PHYTOREMEDIATION OF NAPROXEN IN WASTE WATER USING *Vetiver zizaniodes*

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Abstract

In this study, the capability of *Vetiver zizaniodes* in removing the pharmaceutical-based product, naproxen from wastewater was evaluated. The plant was exposed to naproxen at concentrations ranging from 50, 100, 300 and 500 mgL-1 for duration up to 56 days. Several wastewater parameters including pH, dissolved oxygen (DO, mgL^{-1}) and oxygen-reduction potential (ORP, mV) were recorded during the experimental runs where pH and ORP exhibited significant different ($p < 0.05$). At the end of the exposure, 81.5%, 64.5% and 60.9% of naproxen were removed at 100, 300 and 500 mgL^{-1} concentration, respectively. The interaction between time, system and concentration showed significant naproxen removal ($p < 0.05$) from water and soil. Phytostimulation and phytostabilization played a major role for naproxen removal when there was an activity of the bacteria presence in the soil around the roots of the plants. Hence, *V. zizaniodes* is a potential plant in remediating pharmaceutical-contaminated water and could act as a phytoremediator plant.

Keywords: Phytoremediation, Naproxen, *Vetiver zizaniodes*, Phytotoxicity,
          Rhizobacteria.

1. Introduction

Pharmaceutical micro pollutants are increasingly polluting the environment especially the aquatic environment. These pollutants enter the environment as a result of pharmaceutical industry activities, and improper disposal of unused or expired drugs, waste generated in hospitals and stock raising farms [1, 2]. Most of non-steroidal anti-inflammatory drugs such as ibuprofen, ketoprofen, naproxen

and diclofenac are not fully degraded after application and get into the environment in an unmovable or slightly modified form. Due to their stability, they are not totally eliminated by the sewage treatment plants, thus it can be unintentionally consumed by humans in tap water [2].

The occurence of micropollutants in the aquatic environment has been frequently associated with a number of negative effects, including short-term and long term toxicity, endocrine disrupting effects and antibiotic resistance of microorganisms [3-5]. Onyiri [6] claimed that the pharmaceutical micropollutant have the potential to harm aquatic organisms and disrupt algal productivity. Many studies have also revealed the presence of some of these chemicals in fish tissues, while many literatures are available to date on the effects of pharmaceutical micropollutant on human health/aquatic organisms.

Conventional wastewater treatment plants (WWTPs) are not specifically designed to eliminate micropollutants. Thus, many of these micropollutants can easily pass through the wastewater treatment processes due to their continuous introduction. Phytoremediation represents a promising technology whereby plants and their associated microbial communities (rhizosphere) offers greater potential to remediate various pollutant including naproxen [7]. Phytoremediation can degrade, remove, transform, or immobilize toxic compounds located in soils, sediments, and more recently in polluted ground water and wastewater in treatment wetlands.

Naproxen is one of non-steroidal anti-inflammatory drugs and commonly used for reduction of pain and inflammation, and was selected as micropollutant in the study based on the frequency of use. In 2000, 35 tonnes of naproxen were consumed in England [8] while Picquet [9] announced that Albermarle company produces about 500 tonnes of naproxen per year. Statistical data provided by CVS Pharmacy [10] showed for those aged under 20, they utilized as high as 20.2%, aged 20-40 used 18.7%, aged 40-60 used 34.4% and for those people over 60 they have the usage of 26.6%. Very few plants on literature have wide range of tolerance to extreme conditions and produce a high biomass even growing in the contaminated areas such Vetiver plants [11]. *Vetiver zizanoides* was selected due to their characteristics to withstand the phytotoxicity of micro pollutants. It is a perennial grass with thick fibrous roots, densely tufted and narrow leaves of 1 to 2 m long as shown in Fig. 1. It has a vernacular name based on dialect in India such as Vala, Valo, Kuruveeru and Vettiveru [12].

Fig. 1. *V. zizaniodes.*

The objectives of this study were (1) to determine the phytotoxicity of naproxen using *V. zizanoides*, (2) to determine the types of phytoremediation mechanisms and (3) to determine the naproxen uptake capability by *V. zizanoides* in the removal process. The parameters of pH, dissolved oxygen (DO) and oxidation-reduction potential (ORP) were recorded to observe physicochemical changes in the system. At the end of this experiment, the data obtained was analysed statistically using SPSS Version 20.0 (Chicago, USA).

2. Materials and Method

2.1. Propagation of *Vetiver zizanioides*

The plants were collected from a greenhouse of Universiti Kebangsaan Malaysia (UKM) and cultivated in the plastic crates with dimension 0.45 x 0.30 x 0.15 m. Fine sand was occupied at one third of a plastic crate and with almost similar height of *V. zizaniodes*. The plants were then allowed to grow in the greenhouse for about a month.

2.2. Experimental design of phytotoxicity test

A phytotoxicity test was conducted for 56 days of exposure using sub-surface flow system (SSF) with naproxen concentration were varied from 50, 100, 300 and 500 mg/L. Figure 2 shows the experimental setup for phytotoxicity test.
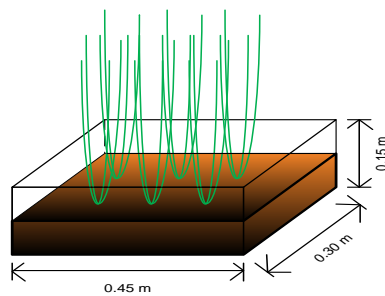

Fig. 2. Plastic crate setup for phytotoxicity test.

17 plastic crates with dimension ($0.45 \times 0.30 \times 0.15$ m) were filled with six healthy *V. zizaniodes* plants, aged two months and spiked with the synthetic naproxen prepared by mixing naproxen sodium (Sigma Aldrich M4015, Malaysia) and distilled water in appropriate dilution. Three replicates (R1, R2, and R3) were used at each concentration, with another four plastic crates for a contaminant control without plants (CC), as shown in Fig. 3. The sampling time were done on week 1, 2, 4 and 8 with physical growth, analysis of water quality, naproxen content in wastewater, soil and plant were also monitored.

2.3. Physicochemical analysis and sample collection

The physicochemical parameters including temperature (T, ˚C), pH, dissolved oxygen (DO, mg/L), oxidation reduction potential (ORP, mV), chemical oxygen

demand (COD, mg/L) and total suspendend solids (TSS, g) were recorded. The samples were measured separately using pH meter (HANNA, USA), DO meter (YSI 550A, US) and ORP meter (HANNA, USA) for pH, DO and ORP readings. The probe were rinsed with distilled water for another sample analysis. The water sample from the growth medium were collected periodically from each plastic crate on the sampling week 1, 2, 4 and 8. Naproxen removal in synthetic wastewater and soil was analysed using high performance liquid chromatoghraphy (HPLC, Agilent/1200 Series, US). The water sample was then filtered through 0.45 μm filter membrane cellulose nitrate and the filtrates were placed in 2 mL vials. For the naproxen concentration in soil, the extraction was performed using 5 g dried sand sample collected from each crate containing different naproxen concentrations (50, 100, 300 and 500 mg/L) and the collected soils were mixed with 20 mL methanol (CH$_3$OH, Merck, Malaysia) and filtered using a vacuum pump before being poured into 2 mL vials. The fine mixtures were filtered using 0.45 μm cellulose nitrate membrane filters. The percentage of naproxen removal on each sampling week was determined using Eq. (1):

$$\text{Removal (\%)} = \frac{\text{Naproxen initial-Naproxen final}}{\text{Naproxen initial}} \times 100 \qquad (1)$$
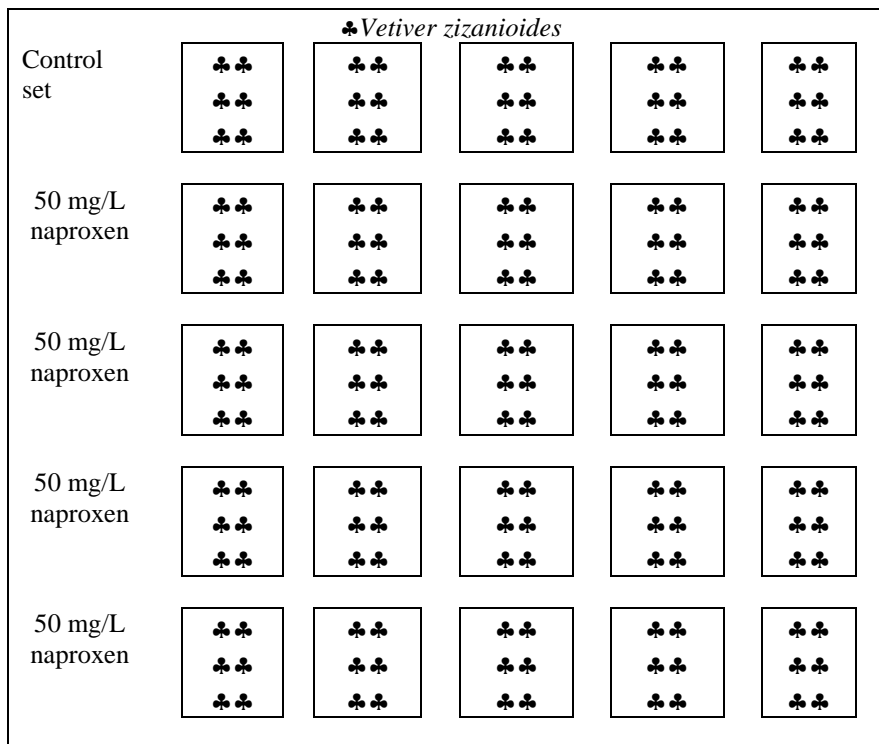

Fig. 3. Experimental design of phytotoxicity test.

2.4. Analysis on physical growth of *V. zizaniodes*

The growth of *V. zizaniodes* in different naproxen concentration (50, 100, 300 and 500 mg/L) was observed over 56 days of exposure. One plant from each crate was

collected on each sampling weeks (1, 2, 4 and 8). The plant was completely rinsed with distilled water. Length and wet weight was recorded immediately when the water was absorbed to tissue paper since plants have a high composition of water that can lead to some drying [13]. Finally, the plant was dried in an oven (MMM Medcenter Venticel, UK) at 105 ^{o}C for 1 h before determining the dry weight.

2.5. Microbial population count

Microbial population count was carried out on plant roots of *V. zizanioides* at the end of plant cultivation. The purpose of conducting the determination was to determine the effects and relationships between different concentrations of naproxen in the soil against bacterial populations [14]. The calculation on the number of bacterial colonies were carried out and recorded.

2.6. Statistical analysis

All experimental data obtained from naproxen removal were analyzed using Statistical Product and Service Solutions version 21.0 (SPSS, Chicago, USA).The sampling and chemical analyses were carried out in triplicates in order to decrease the experimental error [15]. Two way analysis of variance (ANOVA) was used to evaluate the physicochemical parameters, evaluate the significant changes in the physical growth of plant, naproxen content in water, soil and plant with 95% confidence limit. Statistical significant was defined as $p < 0.05$.

3. Results and Discussions

3.1. Physical observation of plant growth

Physicochemical parameters monitored during the removal of naproxen were pH, dissolved oxygen (DO, mg/L) and oxygen reduction potential (ORP, mV)). As shown in Fig. 4, a treatment with and without plants at naproxen concentration of 50, 100, 300 and 500 mg/L were analyzed using t-test on the independent samples. The results of $p = 0.0421$, 0.9888 and 0.0322 for pH, DO and ORP, respectively, indicating that physicochemical parameters between the two systems exhibit statistically significant different ($p < 0.05$) except for DO. The mean values was found to be significantly different for the two systems for pH, DO and ORP. Throughout the 8 weeks, the mean pH values were 7.72 and 7.98 for treatment with plants and without plants, respectively. Between the two systems, there were only small different on pH level. In this comparative study, the value of DO analyzed in water in each crate shows insignificant difference and the DO average were 7.82 and 7.69 mg/L for system with and without plants, respectively. The system with plants showed significantly higher oxidation-reduction potential than the system without plants ($p < 0.05$). The ORP oscillated from -113 and -17 mV for system with plants, while the ORP oscillated from -120 and -51 mV for system with control contaminant, indicating that the system was in reducing state [16]. Thus, treatment with plants shows better ORP than control contaminant treatment where Hijosa-Valsero et al. [17] explained that the removal efficiency of organic contaminants in constructed wetlands was more effectively with higher ORP. This was associated with oxygen and electron acceptor concentrations in treatment medium. Throughout 56 days, dissolved oxygen showed increment in every

week except for 500 mg/L concentration, indicating that *V. zizaniodes* played an important role in phytotoxicity process. Naproxen concentration affected the treatment area and there was no more oxygen dissolved but depleted as concentration increased. Generally, anaerobic processes play a major role in sub-surface flow constructed wetlands [18].
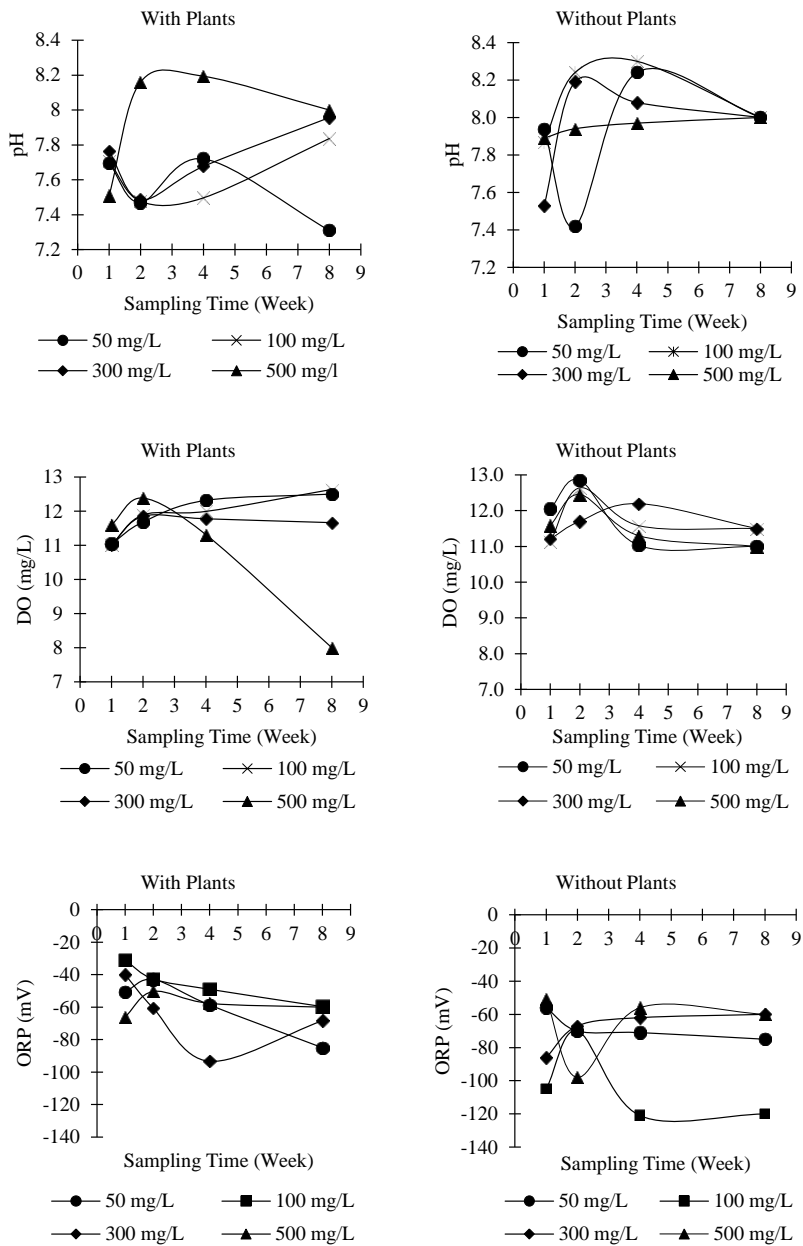

Fig. 4. Physical parameter variations in phytotoxicity test using naproxen as contaminant.

3.2. Adaptation of *V. zizaniodes* to naproxen

In phytotoxicity test, several analyses has been carried out in order to examine the physical growth of the plant which included the color variation in plants,including wet and dry weight analysis and percentage of water content in plants. For *V. zizaniodes* growth over 8 weeks with 50 and 100 mg/L, all plant grew very well and healthily in each crate. However, after 4 weeks of treatment, *V. zizaniodes* started to show impaired growth such as withering, yellowish leaves and a presence of blackish roots. The plants with 300 and 500 mg/L naproxen concentration showed high level of phytotoxicity that had constrained the growth with the brown and blackish spot on leaves. This indicates that *V. zizanioides* could not carry out photosynthesis and eventually died at the end of the exposure due to the high value of naproxen concentration which had inhibited the cycle of oxygen and carbon dioxide during the process.

The tolerance of *V. zizaniodes* was measured based on several parameters such as wet weight and dry weight. All the parameters were recorded after 56 days or 8 weeks exposure to naproxen as depicted in Fig. 5. According to [19], biomass can be an indicator of the overall health of plants growing in the presence of micropollutant. The graph of wet and dry weight measurements of biomass showed fluctuation in 0 mg/L. During exposure to 50 mg/L naproxen concentration, the wet weight showed an increase at earlier week compared to week 8s, the wet weight dropped up to 13 g. The reason was the water content decreased as the plants getting older over weeks of exposure. However, for both wet and dry weight at 100, 300 and 500 mg/L, all the data showed fluctuation and become smaller than the values for control (0 mg/L). Based on the t-test, wet weight and dry weight differ insignificantly ($p<0.05$) between the control.

3.3. Removal of naproxen in water

The percentage removal of naproxen by *V. zizaniodes* was recorded from analysis of High Pressure Liquid Chromatography (HPLC, USA) with different concentrations (50 to 500 mg/L).

From the observation in Fig. 6, it was found that removal rate of naproxen in water progressively decreased as concentration of naproxen increased. Naproxen concentrations decreased from 0.75 to 0.35, 1.35 to 0.25, 2.90 to 0.05 and 4.50 to 0.65 mg/L for 50, 100, 300 and 500 mg/L respectively. The ability of plants to get rid of naproxen decreases since plant cannot resist high concentration of naproxen and subsequently phytoremediation process cannot be executed without healthy plants. The results from statistical analysis using SPSS Version 20.0 (Chicago, USA) showed that there was significant difference ($p<0.05$) between contaminant with plants and without plants. In addition, comparison was done concerning both systems where 21% to 98% were removed with *V. zizaniodes* while only 2% to 32% with the absence of plants. The percentage difference may due to the adsorption by soil during sampling time.

The correlation between factors highlighted was tested and as an outcome, three factors (system*time*concentration) gave momentous effect towards treatment process since $p=0.037$ which is $p<0.05$. In addition, there was a statistically significant interaction at $p<0.05$ between (system*time),

(system*concentration) and (time*concentration) as shown in Table 1. System, time and concentration alone gave significant difference towards the removal.

Table 1. Results between-subject effects (system, time, concentration) and their interaction towards removal of naproxen from water.

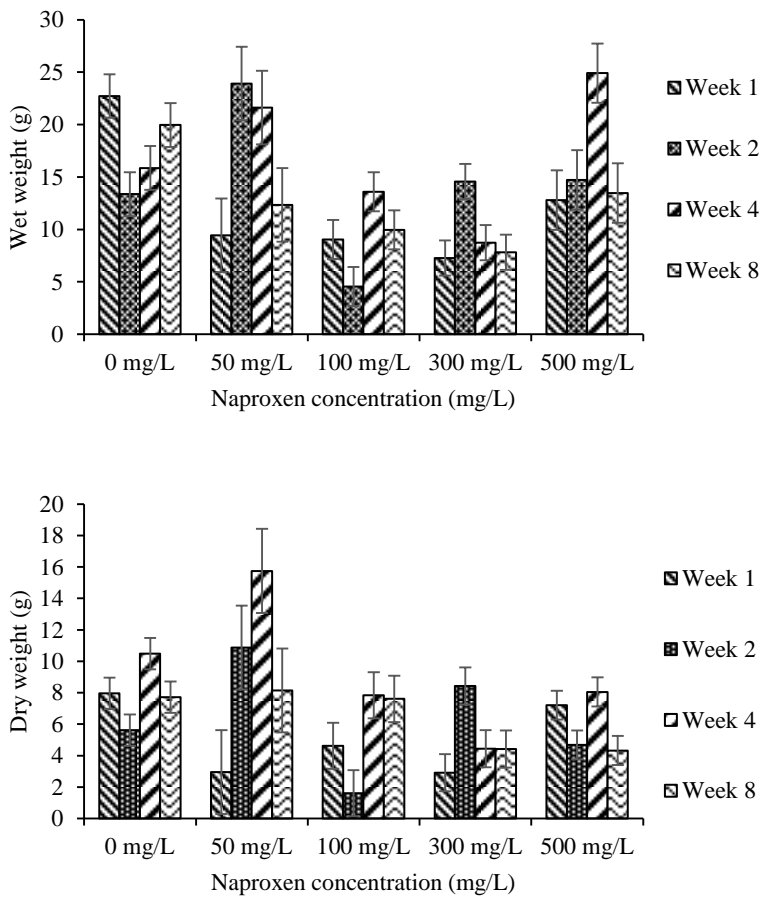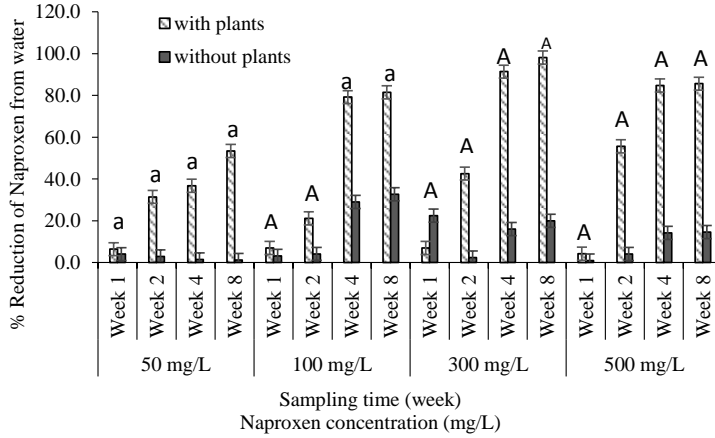| Independent variables	Sig.
System	0.000
Time	0.000
Concentration	0.000
System*Time	0.000
System*Concentration	0.000
Time*Concentration	0.001
System*Time*Concentration	0.037

Fig. 5. Wet and dry weight of *V. zizanioides.*

Fig. 6. Changes of naproxen value in water along phytoremediation process. A: statistically significant removal (*p*<0.05) between with and without plants; a: The reduction of naproxen from water was not statistically significant (*p*>0.05).

3.4. Removal of naproxen in soil

Sand extraction was done in order to obtain percentage removal of naproxen from soil by *V. zizaniodes*. On overall, analysis of naproxen in soil also showed a decline mainly for concentrations of 50 and 100 mg/L (Fig. 7). Over 56 days of treatment, the highest percentage removals achieved were 81.3, 61.4, 36.4 and 60.5% for 50, 100, 300 and 500 mg/L, respectively, giving evidence that the naproxen was removed by the time. From the removal trend at 300 mg/L, the naproxen did not decreased since *V. zizanioides* can no longer withstand high concentration of naproxen, particularly at week 4. Thus, from statistical analysis the removal of naproxen by *V. zizaniodes* in different concentrations (50 to 500 mg/L) was statistically significant for both systems (with and without plants) where *p*<0.05.
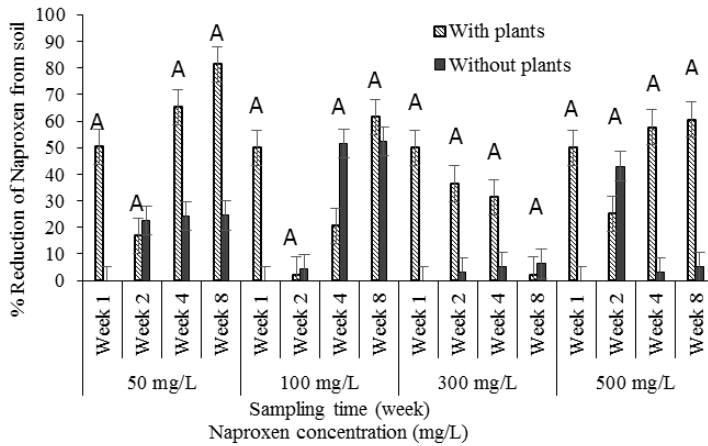

Fig. 7. Changes of naproxen value in water along phytoremediation process. A: statistically significant removal (*p*<0.05) between system with and without plants; a: The reduction of naproxen from water was not statistically significant (*p*>0.05).

The correlation between factors highlighted was tested and as an outcomes, combination of three factors (system*time*concentration) gave momentous effect towards treatment process and it was statistically significant since p=0.037 which is $p < 0.05$. In addition, there was a significant different for two interaction factors at $p < 0.05$ between (system*time), (system*concentration) and (time*concentration) as shown in Table 2. System, time and concentration alone gave significant difference towards the removal.

Table 2. Results between-subject effects (system, time, concentration) and their interaction towards removal of naproxen from water.

Independent variables	Sig.
System	0.000
Time	0.000
Concentration	0.000
System*Time	0.000
System*Concentration	0.000
Time*Concentration	0.000
System*Time*Concentration	0.000

3.5. Profile of microbial population (CFU)

The profile of microbial population was carried out on the last day of the phytotoxicity test. The purpose of calculating the number of bacterial colonies was to determine the role of bacteria in the decomposition of naproxen in different concentrations. Figure 8 shows the number of log bacteria found in waste water containing different concentrations of naproxen ranging from 0 to 500 mg/L. The trend was similar the work carried out by Kaimi et al. [20] where the number of colonies of bacteria for each concentration increases as the concentration of pollutants increased. The log CFU/mL for 0, 50, 100, 300 and 500 mg/L lies on 3.7, 4.0, 5.0, 5.1 and 6.0 CFU/mL respectively. The increasing number of CFU indicated that the contaminated water enhanced the microbial population and increased its communities.
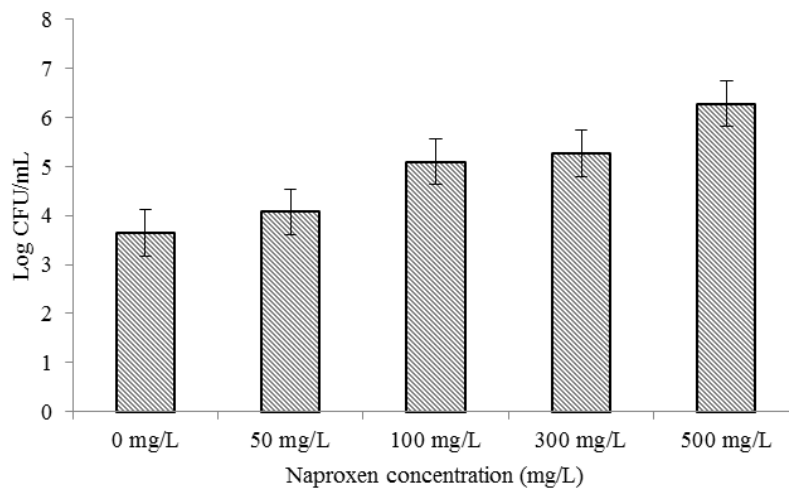

Fig. 8. Population of bacteria during 56 days of naproxen exposure.

4. Conclusions

The results indicated that the removal of micropollutant was enhanced by the presence of *V. zizaniodes*. Rhizobacteria from *V. zizaniodes* roots had an advantageous effect on sustaining the naproxen removal. After 56 days of treatment in a system with and without plants, it was demonstrated that *V. zizaniodes* has the capability to survive and provide good conditions for rhizobacteria to eliminate naproxen at concentration 50, 100, 300 and 500 mg/L. Naproxen removal up to 81.5%, 64.5% and 60.9% were achieved in 100, 300 and 500 mg/L naproxen concentrations, respectively. Phytostimulation and phytostabilization played a major roles for naproxen elimination when there was an activity of the bacteria presented in the soil around the roots of the plants. Results indicated that phytoremediator plant of *V. zizaniodes* was able to remediate micropollutant of naproxen from contaminated water.

Acknowledgement

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