Research article

2,4-D and Glyphosate affect aquatic biofilm accrual, gross primary production, and community respiration

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Abstract: 2,4-Dichlorophenoxyacetic acid (2,4-D) and glyphosate are widely used agricultural herbicides commonly found in surface waters near cultivated land. Field experiments were conducted to determine the effects of 2,4-D and glyphosate on biofilms in a pond next to agricultural land in Athabasca, Alberta. Contaminant-exposure substrates (CES) consisted of GF/C glass fiber or a cellulose filter paper substrates placed on specimen jars filled with agar that contained low levels of nitrogen and phosphorus, and different concentrations (15, 9.0, 1.5 mM) of either 2,4-D or glyphosate. Nutrients and herbicide diffused freely through the agar to the substrate surface. CES arrays were deployed 15 cm below the water surface for 22 days, after which biofilms were collected and biomass (chlorophyll a), autotroph gross primary production (GPP), and heterotroph community respiration (CR) were measured. 2,4-D (15 mM) caused significant decreases in rates of biomass accrual (-22%), GPP (-34%), and CR(-63%). Glyphosate (15 mM) also caused significant decreases in rates of biomass accrual (-50%), GPP (-67%), and CR (-47%). For the contaminant concentrations used, mean flux rates are estimated to be between 50–700 ng cm^{-2} min^{-1}.

Keywords: herbicide; 2,4-dichlorophenoxyacetic acid; glyphosate; contaminant-exposure substrates; lentic; aquatic biofilm; flux rate

1. Introduction

Herbicides are applied to agricultural land in very large quantities. Glyphosate and 2,4-dichlorophenoxyacetic acid (2,4-D) are two of the most widely used herbicides. Glyphosate is a broad spectrum herbicide while 2,4-D is a broadleaf herbicide that leaves grass species, such as cereals, largely unaffected. In 2008, in the province of Alberta, Canada, 6100 tons of glyphosate and

840 tons of 2,4-dichlorophenoxyacetic acid (2,4-D) were sold [1].

Glyphosate and 2,4-D have been detected frequently in surface waters in Canada, at a range of concentrations. An Alberta Environment study [2] showed that 2,4-D was found at a median measurable concentration of 0.043 ppb, and a maximum concentration of 439 ppb. Glyphosate was found at a median measurable concentration of 0.3 ppb, and a maximum concentration of 6.1 ppb. A separate study of glyphosate surface waters in Ontario found a maximum concentration of 40.8 ppb glyphosate [3]. Glyphosate has also been detected in highway runoff [4]. High transient environmental concentrations are most likely at times of mass application of herbicides.

The effects of herbicides on the aquatic environment are not well understood. While herbicides are designed to target unwanted plant species, herbicide compounds can affect a range of aquatic species, including microbes [5]. For example, there are a number of studies that demonstrate that glyphosate and 2,4-D are toxic towards algae [6-9]. Those studies generally focused on single species of phytoplankton, in laboratory conditions.

Aquatic biofilms comprise an important set of microbial communities because they underpin aquatic food webs. Biofilm algae are a basal resource for higher trophic levels, and heterotrophs are responsible for the decomposition of organic matter. Small changes in biofilms induced by herbicides have the potential to alter entire aquatic ecosystems. Thus, herbicides have the potential to influence both aquatic ecosystem structure and function by affecting microbial populations and communities. For example, an herbicide may diminish algal communities, thus changing the structure of biofilms and indirectly affecting the populations of fungi and bacteria [10].

Chemical diffusing substrates (CES) have recently been applied to studying the effects of pharmaceutical and personal care products (PPCPs) on biofilms. CES are a modification of nutrient diffusing substrates, which are an established method for studying nutrient limitation in situ [11-13]. A nutrient diffusing substrate consists of a permeable substrate fastened on top of a volume of agar that has been amended with varying amounts of nitrogen and phosphorus compounds that slowly diffuse out of the agar to the substrate. Diffusing substrates are then placed in the aquatic system of interest, for example on L-bars anchored to a stream bottom. Biofilms grow on the substrates over a chosen time period, after which they can be analyzed for biomass, primary production, and respiration. Bunch and Bernot used CES to study the effects of acetaminophen, caffeine, ibuprofen, or nicotine on aquatic biofilms in streams [14]. Rosi-Marshall et al. showed significant changes to gross primary production in lotic biofilms using CES amended with a range of PPCPs [15]. Shaw et al. reported similar findings on biofilms in a lentic system [16].

Here, we report the results of a contaminant exposure substrate study on glyphosate and 2,4-D in a lentic (lake) system. To our knowledge, this is the first in situ study on the effects of herbicides on aquatic biofilms.

2. Materials and Methods

2.1. Contaminant-exposure substrates (CES)

Each contaminant-exposure substrate (CES) consisted of a 70 mL screw top specimen cup, 6 cm in height, filled with 2% agar (Fisher, granulated) that was amended with 50 mM $NaNO_3$ (Fisher, reagent grade) and 3 mM KH_2PO_4 (Fisher, 99%), (16:1 N:P ratio), and 15, 9, or 1.5 mM of 2,4-D (99%) or glyphosate (isopropylamine salt). Control CES contained agar amended only with $NaNO_3$

and KH$_2$PO$_4$. Either a glass fibre Whatman GF/C (1.2 μm) or cellulose Whatman #1 (11 μm) filter paper was placed on top of the solidified agar, and held in place with a screw top lid through which a 2.5 cm diameter hole had been drilled. This procedure is a modification of the methods of Rosi-Marshall et al. [15] and Tank & Dodds [12], and is also described by Shaw et al. [16]. Autotroph and heterotroph populations grow simultaneously on both types of substrate. Unlike glass fibre substrates, the cellulose substrates can function as food source for heterotrophs. In these experiments, as with Rosi-Marshall et al. [15], analysis of biofilm function on the cellulose substrates was limited to heterotrophs.

CES were placed in two plastic trays that were designed to float 15 cm below the surface. This was achieved by attaching four 8 cm diameter Styrofoam balls to each of the corners with wire, and bolting a galvanized steel angle bracket to the bottom of the tray for ballast. The length of the wires through the foam balls were adjusted to the correct water depth. CES were held inside the tray with a sheet of chicken wire (see Figure 1). CES were organized in a random array pattern using a random number generator. Each CES tray was attached with wire to a metal post that was staked into the bottom of a pond. The CES arrays were left in the pond for 22 days.

Figure 1. Randomized array of CES deployed in pond.

2.2. Field site

CES were deployed in a rural pond adjacent to cultivated farmland, located on the campus of Athabasca University, in Athabasca, Alberta, Canada. Water input to the pond is primarily through drainage and runoff.

Water quality parameters were measured on the first and last day of the field deployment. Temperature, dissolved oxygen and pH (Oakton pH/DO 300), and turbidity (Oakton T-100 Turbidimeter) were measured. Water quality results are presented in Supplementary Information.

2.3. Biofilm function and biomass

GF/C and cellulose substrates were carefully removed from the surface of the agar, folded, and placed in 12 mL screw top vials with septum lids (Thermo Scientific). Two glass beads were added to each vial for mixing. Vials were filled underwater, to prevent bubbles, with pond water that had been filtered at 0.2 μm to remove microbes. Measurements of dissolved oxygen in the vials were

taken with a retractable fibre optic probe passing through a stainless steel needle (Firesting DO meter, Pyroscience). For cellulose substrates, community respiration (CR) was determined by measuring the dissolved oxygen concentration before and after a dark incubation period (1 hour). For GF/C substrates, gross primary production (GPP) was determined by measuring the increase in dissolved oxygen after a 1 hour incubation in a plant growth chamber (Percival, Model E-36LC8) at room temperature, followed by measuring the decrease in dissolved oxygen after a 1 hour dark incubation. Vials were mixed by inversion before each measurement. To correct for background respiration and production, the changes in dissolved oxygen were also measured in 5 sealed vials that were filled with the filtered water only.

For chlorophyll analysis, GF/C substrates were recovered from the vials, and the incubation medium was filtered on a second GF/C filter to collect any biofilm material that had been dislodged from the substrate. Both filters were placed in a 15 mL centrifuge tube. 9 mL of acetone (Fisher, reagent grade) was added to each tube. Sealed tubes were sonicated for 5 minutes, and stored at 4 °C for 24 hours. To each tube, 1 mL of deionized water was added and the tube was centrifuged at 2000 rpm for 7 minutes. A 3 mL portion of each sample was transferred to 1 cm pathlength, quartz cuvettes, and absorbances were measured at 630, 647, 664, 665, and 750 nm with a Thermo Scientific NanoDrop 2000 Spectrophotometer. Following this, 10 μL of 1 M HCl was added to the sample, gently mixed by bubbling air through the sample, and absorbances measured again after 1 minute had elapsed. The amount of chlorophyll *a* in each biofilm sample was corrected for the presence of pheophytin *a* [17]. The chlorophyll *a* content of biofilms on cellulose substrates was not measured.

GPP, CR, and chlorophyll *a* data were analysed by ANOVA with pairwise comparisons (Tukey HSD).

3. Results and Discussion

3.1. Effects of 2,4-D

For autotrophs (i.e., algae) in biofilms grown on CES with inert, sintered glass substrata, 2,4-D caused a reduction in gross primary production and biofilm accrual, as measured by chlorophyll *a* content (Figures 2 & 3). The effect was statistically significant only for 15 mM, the highest concentration of 2,4-D used in this study (34% reduced GPP; 22% reduced chlorophyll *a*). The higher chlorophyll *a* result for 9 mM 2,4-D appears to be an experimental anomaly.

For heterotrophs on cellulose substrata, 2,4-D caused a reduction in community respiration (Figure 4). As in the autotroph GPP and chlorophyll *a*, the effect was only significant for the 15 mM concentration of 2,4-D (−63%).

3.2. Effects of Glyphosate

For autotrophs, glyphosate caused a reduction in GPP and biofilm accrual (Figures 2 & 3). The reduction appeared to be dose-dependent; the highest concentration of glyphosate reduced GPP by 67% (15 mM) and 46% (9 mM). Chlorophyll *a* was reduced at all doses of glyphosate: 50% (15 mM); 30% (9 mM); 25% (1.5 mM).

For heterotrophs, glyphosate caused a reduction in community respiration (Figure 4). This reduction was only significant at the 15 mM glyphosate concentration (−47%).
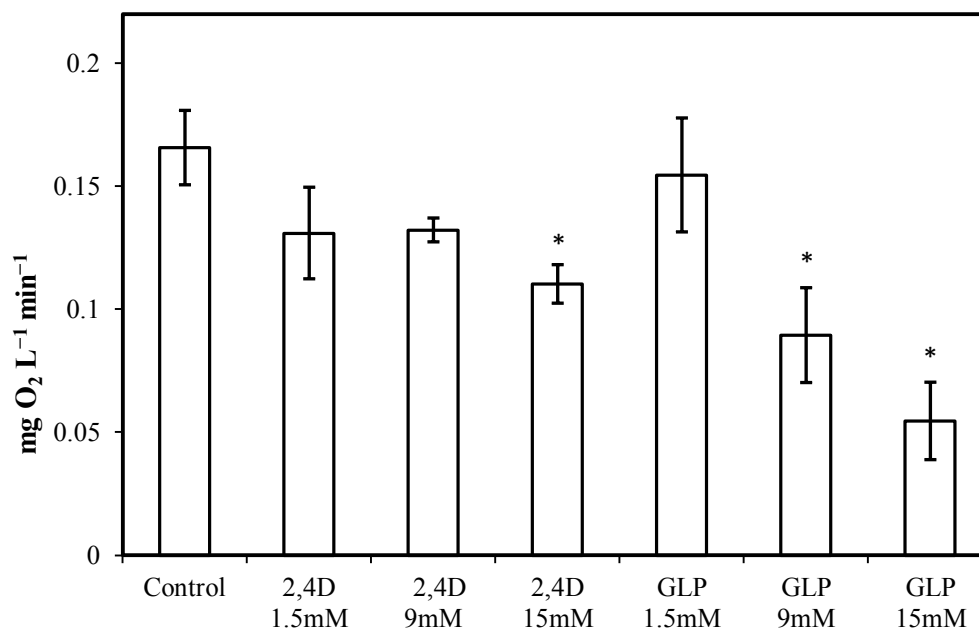
Figure 2. Gross primary production of biofilms grown on glass fibre CES. *Indicates a significant difference relative to the control group. For 2,4-D: 15 mM ($p < 0.0269$). For glyphosate (GLP): 15 mM ($p < 0.0039$), 9 mM ($p < 0.0319$).
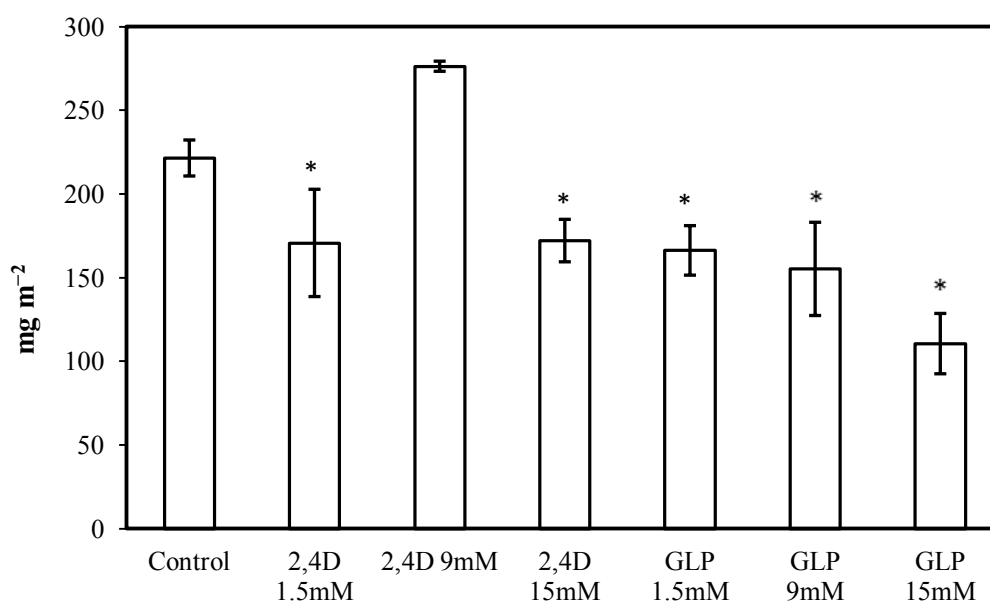

Figure 3. Chlorophyll *a* extracted from biofilms on glass fibre CES. *Indicates significant difference relative to the control group. For 2,4-D: 15 mM ($p < 0.0251$). For glyphosate (GLP), 15 mM ($p < 0.0019$), 1.5 mM ($p < 0.0234$). The 9 mM dose was only significantly different to the control at the 90% level ($p < 0.0683$).
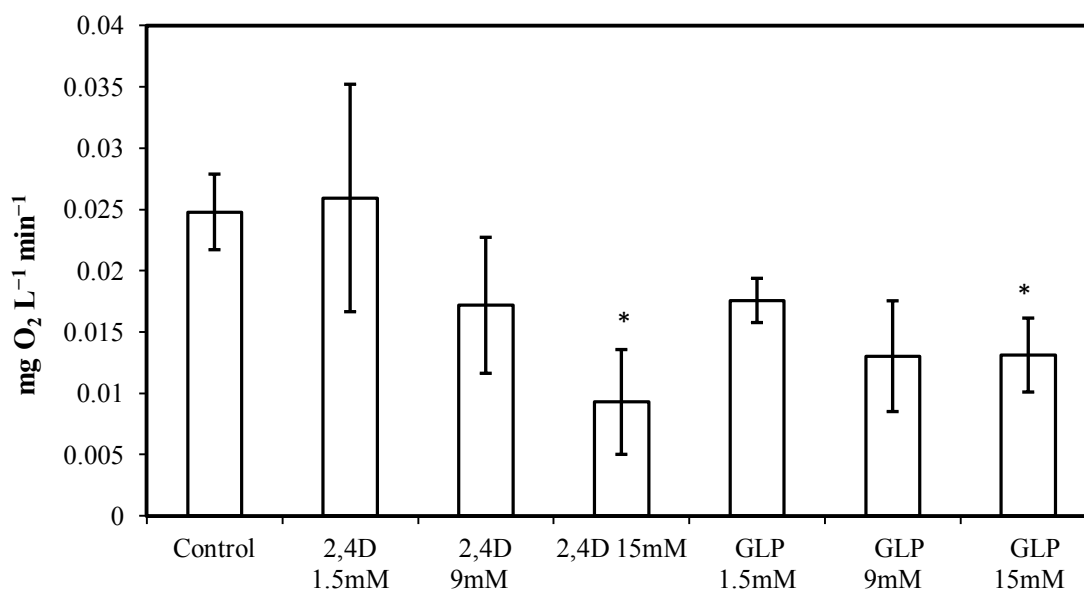
Figure 4. Community respiration of biofilms grown on cellulose CES. *Indicates significant difference relative to the control group. For 2,4-D: 15 mM ($p < 0.0259$). For glyphosate (GLP): 15 mM ($p < 0.0352$).

2,4-D and glyphosate show toxicity towards different species of free phytoplankton [6-9]. As a herbicide, 2,4-D mimics natural auxin and induces abnormal plant growth, leading to plant death at high doses [18,19]. Auxins also have a role in controlling the growth of algae [20-23]. Thus, 2,4-D may interfere with the growth of algae through a similar mechanism to what occurs in plants.

The herbicide action of glyphosate arises from a different mechanism than 2,4-D. In a wide range of organisms, glyphosate inhibits EPSP synthase, which is part of the shikimic acid pathway [24,25]. When EPSP synthase is inhibited, aromatic amino acids cannot be synthesized, which leads to reduced protein synthesis. Ultimately, this leads to reduced growth, and eventually the death of the organism. This is a likely mechanism for the observation of reduced GPP and biomass in biofilms exposed to glyphosate.

We observed that heterotroph CR was also reduced by 2,4-D and glyphosate. The mechanism for this could be as simple as toxicity of the two herbicide compounds towards bacterial and fungal populations in the biofilm. An additional possibility is that 2,4-D and glyphosate separately reduce algal growth, which in turn reduces that amount of biomass available to heterotroph species, thus hindering the growth of heterotrophs [10]. In such a scenario, reductions to both autotroph production and heterotroph respiration would be observed. The relationships among autotrophs and heterotrophs in biofilm communities may be more complicated, involving forms of cooperation that benefit both groups [26]. While it is possible that heterotroph and autotroph populations are directly, and negatively affected by 2,4-D and glyphosate, this could lead to compounded negative effects on the biofilm community as a whole.

Bozeman et al. reported that glyphosate was significantly less toxic towards *Selenastrum* that was immobilized in alginate, as compared with free *Selenastrum* [27]. This brings up questions about the role of the biofilm matrix in protecting microbes from external toxic compounds. The extracellular polymer substances (EPS) in a biofilm matrix can account for over 90% of the dry mass

of biofilms [28].The polysaccharides and proteins can act as a protective barrier from antimicrobial agents such as antibiotics. Further, the EPS can absorb organic compounds [28]. It is likely that the biofilm matrix confers some degree of protection to biofilm microbes from the negative effects of herbicides. In future studies, it may be possible to determine the fates of 2,4-D, glyphosate, and other herbicides in biofilm matrices.

3.3. Flux rates in CES

CES studies have experimental advantages over other forms of toxicity tests, such as flume studies in which a biofilm is exposed to different conditions or toxins in an artificial stream in the laboratory. CES can be placed in situ. CES biofilms are colonized naturally by microbes that are already present in the aquatic habitat, and the biofilms are subjected to natural environmental conditions (sunlight, temperature, dissolved oxygen, etc.). In flume studies, however, it is possible to use concentrations of toxins that are representative of the concentrations found in the environment. In the case of CES, the contaminant diffuses out of the agar. While it is not possible to know the contaminant concentration that the biofilm is exposed to, it is possible to estimate the flux rate through the biofilm [16,29].

The flux rate of the compound can be estimated using the model of Fickian, one-dimensional diffusion from a slab [30]. This has been described by Shaw et al. [16] and Costello et al. [29]. Using equation 1, and assuming a diffusion coefficient of $D = 5 \times 10^{-10}$ m^2 s^{-1}, and $L = 0.06$ m, we can estimate the total mass of 2,4-D and glyphosate released:

$$\frac{M_t}{M_0} = 2 \left(\frac{Dt}{\pi L^2} \right)^{1/2} \quad \text{(Equation 1)}$$

Flux rates correspond to the amount of compound that diffuses out of the amended agar of the CES into the biofilm. Flux rates decrease over time, and are non-linear. Thus, a large pulse (>10%) of compound is released in the first two days.

The estimated mean flux rates are calculated using equation 2. J_{CES} is the mean flux rate; M_t is the total mass at a given time; M_{EQ} is the mass released during an initial equilibration period (2 days); A is the surface area; t is the total exposure time.

$$J_{CES} = \frac{M_t - M_{EQ}}{A \cdot t} \quad \text{(Equation 2)}$$

Mean flux rates (Table 1) are proportional to the initial contaminant concentrations in the CES. This allows for experimental control of the relative flux rate, and the design of studies of dose dependent effects. For example, the results presented in Figures 3–5 are strongly suggestive of a linear dose response—where increasing concentrations of 2,4-D or glyphosate correspond to larger decreases in biofilm function and biomass. More studies are needed to show that this effect is statistically significant over the concentration range used here.

Table 1. Estimated mean flux rates for 2,4-D and Glyphosate from contaminant exposure substrates.

Compound	Concentration in CES (mM)	Mean Flux Rate (ng cm^{-2} min^{-1})
2,4-D	15	670
	9	400
	1.5	67
Glyphosate	15	510
	9	310
	1.5	51

In order to compare exposure of soluble contaminants from CES to typical exposure from chemicals in the water column, it is possible to estimate a dose rate using an approach for calculating O_2 flux into sediments [31]. The flux rate (J) of a solute is given by:

$$J = \frac{D \cdot \Delta C}{Z_\delta} \quad \text{(Equation 3)}$$

where D is the diffusion coefficient (m^2 s^{-1}), ΔC is the concentration change across the DBL, and Z_δ is the height of the diffusive boundary layer (DBL). The size of the DBL above biofilms depends on water velocity, and is typically in the range of 0.1 to 1.0 mm [32]. The largest DBL occurs in a static water column and small DBL in fast-flowing streams.

Using equation 3, a relatively high environmental contaminant concentration of 400 ppb, and a small DBL of 0.1 mm, the expected solute flux rate into the biofilm is 12 ng cm^{-2} min^{-1}. This is approximately 40–60 times smaller than the mean flux rate from the highest concentration CES (15 mM) used in this experiment. In the results, statistically significant decreases in biofilm GPP, chlorophyll *a*, and CR were observed at this concentration for each of 2,4-D and glyphosate. At this time, we cannot conclude whether or not 2,4-D and glyphosate affect aquatic biofilms at flux rates that correlate with the expected exposure rates at known contaminant concentrations in the aquatic environment. Further experimentation using the CES technique will be required to address this question.

Conflict of interest

All authors declare no conflicts of interest in this paper

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