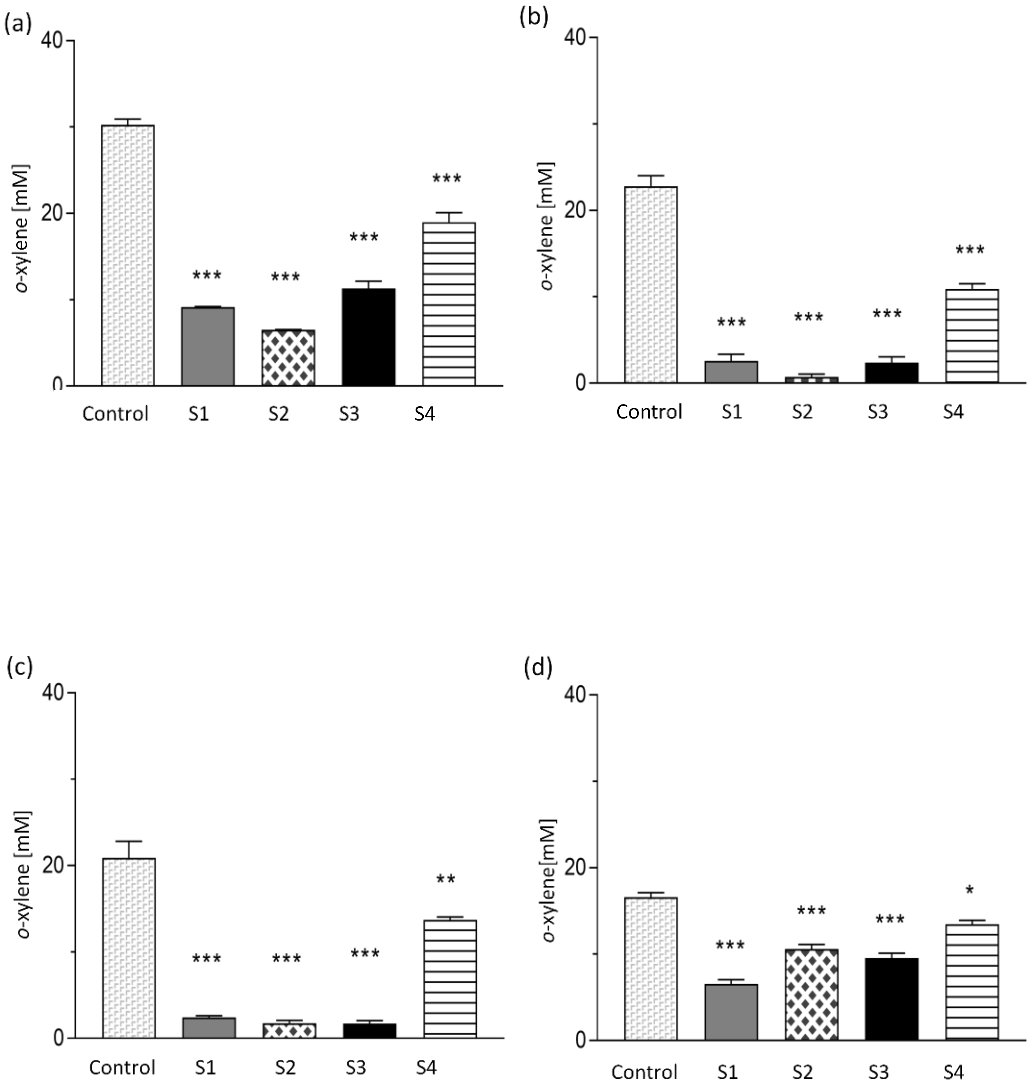

Figure 3: Efficacy of *o*-xylene degradation by indigenous microbial community for each treatment phase and control with (n=3), (abiotic control / without any microbial community, combining phase / S1, activated sludge / S2, settlement tanks / S3 and river discharge / S4). All samples subjected to 50 mM of *o*-xylene and different temperatures ((a) 25°C, (b) 35°C, (c) 45°C and (d) 55°C), incubated at 150 rpm for 14 days.

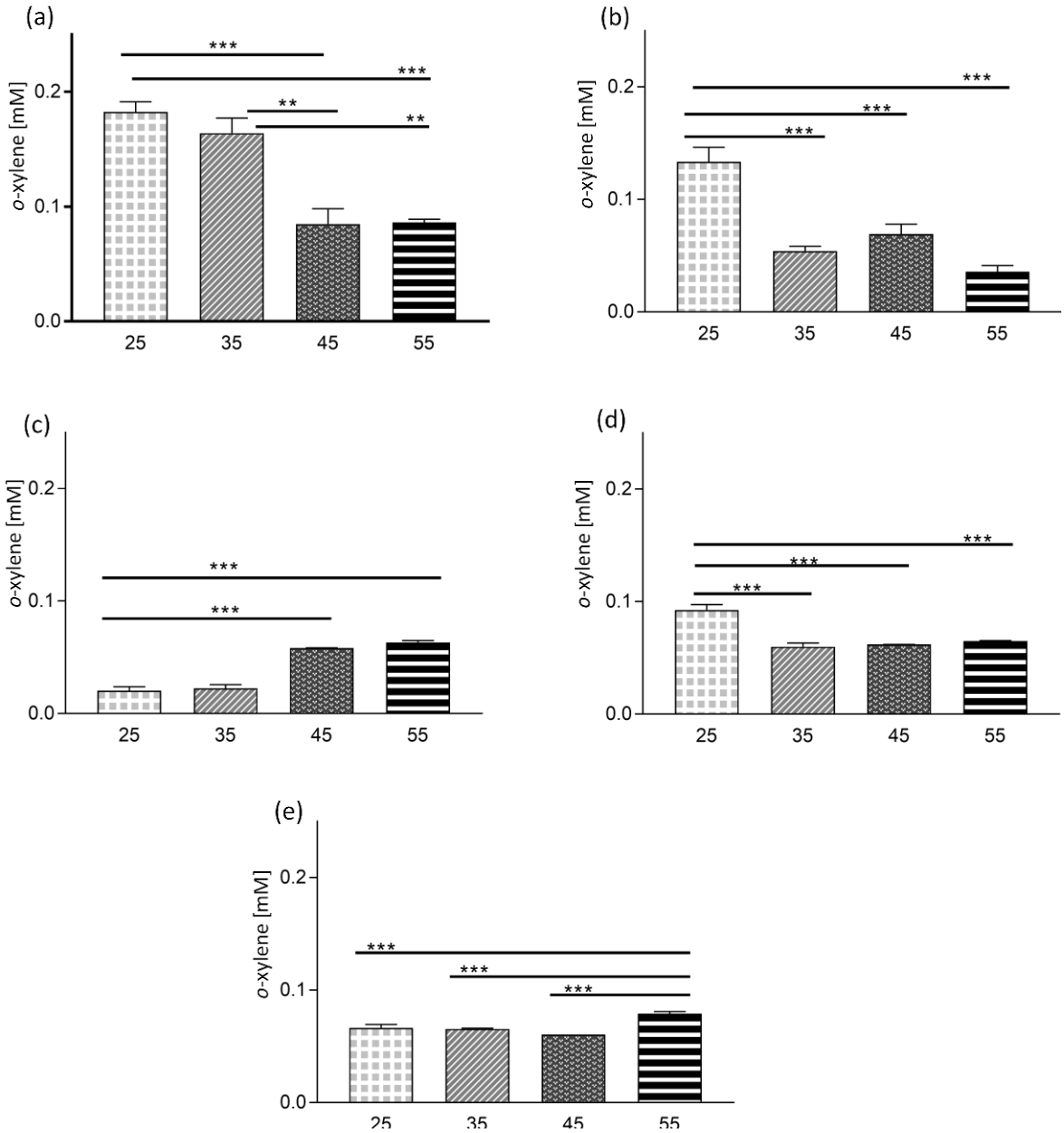

Figure 4: The effect of different temperatures (▭ 25°C, ▨ 35°C, ▩ 45°C and ▬ 55°C) on the efficacy of *o*-xylene degradation by indigenous microbial community from four treatment phases (a) abiotic control (without any microbial community) , (b) combining phase / S1, (c) activated sludge / S2, (d) settlement tanks / S3 and (e) river discharge / S4 (n=3). All samples were incubated at 150 rpm for 14 days subjected to 0.5 mM of *o*-xylene.

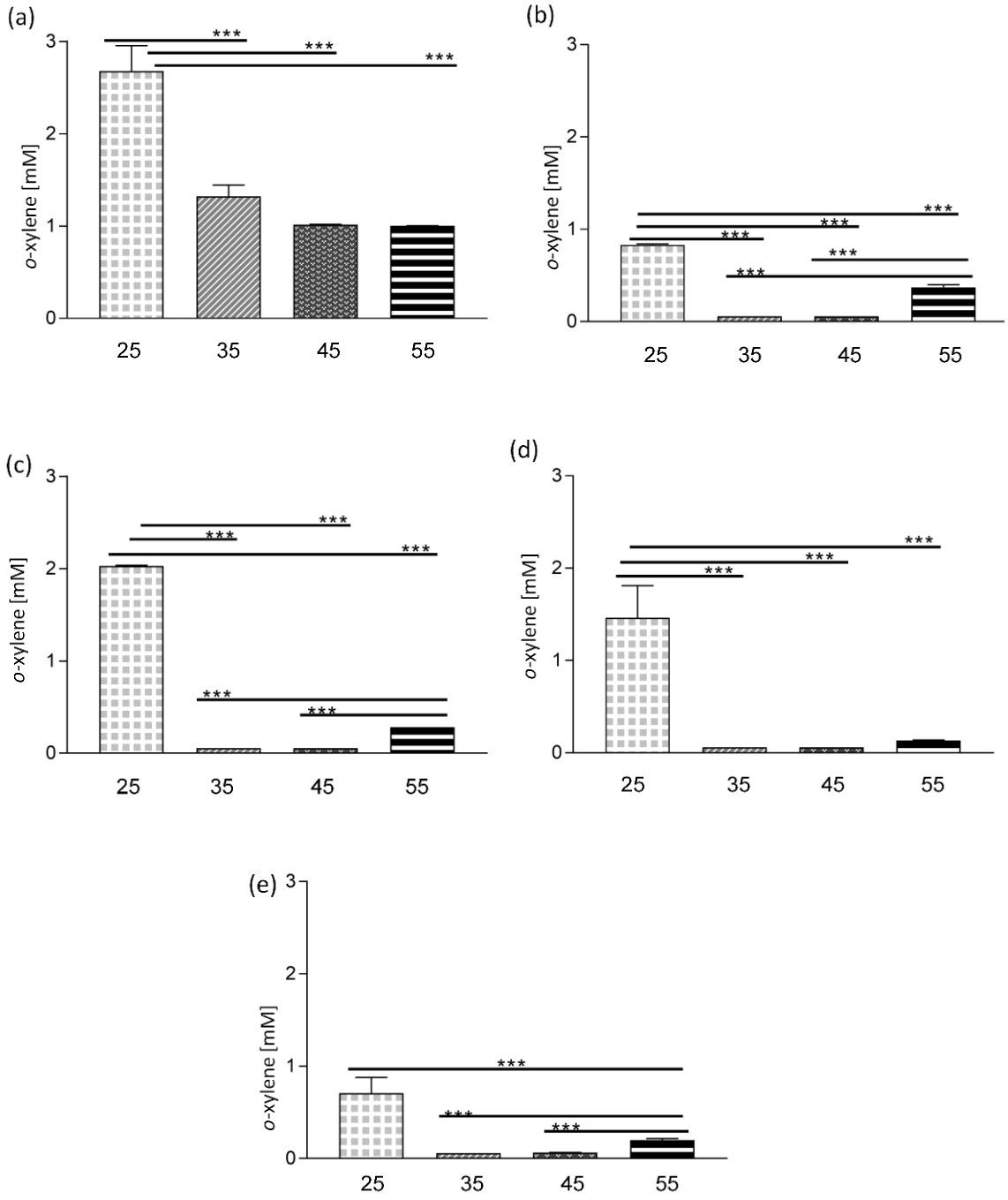

Figure 5: The effect of different temperatures (25°C, 35°C, 45°C and 55°C) on the efficacy of *o*-xylene degradation by indigenous microbial community from four treatment phases (a) abiotic control (without any microbial community) , (b) combining phase / S1, (c) activated sludge / S2, (d) settlement tanks / S3 and (e) river discharge / S4 (n=3). All samples were incubated at 150 rpm for 14 days subjected to 5 mM of *o*-xylene.

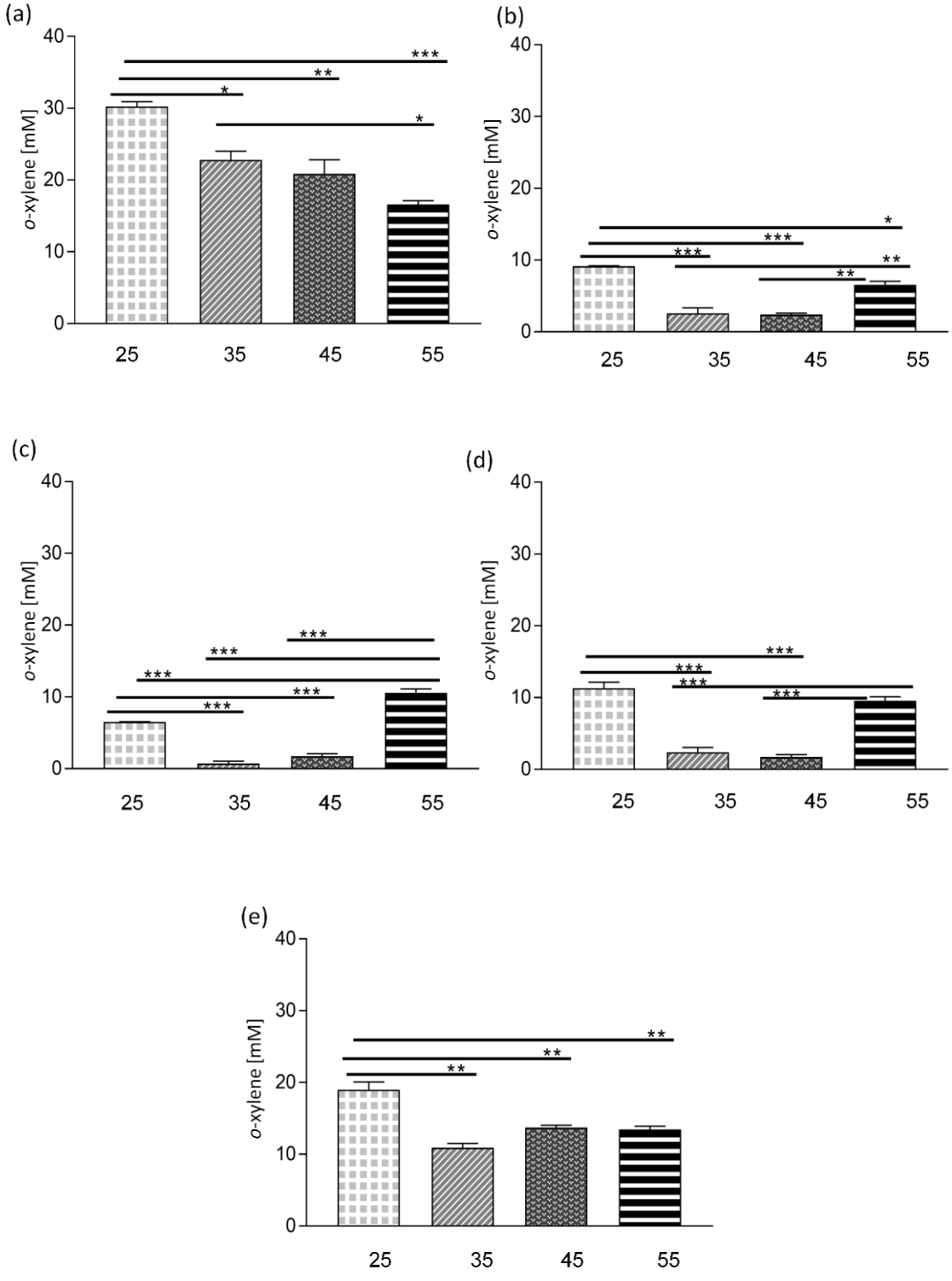

Figure 6: The effect of different temperatures (25°C, 35°C, 45°C and 55°C) on the efficacy of *o*-xylene degradation by indigenous microbial community from four treatment phases (a) abiotic control (without any microbial community) , (b) combining phase / S1, (c) activated sludge / S2, (d) settlement tanks / S3 and (e) river discharge / S4 (n=3). All samples were incubated at 150 rpm for 14 days subjected to 50 mM of *o*-xylene.

3.2 Rustumihia wastewater samples ecological analysis

Richness (^0D)

The fingerprints of the DGGE community were analysed using richness. Regarding ^0D richness taxa, Figure 7 shows a significant statistical difference P<0.0001 for both activated sludge S2 and settelment tanks S3, when comparing the mean effect for each variable with the mean effect of other variable. Also, P <0.0009 resulted for each of river discharge S4 and combining phase S1.

To analyse an ecological sample regarding the interpretations of species diversity, usually two approaches are used:

1. The first comparisons are based on the patterns, shapes or equations of species abundance curves.
2. Second comparisons are based on diversity indices.

The Shannon index (H') combines the variety of evenness (equitability) and the species richness as a component of diversity to measure alpha diversity within a microbial community. The Shannon index is used for this aim because it takes into consideration both evenness and species richness in the distribution of the individuals among the present species [49; 48 & 50].

Figure 7a shows the indigenous microbial community for the combining phase S1 . Shifts in community structure remained consistent in number at 0.5 and 5 mM *o*-xylene concentrations from the beginning of the treatment through the four different temperatures, thus suggesting that these two low concentrations of *o*-xylene have the same effect on the microbial community in combining phase S1 under different temperatures. The highest richness was recorded at 45°

when ^0D = 10 and 8 for both 0.5 and 5 mM respectively. The performance of the microbial community at a high *o*-xylene concentration was different than the low concentration; in this sense, the richness values of the observed microbial community gradually increased between 25°C and 35°C when ^0D= 10 and 15 respectively, decreased at 45°C when ^0D = 11, and then recovered activity with a striking increase at 55°C when ^0D = 20.

Figure 7b illustrates the indigenous microbial community for activated sludge S2. Shifts in community structure remained almost consistent in number at 0.5 and 5 mM *o*-xylene concentrations from the beginning of the treatment course through the four different temperatures, which suggests that these two low concentration of *o*-xylene have the same effect on the microbial community in activated sludge S2 under different temperatures. The highest richness was recorded at 35°C when ^0D = 7 and 9 for both 0.5 and 5 mM respectively. The performance of the microbial community at a high *o*-xylene concentration was different than that at low concentrations. In this sense, the richness values of the observed microbial community gradually increased from 25°C to the highest richness at 35°C when ^0D = 22 and then decreased slightly at, 45°C and 55°C when ^0D =19 and 18 respectively; thus, there was a consistent increase through 35°C, 45°C, and 55°C.

Figure 7c shows the indigenous microbial community for settlement tanks S3. The shifts in community structure remained almost consistent in number at 0.5 and 5 mM *o*-xylene concentrations at 25°C when ^0D = 7 and 9 and 35°C when ^0D = 11 and 13 respectively, and then the richness gradually dropped at 45°C and continued decreasing until 55°C at 0.5 mM, whereas the richness increased at 45°C when ^0D = 15 and 55°C when ^0D = 17 respectively at 5 mM. The performance of the microbial community at high *o*-xylene concentrations fluctuated through different temperatures. In this sense, the lowest richness was recorded at 25°C when ^0D = 2, a remarkable increased was recorded at 35°C when ^0D = 18, and then the richness remained consistent at 45°C when ^0D = 17 before dropping at 55°C when ^0D = 10. Overall, the

best performance for the indigenous microbial community of the settlement tanks S3 and for the three *o*-xylene concentrations was at 35°C.

Figure 7d shows that the indigenous microbial community for the settlement tanks S3 demonstrated shifts in community structure. The highest richness was recorded at 35°C when ^0D = 16.33, 23, and 14.22 for 0.5, 5, and 50 mM respectively, whereas the lowest richness was recorded at 45°C when ^0D = 6 and 8 for both 0.5 and 50 mM respectively. At 5 mM, the highest richness was recorded at 35°C when ^0D = 22 and 45°C when ^0D = 24. Thus, the richness of the indigenous microbial community for S3 was positively affected at temperature 35°C under the 0.5, 5, and 50 mM *o*-xylene concentrations.

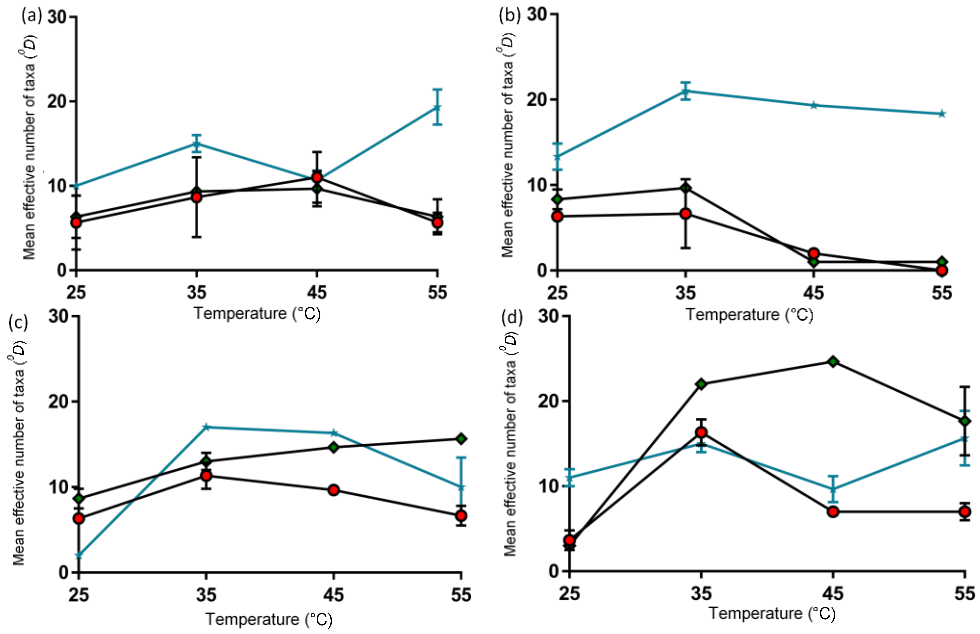

Figure 7: Bacterial richness, as determined by the Hill number ^0D, associated with the S1(Combining phase; (a)), S2 (Activated sludge; (b)), S3 (Settlement tanks; (c)), and S4 (River discharge; (d)) treatment stages of Rustumihia wastewater treatment plant following triplicate incubation in the dark for 14 days at different temperatures and with (0.5, 5 and 50) mM *o*-xylene. Each data point is the mean of triplicate samples. Error bars are SEM.

Hill number Diversity

Diversity of the common community members (^1D)

The diversity of common species ^1D in Figure 8 shows a significant statistical difference P value < 0.0001 for each of combining phase S1, activated sludge S2, settlement tanks S3, and river discharge S4 from the treatment phases.

According to [48], Simpson and Shannon diversity indices limitations are the lack of logical and easily interpretable units and their limited range (0 - 1, Simpson; 0 - 4.5 Shannon), which makes it difficult to interpret results and compare samples, especially when the values are close to the range limits. Another drawback is the absence of the doubling property. In fact, a sample with a Shannon diversity value of 4 is not two times as diverse as a sample with a Shannon index of 2 [49]. As a result, [50] suggested the use of Hill numbers to asses microbial diversity. These indices are mathematical transformations Simpson and Shannon diversity indices that comply with the doubling principle; they use meaningful units at a logical magnitude.

In Figure 8a, the indigenous microbial community shows a similar pattern of the common bacterial member performance at 0.5 and 5 mM of *o*-xylene under four different temperatures. At 50 mM of *o*-xylene, the highest diversity was recorded at 35°C when ^1D = 9 and 55°C when ^1D = 12.66, which suggests that true diversity was induced at 35°C and 55°C and a high *o*-xylene concentration in combining phase S1.

In Figure 8b, the indigenous microbial community also displays a similar pattern of the common bacterial member performance at 0.5 and 5 mM of *o*-xylene under four different temperatures. At 50 mM of *o*-xylene, the diversity started to increase gradually from 25°C when ^1D = 9.66 and continued until 55°C when ^1D = 13.22. Thus, the true diversity of activated sludge enhanced with an increase of temperature under a high *o*-xylene concentration of 50mM.

Figure 8c illustrates the different patterns of the indigenous microbial community and shows the response the common bacterial member to the changes in *o*-xylene concentrations. The highest diversity values were recorded at 35°C under 0.5 and 50 mM of *o*-xylene when ${}^{1}D$ = 9.33 and 15.22 respectively, whereas, at 5 mM, the highest diversity was recorded at 55°C when ${}^{1}D$ = 15.321. The diversity level increased at 35°C and 45°C and then decreased at 25°C and 55°C under 0.5 and 50 mM of *o*-xylene. At 5 mM, the diversity level increased gradually across the four different temperatures, reaching the highest diversity at 55°C.
Figure 8d shows similar patterns regarding the indigenous microbial community response to 0.5 and 50 mM of *o*-xylene, with the highest diversity being recorded at 35°C when ${}^{1}D$ = 7.123 and 9.114 respectively. However, the indigenous microbial community response was different under the effect of 5 mM of *o*-xylene. In this sense, the diversity level gradually increased across 25°C, 35°C, and 45°C, before decreased again at 55°C. Thus, the highest diversity was recorded at 45°C when ${}^{1}D$ = 22.431.

Diversity of the dominant community members (${}^{2}D$)
The dominant community members ${}^{2}D$ (Figure 9) shows a significant statistical difference $P < 0.0110$, 0.0173, 0.0003 and 0.0001, respectively, for each of combining phase S1, activated sludge S2, settlement tanks S3 and river discharge S4 from the treatment phases.
[51 & 50] gave explanations of ${}^{1}D$ as "common species number", and ${}^{2}D$ as "abundant or dominant species number". Considering that these explanations are not rigorous, and the low/high H' values does not necessary imply low/high diversity directly, because it depends both on common or dominant species and their relation to species richness.

The common and dominant species explanation gives insight into the meaning of the inequality factors and evenness. If ${}^{2}D$ is the number of dominant or abundant species, R is the species richness then the evenness factor ${}^{2}D$ /R is "the proportion of dominant species in the community" [52]. If there are some species that are dominant in a community, ${}^{2}D$ only reflects these and "hardly takes into account the rare species in the community" [50].
Figure 9a illustrates the effect of 0.5 and 5 mM of *o*-xylene, as the response of the indigenous microbial community was almost the same across the four different temperatures. Highest diversity recorded at 35°C when ${}^{2}D$ = 4.122 and 5.111 respectively. Highest diversity for the dominant community members recorded at 35°C and 55°C when ${}^{2}D$=7.320 and 8.677 respectively at 50 mM.
Figure 9b the indigenous microbial community displays a similar pattern of the dominant community members performance at 0.5 and 5 mM of *o*-xylene under four different temperatures. Highest diversity recorded at 35°C when ${}^{2}D$=4.121 and 6.123 respectively. While under the effect of 50 mM of *o*-xylene, diversity level increased gradually across the four different temperatures, recording highest values at 45°C and 55°C when ${}^{2}D$=11.21 and 10.87 respectively.

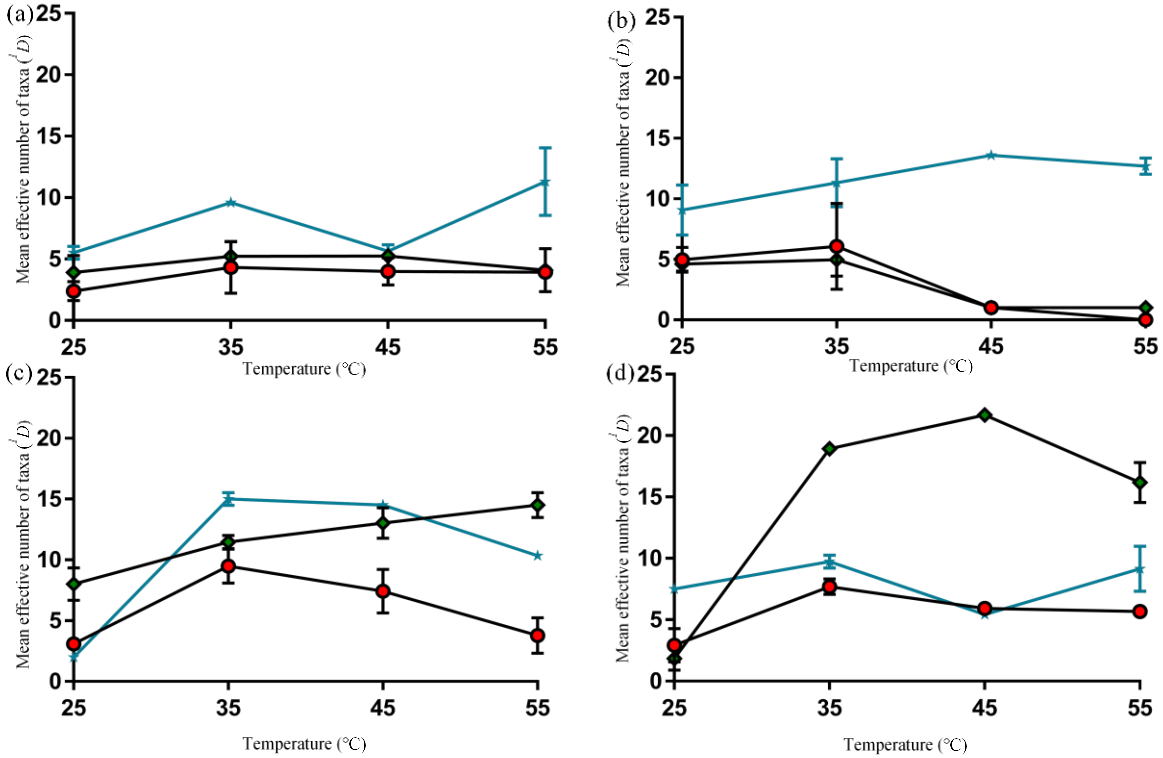

Figure 8: Bacterial diversity, as determined by the Hill number ^1D, associated with the S1(Combining phase (a), S2 (Activated sludge (b), S3 (Settlement tanks (c), and S4 (River discharge (d)) treatment stages of Rustumihia wastewater treatment plant following triplicate. incubation in the dark for 14 days at different temperatures and with (0.5, 5 and 50) mM o-xylene. Each datum point is the mean of triplicate samples. Error bars are SEM.

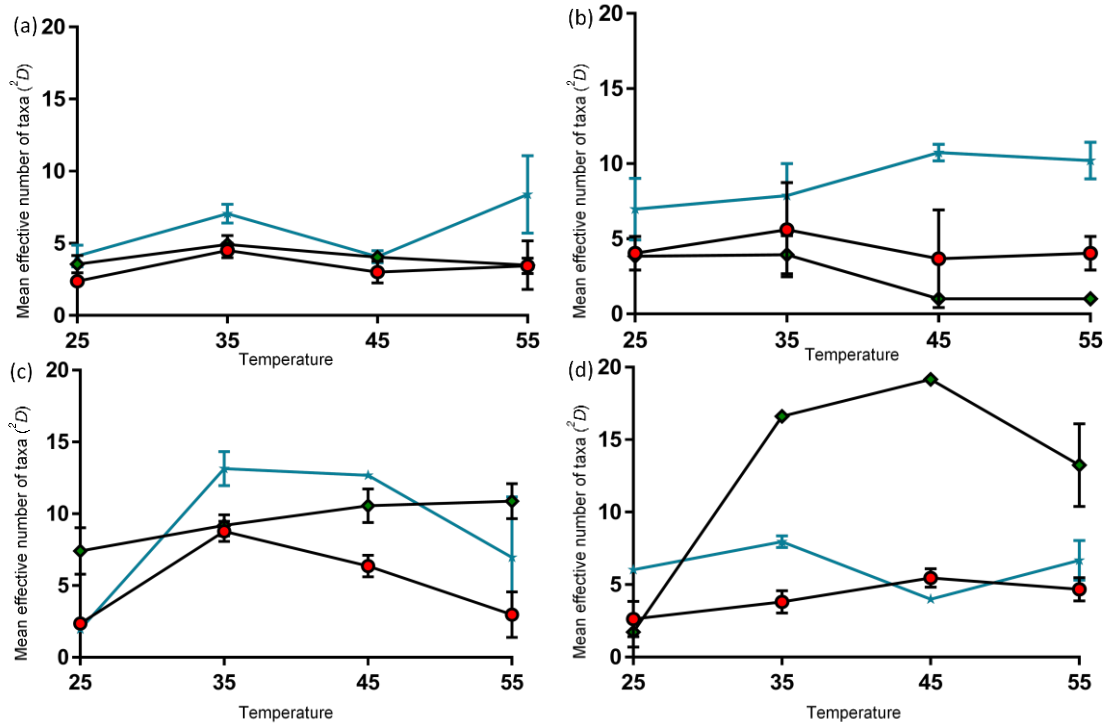

Figure 9: Bacterial diversity, as determined by the Hill number ^2D, associated with S1(Combining phase (a), S2 (Activated sludge (b), S3 (Settlement tanks (c) and S4 (River discharge (d)) treatment stages of Rustumihia wastewater treatment plant following triplicate incubation in the dark for 14 days at different temperatures and with (0.5, 5 and 50) mM *o*-xylene. Each data point is the mean of triplicate samples. Error bars are SEM.

Figure 9c shows similar patterns of the indigenous microbial community response to 0.5 and 50 mM of *o*-xylene. The highest diversity recorded at 35°C when ^2D=7.221 and 13.113 respectively. While under the effect of 5 mM of *o*-xylene, diversity level increased gradually across the four different temperatures, recording highest value at 55°C when ^2D =11.343.
Figure 9d illustrates different patterns of the indigenous microbial community response to the effect of 0.5, 5 and 50 mM of *o*-xylene, Highest diversity recorded for 0.5 and 5 mM at 45°C when ^2D =5.210 and 18.34, respectively. While at 50 mM the highest diversity recorded at 35°C when ^2D =8.24.

Evenness ($^1D/^0D$)
Figure 10 clarifies the evenness ^1D /^0D or Hill ratio, which is the relative dominant taxa in the wastewater environment. Diversity indices are defined mathematically through the calculations of biodiversity, which quantifies to what extent the community is equal numerically. In general, there was a significant statistical difference in activated sludge S2 and settlement tanks S3 with P<0.0001 as evident in Figure 10b and 10c. To contrast this, there was no significant statistical difference in combining phase S1 and river discharge S4 where P<0.1480 and 0.1052, respectively, as shown in Figure 10a and 10d.
Figure 10a shows that the frequency of each taxa of the indigenous microbial community in combining phase is not changing. Diversity is likely the same for the microbial habitat in combining phase under the same growth conditions of different temperatures and *o*-xylene concentrations. Figure 10b illustrates that the indigenous microbial community in the activated sludge was equal regarding taxa frequency at 25°C and 35°C and under the effect of 5 and 50 mM of *o*-xylene, which lead to suggest that these temperatures have the Same effect on the microbial habitat frequency under the effect of *o*-xylene low concentrations. At 0.5 mM the indigenous microbial community was even at 25°C and 35°C, presumably, these two temperatures have the same effect on taxa frequency level

because the microbial habitat stimulated to consume the very low concertation of *o*-xylene. At 45°C and 55°C, the evenness of indigenous microbial community was even under the effect of 5 and 50 mM. While at 0.5 mM the taxa frequency of the indigenous microbial community dropped gradually because of the effect of high temperatures to reach a minimum level when ${}^{1}D/{}^{0}D = 0.0$ at 55°C. Over all 91.2% from the community was evenness at 35°C and 0.5 mM, 74.2% was evenness at 45°C and 55°C and concentration 50 mM and 98.1% was evenness at both 45°C and 55°C and 5 mM.
Figure 10c clarifies that the frequency of each taxa of the indigenous microbial community in settlement tanks is not changing. Diversity is likely the same except a minor affect noticed at 0.5 mM of *o*-xylene at temperature 25°C. Figure 10d explains the indigenous microbial community for the river discharge as the taxa frequency represented by evenness was not changing across four different temperatures and 5 and 50 mM of *o*-xylene. To contrast, a noticed decrease in ${}^{1}D/{}^{0}D$ recorded at temperature 35°C which lead to suggest that this temperature has an effect on the taxa frequency for the indigenous microbial community under very low concentration 0.5mM as evenness was 50.1%.

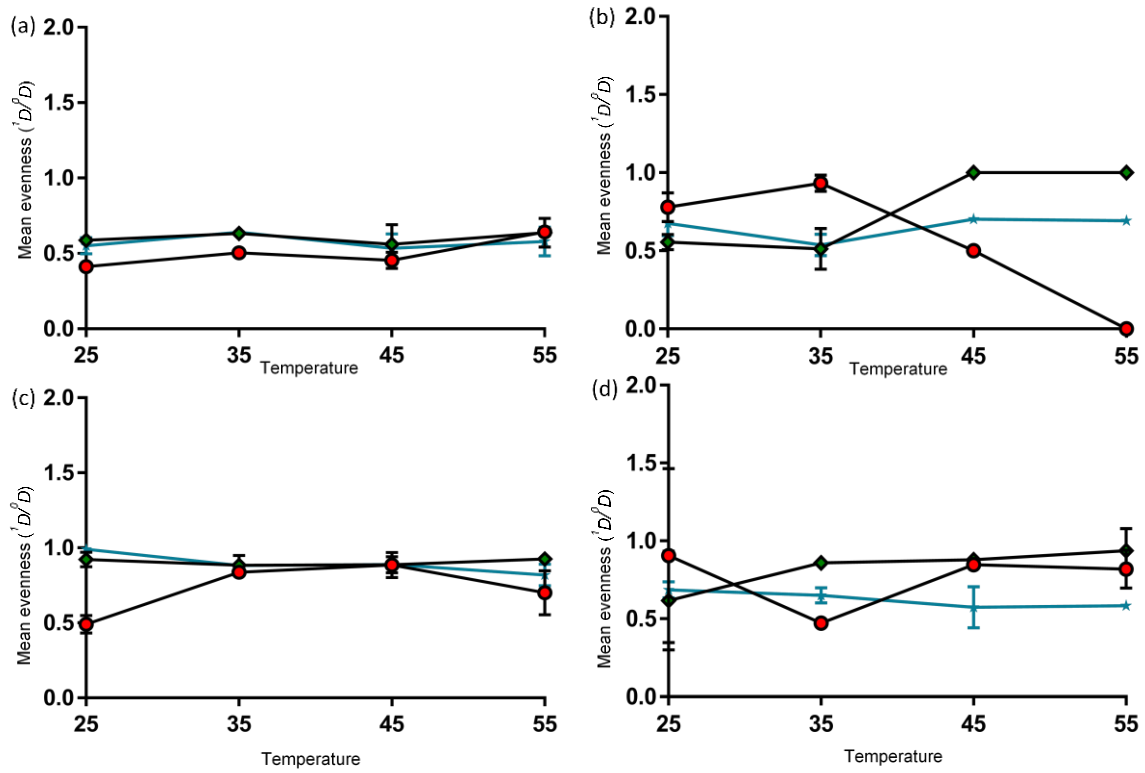

Figure 10: Bacterial evenness, as determined by the Hill number ${}^{1}D/{}^{0}D$, associated with S1 (Combining phase (a) S2 (Activated sludge (b), S3 (Settlement tanks (c), and S4 (River discharge (d)) treatment stages of Rustumihia wastewater treatment plant following triplicate incubation in the dark for 14 days at different temperatures and with (0.5, 5 and 50) mM *o*-xylene. Each data point is the mean of triplicate samples. Error bars are SEM.

Same effect on the microbial habitat frequency under the effect of *o*-xylene low concentrations. At 0.5 mM the indigenous microbial community was even at 25°C and 35°C, presumably, these two temperatures have the same effect on taxa frequency level because the microbial habitat stimulated to consume the very low concertation of *o*-xylene. At 45°C and 55°C, the evenness of indigenous microbial community was even under the effect of 5 and 50 mM. While at 0.5 mM the taxa frequency of the indigenous microbial community dropped gradually because of the effect of high temperatures to reach a minimum level when ${}^{1}D / {}^{0}D$ = 0.0 at 55°C. Over all 91.2% from the community was evenness at 35°C and 0.5 mM, 74.2% was evenness at 45°C and 55°C and concentration 50 mM and 98.1% was evenness at both 45°C and 55°C and 5 mM.

Figure 10c clarifies that the frequency of each taxa of the indigenous microbial community in settlement tanks is not changing. Diversity is likely the same except a minor affect noticed at 0.5 mM of *o*-xylene at temperature 25°C. Figure 10d explains the indigenous microbial community for the river discharge as the taxa frequency represented by evenness was not changing across four different temperatures and 5 and 50 mM of *o*-xylene. To contrast, a noticed decrease in ${}^{1}D / {}^{0}D$ recorded at temperature 35°C which lead to suggest that this temperature has an effect on the taxa frequency for the indigenous microbial community under very low concentration 0.5mM as evenness was 50.1%.

4. Discussion

Temperature is considered to be an important factor by which the bio-concentration of microbial community and xylene concentration are directly influenced [53 & 54]. In the present study, abiotic *o*-xylene concentrations were found to be affected by increases in temperature (Figure. 4a, 5a, and 6a). This is due to volatilization of the molecule, which makes measuring the indigenous microbial community biodegradation
difficult [55]. Therefore, to assess the potential for biological treatment of *o*-xylene at different temperatures for all four treatment samples, the average rates of *o*-xylene biodegradation by Rustumihia microorganisms was measured. To overcome the obstacles associated with volatility, the GC-FID measurements were made in closed microcosms to account for *o*-xylene vapor in the headspace for all the samples subjected to different *o*-xylene concentrations under different temperature [56].

In the current study, the highest degradation efficacy were recorded at 35°C, 45°C and 55°C of 5 mM of *o*-xylene, depending on chemical analysis, and for all of the combining phase S1, activated sludge S2, settlement tanks S3, and river discharge S4 wastewater treatment phases as explained in Figure 2b, 2c and 2d. Therefore, taking into account that temperature and organic compounds concentrations are important factors for stimulating the catabolic activity of Rustumihia microbial communities, Figure 2b, 2c and 2d provided some preliminary evidence that 35°C, 45°C and 55°C were the most effective temperatures for stimulating *o*-xylene degradation for the all phases of the treatment plant. In contrast, at 25°C and 5 mM *o*-xylene, both activated sludge S2 and settlement tanks S3 presented different performances on the ability of indigenous microbial community to degrade *o*-xylene when compared with combining phase S1 and river discharge S4, although both activated sludge S2 and settlement tanks S3 were significant in comparison with abiotic control (Figure 2a). These findings suggested that the indigenous microbial community inside the activated sludge S2 and settlement tanks S3 is different from the indigenous microbial community in combining phase S1 and river discharge S4. In addition to that , the medium concentration of carbon source and low temperature had slight effect on the degradation efficacy of *o*-xylene for activated sludge S2 and settlement tank S3 with recorded values of 20% and 40%, respectively, while for combining phase S1 and river discharge S4 the degradation efficacy of *o*-xylene were 60% and 58.4%, respectively, as shown in Figure 2a.

A mixed bacterial consortium was used, consisting of *Corynebacterium* sp [1]. GS5-66, *Micrococcus* sp. GS2-22, *Bacillus* sp. DS6-86, *Flavobacterium* sp. DS5-73, and *Pseudomonas* sp. DS10-129, to study the efficiency of crude oil biodegradation. They reported that an increase in crude oil concentration from 1% to 10% resulted in a decrease from 78% to 52% in the percentage of utilisation

by the bacterial consortium. Furthermore, maximum biodegradation occurred at the optimum temperature and pH of 30°C and 7.5, respectively.

An ideal environment for biodegradation process is dependent on the optimal growth conditions, including temperature, pH and concentration of *o*-xylene, for the isolated microbial community. Various studies have shown that temperature and different concentrations of *o*-xylene play an important role in enhancing the biodegradation process, which has been supported by the high percentage of *o*-xylene degradation linked to the microbial shifts occurring regarding ecological indices. This ideal environment includes proper nutrients and oxygen to enhance bioremediation and bioaugmentation processes [1; 57; 58 & 59].
Low temperature is one of the obstacles to biodegradation for aromatic hydrocarbon compounds [4]. Research by [60] on the application of bioremediation of total petroleum hydrocarbons under limited growth conditions in Canada, with growth temperatures fluctuating between 10.2°C and 25.6°C outdoors, with -6.2°C as an average temperature, showed that biodegradation process of petroleum hydrocarbon pollutants in *situ* still occurs and that low temperatures (for example lower than 10°C) can delay the degradation process but does not affect the ability of the indigenous microbial community to start organic pollutant biodegradation in *situ* [61]. This matched with what I found where the degradation ability of 5 mM *o*-xylene in activated sludge and settlement tank was not inhibited, but the degradation efficacies of *o*-xylene were affected by the low temperature of 25°C (Figure 2 a).
Low temperatures are considered as a challenging problem in terms of biodegradation, but that another problem also affects the biodegradation process outcome. In contrast, the bioaugmentation effects and the microbial community nature in contaminated locations are not the only factors that affect the oil biodegradation process [62]. There is another crucial factor – optimisation of oxygen supply and nitrogen through the bio-stimulation process – which leads to a significant improvement in bioremediation of oil-pollutants through controlling growth conditions for optimal outcomes; for example, optimising the continuous growth of microbial consortia.

[63] found that oil biodegradation was improved if proper oxygen and nutrient conditions were available. [64] detected that bioaugmentation processes in combination with bio-stimulation and aeration qualify the effective remediation of polluted *situ* by mazut at field scale, reducing remediation efficiency by 94%. Uche and [65] found that the optimum temperature for oil degradation is between 14°C and 45°C and also reported that the optimum pH is between 5.5 and 8.5 and the optimum value for oil degradation is 6.5–8 mgL^{-1} in soil.
Most likely, the indigenous microbial community of Rustumihia WWTP preferred these three *o*-xylene concentrations in four different temperatures and followed the metabolic pathway for *o*-xylene degradation. When the microorganisms were in contact with *o*-xylene compounds this would induce the enzymes as well as the genes which are responsible for *o*-xylene degradation. However, the degradation percentages were variable because of different temperatures, different *o*-xylene concentrations and for each phase have different effects on *o*-xylene catabolic rates. Which seems to be that each treatment phase is mainly characterised by a different indigenous microbial community (Figure 1c and 2a).

The slowest degradation of *o*-xylene by microbial communities from the four phases of the Rustumihia WWTP was correlated with the lowest temperature of 25°C. This might be attributable to the solubility of petrochemical compounds affecting the microorganisms and making them more bioavailable. [66] found that the most optimum temperature for petrochemical waste biodegradation was 30–40°C for microorganisms isolated from soil, while for the microbial community isolated form marine environments the temperature was 15–20°C, and 20–30°C for aqueous environments. Low temperatures have a negative effect on biodegradation processes as propagation and microbial growth slows down, which results in a reduction in the rate of hydrocarbon degradation. Increases in temperature, however, have a positive effect on increasing the efficacy of hydrocarbon degradation

because petrochemical compounds become more soluble in the growth medium, especially if it is supplemented with a growth factor, like yeast extract or glucose [67; 29; 68 & 69].

Microorganisms extract energy from volatile hydrocarbons through a biochemical reaction, which is mediated by monoxygenases and dioxygenases [70 & 71]. Chemical analysis results suggest that the various concentrations of *o*-xylene compounds might change the interactions between the consortium members, which presumably might affect the collective functionality of the metabolism process. This is because the high concentration of the carbon source will enhance microbial growth, which is linked to an increase in the taxa frequency for microbial member in the community. This is represented by high values in the S2 and S3, at 50 mM of *o*-xylene and 35°C, 45°C and 55°C for S2, and 35°C and 45°C for S3 as shown in Figure 8b and for common community members and Figure 9b for dominant community members. Due to the degradation of hydrocarbon molecules, an increase in cell growth occurs, which accelerates the hydrocarbon molecule biodegradation [72 & 73]. This supports the idea that a higher concentration of hydrocarbon load, which simply represents carbon source and energy, could enhance the indigenous microbial community for the four phases of Rustumihia WWTP, encouraging participation in the co-metabolism process to sustain reproduction, as shown in Figure 2a, 2b, 2c and 2d and Figure 3a, 3b, 3c and 3d.

When co-metabolism occurred, the *o*-xylene was degraded. This is because there was a low concentration of energy sources (organic or inorganic compounds and different types of bacteria which were not isolated in enrichments and isolations technique) in the actual wastewater samples. These small amounts of organic and inorganic compounds were degraded by certain enzymes or factors secreted by certain types of microbial community that existed in the original Rustumihia samples. The final result of this degradation was another product that might stimulate another type of bacteria to start the *o*-xylene degradation process. For this reason, there was a difference in degradation efficacy in the original wastewater sample when subjected to the three *o*-xylene concentrations as compared to the samples that were inoculated with the consortium produced from the Rustumihia WWTP. These differences led me to consider that a co-metabolism process might enhance the metabolism of *o*-xylene.

According to [74], the microbial activity and the subsequent biodegradation process are prompted when a hydrocarbon pollutant is added to a natural or laboratory-based ecosystem. For the current study, the cultivation medium, which was based on minimal salts, was nutrient-limited. As a result, the indigenous microbial communities from the four phases of the Rustumihia wastewater treatment plant started to utilise/metabolise the added *o*-xylene at different concentrations. Gradually, the increasing numbers of microorganisms, which had inherent constitutive or inducible metabolising enzyme activity, recorded the highest degradation capabilities at 35°C and 45°C. Similar trends of increased degradation efficiency at 35°C and 45°C were recorded by [75], who noted that, when presenting two mixed isolates of *Pseudomonas* sp. to an SO_2 gas biofilter that contained benzene, toluene and *o*-xylene, the total removal efficiency of mixed compounds (BTEX) was enhanced according to the enhancement of *o*-xylene co-metabolic at 35°C and 45°C [76 & 77]. The indigenous biomasses that are isolated from contaminated *situ* are more capable of dealing with variable local conditions compared to those isolated from other contaminated locations [78]. Consequently, the *Pseudomonas* sp. survived for a long time after treatment because it was originally isolated from its contaminated *situ* later presented in the new inoculum consortia. *O*-xylene enhances the abundance of the *Pseudomonas* group, so when a mixed consortium composed of *Pseudomonas aeruginosa* and *Bacillus subtilis* was used together, a complete removal of BTX could occur. The best recorded temperatures for crude oil biodegradation recorded by a mixed consortium and individual bacterial strains was at 35°C and pH 7 which were originally isolated from oil contaminated area and this is matched with our finding results, 35°C was the best for *o*-xylene degradation by the indigenous microbial community cross over the treatment phases [79]. Although the Rustumihia microbial community maintain their ability for *o*-xylene degradation through different temperatures and concentration but with different efficacies Figure 4, 5 and 6.

In the current study, high degradation efficiencies were recorded for different *o*-xylene concentrations with the help of the Rustumihia indigenous microbial community. This is because, as *o*-xylene was the sole carbon source added to the BMS and was not mixed with other artificial contaminants in the

microcosm, there was less competition between different species of indigenous microbial community – mixed contaminants might demand various microorganism species and various metabolic steps to simplify contaminants and transform them into simple compounds. The presence of *o*-xylene in the BMS promoted a unique bacterial abundance in the microcosm, in agreement with the Hill index results acquired from the DGGE profile. In the current study, the diversity common community member ${}^{1}D$ and most dominant member ${}^{2}D$ improved and increased when high concentrations of *o*-xylene were presented in the microcosm.

It has recently been reported that successful biodegradation is realised when the microbial community that consists of different bacterial species is able to degrade the pollutant as a carbon source [4 &16] and [21]. It can be concluded from the chemical results of the current study that the indigenous microbial communities of the four phases of Rustumihia WWTP were able to degrade *o*-xylene in the microcosm environment and that the nutritional conditions were favourable; for instance, the *o*-xylene concentrations and environmental temperatures were ideal growth conditions [80 & 16].

The applications of bioremediation in contaminated sites were conducted and suggested to be eco-friendly and effective technologies. [43 & 81] recorded by-product formation during the biodegradation process of the three combinations of benzene, toluene, and xylene (BTX), showing that they undergo two main stages. Firstly, the production of catechol, 3-methyl-catechol and the formation of 3, 6-dimethyl-catechol from benzene, toluene and xylene, and secondly, the mineralisation process for intermediaries to produce water and CO_2.

Ecological indices

The DGGE data interpretation was based on applying taxa richness ${}^{0}D$ and diversity regarding common community taxa ${}^{1}D$ and most dominant taxa ${}^{2}D$. The data suggest that the indigenous microbial community drawn from the distinct stages of Rustumihia WWTP responded differently to the changes in growth conditions, which included different carbon source concentrations and incubation temperatures. DNA sequencing was used to identify the microbial species involved and establish whether these DGGE-based community structural changes are linked to compositional shifts.

As stated by [82 & 83], functional properties can elucidate the relationship between ecosystem processes and biodiversity. In the current study, results observed whenever diversity values increased in the microcosm samples regarding common and dominant taxa, which could reflect the real process in actual WWTP. In detail, when diversity values in both the activated sludge and settlement tank were at high concentrations of 50 mM *o*-xylene, at 35°C and 45°C, the functionality of the indigenous microbial community in these two phases and their ability to tolerate high *o*-xylene concentration and temperatures were assessed.

Microbial richness and diversity for waste-activated sludge S2 decreased gradually with changes in temperature and for the two *o*-xylene concentrations of 0.5 and 5 mM (Figure 7b and Figure 8b), respectively. The activated sludge S2, where all the biodegradation processes occurred, was presumed to be colonised mainly by a microbial community that was affected by different concentrations of *o*-xylene as well as different temperatures. In the current study, chemical results revealed that there was no significant difference for the indigenous microbial community for activated sludge S2 when temperature 25°C versus 35°C, also when 45°C versus 55°C as this was at 0.5 mM as shown in Figure 4c. This is linked to the results as shown in Figure 8b that explained the performance of common indigenous microbial community were the same when 25°C versus 35°C, also when 45°C versus 55°C at 0.5 mM only. While chemical results illustrated that there was no significant difference for the indigenous microbial community for activated sludge S2 when temperature 35°C versus 45°C only at 5 and 50 mM as shown in Figure 5c and Figure 6c. This is linked to the results shown in Figure 9b, which shows the performance of most dominant community member were similar under the effect of temperature 35°C and 45°C. Also, microbial richness was the highest at 35°C in the presence of 50 mM *o*-xylene, while the highest diversities were recorded at 35°C and 45°C for 50 mM of *o*-xylene. For settlement tanks S3 and river discharge S4, microbial richness was the highest at 35°C and 45°C and, in the combining phase S1, at 55°C.

The ecosystems of Rustumihia WWTP, under stressed growth in conditions of high temperatures 55°C and high *o*-xylene concentration (50 mM) were more stable. This could potentially be attributed to microbial diversity levels that enhanced the WWTP ecosystem efficiency where the functionality of

hydrocarbon degradation was stimulated, especially when 55°C versus 25°C, 35°C and 45°C in combining phase S1 and activated sludge S2 as shown in Figure 6b, 6c. This suggested that the indigenous microbial community for both combining phase S1 and activated sludge S2 maintaining its viability to degrade high *o*-xylene concentration under high temperatures. While in settlement tanks S3 the indigenous microbial community performance was significant when 55°C versus 35°C and 45°C but not 25°C as shown in Figure 6d. In river discharge S4 there was no significant effect between 55°C versus each 35°C and 45°C, although a significant effect was recorded when 55°C versus 25°C as illustrated in Figure 6e. The results indicated that high concentrations of *o*-xylene positively affected the diversity of hydrocarbon clastic bacteria in the combining phase S1, activated sludge S2 and settlement tanks S3.

Overall, the community structure changed remarkably along the gradients of the hydrocarbon concentration; for example, *o*-xylene and temperatures [84;54 & 21]. Clear differences were detected in the community composition, as shown by the differential diversity regarding ${}^{0}D$, ${}^{1}D$ and ${}^{2}D$. The wastewater communities were among the most diverse and complicated environments [35 and 85] and [11]. It was found that the differently used *o*-xylene concentrations resulted in an increase in the bacterial diversity under stressful growth conditions; this result applied to all four phases of the treatment plant water [86; 3; 87 & 88].

5.Conclusion

Biological treatment in a wastewater treatment plant is one of the essential functions of the industrial sewage system. It usually consists of organic and inorganic contaminants. Various studies have focused on biodegradation and bioremediation as cost-effective processes, rather than on the physical and chemical processes. Biological treatment has an economic advantage as it employs a range of different microorganisms that behave as a community, sometimes by forming microbial biofilms. A diverse range of micro-niches, which are derived from microbial biofilms, provide protection against physical agitation to the microbial communities in order to strength their functional stability and the potential metabolic process. Nowadays, in the wastewater treatment environment, biofilms are extensively applied in developed countries. Their advantage is that they degrade toxic wastes in soil and water and create numerous commercial products. Although biofilm is extremely important in developing countries, where it has been proven theoretically to be a promising biotechnology, it currently has limited application in reality (86; 11& 88).

The degradation of volatile hydrocarbons was investigated under mesophilic growth conditions. Microbes like bacteria and fungi show optimum activity for the bioremediation process at mesophilic temperature 35°C [89]. However, very little information is available on the hydrocarbon-degrading microorganisms that favour thermophilic temperatures. This is surprising, especially as specific thermophilic bacteria are considered important for biotechnology applications, such as the sourcing of thermostable enzymes and other products of industrial interest [90]. Against this background, a key question arises of why high temperatures are applied in the Rustumihia wastewater treatment site for the current research programme. Iraq is one of the hottest countries in the world, especially during the summer season. Temperatures are between 20°C and 28°C during the winter, and between 30°C and 60°C in the summer [91& 92]. Therefore, the applied growth temperatures ranged between 25°C and 55°C; this range covers all microbial community growth conditions that have the ability to tolerate heat and to degrade *o*-xylene at different concentrations. The solubility of *m*-xylene as a substrate increased with increasing temperatures and the risk of pollution by pathogenic microorganisms decreased as growth rate decreased as the temperature was lowered from 30°C to 25°C [93].

The results demonstrated tolerance towards the higher concentrations of *o*-xylene and temperatures at the same time. According to the Hill number, diversity and richness should be considered the increased that is occurred in common and most dominant taxa values the results demonstrated tolerance towards the higher concentrations of *o*-xylene and temperatures at the same time. According to the Hill number the evenness values at 50 mM and 55°C, as ${}^{1}D/{}^{0}D$ for combining phase S1=63.3%, activated sludge S2=69%, settlement tanks S3=82% and river discharge S4=58%. These results

indicated that Rustumihia microbial community were able to tolerate and degrade *o*-xylene at 55°C, results that are supported by GC-FID results.

All cultural growths in BMS were observed at a concentration of 0.5 mM as an initial step; this was followed by chemical analysis in addition to molecular analysis. The wastewater environment is complex and heterogeneous; hence its description still poses a significant challenge for researchers. In a well-controlled and organised wastewater system, microbial community structure, microbial biomass and their functions are generally regarded as markers for wastewater quality. As indicated previously, in this study, wastewater microbial diversity was analysed in terms of its genetic diversity. In terms of measuring genetic diversity, 16S rDNA and DGGE analysis are considered as reliable and fast methods because large numbers of samples can be analysed simultaneously. However, these methods are sensitive towards PCR biases and sample handling. Overall, the DGGE method has its own advantages and disadvantages; it can be chosen based on the needs and aims of the study of wastewater microbial diversity. As a result, it is essential to investigate the microbial community structure in depth.

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