Determining the impacts of environmental parameters on model microbial community dynamics isolated from Rustumihia WWTP/Iraq

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Determining the impacts of environmental parameters on model microbial community dynamics isolated from Rustumihia WWTP/Iraq

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Abstract: The composition of Rustumihia microbial community and their diversity with o-xylene-contaminants were investigated by applying molecular techniques, Polymerase chain reaction and denaturing gradient gel electrophoresis (PCR and DGGE) via investigating 16S rRNA gene fragments and understand the interrelationships between microbial community composition and structure for established microbial model community isolated from Rustumihia WWTP. To this end, that the established consortium could be used to assess the microbial response as defined by diversity and richness shifts, which are linked to changes in growth conditions. In this research paper a synthetic consortium was created by isolating indigenous microbial community members from the Rustumihia WWTP and subjecting consortium to different pH of (6.5, 7.0 and 7.5) and *o*-xylene concentrations of (0.5, 5 and 50 Mm) and temperatures (25°C, 35°C, 45°C and 55°C).

The results of this study indicated that the high *o*-xylene concentration of 50 mM was tolerated and degraded effectively at 35°C and 55°C, and pH 6.5 ($P < 0.001$). Bacterial richness and diversity were recorded according to the Hill parameters of ${}^{0}D$, ${}^{1}D$ and ${}^{2}D$ under each of the growth conditions, and then linked to the *o*-xylene degradation efficiency. At 35°C and pH 6.5, the consortium achieved high degradation percentage for each of 0.5, 5 and 50 mM of *o*-xylene with values 73.1%, 94.8% and 63.08%, respectively. The current study is the first of its kind in Iraq. It investigates the enrichment, isolation, and identification of a microbial community from the Rustumihia WWTP and determines the efficiency of the isolates to tolerate and degrade o-xylene, highlighting their sole source of hydrocarbon. This research underscores the usefulness of molecular techniques for both diversity and richness to understand the ecological impact of *o*-xylene as a contaminant and to identify potential molecular techniques for detection of gene that is responsible for *o*-xylene degradation.

1.Introduction: *O*-xylene isomer is an aromatic hydrocarbon that is one of the crude oil components [1 & 2] and [3]. The uncontrolled use of *o*-xylene and its release into wastewaters has resulted in considerable harm to the environment [4 & 5]. *O*-xylene, in comparison with other oil components, is soluble in water at 175 (± 8) g of hydrocarbon in 10^6g of water [6]. Although it has carcinogenic properties [7; 8 & 3], it is biodegradable by numerous types of microorganisms [9; 10 & 3] and [11] under favourable growth conditions. What is increasingly interesting is the use of microbial communities and their genomes for developing environmental biotechnological processes (biodegradation, bioremediation, and bioaugmentation) that deal with aromatic

compounds [12; 13 & 2]. Various bacterial species have been confirmed that could metabolize the chemical components found in oil, [14 & 15]. For example, literature recorded that a consortium adapted to unleaded gasoline, which consisted of *Pseudomonas*, *Burkholderia*, *Rhodococcus*, *Shewanella*, *Bacillus*, and *Alcanivorax* species that were characterised via 16S rDNA, can degrade around 95% of the total Benzene, Toluene, Ethylbenzene and Xylene At the same time, a *Pseudomonas putida* strain, that was isolated from that consortium, could degrade approximately 90% of the total BTEX [16]. Additionally, the bacteria belonging to the genus *Rhodococcus* were capable of growing on a wide range of toxic hydrocarbon compounds including aromatic and aliphatic hydrocarbons [17]. Other microorganisms that can degrade toluene are *Pseudomonas mendocina*-KR1 and *Pseudomonas putida*-DOT T1E [18]; *Pseudomonas fluorescens*-CA-4 removed ethylbenzene [4] while *Pseudomonas putida* F1 removed benzene, ethylbenzene and toluene [3]. The ability to utilize *o*-xylene by microorganisms is mainly attributed to the expression of enzymes known as dioxygenases [19], which can cleave the aromatic-rings and thus oxidise toxic compounds. *O*-xylene is considered as one of the most recalcitrant compounds. As a result, specific microbial strains and/or communities are required for its controlled biodegradation [20]. Nevertheless, several bacteria like *Comamonas* sp. JB [21], *Cladophialophora* sp. T1 [22], *Janibacter* sp. SB2 [23], *Pseudomonas putida* BCNU106 [24], *Pseudomonas Putida* F1 [25], *Pseudoxanthomonas spadix* BD-a59 [25], *Rhodococcus* sp. BTO62 [20], *Pseudomonas putida* OX1 and *Pseudomonas* sp. FMB08 [27], were isolated and identified for their ability to degrade *o*-xylene although their catabolic rates were low (0.004 - 5 mg L^{-1} h^{-1}).

The slow degradation rates are considered obstacles considering the large-scale applications of *o*-xylene in different fields of life. According to the literature, the *o*-xylene degradation pathway depends upon the types of inoculated microbes that are used to initiate the degradation process. For example, *Cladophialophora* sp. T1 transformed *o*-xylene to phthalates as end metabolites [22]. *Corynebacterium* sp. C125 and *Nocardia* sp. can degrade *o*-xylene during an initial aromatic dioxygenase process to produce a cis-dihyrodiol [28]. Two metabolic pathways were found for *Rhodococcus* sp. B3 where the monooxygenase enzyme was responsible for initiating both. Under aerobic conditions, one pathway was found to include the oxidation of the methyl group to produce 2-methylbenzyl alcohol. The other was observed through the oxidation process of the aromatic ring to generate 2, 3-dimethylphenol [29]. In *Pseudomonas stutzeri* OX1, the situation is different as the mono-oxidation enzymes act simultaneous on the aromatic ring. They generate 2, 3-dimethylphenol and 3, 4-dimethylphenol during *o*-xylene degradation, which are lastly transformed into 3, 4-dimethylcatechol and 4, 5-dimethylcatechol, respectively [30].

Consequently, the primary objective of this study was to establish a microbial consortium that could be used to assess the microbial response as defined by diversity and richness shifts, which are linked to changes in growth conditions. The synthetic consortium was created by isolating indigenous microbial community members from the Rustumihia WWTPs in 0.5 mM of *o*-xylene at 25°C and pH 7.0, and then selecting taxa that produced unique bands on DGGE gels with the highest degradation percentage, according to GC-FID. Later, the consortium was subjected to four different temperatures, three different *o*-xylene concentrations and three pH conditions. With the aid of chemical analysis using the GC-FID method, the *o*-xylene removal efficiency was measured. The optimal temperature and pH for *o*-xylene degradation was identified according to ecological indices of richness and diversity, which were correlated subsequently with chemical analysis results. The relationships among the most dominant consortia taxa will be highlighted via the use of ecological indices and assessment of *o*-xylene removal efficiency.

This research paper is an overview of the microbial genera inside the Rustumihia wastewater treatment system. It will also develop an understanding of the microbial structure of a model consortium, which is capable of *o*-xylene degradation. Molecular techniques are used to assess the microbial community regarding structure and function *in vitro*. Quantitative chemical analysis that is based on GC-FID is used to determine the degradation rates of *o*-xylene. Therefore, the current study was conducted to test the hypothesis that the developed microbial consortium from Rustumihia WWTP will respond differently to stressful growth conditions such as variations in temperature, pH and alongside with different carbon source (*o*-xylene) concentrations.

2.Experimental design: A bacterial consortium was prepared by selecting 15 unique monocultures according to their DGGE band positions and GC-FID results. Fresh cultures were prepared by refreshing the frozen stock where (1 mL) of each isolate stock was inoculated into (9 mL) of basic mineral salt solution, and then subjected to different growth conditions like four different temperatures (25°C, 35°C, 45°C, 55°C) incubated at dark and on a rotary shaker at (150 rpm) for 14 days, with the presence of three different concentrations of *o*-xylene: 0.5, 5 and 50 mM and three different pH 6.5, 7.0 and 7.5. All experiments were done in triplicate to achieve accurate results. After DNA extraction from monocultures via using Fast DNA™ Spin Kit for Soil (MP Biomedicals, U.S.A.), according to the manufacturer's instructions, all ecological indices were calculated using ecological equations (1-4). Colony DNA extraction for the 15 monocultures were applied and the PCR products were sent to Genius Laboratories Limited for 16S rDNA sequencing. PCR programme aimed to target the V3 region of the bacterial 16S rRNA genes, from location 341 to 534 which was amplified according to [31] using the forward primer: 5`-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3` and the reverse primer 5`-ATT ACC GCG GCT GCT GG-3`. The reaction mixture was composed of 2 µL each DNA extract, 2X PCR Master Mix, 0.5 µL of both the forward and reverse primers, 1.25 µL L^{-1} BSA and molecular grade water up to a final volume of 25 µL. Thermal-cycling programme consisted of 1 cycle at 95°C for 2 minutes, 35 cycles of: denaturation at 95°C for 1 minute; annealing at 60°C for 1 minute; extension at 72°C for 1.5 minutes; and final extension at 72°C for 30 minutes. The amplicons were then checked on 1.5% (w/v) agarose gels as above.

3.Results:

3.1 A developed wastewater microbial consortium

The microbial consortium, which consisted of 15 isolates from distinct stages of the Rustumihia treatment facility, responded differently to changes in temperatures and carbon source concentrations. The ecological indices suggested a new way to assess the big scale stability, which was also complemented by 16S rRNA sequencing of the 15 isolates.

3.2 Temperature

The influence of temperature (ranging from 25°C to 55°C) on a developed wastewater microbial consortium, regarding *o*-xylene degradation capacity and efficiency, was investigated with a qualitative assessment of key ecological indices. Specifically, Hill numbers (equations 1-4).

Equation 1

$$ {}^{q}D = \left(\sum_{i=1}^{s} pi^{q}\right)^{1/[1-q]} $$

General Equation

Equation 2

$$ {}^{0}D = \sum_{i=1}^{s} pi^{0} $$

Equation 3

$$ {}^{1}D = e^{H'} = e^{-\sum_{i=1}^{s} pi \ln pi} $$

Equation 4

$$ {}^{2}D = 1/\sum_{i=1}^{s} pi^{2} $$

Where q = the exponent of species frequencies; s = number of species; pi = proportion of total sample belonging to its species, e = the base of the natural logarithm

3.3 Ecological indices profile

Richness (^0D)

In Figures 1a, 1b, 1c and 1d a noticeable change was observed in the taxa richness under different *o*-xylene concentrations and temperatures. For biomass prediction, taxa richness is not a significant indicator that ecologists can rely on [9 and 10]. Notwithstanding this, a positive relationship appeared for community richness (^0D) when different *o*-xylene concentrations were applied to the consortium under four different temperatures. This suggested different effects on *o*-xylene utilisation, possibly via different metabolic pathways, by individual members of the synthetic consortium. The different growth conditions revealed some idiosyncratic patterns. In Figure 1b, the ecological index of ^0D was a good predictor of the environmental variation because its fluctuations represented the consortium's catabolic response and were consistent with their degradation efficacy.

In general, Figure 1 demonstrated a significant statistical difference regarding taxa richness at 25°C, 45°C and 55°C with P<0.0001. For 35°C, P was <0.0274.

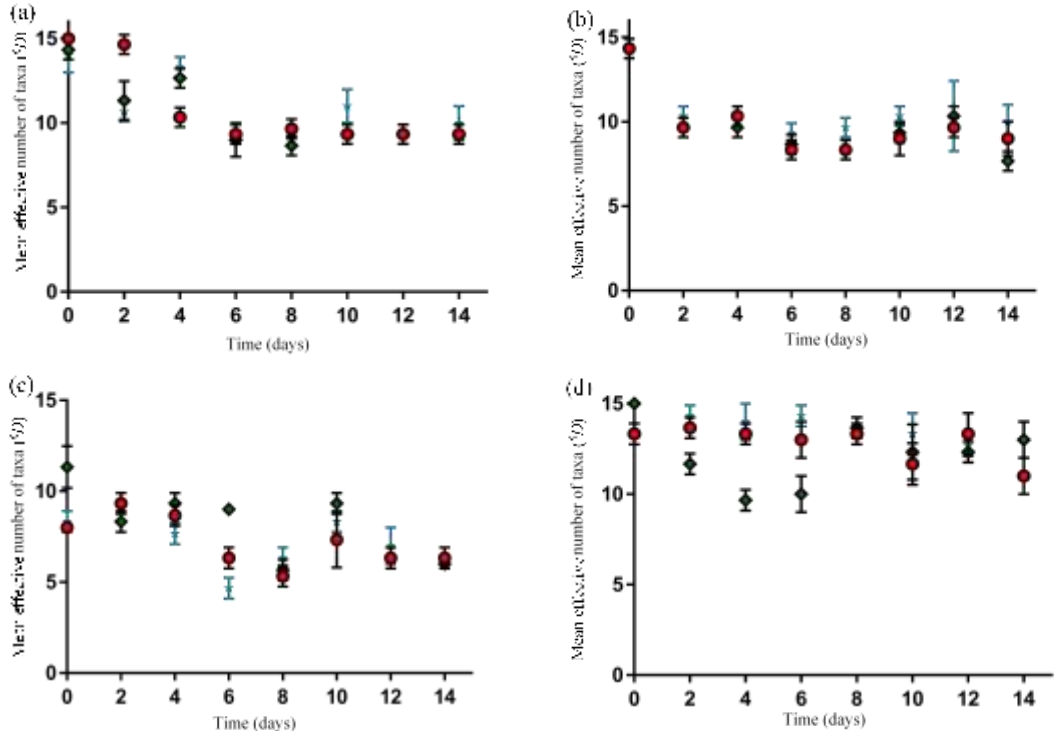

Figure 1: Consortia taxa richness, as determined by the Hill number ^0D, associated with 25°C (a), 35°C (b), 45°C (c) and 55°C (d) following triplicate incubation in the dark for 14 days and with(0.5 (●), 5 (◆) and 50 (★) mM *o*-xylene. Each datum point is the mean of triplicate samples. Error bars are SEM.

Diversity of the common community members (^1D)

Figure 2a, 2b, 2c and 2d showed the diversity of common community members of the synthetic consortium in the presence of *o*-xylene at 0.5, 5 and 50 Mm. Statistically significant differences were recorded for 25°C, 35°C, 45°C and 55°C with P<0.0001.

An observed change was recorded for the consortium, during the first two days of incubation in 0.5 and 5 mM *o*-xylene (Figure 2d). On day 4, 6 and 10, the consortium members recorded the highest

value for at 50 mM as ^1D = 9.934, 10.206 and 11.163, respectively, and then steady until day 14. In contrast, for 0.5 mM, the consortium recorded.

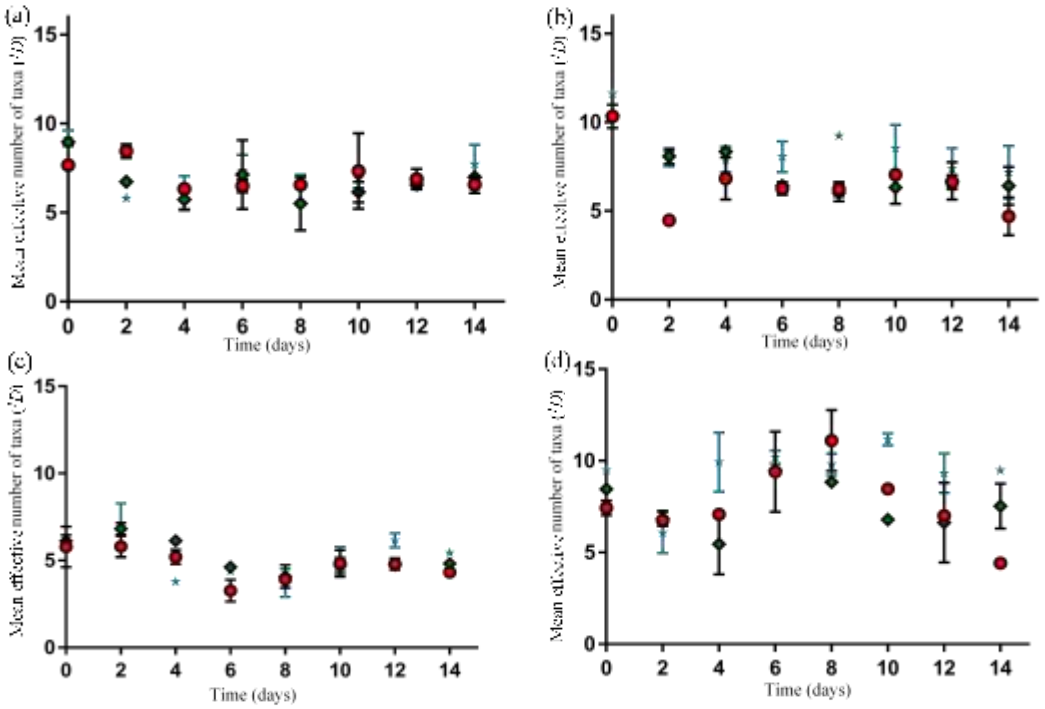

Figure 2: Consortia diversity, as determined by the Hill number ^1D, associated with 25°C (a), 35°C (b), 45°C (c) and 55°C (d) following triplicate incubation in the dark for 14 days and with 0.5 (●), 5 (◆) and 50 (★) mM o-xylene. Each datum point is the mean of triplicate samples. Error bars are SEM.

The highest values at day 6 and 8 as ^1D = 9.499 and 11.106, respectively, from day 8, the consortium diversity was reduced until the end of the incubation course. For 5 mM, the highest ^1D values were on days 6 and 8 as 10.206 and 8.846, respectively, after which the consortium remained static from day 10 until day 14.

Diversity of the dominant community members (^2D)

In general, Figure 3a, 3b, 3c and 3d, highlighted a significant statistical difference for the diversity of dominant community members. When temperatures 25°C, 35°C 45°C and 55°C applied on developed consortium and P value were <0.0001.

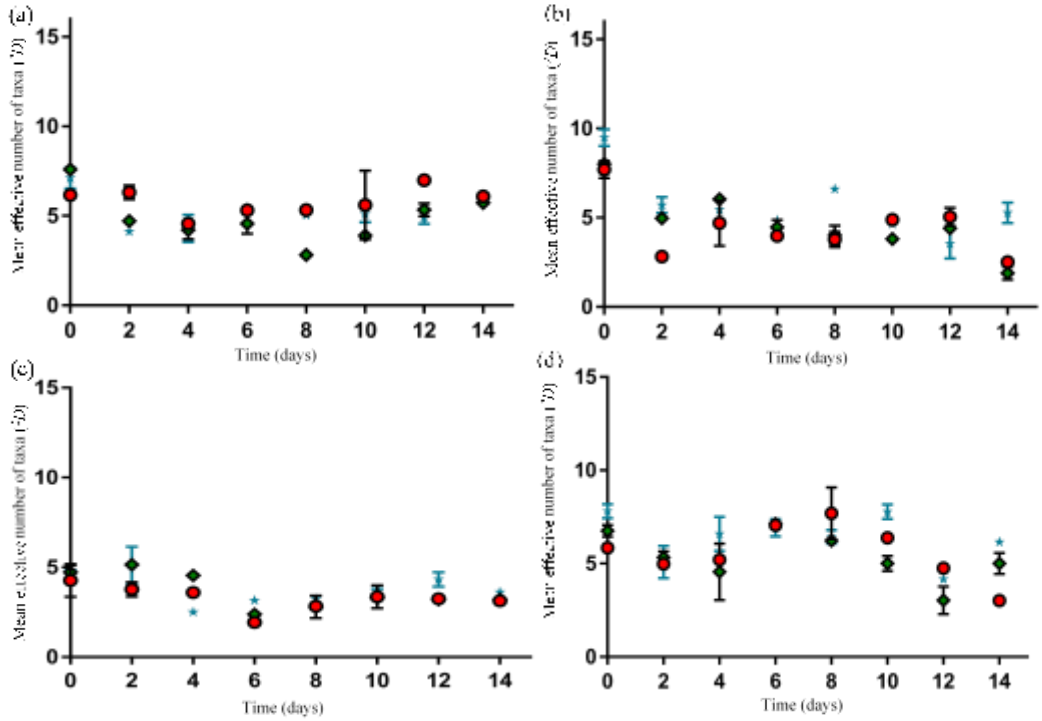

Figure 3: Consortia diversity, as determined by the Hill number ^{2}D, associated with 25°C (a), 35°C (b), 45°C (c) and 55°C (d) following triplicate incubation in the dark for 14 days and with 0.5 (●), 5 (◆)and 50 (★) mM *o*-xylene. Each datum point is the mean of triplicate samples. Error bars are SEM.

Evenness ($^{1}D/^{0}D$)

Figure 4a, 4b, 4c and 4d demonstrate a significant statistical difference for evenness at 25°C, 35°C 45°C and 55°C applied on developed consortium and 0.5, 5 and 50 mM recording P value <0.0001. According to $^{1}D/^{0}D$, the consortium was uneven at 25°C (Figure 4a).

In Figures 4b and 4d, under temperatures of 35°C and 55°C, the consortium showed a consistent effect of the three *o*-xylene concentrations except for day 2 as there was a decrease in $^{1}D/^{0}D$ value as it was 0.465 at 35°C under 0.5 mM and 0.320 for 55°C under 50 mM. Figure 4c revealed a decrease in $^{1}D/^{0}D$ in the presence of 0.5 mM *o*- xylene from the start point of inoculation until day 6. There was a fluctuation in $^{1}D/^{0}D$ for both 5 and 50 mM *o*-xylene starting from day 0 of inoculation until day 6. The consortium started to balance again after day 6 and continued to be stable until the end of the incubation course.

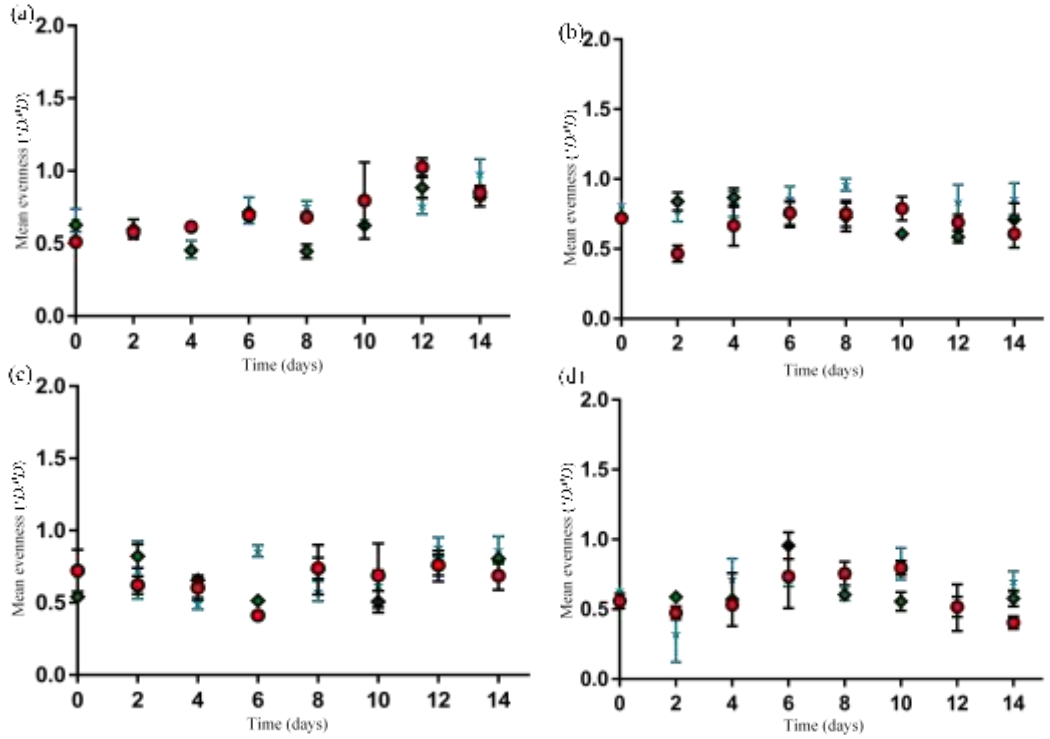

Figure 4: Consortia evenness, as determined by the Hill number $^1D/^0D$, associated with 25°C (a), 35°C (b), 45°C (c) and 55°C (d) following triplicate incubation in the dark for 14 days and with 0.5 (●), 5 (◆) and 50 (★) mM o-xylene. Each datum point is the mean of triplicate samples. Error bars are SEM.

3.3.1 Effect of pH regarding ecological indices profile

Richness (^0D)

Figure 5a, 5b and 5c a displayed a significant statistical difference regarding the consortia richness for pH 6.5 and 7.0 with P<0.0001. Furthermore, the P value was different for pH 7.5 as it was<0.0009.

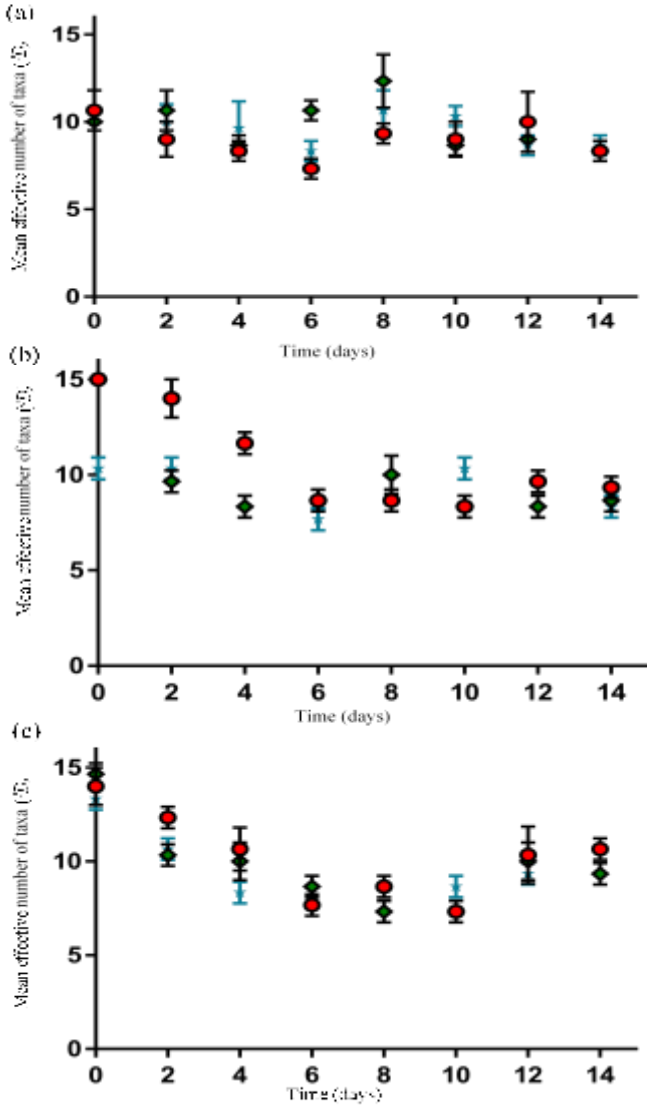

Figure 5: Consortia richness, as determined by the Hill number ${}^{0}D$, associated with pH 6.5 (a), 7.0 (b) and 7.5 (c) at °C 35 following triplicate incubation in the dark for 14 days and with 0.5 (●), 5 (◆) and 50 (★) mM *o*-xylene. Each datum point is the mean of triplicate samples. Error bars are SEM.

Diversity of common community members (${}^{1}D$)

A significant statistical difference in Figure 6a, 6b and 6c revealed consortia taxa richness for common community members at pH 6.5 and 7.0 with $P<0.0001$. The P value for pH 7.5 was <0.0019.

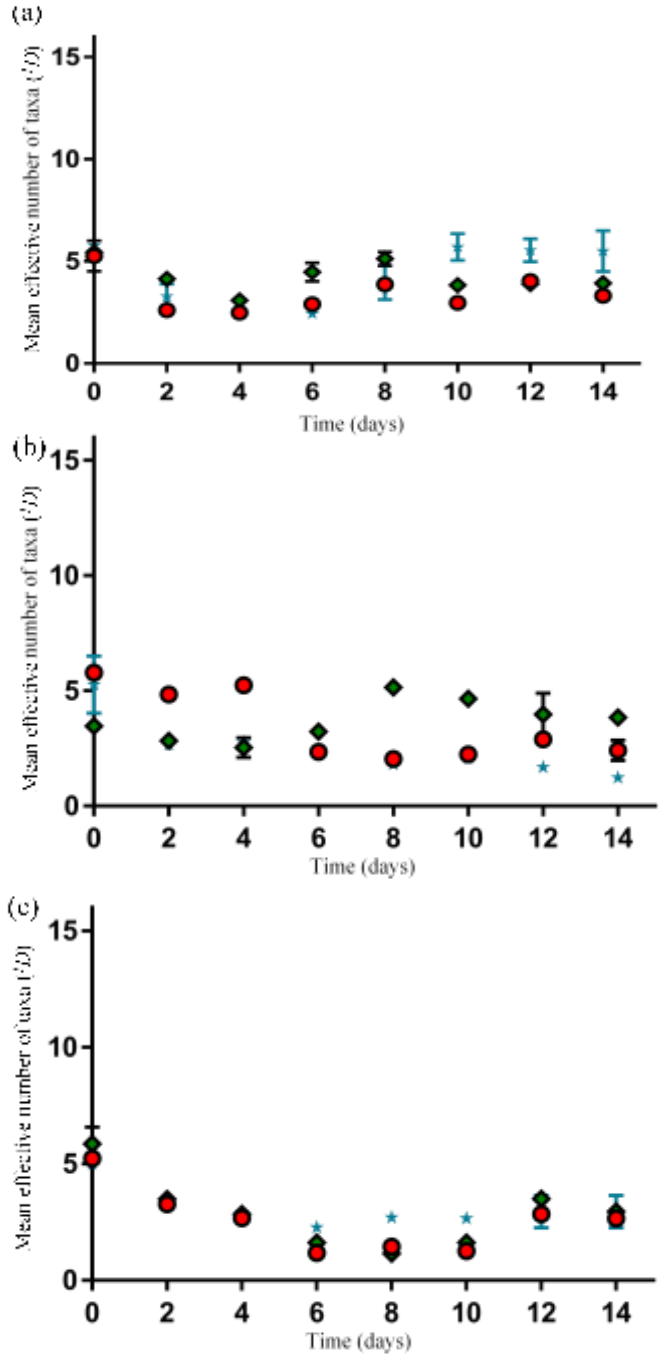

Figure 6: Consortia diversity, as determined by the Hill number ^{1}D, associated with pH 6.5 (a), 7.0 (b) and 7.5 (c) at 35°C following triplicate incubation in the dark for 14 days and with 0.5 (●), 5 (◆) and 50(★) mM *o*-xylene. Each datum point is the mean of triplicate samples. Error bars are SEM.

Diversity of the dominant community members (^{2}D)

A significant statistical difference was apparent for the consortia taxa diversity for pH 6.5, 7.0 and 7.5 with P<0.0001 in Figure 7a ,7b and 7c. There was a remarkable increase in the dominant taxa value

with ^{2}D 4.460 at 50 mM on day 4, this decreased again on day 6 with ^{2}D value= 2.008, which then remained static until the end of the study.

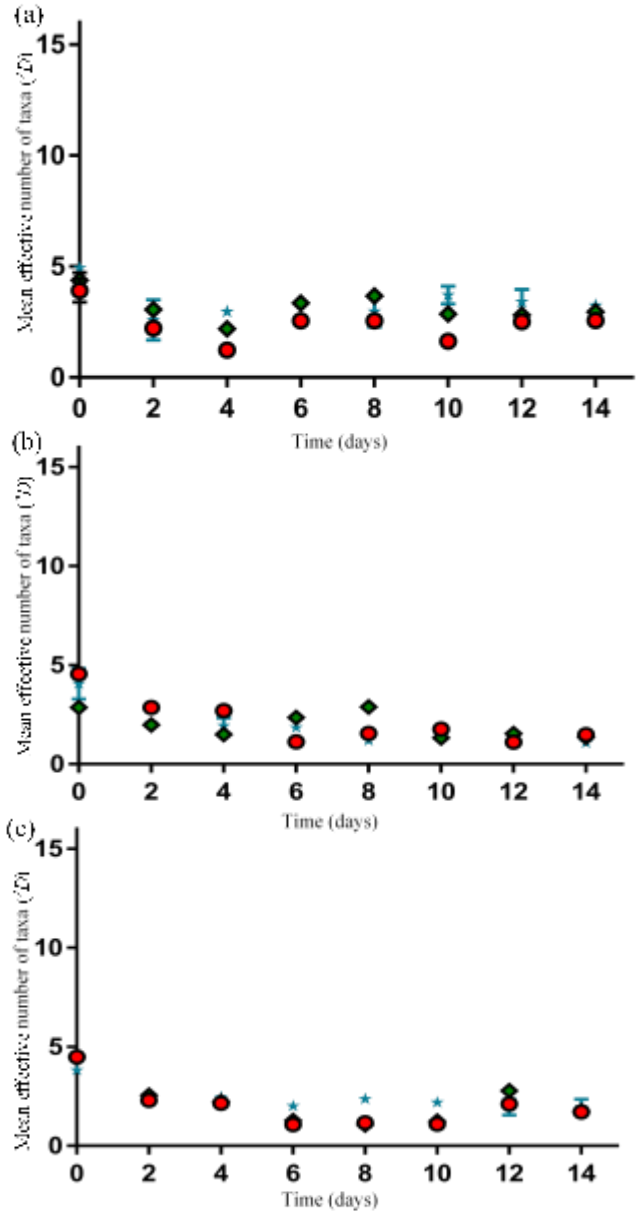

Figure 7: Consortia diversity, as determined by the Hill number ^{2}D, associated with pH 6.5 (a), 7.0 (b) and 7.5 (c) at 35°C following triplicate incubation in the dark for 14 days and with 0.5 (●), 5 (◆) and 50 (★) mM *o*-xylene. Each datum point is the mean of triplicate samples. Error bars are SEM.

Evenness ($^{1}D/^{0}D$)

In general, Figure 8a, 8b and 8c displayed a significant statistical difference regarding the consortia evenness for pH 6.5, 7.0 and 7.5 with P<0.0001. The highest concentration of *o*-xylene 50 Mm showed that the consortium started with $^{1}D/^{0}D$ value 0.372 to record a highest value at day 4 and 8 with 0.432. 0.461, respectively. Evenness then fluctuated and reached $^{1}D/^{0}D$ value 0.271 at day 14.

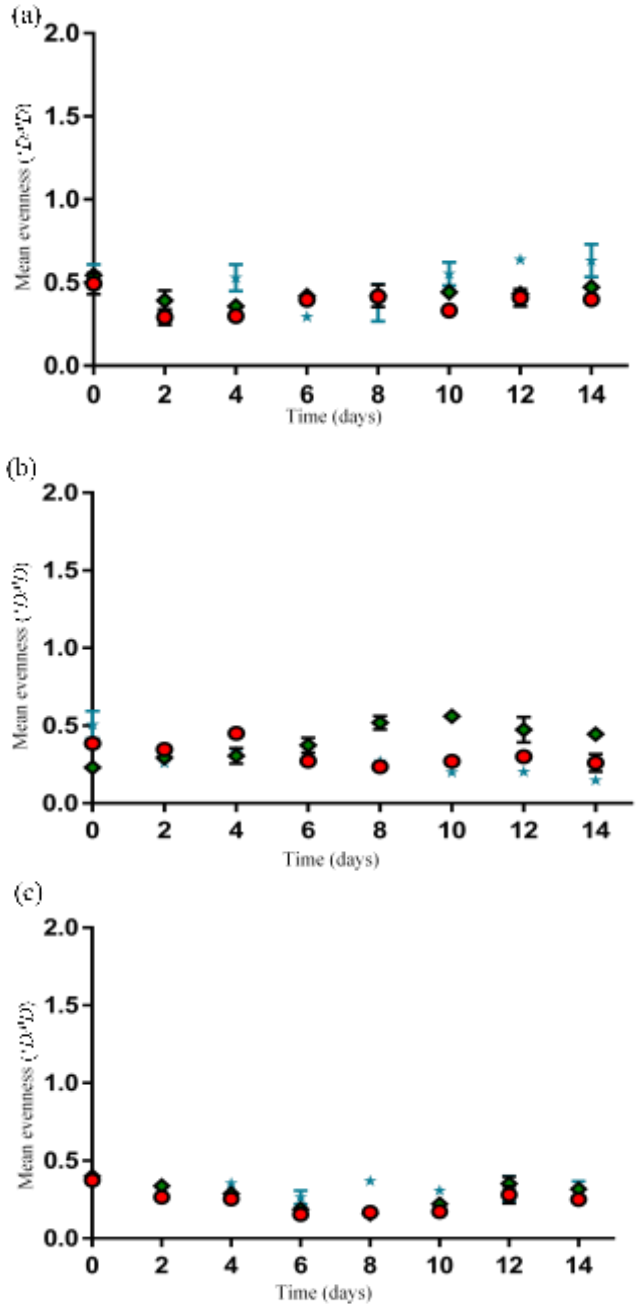

Figure 8: Consortia evenness, as determined by the Hill number ${}^{1}D/{}^{0}D$, associated with pH 6.5 (a), 7.0 (b) and 7.5 (c) at 35°C following triplicate incubation in the dark for 14 days and with 0.5 (●), 5 (◆) and 50 (★) mM *o*-xylene. Each datum point is the mean of triplicate samples. Error bars are SEM.

3.3.2 Changes of o-xylene degradation by a developed consortium

The biodegradation of *o*-xylene by the mixed consortium was carried out at an initial concentration of 0.5, 5 and 50 mM of *o*-xylene at pH = 7.0 The results are exhibited in Figures 9, 10 and 11 respectively. Figure 9a, 9b, 9c and 9d revealed that the consortium had good removal efficiencies of *o*-xylene at four temperatures and different *o*-xylene concentrations. The consortium grew between 25°C and 55 °C but the maximum removal of *o*-xylene occurred at 45°C firstly, 35°C and then 55°C secondly.

Figure 10 a, 10b, 10c and 10d revealed that the consortium preformed a good removal efficiency of *o*-xylene at 25°C and 35°C with 5 mM of *o*-xylene.

Figure 11a, 11b, 11c and 11d revealed the P value <0.0001 with 50 Mm of o-xylene and temperature 35°C had an effect where the *o*-xylene degradation efficacy is as explained with Figure 12c. The four different temperature were significant regarding *o*-xylene degradation efficacy.

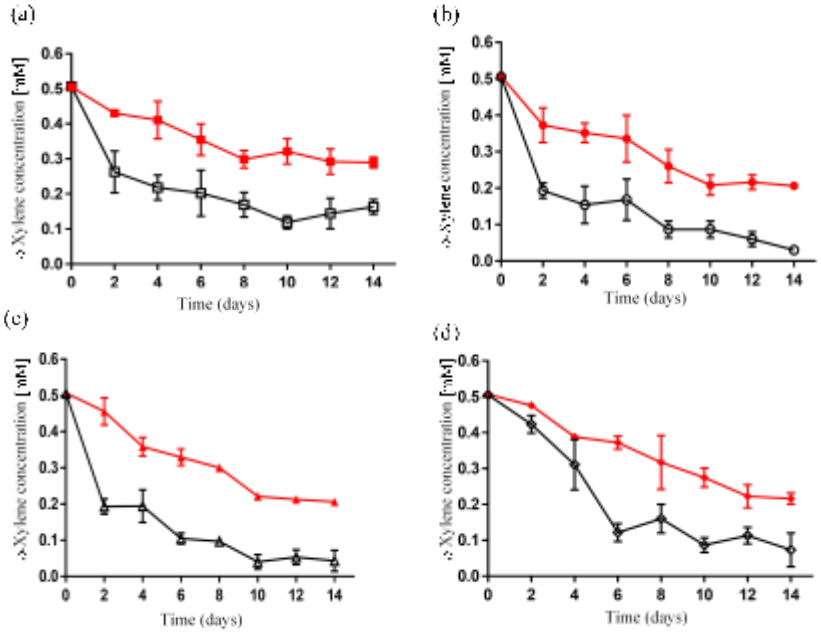

Figure 9: Changes in *o*-xylene concentrations at 25°C (a; consortium, Ab), 35°C (b; consortium, Ab), 45°C (c; consortium , Ab ▲), and 55°C (d; consortium , Ab). Consortium and abiotic controls (Ab) were subjected to 0.5 mM *o*-xylene and incubated at 150 rpm and different temperatures for 14 days. Each datum point is the mean of triplicate samples. Error bars are SEM.

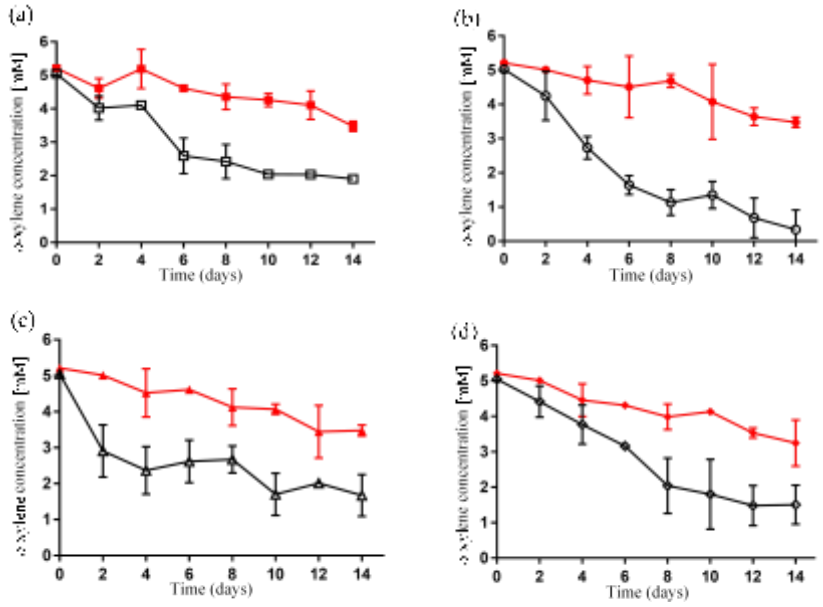

Figure 10: Changes in *o*-xylene concentrations at 25°C (a; consortium ⊟, Ab ■), 35°C (b; consortium ⊖, Ab ●), 45°C (c; consortium ⧋, Ab ▲), and 55°C (d; consortium ◈, Ab ◆). Consortium and abiotic controls (Ab) were subjected to 5 mM *o*-xylene and incubated at 150 rpm and different temperatures for 14 days. Each datum point is the mean of triplicate samples. Error bars are SEM.

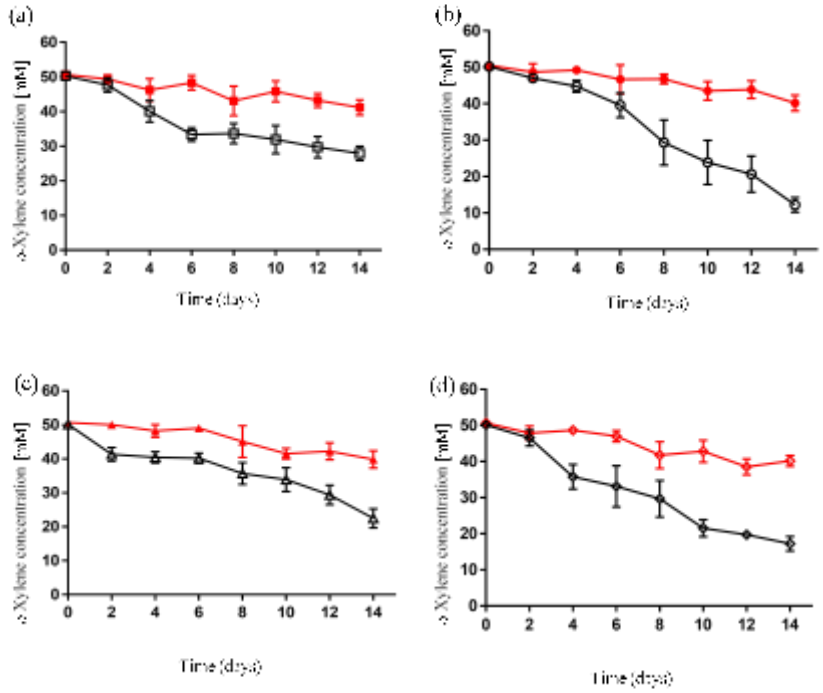

Figure 11: Changes in *o*-xylene concentrations at 25°C (a; consortium ⊟, Ab ■), 35°C (b; consortium ⊖, Ab ●), 45°C (c; consortium ⧋, Ab ▲) and 55°C (d; consortium ◈, Ab ◆). Consortium and abiotic controls (Ab) were subjected to 50 mM *o*-xylene and incubated at 150 rpm and different temperatures for 14 days. Each datum point is the mean of triplicate samples. Error bars are SEM.

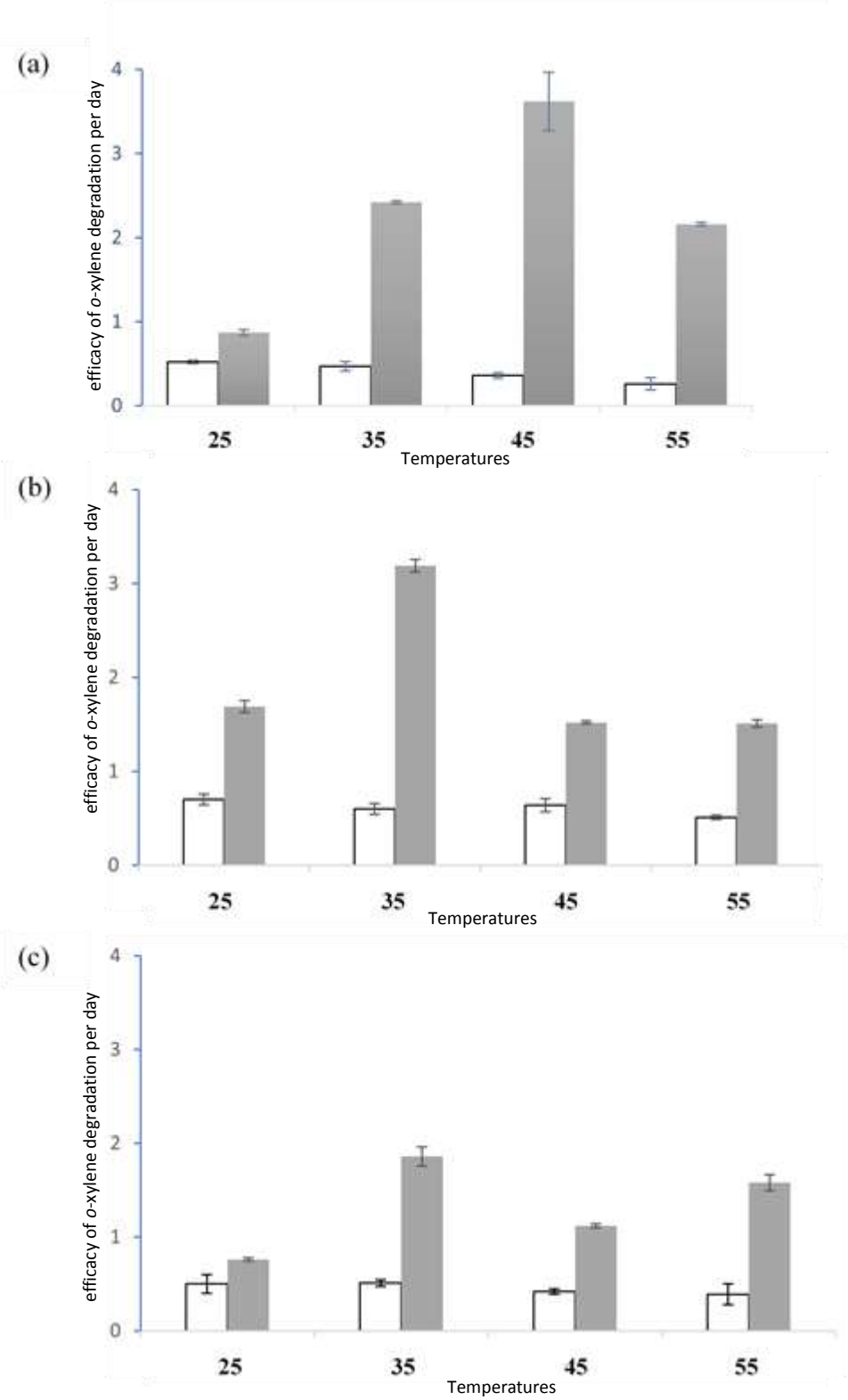

Figure 12: The efficacy of *o*-xylene degradation per day (control □ and consortium■). Efficacy calculated by fitting an exponential trend line to the data for (a) 0.5, (b) 5 and (c) 50 mM of *o*-xylene at different temperatures.

3.4 Sequencing for members of the developed consortium

Wastewater enters the Rustumihia plant and contains high concentrations of inorganic and organic contaminants. This was revealed by COD levels, according to original data collected from the same plant [32 & 33]. Generally, wastewater microbial community diversity is important since different variables such as pH, COD and BOD ratios are drivers of variation in stable wastewater treatment plants. For this study, individual members of the synthetic microbial community, which were drawn from distinct stages of the Rustumihia treatment plant, responded differently to changes in temperature and carbon source concentration. As a result, it was essential to establish if the observed structural changes of the consortia were linked to compositional shifts.

All the 15 isolates that generated unique DGGE bands and had high efficiencies to degrade *o*-xylene were identified via comparing their 16S rRNA consensus sequence data to information in the National Centre for Biotechnology Information (NCBI) GenBank databases with the aid of n-BLAST. The sequences with a score of more than 99% were considered to be of the same bacterial species as those identified in the GenBank. Thus, DNA sequencing results identified the microbial species that have the ability to tolerate 0.5 mM *o*-xylene as evident in Table 1.

Overall, the 15-member synthetic consortium consisted of six Gram-negative bacteria: Serratia sp., *Echerichia coli*, *Klebsiella* sp., *Pseudomonas* sp., *Bacillius* sp. and *Stenotrophomonas* sp. and one Gram-positive *Streptomyces* sp. The 16S rRNA consensus sequence data revealed *Stenotrophomonas* sp., *Serratia* sp, and *Bacillus* sp to be the most common bacterial classes across all four stages of the Rustumihia treatment plant. All of the consortia species had the ability to tolerate *o*-xylene at a concentration of 0.5 mM.

Table 1: 16S rRNA consensus sequence data compared with the information in the NCBI GenBank data bases via n-BLAST.

Sample type	Bacterial species	Total score	Query cover	Identity	Accession Number
S1/1	*Serratia ficaria* strain NCTC12148. Genome assembly; chromosome 1	21697	100%	99%	LT906479.1
S1/2	*Echerichia coli* strain ST540. Complete genome.	29823	99%	99%	CP007390.1
S1/3	*Stenotrophomonas* sp. LM091. Complete genome.	4798	100%	99%	CP017483.1
S1/4	*Echerichia coli* strain C8. Complete genome.	24733	100%	99%	CP010125.1
S1/8	*Bacillus subtilis* strain MJ01. Complete genome.	19804	100%	99%	CP018173.1
S2/13	*Klebsiella variicola* strain HKUOPIA	24995	100%	99%	CP012252.1
S2/17	*Serratia ficaria* strain NCTC12148. Genome assembly; chromosome 1.	21025	99%	99%	LT906479.1
S3/18	*Pseudomonas* sp. STFLB209 DNA Complete genome.	17525	100%	99%	AP014637.1
S3/19	*Bacillius subtilis* starin GQJK2. Complete genome.	14427	97%	100%	CP020367.1
S3/22	*Serratia marcescens* SM39 DNA. Complete genome.	20192	100%	99%	AP013063.1
S3/24	*Streptomyces* sp. Strain RKND-187 16S ribosomal RNA gene. Partial sequence.	3117	100%	100%	KY362392.1
S3/25	*Stenotrophomonas rhizophila* strain DSM14405 genome.	9324	100%	99%	CP007597.1
S3/28	*Stenotrophomonas* sp LM091. Complete genome.	9374	100%	99%	CP017483.1
S4/38	*Bacillus subtilis* starin MJ01. Complete genome.	29945	100%	99%	CP018173.1
S4/41	*Pseudomonas aeruginosa* S86968. Complete genome.	12393	100%	100%	CP008865.2

4. Discussion

In the present study, the performance of a synthetic bacterial consortium was assessed with respect to the ecological indices of richness, diversity and evenness, with the aid of denaturing gradient gel electrophoresis as a molecular microbial ecology technique. *O*-xylene degradation was evaluated via GC-FID analysis as a quantifying method. The environmental conditions that affect the structural composition of the consortium were also determined. The microbial consortium reacted differently under various ecological stress conditions of temperature, pH and *o*-xylene concentration. This supports the concept of shaping the mixed microbial consortium.

4.1 Effects of temperature

Understanding the diversity patterns of wastewater microbial community structure and succession remains a principal aim in microbial ecology science [34]. The diversity of the bacterial assemblage in wastewater microbial community are believed to control the ecosystem function [35] and [34]. Microbe's associations in different ecosystems such as, wastewater, soil and marine ecosystem can be randomly determined by assuming the equal fitness for all the species sharing one microbial ecosystem, as well as the changes in structure that could arise from random events of ecological accumulations. Alternatively, the associations can be affected by deterministic factors [36] when specific communities are shaped as a result of niche diversity formed by biotic or a biotic factor [37]. The dynamics of random or deterministic events of ecological accumulations have been reported to concurrently act in driving diversity patterns of any microbial community structure observed in nature [38;39 & 34]. This encouraged a scientific discussion including modelling of experimental data [40] and [34] both manipulative and observational of experimentation in a variety of microbial ecosystems. For example, groundwater [41], deserts on a global scale [42], soil plant–fungi associations [43], sludge bioreactors [44;45 & 46] and water ponds [47].

The manipulation in any of growth factors for a group of microbial assemblage is considered as a disturbance. In ecology, disturbance could be defined as an event that physically injures, inhibits or kills some members that are part of the community, making chances for other members to reproduce and grow [34]. The main factor that influences the species variations in diversity is disturbance (Mackey and Currie, 2001), as well as structuring the microbial ecosystems [48 & 49]. However, there is still a lack of a clear understanding of the disturbance outcomes [50]. According to [34], microbial diversity can reach a peak when disturbance is applied for short term in any factors that control microbial ecosystem. This was because of the trade-offs between species that could tolerate the applied disturbance and species with the ability to compete and colonize ecological niches. In the current study, several disturbance factors (different concentrations of *o*-xylene, different pH and different temperatures) were applied on a consortium of selected species that were isolated from four different sites of Rustumihia WWTP. For example, different patterns of diversity were observed with increasing disturbance frequency with biomass destruction in freshwater and soil bacterial communities [51]. Today, the mechanism behind the observed patterns of diversity under disturbance still need more explanation and clarification. [52 and 34].

Diversity and richness for the mixed microbial consortium dropped gradually under three temperatures of 25°C, 35°C and 45°C and two lower concentrations of 0.5 and 5 mM *o*-xylene (Figure 1; Figure 2a – 2c). In contrast, the richness at 55°C remained constant with 0.5 mM *o*-xylene, while the diversity as determined with ${}^{1}D$ and ${}^{2}D$ dropped and then recovered again on days 6 and 8 as evident from Figures 2d and 3d.

A consortium which consisted of 15 bacterial isolates was employed to analyse the changes in the ecosystem function over an incubation time of 14 days, by measuring the degraded *o*-xylene that was added at different concentrations and was the only sole source of carbon in BMS. The community structure changes in microcosm were examined via 16S rRNA gene fingerprinting techniques. These changes were evaluated by diversity tools for both common and dominant species. Also, we explored how diversity was related to bacterial function emphasising on microbial community shifts through bacterial trade-off.

Ecological indices showed that, consortium was affected by different *o*-xylene concentrations at different temperatures. At 50 mM of *o*-xylene, the highest microbial consortium richness at 25°C, 35°C, 45°C and 55°C was recorded when ${}^{0}D$= 13.66,11.66, 8.66 and 13.33, respectively.

[53] suggested that "low evenness at high disturbance levels could be caused by the dominance of a few disturbance specialists". Additionally, the use of richness indices for any microbial communities is not dependable [54] because it is heavily restricted with the way of measurement [55], which makes it difficult to compare between results for different studies using this metric. Moreover, for complex communities like wastewater or sludge there are often huge differences between the abundance of common taxa and dominant species. For this reason, for microbial systems, it is reasonable to evaluate diversity in terms of more robust compound indices rather than richness, and this is the reason why we focused on ${}^{1}D$ and ${}^{2}D$ to assess diversity for Rustumihia wastewater samples.

Diversity in terms of common and dominant species resulted as the following: ${}^{1}D$ = 8.136, 9.246, 6.122, 11.134 and ${}^{2}D$ = 5.301, 7.105, 5.331, and 7.565. The degradation efficacy was 32.6%, 63.08%, 42.5% and 58.3%. These findings led to the conclusion that some or all members of the mixed consortium were able to tolerate and degrade high *o*-xylene concentrations, and under variable temperatures (Figure 1b, Figure 2b, Figure 3b, Figure 4b, Figure 11b and Figure 12c).

Shifts in the synthetic consortium might be due to the short incubation time and different *o*-xylene concentrations alongside with different temperatures. Results in Figure 2b and d, and Figure 3b and d, suggest an increased α-diversity as a type of response to the disturbance in the established microcosm, because of the adaptation to the changes in the growth factors inside the environment. Consequently, samples triplicates displayed a higher and robust results in terms of both community structure and function. Overall, the results inferred that the high concentration of *o*-xylene affected positively the richness diversity of hydrocarbon clastic bacteria within the synthetic consortium.

Diversity levels might stimulate the productivity and functionality of hydrocarbon degradation (Figures 1d, Figure 2d, Figure 3d, Figure 4d, Figure 11d and 12c). This suggests that a highly diverse microbial consortium may promote the functional diversity and, therefore, degrade a wide range hydrocarbon concentrations and types. Finally, diversity for the four different temperatures at a very high *o*-xylene concentration positively impacted the findings and correlated with the common species (Figure 2d).

The observed patterns of α-diversity assessed after 14 days of incubation suggested that the diversity increased over time with respect to the consortium inoculum. Such a temporal increase in diversity was higher at 50 mM of *o*-xylene and 35°C (Figure 12c). These results were interpreted alongside with the GC-FID results in confirming the higher removal for 50 mM as explained in Figure 11d.

Hill numbers ${}^{0}D$ and ${}^{1}D$, which give higher weight to higher abundant operational taxonomic units (OTUs), represent the same pattern. Welch's ANOVA tests were statistically significant for all Hill numbers ($P < 0.01$). In addition to that, there were robust correlations between Hill numbers diversity and degradation ability of indigenous microbial consortium as represented by GC-FID results.

Temporal dynamics were expected to occur in the synthetic microcosm since the isolated bacterial species from the four treatment phases experienced an initial perturbation after transfer from the Rustumihia wastewater treatment plant environment to our laboratory microcosms. For the synthetic microcosms, this implied changes in growth condition, different concentrations of carbon source (*o*-xylene), and transferring isolated species from open to closed synthetic system. This was a succession scenario in which synthetic microcosm had to adapt to such changes along with the designed disturbance array. The dominant species which were represented with ${}^{2}D$ decreased over the first week of incubation for 25°C, 35°C and 45°C, probably because the dominant species in the synthetic microcosms might represent the most predictable environments within our disturbance range from various temperatures and xylene concentration. Moreover, both ${}^{1}D$ and ${}^{2}D$ were positively correlated with *o*-xylene degradation results, suggesting that higher community evenness favours functionality including ability to degrade *o*-xylene under selective pressure [56] Therefore, I suggest that more diverse microbial communities have enhanced functionality without considering trade-offs. This supports the idea that higher α-diversity might imply a "better" [57].

Temperature is regarded as one of the most important factors that affect the dominant of hydrocarbon tolerant bacteria and probably, therefore the balance of all the species in the artificial microcosm. At

35°C, the consortium achieved high degradation percentage for each of 0.5, 5 and 50 mM of *o*-xylene with values 73.1%, 94.8% and 63.08%, respectively, as evident in Figure 9b, 10b and 11b. Additionally, the efficacy of *o*-xylene degradation was highest at 5mM (Figure 12b) at the same temperature. This suggests that a combination of a medium temperature of 35°C, with different concentrations 0.5, 5 and 50 mM of *o*-xylene, may favour certain species and make them more dominant than others that formed the initial consortium. The temperature influenced the sensitivity of the hydrocarbon-tolerant bacteria, raising it to a high *o*-xylene concentration. The high value of the species richness of the consortium possibly indicated a high catabolic capacity of *o*-xylene. These results may indicate the presence of a high number of common species of heterotrophic bacteria that dominate the consortium.

The developed consortium demonstrated a very good degradation efficiency of *o*-xylene at 35°C and 0.5, 5 and 50 mM, which suggested the potential application of the consortium for wastewater bioremediation. On the basis of literature [16] and sequencing data from the current study (Table 1), it is predicted that *Pseudomonas* sp. and *Bacillus* sp. were the main functional bacteria at 50 mM of *o*-xylene concentration considering that *o*-xylene was the sole contaminant in the enrichment media. This will be assessed further with next-generation sequencing. Presumably, the other species existing in the consortium were related to the main functional bacteria via the adhesion process [58 & 59] and [60]. The consortium has the benefits of being well adapted to the heavy polluted environment and to the efficient degradation of *o*-xylene at a high concentration; that is consistent with what [61] concluded.

4.2 Effects of pH

The impact of pH on the consortium structure was investigated under different *o*-xylene concentrations at 35°C. Ecological indices were used to assess richness and diversity in terms of common and dominant species. The results displayed that 6.5 was the best pH. They also confirmed that pH plays an important role in *o*-xylene degradation, pH 6.5 affected the activity of the consortia richness and evenness as evident in Figure 5a, Figure 6a, Figure 7a and Figure 8a.

The DGGE results illustrated that the microorganisms which were used in the developed consortium, tolerated the low pH levels as they could survive and grow in acidic conditions [62]. Bacillus is able to tolerate harsh and stressful environmental conditions, which may explain why the organisms can reduce themselves to stabilize in a dormant situation during the 14 days of incubation time [9; 63; 64; 65 & 66]. The consortium richness differed, depending on the observed DGGE band. The reason for this was competition between the different bacterial species under diverse *o*-xylene concentrations alongside the different pH.

Also, some members of these genera are identified as spore-forming bacteria like *Bacillus* sp, *Stenotrophomonas* sp., that proved their resistance in the stressful environmental conditions; for example, high temperature and high *o*-xylene concentrations. *Stenotrophomonas* sp. and *Serratia* sp. are Gram-negative bacteria and they are well-known as aerobic soil organisms. *Serratia* sp. *is considered as a* suitable strain for bioremediation processes in polluted soil with the organophosphorus pesticides (OPPs). The genera *Stenotrophomonas* and *Serratia* are identified for their ability to utilize petroleum hydrocarbons [67; 68 & 69], due to the presence of two enzymes that are involved in the enzymatic reaction processes of petroleum hydrocarbon degradation and *o*-xylene in particular - firstly, monooxygenase and secondly, dioxygenase enzyme [70]. The current study also suggests that these two species may have the ability to oxidize *o*-xylene (see Table 1).

4.3 Effects of o-xylene concentration

The developed microbial consortium changed at 0.5, 5 and 50 mM for both 25°C and 45°C. Over the biodegradation process in the mineral salt media, the degradation efficiencies were 33.3%, 51.2% and 32.6% at 25°C (Figure 9a, Figure 10a and Figure 11a), respectively, while at 45°C they were 96.3%, 48.8% and 42.5% (Figure 9c, Figure 10c and Figure 11c), respectively. The efficacy of *o*-xylene degradation was significant for 0.5 and 50 mM at 25°C and 45°C as evident in Figure 12a and 12c. Richness and diversity indices were observed as related to common and dominant species. They were constant. Presumably, the reason for this observation is that the microbial activity of biodegradation

was induced by the added *o*-xylene, although the microbial biomass remained stable. For this reason, the number of microorganisms changed very little, but it induced the activity of the biocatalytic enzyme to reach the maximum degradation efficiency.

The consortium members that could degrade *o*-xylene were likely to increase in their richness (^0D) during incubation in the presence of *o*-xylene. This was true, in some cases, where bacterial dominance showed a robust connection to *o*-xylene degradation by the mixed consortium. This was linked potentially to some strains under their favourable degradation temperature. In certain cases, and for specific microbial strains, this could not be true, especially when dominance was not connected to *o*-xylene degradation. This is explained as by the bacterial strains that may utilize *o*-xylene as a sole carbon source during growth and favour certain temperatures. In contrast, other bacteria may grow on the metabolites or *o*-xylene degradation by-products generated by other strains in a phenomenon known as commensalism. As a result, the *o*-xylene degradation activity depends on the strains dominant. However, in some cases, the strains might increase in their richness value even though they are unable to degrade *o*-xylene. Therefore, a single isolated strain in a mixed bacterial community might presumably have different behaviour towards the *o*-xylene degradation process.

Figure 2a presented the consortium diversity regarding the common species (^1D) under the three *o*-xylene concentrations of 0.5, 5 and 50 mM, which showed no remarkable change. This might be attributed to the low temperature 25°C that was applied during that experiment. This leads to the prediction that there was no competition between the consortium members because of the low temperature that might affect the catabolic gene functionality. According to [61], the removal efficiency of *o*-xylene by *Azotobacter chroococcum* was linked to low temperatures directly as the degradation rate decreased between 15°C and 25°C, while the best contaminant removal was at 30°C. Also, the highest value of ^1D = 9.249 at 50 mM suggested that the common members of the consortium could adapt to the high *o*-xylene concentrations for a long time, a process which might increase the consortium efficacy to degrade *o*-xylene.

The degradation ability of the consortium was assessed for the three *o*-xylene concentrations in BMS. The *o*-xylene loss during treatment in culture was negligible in the abiotic control without a microbial inoculum compared to the consortium sample. The synthetic microbial consortium exhibited higher degradation ability at 5 mM of *o*-xylene as evident in Figure 10b and Figure 12b, especially at 35°C as also observed by [61]. [71] added yeast extract at different concentrations to media that were supplemented with different concentrations of BTEX in order to improve the degradation ability for the used consortium. According to [72] and [71], the yeast extract served as another carbon source for the consortium and potentially contained stimulator factors that were important for the efficient expression of the BTEX-degrader genes. In case of the mixed consortium that was developed and used in the current study, *o*-xylene was the sole source of carbon and the consortium showed a sufficient capacity for *o*-xylene degradation at three different concentrations and under four different temperatures.

The decrease in $^1D/^0D$ between days 0 and 6 due to 0.5 mM *o*-xylene Figure 4c suggested that the low concentration under high temperature had a negative effect on the consortium evenness. Presumably, the consortium did not prefer 45°C at the start of the incubation. However, after day 6, the consortium adapted itself to the high temperature at the same low concentration.

The ecosystems of the synthetic consortium used in the current research programme, responded positively in terms of the ecological indices to the stress growth conditions of medium and high temperatures of 35°C and 55°C, and high concentration of *o*-xylene at 50 mM. Furthermore, the consortium recorded the maximum diversity on BMS containing 0.5, 5 and 50 mM *o*-xylene as a sole carbon source at 25°C, 35°C, 45°C and 55°C. Finally, there was a significant statistical difference in the microbial consortium regarding richness (^0D), common community member species (^1D) and dominant community member species (^2D).

There are different bacteria from the *Pseudomonas* genus with demonstrably diverse metabolic activity and are known for their organic-degrading abilities [73]. [74 & 75] confirmed Gram-negative strains that were capable of metabolising aromatic hydrocarbons and ethylene, benzene, toluene and xylene as their only carbon source. *Pseudomonas* sp. belongs to a heterotrophic group known for its ability to degrade *o*-xylene [73]. *Bacillus cereus* is also known for its ability to degrade *o*-xylene and

other organic compounds, and to grow in acidic environments [76]. According to [77], a consortium consisting of two members, for example, *Pseudomonas aeruginosa* and *Bacillus subtilis;* was capable of degrading a mixture of BTX completely.

For the current study, isolates S3 18 and S4 41 (Table 1), which were isolated from the settlement tanks and the river discharge, respectively, were identified as *Pseudomonas* sp. STFLB209 (99%) and *Pseudomonas aeruginosa* S86968 (100%), respectively. Similarly, [78] isolated *Pseudomonas putida* and *Pseudomonas stutzeri* from oil refinery wastewater. Their results proved the ability of these two bacteria to degrade a combination of benzene, toluene and xylene and to tolerate the high concentrations of these compounds, especially when mixing two *Pseudomonas* species and adding the BTX compounds to the enrichment media. Further to this, [79 & 80] observed that the efficiency for removing *o*-xylene was not only enhanced, but also increased parallel with the removal of benzene and toluene [73]. The *Pseudomonas* sp. STFLB209 and the *Pseudomonas aeruginosa* S86968 are indigenous species isolated from the Rustumihia WWTPs. They are probably more adapted to the Iraqi local environmental conditions. Therefore, these two *Pseudomonas* species survived and remained predominant during the course of treatment.

5.Conclusions

The results of this study indicated that the high *o*-xylene concentrations of 50 mM could be tolerated and degraded effectively at 35°C and 55°C, and pH 6.5. Bacterial richness and diversity were recorded according to the Hill parameters of ${}^{0}D$, ${}^{1}D$ and ${}^{2}D$ under each of the growth conditions, and then linked to the *o*-xylene degradation efficiency. They ecological indices were related directly to the concentration of *o*-xylene and the incubation temperature. The sole carbon source supported the diversity of the model microbial consortium at the highest *o*-xylene concentration. Future work should investigate more closely the potential of the consortium for *o*-xylene degradation during the first 24 hours of incubation under the optimum conditions of 35°C and pH 6.5. It should also examine the catabolic gene that is responsible for *o*-xylene degradation.

According to the sequencing results, our synthetic consortium consisted of Gram-negative bacteria from the Serratia, *Echerichia*, *Klebsiella*, *Pseudomonas*, *Bacillius*, *Stenotrophomonas* genera and a Gram-positive member from the *Streptomyces* genus. This mixed consortium, as isolated from the four stages of the Rustumihia WWTPs, was capable of tolerating and degrading different *o*-xylene concentrations under diverse growth temperatures. These findings support the hypothesis that *o*-xylene was used as the sole source of carbon for cell growth and energy. Additional focus is required on more specific experiments that deal with the catabolic genes responsible for the *o*-xylene degradation. According to [81], a consortium culture including six Gram-negative bacteria (*Chryseomonas* sp., *Agrobacterium* sp., *Pseudomonas* sp., *Serratia* sp., *Flavobacterium* sp. and *Xanthomonas* sp.) and three Gram-positive bacteria (*Micrococcus* sp., *Bacillus* sp. and *Arthobacter* sp.) could biodegrade and tolerate each of the benzene, toluene, ethylbenzene and xylene (BTEX). The bioremediation processes of wastewater employing microorganisms and their aggregates, which are identified to be cost-effective biological treatments correlate with each other at a relatively low cost, compared to the conventional chemical and physical treatment processes. The microorganism can be employed in the following two ways:

1. There could be a direct mixture of wastewater with free microorganisms, as there is no separation between the treated water and microorganisms.
2. The microorganism could be encapsulated within a matrix of immobilized and embedded materials, keeping a distinct separation between the treated water and the microorganisms.

[82] reported that the immobilized bacterial cells like *Pseudomonas putida, Bacillus* sp., *Pseudomonas* sp., *Serratia* sp. gave enormous promises to clean up an extensive range of contaminants counting hydrocarbons, phenolic organic compounds, inorganic dyes, N-dimethylformamide, pyridine, propionitrile, benzene, toluene and xylene (BTX).

Biodegradation has been used for *in situ* clean-up of sites contaminated with the mixed hydrocarbon isomers benzene, toluene, and *o*-xylene [83] and [84]. Recently, it was reported that successful biodegradation was dependent on inoculated consortia consisting of different bacterial species that

degraded hydrocarbon molecules [85;86 & 87]. A single organism is capable of mineralizing *o*-xylene with a certain activity. However, it has been reported that use of consortia of mixed bacteria with different abilities to degrade hydrocarbons is an effective alternative method for the remediation of contaminated soils or wastewaters [9; 89;70 & 21].

There are 31 hospitals in Baghdad, three of which employ only primary treatment units before discharging wastewaters into the main drain. The remaining 28 hospitals dispose effluents directly to the main drain. Environmental awareness among most Iraqi families has deteriorated in recent years, affecting the quality and type of bacteria in the sewage of domestic households [90]. The sewage from domestic households and hospitals in Iraq differ in their quality and type of bacteria [90 & 87]. For the Rustumihia WWTPs, all the wastewater comes from hospitals, households and the industrial sector. For this reason, different types of microorganisms are usually generated and accumulated. Most of the motile bacterial species are associated with traps and washbasin wastewater pipes particularly *P. aeruginosa* and other Gram-negative species, which are consistent with our findings.

The observed changes in OTU diversity, that was evident by the variation in microbial ecosystem function along the disturbance range studied, proved that high evenness lead to robust ecosystem (Fig 4 b and 12 b). These findings suggest that higher α-diversity at high *o*-xylene concentrations, which caused disturbance in species frequencies inside the synthetic microcosm, is the result of robust stabilizing mechanisms (niches). It is proposed that infrequent or too-frequent disturbances, for example high *o*-xylene concentrations, different pH and different temperatures, will lead to some species being favoured over others. Since the underlying assembly mechanisms would affect taxa abundance distributions, frequencies increased the competitive ability of previously common taxa that were measured by ^{1}D. As a result of that, the mechanisms of ecological drift could then play a critical role in shaping the emerging structure of microbial communities [91].

Where random processes of reproduction, birth and death for microbial communities can influence which of these low abundance taxa, and which will be more beneficial because they occupy opened niches, and this could be explained as early disturbance could reduce the availability of food resources to other taxa whose abundance will then be restricted. Higher α-diversity in this study was found at *o*-xylene concentrations of 5 and 50 mM and temperature 35°C. Disturbances into wastewater ecological system could be represented by different carbon concentrations and various temperatures. All these factors together could promote and control assemblages of the Rustumihia wastewater microbial communities, especially when temporal disturbance occurs in wastewater microbial environment. This will result to favour the random assembly leading to a different final community after the perturbation [48]. This might influence the expected ecosystem function. For Rustumihia WWTP, there is a need to identify such scenarios in practice. This study described how different frequencies of disturbance affected ecosystem function and diversity of bacterial community as well as, their assembly in a synthetic microcosm. Communities were evaluated through molecular methods and DGGE techniques. Moreover, besides the Rustumihia WWTP microbial community, other complex microbial systems (e.g., oil contaminated soil) might demonstrate similar responses to ecological disturbance. In this chapter, the argument is that changes not only in diversity because of favouring certain concentrations, pH and temperature, but also in the underlying deterministic–random assembly mechanisms, were assessed in this study of the effects of disturbance on such systems.

There is still an urgent need for broad and multifaceted studies in this field to enhance treatment efficiencies and to improve the waste treatment technology with an integrated system. To the best of my knowledge, the current study is the first of its kind in Iraq. It investigates the enrichment, isolation, and identification of a microbial community from the Rustumihia WWTP and determines the efficiency of the isolates to tolerate and degrade *o*-xylene, highlighting their sole source of hydrocarbon. Consequently, there is a need for research studies that: (i) explore in-depth the degradation activity of each isolate from the current research programme; and (ii) screen the other reported catabolic genes that are responsible for biodegradation. These are essential steps towards the enhancement of the bioremediation strategies for the Rustumihia WWTP in particular.

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