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A multiphasic characterization of the impact of the herbicide acetochlor on freshwater bacterial communities

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Acetochlor is the third most frequently detected herbicide in natural waters; however, it is unknown if exposure to environmentally relevant concentrations of acetochlor will impact bacterial community structure and function. This study examined the impact of acetochlor on freshwater heterotrophic bacteria number, and community structure and function using direct counting, community level physiological profiling (CLPP) and denaturing gradient gel electrophoresis (DGGE) analysis. Acetochlor concentration did not appear to correlate with the number of total ($P = 0.69$) and viable ($P = 0.80$) bacteria, even at concentrations up to 500 µg l^{-1}. However, CLPP indicated that acetochlor increased functional diversity as shown by (i) an increase in the number of carbon sources utilized by the microbial community, relative to nonexposed controls and (ii) increased functional evenness within the heterotrophic bacterial community. Conversely, DGGE fingerprints suggested that exposure to acetochlor generally decreased the community complexity, as the average number of DGGE bands in most treatments was significantly less than in the control treatment. Cluster analysis of DGGE fingerprints revealed three distinct, dose-dependent clusters (i) communities exposed to 0, 1 and 5 µg l^{-1}; (ii) 50 and 100 µg l^{-1} and (iii) 500 µg l^{-1}, indicating a relationship between acetochlor concentration bacterial community changes. This study indicated that while exposure to environmentally relevant concentrations of acetochlor resulted in no significant impact to the number of freshwater bacteria, impacts to the function and structure of the community were revealed by adopting a multiphasic approach.

Introduction

Acetochlor (2-chloro-*N*-(ethoxymethyl)-*N*-(2-ethyl-6-methylphenyl) acetomide) is a chloracetanilide herbicide registered for pre- and early postemergent control of annual grasses and small-seeded broadleafed plants (Vasilakoglou *et al.*, 2001; Bunting *et al.*, 2003; Zheng and Ye, 2003). It is commonly used in the United States and countries throughout Latin America, Europe and Africa in field, pop, seed, sweet and silage corn, sunflowers and soybean agriculture (Battaglin and Goolsby, 1999; Clark *et al.*, 1999; Vasilakoglou *et al.*, 2001; Zheng and Ye, 2003). Recent data show that acetochlor use is increasing, a trend that is expected to continue as new acetochlor-based products replace older chloracetanilide herbicides.

The prevalence of acetochlor in the environment has become a significant topic of interest following its introduction in the United States in 1994. As a result of its propensity for off-site movement, acetochlor is the third most frequently detected herbicide in natural waters behind atrazine and metolachlor (Rebich *et al.*, 2004). In natural waters, chloracetanilide herbicides are degraded primarily by photolytic- (Houston and Pignatello, 1999; Zheng and Ye, 2003) and to a lesser extent, microbial (Ensz *et al.*, 2003) activities. Degradation of chloracetanilide compounds results in the removal of a chlorine atom and the addition of a sulfonic acid functional group, which greatly increases its water solubility relative to the parent compound. In the case of acetochlor, commonly detected degradation products include its ethane sulfonic (ESA) and oxanilic (OA) acids, which are commonly detected in surface waters (Kalkhoff *et al.*, 1998; Rebich *et al.*, 2004). ESA is likely a by-product of glutathione

conjugation occurring in plants, algae and micro-organisms (Kalkhoff et al., 1998). The movement of acetochlor and its transformation products is mediated through several pathways including leaching, soil erosion (Baran et al., 2004) and surface runoff, which has been shown to facilitate the movement of up to 1% of applied acetochlor following natural rainfall conditions (Ferenczi et al., 2002). As a result of these transport mechanisms, it is estimated that up to 96% of freshwater streams in agricultural environments contain detectable quantities of acetochlor (Kolpin et al., 1996; Battaglin et al., 2000; Rebich et al., 2004). The widespread prevalence of acetochlor (and related compounds) in natural waters likely results from the herbicide's high stability and persistent nature in the aquatic environment. For example, Cavalier et al. (1991) reported that alachlor in groundwater samples exhibited a half-life of 808–1518 days, while Pothuluri et al. (1990) observed half-lives of acetochlor in aquifers ranging from 320 to 324 days.

The public and environmental health impacts of acetochlor are not well established. Acetochlor is listed as a suspected endocrine disruptor by the US EPA and European Union, as it has been shown to exhibit activity in a wide range of vertebrates (Cheek et al., 1998; Tilson, 1998; Zacharewski, 1998). Acetochlor has also been classified by the US EPA as a probable human carcinogen (US EPA, 1994). In light of the established dose-dependent impacts of acetochlor on animal populations, the prevalence of acetochlor in natural waters warrants investigation of its ecological impact on freshwater organisms. Bacteria represent a key trophic level in aquatic environments and general disturbances to this group impact organisms at higher trophic levels in addition to processes that contribute to overall water quality, including the maintenance of clean water, nutrient cycling and energy flow through the ecosystem (Verrhiest et al., 2002; Ensz et al., 2003). A small number of studies have shown that freshwater bacterial communities respond to acetochlor exposure. For example, acetochlor exposure can select for streptomycete communities that possess the ability to degrade the parent compound (Sette et al., 2004), while repeated exposure can result in enhanced acetochlor biodegradation (Anderson et al., 1994; Staddon et al., 2001). Despite previous characterization of the potential for microbial degradation of acetochlor, it is unknown if exposure to environmentally relevant concentrations of acetochlor, or concentrations representative of extreme contamination events, will result in broader impacts to the bacteria community, namely impacts to bacterial community structure and function.

The characterization of microbial communities has become increasingly successful by combining culture-dependent and -independent microbiological methods. Therefore, a multiphasic approach that employs complementary methodology might provide a comprehensive view of how acetochlor impacts freshwater bacterial communities. In particular, denaturing gradient gel electrophoresis (DGGE) analysis provides descriptive community DNA fingerprints of complex bacterial assemblages by separating PCR-amplified DNA fragments that represent functional genes or taxonomic biomarkers. The use of DGGE can not only reveal differences in microbial communities inhabiting different freshwater environments (Gao et al., 2005) but can also be used to characterize the impact of contaminants, including herbicides, on microbial community structure (Seghers et al., 2003; Chinalia and Killham, 2006; Lu et al., 2006; Oliveira and Goulder, 2006). While molecular methods have been shown to eliminate much of the bias associated with culture-dependent community analyses, it has been recognized that culturable bacteria can play key roles in many environmental processes and should also be regarded as an important component of the microbial community. In light of this consideration, community level physiological profiling (CLPP) can be used to provide a characterization of community function by assessing the potential of the culturable community, as a whole, to degrade an array of carbon sources (Konopka et al., 1998). Commonly used in a microplate format, respiration of any carbon source by a member of the community results in a concomitant reduction of a tetrazolium dye and the formation of purple coloration in the microplate well that contained the utilized carbon source. The patterns and intensity of color formation have been used to show that changes in bacterial communities occur in response to addition of (i) glyphosate and atrazine to soil (Busse et al., 2001; Marchand et al., 2002), (ii) diamuron and bensulfuron to freshwater environments including rice paddies (Itoh et al., 2003) and (iii) mecoprop and isoproturon injection in subsurface aquifers (de Lipthay et al., 2004). Although the combination of DGGE and CLPP can effectively characterize the structure and functioning of bacterial communities, the use of these methods in concert to characterize simultaneously the impact of acetochlor on freshwater bacterial community structure and activity is yet to be performed.

Despite the frequent occurrence and persistence of acetochlor in stream waters and the established deleterious potential of acetochlor on higher organisms, little information exists regarding the impacts of acetochlor on freshwater bacterial communities. Therefore, the overall objective of this study was to characterize the influence of acetochlor on freshwater bacteria density in addition to community structure and function.

Materials and methods

Sample collection
Approximately 10 l of water from the surface (0–20 cm) of an agricultural drainage stream in Oregon,

OH (USA) was collected in sterile plastic bottles. The bottles were immediately placed on ice prior to transport to the laboratory. Samples were stored at 4 °C prior to analysis, which was performed within 48 h for all experiments. The overall methodological approach is depicted in Figure 1.

Impact of acetochlor exposure on the abundance of total and viable bacteria
Sixty milliliters of stream water were added to six foil-wrapped, 250-ml Erlenmeyer flasks (in triplicate, for a total of 18 flasks) and then supplemented with 0.6 ml of 0, 0.1, 0.5, 5, 10 or 50 $\mu g\,ml^{-1}$ acetochlor stock solutions. This resulted in a final concentration of 0, 1, 5, 50, 100 or 500 $\mu g\,l^{-1}$, in each set of triplicate flasks, respectively. This range of acetochlor concentrations encompasses commonly reported, environmentally relevant concentrations (0, 1 and 5 $\mu g\,l^{-1}$) as well as those that represent abnormally high concentrations that might occur following unintentional acetochlor release (50, 100 and 500 $\mu g\,l^{-1}$). Stock acetochlor solutions were prepared in 95% ethanol (the 0 $\mu g\,ml^{-1}$ was 95% ethanol containing no acetochlor), resulting in a final ethanol concentration in each flask of $\sim 1\%$ (v/v). This concentration of ethanol is generally nontoxic to most bacteria (Price, 1939) and was found to be nontoxic to culturable bacteria following dilution-plating assays of the stream water on R2A agar (data not shown). Following the addition of acetochlor, the microcosms were mixed thoroughly for 30 min on an orbital shaker (200 r.p.m.) and incubated with shaking at 25 °C for 96 h.

Enumeration of total and viable (membrane intact) bacteria following exposure to acetochlor was assessed by collecting four 1-ml aliquots of water from each microcosm following microcosm setup (referred to as the background value) and then again following the 96-h incubation. Samples were fixed by transferring an aliquot (1 ml) to a sterile, 1.5-ml microcentrifuge tube, and then adding formaldehyde (2% v/v). Samples were mixed and stored at −20 °C until enumeration was performed. For visualization of bacteria, 500 μl of each fixed sample was treated with propidium iodide (4 μl, final concentration of 5 $\mu g\,ml^{-1}$), which stains bacteria exhibiting damaged membranes (nonviable), followed by incubation at 20 °C in the dark for 10 min. PicoGreen (2.5 μl, final concentration of 3 $\mu g\,ml^{-1}$) was added to each sample and further incubated at 20 °C for 5 min (total staining time of 15 min) to enumerate total bacteria. Each sample was vacuum-filtered through black polycarbonate membranes (0.22 μm pore size, 25 mm diameter) and placed on a glass microscope slide. A drop of immersion oil was added to the sample, and glass coverslides were placed over the membranes before storage in the dark at 4 °C for up to 2 h. Slides were viewed at $\times 1000$ magnification for enumeration of total bacteria (PicoGreen excitation 425–525/emission 475–650 nm) and nonviable bacteria (propidium iodide excitation 300–650 nm/emission 550–750 nm). A minimum of 10 filter fields and 200 bacteria per filter were counted to obtain the average number of bacteria per field, which were distinguished from other types of cells based on size, shape and intensity of emitted light. The number of viable bacteria was calculated by subtracting the number of nonviable bacteria from the number of total bacteria. The average number of total and viable bacteria in background samples and in those exposed to acetochlor for 96 h was calculated and expressed as the average and standard error of the mean bacteria number per ml water. The statistical difference between treatment means was determined following single-factor ANOVA ($\alpha = 0.05$) followed by a Tukey test of the treatment means ($\alpha = 0.05$). Pearson's product moment correlation analysis was performed to determine the relationship between acetochlor concentration and cell number.

Impact of acetochlor on bacterial community structure
The impact of acetochlor exposure on the freshwater bacterial community structure was assessed by DGGE analysis. Specifically, bacterial communities were concentrated by filtering stream water (200 ml) through each of 18 mixed cellulose ester filters (0.45 μm pore size, 47 mm diameter). This pore size was preferred over smaller pores to avoid clogging of the filter, which was evident during preliminary experiments to test the amount of water that could be passed through the filter. Membrane filters were placed on filter pads (47 mm diameter, in triplicate) that had been soaked with 1 ml of sterile acetochlor solution (mixed in 10 mM phosphate buffer) that represented six exposure levels (0, 1, 5, 50, 100 or 500 $\mu g\,l^{-1}$). This setup provided the bacteria concentrated on the membrane filter with continual exposure to acetochlor. Filters and pads were incubated at 25 °C for 96 h in 47 mm, tight sealed Petri dishes (Millipore, Billerica, MS, USA) wrapped in foil. During incubation, the dishes were visually inspected throughout the incubation period to ensure that the filters and pads did not desiccate. Following incubation, bacteria were released from the filters by vortexing the filter (1 min, medium speed) in a 15-ml falcon tube containing 6 ml of
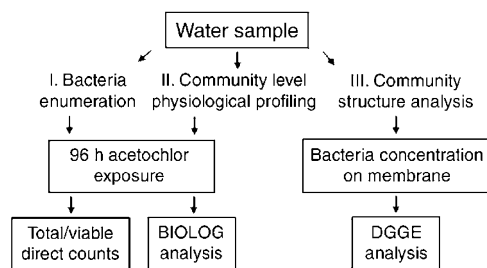

Figure 1 Methodological approach used to determine the impact of acetochlor on freshwater microbial communities.

10 mM phosphate buffer and six sterile glass beads (6 mm diameter). The solution was centrifuged (10 min at 4500 g) and DNA was isolated from the bacteria pellet as described by Sigler and Zeyer (2002). PCR amplification of a portion of the 16S rRNA gene was performed as described by Muyzer et al. (1993) including a negative PCR control containing sterile water instead of DNA. A GC clamp (Muyzer et al., 1993) was attached to the 5′ end of the forward primer to facilitate DGGE-based separation of the resulting PCR products. Amplification of the proper gene segment (~190 bp) was confirmed by agarose gel electrophoresis.

Denaturing gradient gel electrophoresis analysis of the PCR products was performed according to the method of Sigler et al. (2004). Gels were stained for 20 min with a 1:10 000 dilution of GelStar nucleic acid stain, and fingerprints were visualized with a Kodak Gel Logic 200 imaging system. DGGE fingerprints were normalized using a custom marker of PCR products that were distributed along the length of the DGGE gel following electrophoresis. The marker was loaded such that a maximum of five samples separated each marker lane. Fingerprints were analyzed with GelCompar II software (version 3.5, Applied Maths, Austin) using an optimization parameter calculated automatically by the software to account for electrophoretic shifts between any two identical bands/patterns. Two parameters to describe the effect of acetochlor exposure on the bacterial community were measured. First, to identify changes in the bacterial community complexity, the number of bands in each fingerprint was determined using the band-matching function in GelCompar II. Second, bacterial community structure was assessed by comparing fingerprint patterns. Specifically, a similarity matrix that quantified the relationships among the resulting DGGE fingerprints was calculated by the software using the Pearson's product moment correlation coefficient (Jobson, 1991). Cluster analysis was performed on the resulting similarity matrix using the unweighted pair group method with arithmetic means (UPGMA) algorithm, resulting in dendrograms that graphically displayed the similarities among fingerprints. The similarity of the fingerprints contained within a given cluster was reported as the *within-cluster* similarity, which provided a measure of the community similarity within the cluster. Equally important as the within-cluster similarity is the *among-cluster* dissimilarity or depth of branching. This was quantified by subtracting the node similarity of any two clusters from their average within-cluster similarity. The resulting value was used to characterize the degree to which two clusters differed, and therefore, the impact of acetochlor treatments. To further validate relational inferences represented by dendrogram clustering, cophenetic correlation coefficients were also calculated with GelComparII software.

Impact of acetochlor on bacteria function
The impact of acetochlor on the function of freshwater bacterial communities was assessed using CLPP. Four sets of triplicate BIOLOG Ecoplates (31 carbon-source-containing wells and one control well containing no carbon source, in triplicate) were each inoculated (100 μl per well) with water from the microcosms that contained the low (0 and 5 μg l^{-1}), high (50 μg l^{-1}) and extreme (500 μg l^{-1}) concentrations of acetochlor. The plates were placed in a humidified container and incubated aerobically at 25 °C for 96 h. Following incubation, the contents were homogenized by mixing and the optical density (OD$_{490}$) of each well was measured with a microplate reader. The absorbance value of the control well of each triplicate was subtracted from the value measured from each of the 31 remaining wells. The number of carbon sources utilized by the community, a measure of functional diversity (Zak et al., 1994; Konopka et al., 1998), was calculated by summing the number of wells that exhibited absorbance greater than 0.25 OD$_{490}$ (Garland, 1996). The proportional metabolism of each carbon source was also calculated as the ratio of the absorbance value, each carbon source to the sum of all absorbance values. The Shannon index (H′) (Magurran, 1988) of carbon source utilization was calculated as $H' = -\sum p_i \ln(p_i)$, where p_i is the proportional usage of each carbon source. H′ was subsequently used to calculate the evenness (E) of substrate usage, as $E = H'/\log S$ (Magurran, 1988), which was compared among treatments with ANOVA followed by pairwise multiple comparisons using the Student–Newman–Keuls method ($\alpha = 0.05$). High evenness implies that each of the 31 carbon sources was utilized at the same proportional abundance (each used at 3.2% of the total carbon usage, that is each used at a ratio of 1:31), while low evenness indicates the preferential use of some sources, at the expense of other sources.

The degree to which the bacterial communities utilized each of the carbon source guilds represented in the Ecoplate (polymers, carbohydrates, carboxylic acids, amino acids, amides/amines and miscellaneous) was determined by summing the OD$_{490}$ of the wells containing substrates belonging to each guild that exhibited absorbance greater than 0.25 OD$_{490}$. Kruskal–Wallis one-way ANOVA on ranks was conducted to determine significant differences in carbon usage for each guild that resulted from exposure to differing acetochlor concentrations ($\alpha = 0.05$).

Results

Total and viable bacteria
Significant differences in the number of total and viable bacteria were observed between several treatments following incubation of water samples with differing concentrations of acetochlor (total:

$F = 24.28$, $P < 0.0001$, d.f. $= 6, 15$; viable, $F = 31.26$; $P < 0.0001$, d.f. $= 6, 15$), however regression analysis and Pearson's product moment correlation indicated no significant correlation of cell number with acetochlor concentration (total cells: $R = 0.21$, $P = 0.69$; viable cells: $R = 0.13$, $P = 0.80$) (Figure 2). In general, the number of viable bacteria was $10 \pm 4\%$ of the number of total bacteria and the densities of both total and viable bacteria followed similar trends in response to acetochlor exposure. Specifically, the number of total and viable bacteria increased with respect to non-amended controls following incubation with $1 \mu g l^{-1}$ acetochlor, decreased to a minimum in the microcosm containing $50 \mu g l^{-1}$ acetochlor and then increased again in the microcosms containing 100 and $500 \mu g l^{-1}$ acetochlor. The total bacteria density in the stream water prior to incubation was 1.24×10^6 cells ml^{-1}, which increased to a maximum number of 5.68×10^6 cells ml^{-1} (total bacteria) and 9.88×10^5 cells ml^{-1} (viable) in the $1.0 \mu g l^{-1}$ acetochlor. The minimum number of bacteria was observed in the microcosm containing $50 \mu g l^{-1}$ acetochlor (total bacteria: 6.74×10^5 cells ml^{-1}; viable: 5.31×10^4 cells ml^{-1}).

Impact of acetochlor on bacterial community structure
DGGE fingerprints were successfully generated from all DNA samples and comprised between 11 and 25 dominant bands (Figure 3). The number of bands detected in the fingerprints within a given acetochlor treatment was consistent, as the standard error of the mean band number within a treatment never exceeded 8% (data not shown). In general, exposure to acetochlor appeared to decrease the complexity of the bacterial communities. For example, the average number of bands detected in the 1- (13 bands), 5- (13 bands), 50- (11 bands) and 100-$\mu g ml^{-1}$ (14 bands) treatments was significantly less than in the control treatment (19 bands) (one-way ANOVA, $P < 0.05$).
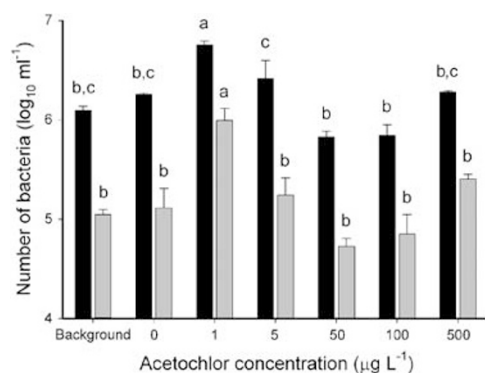

Figure 2 Total (black bars) and viable (gray bars) bacterial densities following exposure (96 h) to acetochlor at concentrations of 0, 1, 5, 50, 100 and 500 $\mu g l^{-1}$. 'Background' label refers to the total and viable bacteria concentration in water samples prior to incubation. Same letter designation above each bar indicates that no statistical significance was observed in either total or viable bacteria counts (Tukey test, $\alpha = 0.05$). Error bars represent the standard error of the mean ($n = 3$).

The number of bands detected in fingerprints from communities exposed to $500 \mu g ml^{-1}$ acetochlor (23 bands) was higher than in control samples, although the difference was not significant ($P = 0.19$).

Cluster analysis of the DGGE fingerprints indicated that communities exposed to a common treatment were generally of high similarity ($90 \pm 2.6\%$). Analysis of among-treatment fingerprint similarity revealed that the presence of acetochlor caused distinct changes in the freshwater bacterial communities. Specifically, three main clusters of fingerprints were observed that represented communities exposed to (i) control-level or environmentally relevant- (0, 1, and $5 \mu g l^{-1}$), (ii) high- (50 and $100 \mu g l^{-1}$) and (iii) extreme- ($500 \mu g l^{-1}$) acetochlor concentrations (Figure 3). Specifically, bacterial communities exposed to control- and environmentally relevant concentrations were 88% similar as a group, while the fingerprints representing communities exposed to high and extreme concentrations were 96 and 86% similar, respectively. The among-cluster dissimilarity of the control/environmentally relevant concentration and high concentration clusters was 11.5%, while the dissimilarity of these clusters, as a group, to the extreme concentration cluster was 50.5%. The integrity of the dendrogram organization (branching and structure of the resulting clusters) was validated by the calculation of cophenetic correlation coefficients, which were always greater than 0.85 (maximum is 1.0).

Impact of acetochlor on bacteria function
The impact of acetochlor on the function of freshwater bacteria was assessed by determining the number of different carbon sources that the bacteria assemblage could utilize following exposure to four acetochlor concentrations (0, 5, 50 and 500 μg acetochlor l^{-1}). Following 96 h of incubation, microbial communities in microcosms containing 5, 50 and 500 μg acetochlor l^{-1} used a significantly greater number of carbon sources than communities in the control microcosms (one-way ANOVA, $P < 0.05$) (Figure 4). Specifically, communities in the microcosms containing 0, 5, 50 and 500 μg acetochlor l^{-1} utilized an average of 9.5, 24.5, 25 and 25 carbon sources, respectively.

To further investigate the impact of acetochlor exposure, we compared the pattern of proportional utilization of each carbon source across the four acetochlor treatments. Proportional utilization analysis indicated that in addition to increasing the number of carbon sources utilized, the addition of acetochlor effectively stimulated a trend in the substrate-use patterns toward the equitable use of the carbon sources (Figure 5). Specifically, with the exception of the community exposed to $500 \mu g ml^{-1}$ acetochlor, evenness was significantly higher in all communities following incubation with acetochlor, relative to the untreated, control communities ($P < 0.05$) (Figure 4).
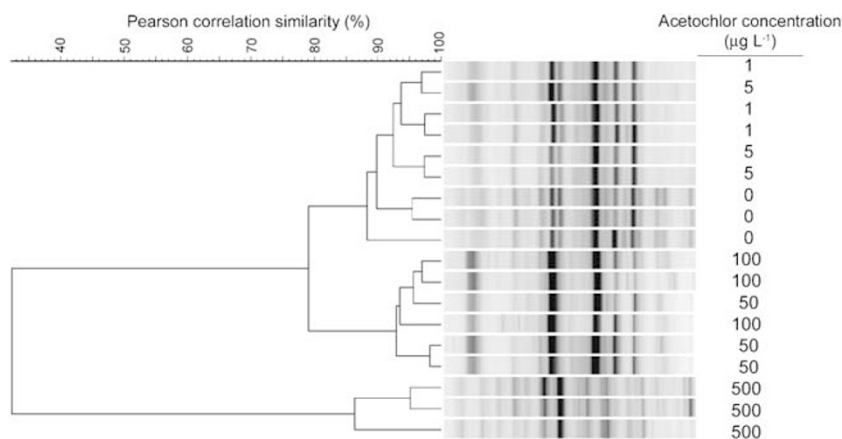
Figure 3 Cluster analysis of triplicate denaturing gradient gel electrophoresis fingerprints of bacterial communities exposed to acetochlor (96 h) concentrations of 0, 1, 5, 50, 100 and $500 \mu g \, l^{-1}$. The dendrogram scale represents the percentage of similarity between clusters as calculated by Pearson's correlation method.
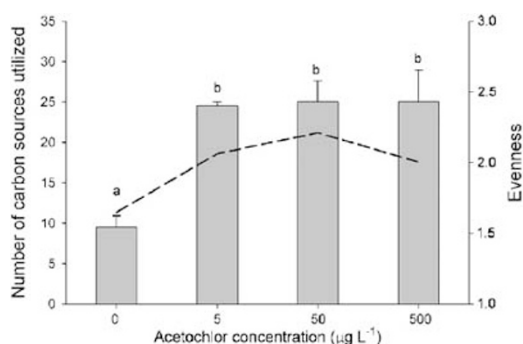

Figure 4 Impact of exposure (96 h) to acetochlor concentrations of 0, 5, 50 and $500 \mu g \, l^{-1}$ on (i) the number of carbon sources utilized by the bacterial community (bars) and (ii) community evenness (line). Error bars represent the standard error of the mean number of utilized carbon sources ($n = 3$), while differing letters above the bars represent significant difference in the mean number of substrates utilized.

Acetochlor exposure resulted in no significant impact on the use of the carbon substrate guilds, as compared to control communities ($P = 0.267$, 0.267, 0.267, 0.210, 0.324 and 0.171 for polymers, carbohydrates, carboxylic acids, amino acids, amides/amines and miscellaneous, respectively) (Figure 6).

Discussion

Following land application, acetochlor has been shown to exhibit substantial off-site movement to natural waters via overland runoff and leaching (Ferenczi *et al.*, 2002; Baran *et al.*, 2004). The established negative impacts of acetochlor exposure to aquatic organisms combined with the importance of bacteria consortia to freshwater food chain processes such as decomposition and nutrient cycling, warranted an investigation of the impacts of acetochlor on freshwater microbial communities. Since microorganisms adapt rapidly to changing environmental conditions, alterations in bacteria
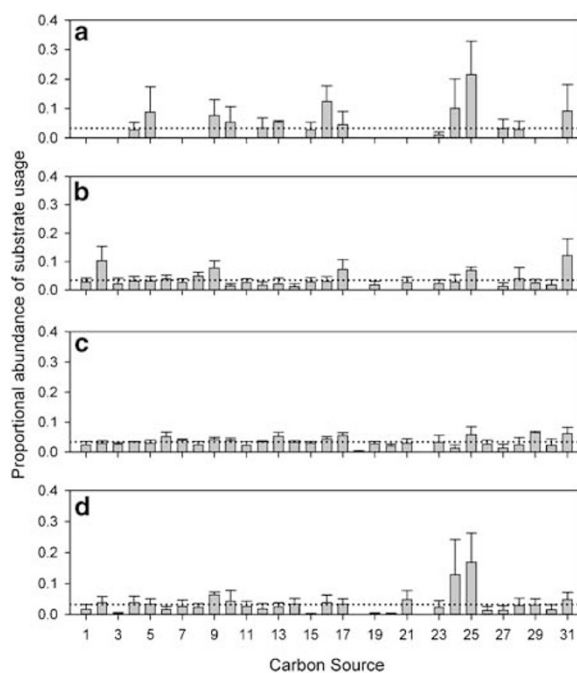

Figure 5 The proportional abundance of carbon substrate usage by bacterial communities exposed to (**a**) 0, (**b**) 5, (**c**) 50 and (**d**) $500 \mu g \, l^{-1}$ of acetochlor for 96 h. Error bars represent the standard error of the mean proportional abundance ($n = 3$). The broken line represents a theoretical proportional abundance that would result from equal usage of all the carbon substrates (proportional abundance of 0.032). Deviation from this threshold represented preferential or limited utilization of a particular carbon source.

number, community structure and function can serve as effective indicators of overall changes in environmental quality (Kennedy and Papendick, 1995; Pankhurst *et al.*, 1997). Therefore, we adopted a multiphasic approach that combined enumeration, and bacterial community structure and function analyses to characterize the impact of environmentally relevant and greater concentrations of acetochlor on freshwater microbial communities.
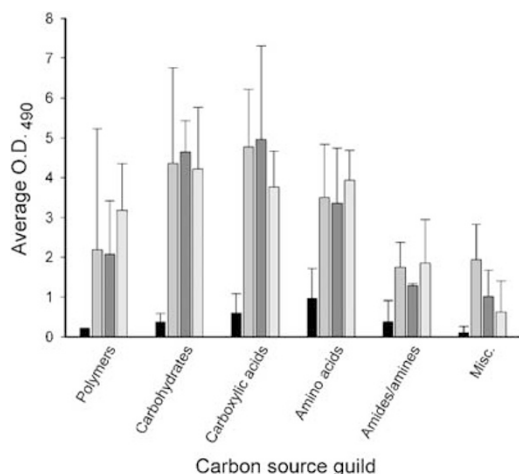
Figure 6 The use of carbon substrate guilds (divided into guilds representing polymers, carbohydrates, carboxylic acids, amino acids, amides/amines and miscellaneous substrates) by bacterial communities following exposure (96 h) to acetochlor. Legend: ■, $0 \mu g l^{-1}$, ▨ $5 \mu g l^{-1}$, ▨ $50 \mu g l^{-1}$ and ▨ $500 \mu g l^{-1}$). The error bars represent the standard error of the mean color production (expressed as A_{490}) in the microplate wells representing the defined guilds.

Although some significant differences in the number of bacteria resulted from acetochlor exposure, acetochlor concentration did not appear to correlate with the number of total ($P = 0.69$) and viable bacteria ($P = 0.80$), even at concentrations up to $500 \mu g l^{-1}$ (Figure 2). The impact of acetochlor concentrations greater than $25 \mu g l^{-1}$ on the number of freshwater bacteria has not been reported previously. However, our result suggests that acetochlor inputs greater than $25 \mu g l^{-1}$ can be tolerated by freshwater bacteria assemblages without a significant decrease in bacterial density. It was expected that our extreme treatment ($500 \mu g l^{-1}$) would elicit a toxic effect; however, no evidence of toxicity with regard to bacteria number was observed. To our knowledge, no studies have been performed that characterize the toxicity of acetochlor to bacteria, although studies investigating the impact of metolachlor, another chloroacetanilide herbicide, have been performed. Metolachlor and acetochlor differ in their molecular structure, but the two herbicides have been shown to share toxicological mechanisms in animal models (USEPA, 2001). Therefore, it is possible that data concerning the toxicity of metolachlor to bacteria could be used to predict the behavior of acetochlor. Specifically, metolachlor was shown to decrease the number of bacteria in soil when applied at a concentration of $100 \, mg \, kg^{-1}$ soil (Chang et al., 2001). Furthermore, assays of the impact of metolachlor on the model bacterium V*ibrio fischeri* revealed EC_{50} values of between 5 and $30 \, mg \, l^{-1}$ water (Gaggi et al., 1995; Karuppiah et al., 1997; Tsui and Chu, 2003; Sakkas et al., 2004). While toxicological effects on bacteria populations were demonstrated in these studies, it should be noted that the concentrations of metolachlor

exceeded those normally observed in the aquatic environment, as well as the one used in the current study, by approximately 1000-fold.

DGGE analysis was used to observe changes in band number (a proxy for community complexity) and band patterns (a gross indicator of community structure) that resulted from exposure of bacterial communities to acetochlor. Although DGGE is an established method for examining the impact of herbicide exposure in soils (Seghers et al., 2003; de Lipthay et al., 2004; Saeki and Toyota, 2004; Seghers et al., 2005; Ros et al., 2006; Valle et al., 2006), few studies have used DGGE analysis to examine the impact of herbicides on aquatic microorganisms. Our data revealed that exposure to acetochlor decreased bacterial community complexity, as the fingerprints collectively representing water samples exposed to 1, 5, 50 and $100 \mu g$ acetochlor l^{-1} contained significantly fewer bands relative to those representing the control treatment. Despite this difference, band number was not significantly correlated with acetochlor concentration ($P = 0.627$). This result suggested that changes in community complexity were not dose dependent, but occurred regardless of acetochlor concentration. Cluster analysis of community fingerprint band patterns revealed three, distinct, dose-dependent clusters of fingerprints (Figure 3), indicating a predictable relationship between acetochlor concentration and alterations to the bacteria community. The fingerprints that represented communities exposed to the control treatment ($0 \mu g l^{-1}$) and the environmentally relevant acetochlor concentrations (1 and $5 \mu g l^{-1}$) clustered with relatively high (88%) similarity. Therefore, since acetochlor is most often detected in environmental waters at concentrations less than $25 \mu g l^{-1}$ (Kolpin et al., 1996; Clark et al., 1999; Battaglin et al., 2000; Rebich et al., 2004), our fingerprinting analyses suggest that exposure to relevant levels of environmental contamination will result in limited detectable alteration to the indigenous bacterial community. In contrast, exposure to acetochlor at concentrations that were (i) higher than environmentally relevant (50 and $100 \mu g l^{-1}$) and (ii) extreme ($500 \mu g l^{-1}$) resulted in bacteria communities that were less than 80 and 35% similar, respectively, to the control communities. Since all environmental factors other than acetochlor concentration were standardized throughout the exposure period, it is assumed that community structure changes resulting from increased acetochlor bioavailability. Several studies have shown that the content and nature of organic matter can play a key role in the availability of applied pesticides (Hamaker and Thomson, 1972; Stevenson, 1972). The total organic carbon concentration in the water samples used in the current study was relatively low ($10.72 \, mg \, l^{-1}$). Therefore, it is likely that few sites for acetochlor adsorption were available, which in turn, facilitated increased bioavailability when higher concentrations of acetochlor

were assayed. Adsorption studies involving soil assays have indicated that as the initial concentrations of acetochlor increased, the percentage adsorbed by the soil decreased (Nemeth-Konda *et al.*, 2002). This characteristic is likely enhanced in an environment featuring limited organic carbon, which could have further facilitated increased acetochlor bioavailability and subsequent impacts to the bacteria communities observed in this study.

Analysis of community complexity and structure can provide a gross representation of how exposure to a xenobiotic compound might impact a microbial community. However, structure analyses do not necessarily give insight to changes in the functional qualities of a community. Therefore, we assayed the impact of acetochlor exposure with community level physiological profiling, which revealed significant changes in the functional diversity of the heterotrophic bacterial community. Specifically, our results indicated that acetochlor increased functional diversity in two ways. First, acetochlor exposure resulted in an increase in the number of carbon sources utilized by the microbial community, relative to nonexposed controls (Figure 4). This result was not surprising, as the stimulation of microbial metabolism following exposure to chloroacetanalide herbicides has been previously shown. For example, growth of *Acinetobacter* spp and *Psuedomonas* spp has been shown to result from the dehalogenation of propachlor and utilization of the herbicide as a sole carbon source (Villarreal *et al.*, 1991; Martin *et al.*, 1995, 1999; Xu *et al.*, 2006). Since acetochlor and propachlor are structurally similar (they share the same molecular composition ($C_{14}H_{20}ClNO_2$) with a ClNO functional group single bonded to the benzene, however, with fewer functional groups in the case of propachlor), the same mechanisms used to assimilate energy from propachlor might also drive the metabolism of acetochlor, resulting in the stimulation observed in the current study. Second, acetochlor exposure increased functional evenness within the heterotrophic community. While evenness is a term historically reserved as a descriptor of a community's equity of species abundance (Magurran, 1988), the use of evenness to describe a microbial community's equity of carbon substrate usage has also been established (de Lipthay *et al.*, 2004). Microbial communities exhibited significantly increased functional evenness following exposure to 5 and 50 μg l^{-1} acetochlor (relative to the control) (Figures 4 and 5), and an insignificant increase following exposure to 500 μg ml^{-1} acetochlor. At this time, the mechanism by which acetochlor modulates functional diversity and evenness of bacterial communities is unclear. However, our results provide evidence that the observed physiological response of the community is a combination of neutral and positive effects. Acetochlor appeared to stimulate carbon usage by a subset of the community, without promoting bacterial growth, as interpreted by nonsignificant changes in bacteria number (Figure 2). Furthermore, acetochlor did not appear to select for specific functional groups, which was reflected by the consistent use of carbon source guilds, regardless of acetochlor concentration (Figure 6). Consistency of substrate guild usage following herbicide application was also observed previously. For example, Ros *et al.* (2006) observed nonsignificant differences in guild preference following long-term exposure (45 days) of soil bacteria communities to low-to-moderate concentrations of atrazine (1–10 mg kg^{-1} soil), with significant differences occurring only after exposure to high atrazine concentrations (100 and 1000 mg kg^{-1}). Despite the apparent impact to the community guild preference, the concentration of atrazine utilized by Ros *et al.* (2006) exceeded the environmentally relevant acetochlor concentrations used in the current study by approximately 1000-fold.

While DGGE analysis effectively differentiated the impacts of exposure to acetochlor, some noteworthy methodological limitations should be noted. For example, DGGE fingerprints were generated by amplifying the 16S rRNA gene, which can be present in multiple copies of differing sequence in some bacteria (Nübel *et al.*, 1996). This can result in multiple bands that represent a single phylotype, while the comigration of bands containing DNA of different sequences but similar melting behavior can lead to misinterpreted community structure and an underestimation of community complexity (Casamayor *et al.*, 2000). The effective use of DGGE also relies on selecting an appropriate method to compare fingerprint patterns. Despite the popular use of band-based similarity measures (for example, Dice and Jaccard), we used the Pearson's product moment correlation coefficient, which accounts for the entire data set describing each fingerprint by correlating points along a densiometric curve generated by the GelCompar II software. The use of Pearson's correlation was preferred with respect to band-based methods because (i) it is insensitive to relative differences in intensity and background, features common to DGGE fingerprints of environmental samples, and (ii) more of the data contained in each fingerprint is used vs band-based algorithms (Hane *et al.*, 1993). Furthermore, when Dice similarity analysis was performed on these fingerprints (data not shown), it was often necessary to correct manually for bands misidentified by the software, which introduced an unwanted degree of bias to the analysis. Fingerprints generated in our analyses exhibited average cophenetic correlation coefficients greater than 0.85, indicating that the relationships reflected by the dendrogram were appropriate and that fingerprint differences could be interpreted as real differences in the communities.

The use of a multiphasic approach to determine the environmental impact of xenobiotic contamination is an increasingly popular trend, as the combination of methods can draw upon the

strengths of each individual method. In this study, each method revealed details concerning a differing subset of the bacterial community. While direct counting effectively enumerated most bacteria cells, regardless of phylogenetic or metabolic characteristics, DGGE and CLPP analyses assayed contrasting subsets of the overall community. For example, DGGE analysis has been shown to effectively characterize changes in dominant bacteria populations. However, it is less sensitive to changes in the nondominant populations (Felske *et al.*, 1998) and might not detect subtle changes occurring overall. In contrast, CLPP analysis does not select for dominant bacteria, but for those that will exhibit growth on a defined substrate in high concentrations, relative to the *in situ* environment (Konopka *et al.*, 1998). Therefore, CLPP analysis might not reflect the contribution of difficult to culture bacteria, such as oligotrophic bacteria and obligate anaerobes. Nonetheless, CLPP can provide a valuable index of relative community functional potential and an effective means to compare the culturable subsets of different communities (Haack *et al.*, 1995). Taken together, our enumeration, and structural and functional description of the impact of acetochlor exposure demonstrate the necessity for a multiphasic approach in which complementary methods can be used to comprehensively describe bacterial communities.

The results of this study indicated that the impact of environmentally relevant concentrations of acetochlor on freshwater bacteria is not universal, but dependent on the subset of the bacterial community in question. Acetochlor exposure at concentrations as high as $500 \mu g \, l^{-1}$, which is approximately 50-fold greater than concentrations commonly encountered in the environment, exhibited little impact to the number of total and active bacteria and on the types of carbon utilized by the bacterial community. Similarly, cluster analysis of community fingerprints showed that communities of bacteria exposed to environmentally relevant concentrations were most similar to control communities. In contrast, exposure to environmentally relevant acetochlor concentrations resulted in decreased bacterial community complexity, but stimulated functional potential. Overall, acetochlor exposure appears to result in complex and varied community responses, the mechanisms of which are still unidentified. Our results show that the methods used in this study each described different aspects of the bacterial community and demonstrated the importance of adopting a multiphasic approach to produce a comprehensive picture of bacterial community structure and function in response to environmental stresses.

Acknowledgements

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