

Effects of Selected Pharmaceuticals on Freshwater Algal Biofilms in Different Organic Loads

Waqar Ahmed¹, Aneela Yasmin^{1,2a}, Ayesha Tajammul^{2b}, Najeebullah Channa³,
Jamil Ahmed^{2c}

RECEIVED ON 24.07.2018, ACCEPTED ON 26.07.2019

ABSTRACT

The growth pattern of different algal biofilms investigated in the presence of Ibuprofen and Benzoyl Metronidazole in batch reactors at varying organic loads. Study suggests that the inhibition of algal growth is correlated to organic loads in water rather than the concentrations of PhCs (Pharmaceuticals). Mortality of algae was recorded in BG11 media with binary mixtures of PhCs and no growth inhibition was monitored in the activated sludge with PhCs. These outcomes indicate that the presence of PhCs as pollutants in drinking water have severe effect on algal biofilms due to low organic load thus can alter the aquatic ecosystem. Additionally, PhCs might not adversely hinder the growth of microbes in WWTP (Wastewater Treatment Plants) or in waters with high organic loads. Nevertheless, inference of these results to a full-scale WWTP need more evidence through extensive studies using variable organic substrates and PhCs with numerous ranges of organic loads. These findings suggest that PhCs as water contaminant are able to shift microbial ecology in water bodies.

Key Words: Ibuprofen, Benzoyl Metronidazole, Algal Biofilms, Freshwater, Pharmaceuticals.

1. INTRODUCTION

In Pakistan, about four MAF (Million Acre Feet) per year household and industrial wastewater is dumped into the water bodies with only 3% as treated effluent [1]. This untreated sewage, domestic and/or industrial waste contains trace elements, pesticides, and detergents [2]. Among the emerging pollutants presence of the traces of PhCs compounds in surface water is quite disturbing and the route of these compounds in drinking water is disposal of sewage directly in water bodies [3]. PhCs are used by patients for longer periods until these reach to therapeutic effect and during that time majority of

these compounds excreted in the environment [4]. According to Daughton and Ternes [5] hundreds of PhCs are detected in river water at the levels of ng L^{-1} to $\mu\text{g L}^{-1}$. These have the ability to affect biochemical processes at low concentrations in human body thus can also affect aquatic life however, the fate and the impacts of these compounds is not well understood [6].

Huerta *et. al.* [6] demonstrated the bio-absorption of PhCs in fish and invertebrates, respectively. Numerous reports demonstrated that the PhCs have negative effect on biofilms, the primary producers, by

¹ Department of Biotechnology, Sindh Agriculture University Tando Jam.

Email: waqarahmeddasti@gmail.com

² US-PCASW, Mehran University of Engineering and Technology, Jamshoro, Pakistan.

Email: ^aaneelayasmin@yahoo.com (Corresponding Author), ^bayesha.uspcasw@faculty.muet.edu.pk,
^cjamilahmed.uspcasw@faculty.muet.edu.pk

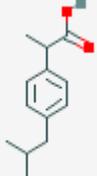
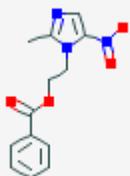
³ Institute of Environmental Engineering and Management, Mehran University of Engineering and Technology, Jamshoro, Pakistan. Email: Najeebchanna@outlook.com.

fluctuating its metabolism and consequently community structure [7]. Nonetheless, biofilms also purify water by this bioaccumulation ability [8]. Freshwater biofilms play a vital role of nutrient retention in river ecosystems [3]. Bioaccumulation of PhCs or other contaminants means traces of compounds inside the members of biofilms namely bacteria, algae and fungi and in the organic matrix embedding all species together led by active biological uptake or passive physical sorption. Bioaccumulation capacity of biofilms demonstrated for many contaminants like pesticides, metals, hormones, surfactants and a psychiatric drug [9-10]. It is important to understand that as the biofilms can accumulate contaminants, they can also transfer these pollutants to higher levels of river food-web.

However, the primary goal of the present study was to determine the effect of PhCs on the growth of algal biofilms and shifts in their microbial community in the presence of different organic loadings. Two commonly used PhCs in Tandojam Community i.e. Ibuprofen and Benzoyl Metronidazole were selected for this study according to a survey at the Medical Health Facility, Sindh Agriculture University, Tandojam, Pakistan (un-published data).

2. MATERIALS AND METHOD

Erlenmeyer flasks (250 mL) with 50 mL of BG11 medium or activated sludge were used as batch reactors and sterilized by autoclaving. The properties of activated sludge were TDS: 650 mgL⁻¹, EC: 1300 μs cm⁻¹, pH: 6.86, turbidity: 34.13 NTU, COD: 235.5 mgL⁻¹. Ibuprofen and Benzoyl Metronidazole (PhCs) were used in this study. Details of both PhCs like CAS number, molecular formula, molecular weight, IUPAC number, chemical formula, classification and uses are compiled in **Table 1** from Open Chemistry database. The stock solutions of selected PhCs were prepared by dissolving each PhC in pure methanol or ethanol. The final concentrations of both PhCs were 0, 50 and 100 μM in BG11 media or in activated sludge corresponding to single or binary mixtures. In total nine different treatments of PhCs were tested and the description of treatments studied in present investigation is presented in **Table 2**. Triplicates of

PhCs Name and Properties	Ibuprofen	Metronidazole Benzoate
CAS number	15687-27-1	13182-89-3
Molecular formula	C ₁₃ H ₁₈ O ₂	C ₁₃ H ₁₃ N ₃ O ₄ ;
Molecular weight	206.285 g/M	275.264 g/M
IUPAC name	2-(4-Isobutylphenyl) propanoic acid	2-Methyl-5-nitro-1H-imidazole-1-ethanol 1-Benzoate
Chemical formula		
Chemical classification	propionic acid derivate	benzoate ester of metronidazole, a synthetic nitroimidazole derivative
Use	NSAID (Nonsteroidal Anti-Inflammatory Dru) with anti-inflammatory, analgesic, and antipyretic effects	antiprotozoal and antibacterial activities
Source: Open Chemistry Database; https://pubchem.ncbi.nlm.nih.gov/compound/		

Pharmaceuticals	Metronidazole Benzoate (0.0 μM)	Metronidazole Benzoate (50 μM)	Metronidazole Benzoate (100 μM)
Ibuprofen (0.0 μM)	T1	T2	T3
Ibuprofen (50 μM)	T4	T5	T6
Ibuprofen (100 μM)	T7	T8	T9

each treatment condition were prepared. Additionally, proper negative controls were prepared to verify and compare the observations. Equal amount of algal biofilms (1-gram) was added to the nine treatments. The algal biofilms collected from freshwater

waterways and canals of Tando-jam and maintained in laboratory conditions. For uniform oxygen and nutrient distribution the reactors were kept on a shaker at 100 rpm and 22 ± 3 °C. The source of Ibuprofen and Metronidazole benzoate was Merck, International. Algal growth measurements were recorded every 7 days for 5-weeks on weighing balance (LIBROR EB-340 S, Japan). Statistical Analysis was carried out using Microsoft Excel (2010). Mean, standard deviation and ANOVA (Analysis of Variance) were performed.

3. RESULTS AND DISCUSSION

Pharmaceuticals (PhCs) in drinking water are a threat to aquatic ecosystem. PhCs are agents to adjust metabolism in living organisms at lower doses and remain in the body until attain a therapeutic effect hence during that time these are excreted in the environment and reach to freshwater bodies through sewage. On the other hand biofilms are primary producers in aquatic ecosystem and are severely affected by polluted water. They absorb pollutants and transfer those to food chain. In present study the effect of two PhCs (Trade names- Brufen and Flygal; Table 1) on biofilms was evaluated in two different media conditions i.e. low organic load that actually simulate the condition of our surface water bodies and second the wastewater with high organic load. In Pakistan, unfortunately wastewater is directly dumped into surface water bodies without prior treatments, thus creating a gradient of organic loads in water streams. Our study focused the presence of PhCs in that gradient of organic load and its effect on primary producers i.e. algal biofilms. In total nine different concentrations of two PhCs in two different media were evaluated for their effect on algae growth and data, as the wet-biomass, are presented in table 3 and 4 whereas table 5 shows the ANOVA of wet biomass of algae in two different media with nine different single and binary concentration mixtures of PhCs.

As expected, the algal growth was lowered in the presence of Ibuprofen and Metronidazole Benzoate at the tested concentrations (50 and 100 μ M) as compared to control in the presence of BG11 media (**Table 3**) with low organic load. Maximum growth inhibition was recorded after week 4 and 5 in the

presence of Metronidazole Benzoate (T2, T3) and in binary mixtures of both PhCs (Table 1: T5, T6, T8 and T9). It is obvious from the table 3 that the observed damage was 100% mortality due to complete disassemblage of algal biofilms during the week-5. Week wise discrete means also revealed that the every week the mass of biofilms was reduced as compared to previous week (**Table 3**). However, no effect was observed in the control (T1) and algae flourished during the whole experiment in control conditions. In short PhCs as pollutants of drinking water have severe effect on algal biofilms in drinking water with low organic load thus can alter the aquatic ecosystem. This is due to the therapeutic biocidal effects of both PhCs. Flagyl has Benzoyl Metronidazole as an active ingredient that can kill various pathogenic microorganism which include bacteroides, clostridia, fusobacteria, eubacteria, gardnerella vaginalis, trichomonas, giardia lamblia, entamoeba histolytica, and balantidium coli by interfering with DNA synthesis [11; Fact sheet provided by supplier]. On the other hand Brufen is a non-steroidal anti-inflammatory drug (NSAID) with Ibuprofen as an active part that has biocidal activity too [12; Fact sheet provided by supplier]. Sanoh et al. [11] detected 6-metabolites of ibuprofen in the urine of humans however they did not detected its excretion in stools whereas Benzoyl Metronidazole is found in urine (77%) and faeces (14%) after administration [10]. During this study algae resisted to the adverse conditions to the point that it changed the pH of medium from 6.00 to 8.5 however in the presence of PhCs, biofilms showed reduction in biomass and spread with simultaneous reduction in biofilm pigmentation, opacity and microbial community followed by 100% mortality.

Note: PhCs include Ibuprofen and Metronidazole Benzoate. Details of the different treatments are given in **Table 1**. The weight of initial inoculated biofilm was 1g and the increase in the weight of biofilm was calculated as the difference of W_0 - W_1 recorded every week. The displayed data represent mean values of three replicates of each treatment.

Algal biofilms are formed when high amount of nutrients and organic matter such as nitrogen and phosphorus enter in the slow moving water streams [13]. These nutrients are fertilizers for algae and when

TABLE 3. EFFECT OF SELECTED PHCS ON THE WET-BIOMASS (g L⁻¹ DAY⁻¹) OF ALGAL BIOFILMS GROWING IN THE BG11 MEDIA AS GROWTH MEDIUM

Weeks	T1	T2	T3	T4	T5	T6	T7	T8	T9	Mean
1	1.213	0.342	0.350	0.366	0.227	0.279	0.393	0.217	0.160	0.394
2	0.448	0.236	0.170	0.314	0.198	0.078	0.425	0.144	0.073	0.232
3	0.444	0.444	0.176	0.055	0.435	0.072	0.013	0.245	0.043	0.214
4	1.255	0.114	0.000	0.347	0.034	0.000	0.022	0.022	0.000	0.199
5	0.423	0.004	0.000	0.377	0.000	0.000	0.215	0.000	0.000	0.113
Mean	0.757	0.228	0.139	0.292	0.179	0.086	0.214	0.126	0.055	0.231
Standard Deviation	0.436	0.175	0.146	0.135	0.174	0.114	0.196	0.111	0.066	0.102

TABLE 4. EFFECT OF SELECTED PHCS ON THE BIOMASS (g L⁻¹ DAY⁻¹) OF ALGAL BIOFILMS GROWING IN THE ACTIVATED SLUDGE AS GROWTH MEDIUM

Weeks	T1	T2	T3	T4	T5	T6	T7	T8	T9	Mean
1	1.213	0.442	0.437	0.660	0.327	0.355	0.413	0.370	0.260	0.497
2	0.848	0.190	0.238	0.523	0.481	0.281	0.410	0.343	0.173	0.388
3	0.944	0.522	0.227	0.662	0.330	0.238	0.501	0.345	0.276	0.449
4	1.001	0.429	0.351	0.558	0.343	0.251	0.566	0.307	0.333	0.460
5	0.923	0.524	0.461	0.655	0.390	0.251	0.641	0.421	0.344	0.512
Mean	0.986	0.421	0.343	0.612	0.374	0.275	0.506	0.357	0.277	0.461
SD	0.138	0.136	0.109	0.066	0.065	0.047	0.100	0.042	0.068	0.049

algae bloom, they can change the chemistry of water specially the pH and dissolved oxygen. Interestingly, activated sludge as high organic load, distinctively affected the algal growth positively and no mortality was observed even at the highest PhCs concentration like 100 µM for the both compounds (Table 4). Our data suggest that the presence of PhCs in wastewater can slow down the growth of algae in high organic loads however algal mortality is correlated to the presence of biodegradable carbon present in the media with high organic load rather than the absolute concentration of PhCs (Table-4). Control treatment T1 of activated sludge without PhCs (Table 4) revealed wet-biomass 0.986 g L⁻¹ day⁻¹ that is significantly higher as compared to T1 of BG11 media without PhCs (Table 3) i.e. 0.757 g L⁻¹ day⁻¹ on the basis of ANOVA and DMRT at p = 0.05 (Table 5). Moreover, PhCs in activated sludge also revealed the short term

negative effects on algal growth and the drop in algal growth was found prominent after the end of 2nd week. Later, biofilms recovered and growth increments were recorded that were obviously significantly lower than the control (Table 4). Table 5 reveals the effect of time in weeks and treatments on the growth of biofilms that came out to be highly significant at the level of p= 9.59E-10 for weeks and p= 3.63E-15 for treatments when tested through ANOVA. However, the interaction of time and treatments was found non-significant on the basis of ANOVA thus showing that the treatments were lethal for the growth of biofilms since the first exposure and time also played a critical role on the growth of biofilms nevertheless the resultant growth decrease is not an additive result of both variables because these were not depending on each other.

TABLE 5. TWO WAY ANOVA WITH REPLICATIONS FOR THE ALGAL BIOMASS OF BIOFILMS GROWING IN TWO DIFFERENT MEDIA WITH SELECTED PHCS

ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Weeks (W)	1.19	1	1.19	49.47	9.59E-10	3.97
Treatments (T)	3.74	8	0.47	19.31	3.63E-15	2.07
WxT Interaction	0.043	8	0.01	0.22	0.986511	2.07
Error	1.74	72	0.03			
Total	6.73	89				

These findings have implications because it suggests that the PhCs might not adversely hinder the growth of microbes in wastewater treatment plants (WWTP) or where water bodies have high organic loads. At such points algal growth flourish, utilize nutrients as food and accumulate the hazardous chemicals like PhCs. When other aquatic organisms eat those biofilms the accumulated chemicals are transferred to the higher food chains and reach to our plates. Further, presence of PhCs in drinking water with lower loads of organic matter is found fatal for these algal biofilms thus changing the complete microbial ecology of our water bodies. Lawrence *et al.* [3] examined biofilms growing under selected PhCs and found clear changes in the structure and community arrangement, microscopically. They found significant changes in the microbial groups like bacteria, cyanobacteria and algae. However they did not find change in algal biomass and spread but found significant reduction in the biomass of cyanobacteria. Nonetheless they tested caffeine, ibuprofen, carbamazepine and furosemide. Carbamazepine revealed algal dominance as compared to bacterial dominance in control. Biofilm thickness was also found affected by these PhCs and only caffeine displayed more thickness as compared to control. In contrast we recorded the reduction in biomass and spread due to Brufen and Flaygl in low organic conditions. These PhCs also reduced opacity of biofilms with time. 100% Mortality was recorded in our case. Mortality represents the effect of chemicals on the survival rate of biofilms that was recorded on the basis of changes in the pigmentation, opacity and deterioration of biofilms. The microscopy was also used to record the change in microbial communities of biofilms that was found decreasing with time due to the adverse effects of PhCs.

In agreement to our studies, Ibuprofen is documented as an antimicrobial agent that has the ability to reduce bacterial biomass at the concentration of 10 g L⁻¹ during 8-weeks particularly by killing the algae, proteobacteria and gram positive bacteria [14, 15]. In the presence of different PhCs the algal biomass, community composition and taxa is decreased however to the best of our knowledge, there are not many studies done considering Ibuprofen and Metronidazole Benzoate with different organic loads. Though, it is reported that the sensitivity of algae to

PhCs is due to cellular organization [16]. Although, [17] demonstrated that the biofilms of WWTP show higher tolerance to diclofenac and ibuprofen due to receiving chronic inputs of PhCs and [18, 19] have also reported that this community tolerance usually develop with time this was not fully observed in our study; probably because of high organic loads that help biofilms to resist and tolerate PhCs. In high organic loads algae not only have the nutrients in excess but also a broth like environment around the cells that provide them cellular stability. Nonetheless, microbial destruction was obvious in low organic loads (**Table 3**) and [18, 19] also reported the comparable results. [20] revealed the fact that when biofilms exposed to PhCs, the microbial community shifts are the most prominent effects and sensitive species are replaced by the tolerant ones whereas in our case both PhCs have the properties of biocide as described earlier. The active ingredients of Ibuprofen and Metronidazole Benzoate have the ability to kill microbes thus mortality of biofilms was observed.

Nevertheless, inference of these results to a full-scale WWTP or water bodies getting untreated wastewater, as in Pakistan, need more evidence through extensive studies using variable organic substrates and PhCs with numerous ranges of organic loads. It is reported that the high organic load promote rapid growing microbes thus outgrowing slow growing ones in the systems with elevated organic loads (21, 22). It can be concluded that the increase in organic loads possibly promotes the growth of some microbes and mask the mortality of others. However, further data are required to verify this assumption. In short the effect of two PhCs was evaluated on the algal biofilms and results revealed very disturbing phenomenon of mortality of biofilms in low organic loads. If such chemicals will continually accumulate in water bodies thus will seriously affect the structure and function of aquatic ecosystem and food web.

4. CONCLUSION

In present study the effects of two PhCs i.e. Ibuprofen and Metronidazole Benzoate on algal biofilms were evaluated. Algal biofilms are primary producers of food in aquatic food chain. When wastewater is dumped without treatment in rivers, it creates a

gradient of organic loads along the moving streams, posing multi stress conditions for biofilms. Our laboratory scale study showed that the both PhCs (ibuprofen and Metronidazole Benzoate) are lethal for algal biofilms at doses like 50 and 100 μM in surface water bodies with low organic loads and the absence of algae from aquatic ecosystem disturbs the whole food web and functionality of the aquatic ecosystem whereas at higher organic loads biofilms resist the biocide effects of PhCs and flourish, though accumulates the drugs and transfer these to higher aquatic animals like fish to human. In summary, the presence of tested PhCs in surface water bodies is an ecological risk, even at the concentration of as low as 50 μM .

ACKNOWLEDGEMENT

Authors are grateful for the financial support and space provided by the Sindh Agriculture University, Tando Jam, Pakistan, to conduct this study.

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