Herbicide contamination of freshwater ecosystems: Impact on microbial communities

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Herbicide Contamination of Freshwater Ecosystems: Impact on Microbial Communities

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1. Introduction

Human society relies on surface freshwater ecosystems for many goods and services (drinking water, recreational facilities…), which places these systems at the center of a web of ecological, economic and political interests (Wilson & Carpenter, 1999). They are also being subjected to increasing pressure resulting from anthropogenic activities, including contamination by a variety of mineral and organic pollutants. Most of the organic pollutants are herbicides (e.g. Kreuger, 1998; Dorigo et al., 2007), which are used not only in agriculture but also for many other purposes (ranging from domestic use in gardens to maintaining railway tracks weedfree, for example). These herbicides can enter aquatic ecosystems as a result of terrestrial runoff, and to a lesser extent, of direct application and aerial spraying (e.g. Carter, 2000). Microbial communities in freshwater ecosystems are not directly targeted, but these communities are exposed to herbicides and can be directly or indirectly affected by these compounds. For instance, many commercial herbicides act by binding to Photosystem II (PSII), which is a pigment-protein membrane complex (see e.g. Schuler & Rand, 2008). PSII inhibitors have a direct impact on photosynthetic aquatic microorganisms that contain the same PSII apparatus as the terrestrial weeds targeted by these herbicides. In addition to this direct impact on photosynthetic microorganisms, as a result of the strong interactions that occur between all these aquatic microorganisms, herbicides can also have an indirect impact on non-photosynthetic species that are not susceptible to PSII inhibitors.

These effects on microbial communities can have a critical impact on the overall functioning of freshwater ecosystems, because prokaryotic and eukaryotic microorganisms are major players in these systems. Indeed, microorganisms contribute to most of the primary production in these systems, which is closely related to the total productivity (Aoki, 2003). They are also involved in nutrient cycling and decomposition, via the microbial loop (see, for example, the review of Fenchel, 2008). In lentic ecosystems (lakes, reservoirs, ponds…), most of these processes (primary production and nutrient cycling/decomposition) occur in the water column, and involve planktonic communities. On the other hand, in lotic ecosystems (rivers, streams...), these processes occur mainly in the biofilms located on the surface of sediments and plants, which involve benthic microbial communities. Depending on the kind of freshwater ecosystem concerned, both these communities must be considered when attempting to evaluate the impact of herbicides on microbial communities.

In this paper, we will start by brief overview of herbicide contamination of freshwater ecosystems, focusing particularly on a few specific countries in different geographical zones. We will then go on to present the main methods used to assess the impact of herbicides on microbial communities, ranging from single species tests to field studies. The third part of this review will focus on molecular methods that have been used in the last few years to evaluate the impact of herbicides on freshwater microbial communities. The fourth section will provide a brief summary of what is known about the impact of herbicides on freshwater microbial communities, and also on how these communities respond to this selective pressure. We will then look at the data available on glyphosate, which is one of the herbicides most often found in freshwater ecosystems. Finally, we will try to relate herbicide contamination and its impact on freshwater microbial communities to global changes, which are also impacting on these communities.

2. A rapid review of the contamination of aquatic ecosystems

2.1 General aspects

The information available about herbicide contamination of surface freshwater ecosystems varies considerably from country to country. A lot of information is available for countries in Europe and North America, but the situation is more patchy for Asia and South America, and there is almost no general or local data for Africa. This can be explained by the fact that so far the heavy use of herbicides has largely been confined to North and Latin America, Europe, Japan, and Australia, although their use is rapidly increasing in many developing countries (Ware & Whitacre, 2004). From all these data, it can be seen that even though the amount of herbicides entering the surface water varies considerably between regions, all surface freshwater ecosystems are now contaminated by herbicides. However, the concentrations of these herbicides in water vary considerably depending on the size and land use of the watersheds (e.g. Neumann et al., 2003), and also on season and climatic events. For example, peak contamination of rivers occurs just after discharge events, with relatively low levels between these events (e.g. Spalding & Snow, 1989; Botta et al., 2009). In agricultural landscapes, the amount of pesticides contaminating surface water also depends on the methods and levels of application, and more generally on agricultural practice (Huber et al., 2000). Finally, in a recent paper of Wittmer et al. (2010), it has been shown that five types of pesticide concentration patterns in surface water samples can be distinguished, and that these patterns reveal the relative contributions made by urban and agricultural land to the contamination of water by herbicides. This paper also showed that in mixed land use catchment areas, the contamination of surface water by biocides from urban areas was at least as great as that from agricultural areas. Thus, both urban and agricultural contaminations contribute to the contamination of surface water, although the most dramatic levels occur in areas characterized by intensive agriculture (see below).

2.2 Water contamination in some specific countries

In France, the "Institut Français de l'Environnement" (French Institute for Environment; now known as the Service de l'Observation et des Statistiques, SOeS) has published data on the contamination of water by pesticides for every year since 1998. Their annual report is based on monitoring 453 pesticides at 2023 sampling points (groundwater and rivers). In 2007, pesticides were detected at almost 91% of the sampling points, but usually at mean

annual concentrations of <0.5 µg/L. The highest concentrations were found in regions with intensive agriculture (South-West, Center-North and North of France) and the lowest in regions (South-East and South of Massif Central) characterized by less intensive agriculture or by the presence of large areas of natural environments. The pesticide most often detected in French streams was AMPA (aminomethyl phosphonic acid), which is the primary degradation product of glyphosate. The second and the third most frequently identified pesticides in streams were diuron and glyphosate, which are both herbicides. More generally, the 15 most frequently occurring pesticides were all herbicides or degradation products of herbicides. Finally, changes in herbicide concentrations in French streams from 1997 to 2007, indicate that there has been no significant decrease in the herbicide pollution of streams, but that there have been changes in the dominant compounds found in these ecosystems. For example, atrazine, which was the most frequently-found herbicide in 1997, have been less often detected since it has been banned and replaced by glyphosate (Dubois et al., 2010). Data of this type is not available for lentic ecosystems, for which only occasional analyses have been performed. To compare the situation in France with that in another European country, a recent review of Greek freshwater ecosystems has shown that atrazine, simazine, metolachlor and alachlor were the most frequently detected herbicides (Konstantinou et al., 2006). Glyphosate was not mentioned in this review, but we do not know if this was because most of the data were obtained before massive use of this herbicide began, or because Greece does not have large areas of corn production. However these studies have shown that the levels of contamination of major European rivers by herbicides such as atrazine or simazine, are similar in all countries.

We do yet have overall data for the level of contamination of surface freshwater ecosystems by herbicides in China, but three papers have provided some indications of the level of herbicide contamination in Chinese surface freshwater. Gfrerer et al. (2002a,b) have shown that triazines were detected throughout the year in three rivers from China, with a concentration peak in June due to their use in agriculture. But, apart from the June values, the concentrations of triazine were quite low compared to those in European rivers. On the other hand, in an analysis of 14 different organic pesticides and their metabolites in Lake Taihu, Na et al. (2006) reported very high concentrations of atrazine. This herbicide has been widely used in China since the 1980s, with an annual increase of 20% (*in* Na et al., 2006). More data are available for another Asian country, Japan. For example, in a study on the contamination of the lake Biwa, Sudo et al. (2002 & 2004) have shown that three of the pesticides most frequently found in this lake and in the rivers flowing into it were herbicides (simetryn, molinate and bromobutide), which are all used in paddy fields, and so found in numerous rice producing areas.

It appears that triazines and in particular atrazine and simazine, are very frequently found in surface freshwater ecosystems worldwide, as are glyphosate and its degradation product, AMPA. But, local differences do occur, and are related to the kind of agriculture or to the banning of specific compounds.

3. The main methods used to estimate the impact of herbicides on microbial communities

Ecotoxicology deals with the evaluation of the processes and mechanisms by which toxicants are dispersed, and with their effects on populations, communities and ecosystems. This implies the need for studies at different geographical and time scales, which has resulted in the development of a wide variety of methods (Fig. 1)
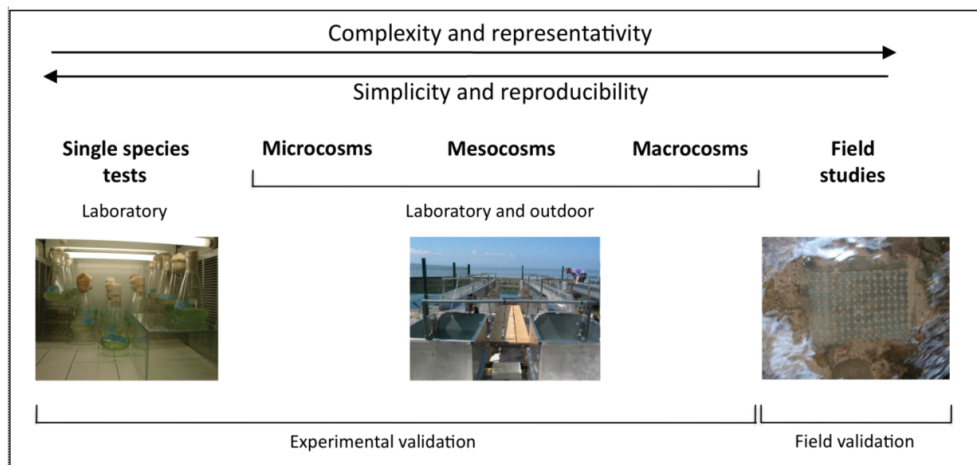
Fig. 1. Various levels of biological complexity in the experimental methods used in ecotoxicology (from Caquet et al., 1996)

Many studies are based on single-species tests, and these are usually considered to concern environmental toxicology rather than ecotoxicology (Ramade, 2007). These studies are fundamentally reductionist, and they give priority to the reproducibility of the data acquired (single-species tests and microcosm experiments). In such studies, the number of experimental parameters is limited, which makes them easier to control. In contrast, studies conducted in mesocosms or in macrocosms are closer to those performed in ecosystems, which makes them more realistic. They are designed to provide an appropriate way to assess the real impact of a pollutant in a natural ecosystem. However, these studies are more difficult to conduct, and often less reproducible, because of the large number of factors and processes involved, many of which are not easy to control.

3.1 Single species tests

Ecotoxicity tests are a useful way of determining the effect of one or more products on selected organisms, under defined conditions (Keddy et al., 1994). These tests can be classified according to their duration, compared to the life cycle of the organism, and to the complexity of the biological community studied. Acute toxicity tests cover a relatively short part of the life cycle of the organism. Bacteria, protists and algae have life cycles lasting 24-48 hours. In contrast, chronic toxicity tests involve the repeated exposure of organisms to low doses of a contaminant over a long period.

In order to characterize the toxicity of a substance in a regulatory context, it is necessary to evaluate both its acute and chronic toxicity at several trophic levels. Various estimators have been defined in order to do this:

- EC_{50}: The "half maximal effective concentration" is the concentration of a toxicant that induces a response halfway between the baseline and maximum response after some specified exposure time.
- LC_{50}: The Lethal Concentration 50 is the concentration of a toxicant that causes 50% mortality

- NOEC: No Observed Effect Concentration.
- LOEC: Lowest Observed Effect Concentration.

In addition to these indicators, water resource managers also use the concepts of:
- PNEC: Predicted No Effect Concentration
- PEC: Predicted Effect concentration

The EC_{50} value is estimated using dose-response relationships. Generally, increasing the dose of a toxicant causes a proportional increase in its toxic effect on the biological parameters investigated. The dose-response curve is based on the following assumptions:
- The response increases with dose,
- There is a threshold dose below which there is no measurable effect.

In aquatic ecosystems, algae are known to be sensitive to many chemicals, and using these organisms in test batteries has been shown to improve the capacity to predict the most sensitive ecosystem responses. The importance of these organisms in the primary production of most aquatic ecosystems justifies their choice for risk assessment. The usefulness of single or multi-species tests to characterize the ecotoxicological properties (EC50, NOEC, LOEC...) of potentially polluting molecules has been proven. Performed in the laboratory under controlled conditions, they meet repeatability, reproducibility, reliability and robustness criteria. However, their use for assessing and predicting effects on natural ecosystems is limited because of their lack of ecological realism. To address this aspect, it is necessary to work at a higher level of biological organization, i.e. with more complex experimental systems.

3.2 Microcosms, mesocosms and macrocosms

All these experimental systems, which differ by their volume (ranging from several mL to several m^3) and their biological complexity (from a few strains to complete trophic networks), are used to study the impact of a toxicant on communities. These systems take into account both positive and negative interactions between organisms and also those with various abiotic factors. They can be used to establish an "exposure-response" relationship, rather than just a "concentration-effect". Because of the relatively large size of these systems, the tests can be conducted with long exposure times, which makes them more suitable for studying the effects of contaminants on the dynamics of species succession, and on ecological processes, such as the cycling of matter and energy flows (Guckert, 1993). These systems can also be used to evaluate the impact on diversity and functioning of the intensity of the contamination (concentration and form of contaminants) and the exposure dynamics (duration, frequency), whilst simultaneously investigating the fate and the effects of pollutants (Belanger et al., 2000). These methods can also be used to identify the temporary, progressive or persistent effects of contaminants on communities (Rand et al., 2000; Belanger et al., 2002), by measuring their resilience, and also to distinguish between the direct and indirect ecological effects of disturbances caused by contaminants (Belanger et al., 2000; Culp et al., 2000; Hense et al., 2003).

Just to provide recent examples of the variety of systems used, 3L liter Pyrex Erlenmeyer flasks have been used to study the response of microbial communities following exposure to glyphosate (Pesce et al., 2009a), indoor experimental channels have been used to study the combined effect of physical factors and exposure to diuron on benthic microbial communities (Villeneuve, 2008), and artificial outdoor mesocosms (surface area 25 m^2) have been used by Vera et al. (2010) to evaluate the impact of Roundup on periphyton communities.

To conclude, all these systems can be defined as essential intermediates between single-species tests and *in situ* studies (Caquet et al., 2000) in exploring the effects of toxicants and their potential interaction with environmental factors.

3.3 Field studies to evaluate the impact of contaminants on microbial communities

Numerous studies have attempted to evaluate the impact of herbicide contamination on microbial communities in freshwater ecosystems (see, for example, the reviews of DeLorenzo et al., 2001; Ricciardi et al., 2009). From these studies, it appears that one of the difficulties that arises is that microbial communities are subjected simultaneously to herbicides and to many other environmental factors and processes (see §5.3). In order to evaluate the direct impact of herbicides on microbial communities, Blank et al. (1988) have developed a very interesting concept, known as PICT (Pollution Induced Community Tolerance). This concept is based on the fact that communities contain species with differing sensitivities to various chemicals (see for example for diatoms, Debenest et al., 2009). After exposure to these pollutants for long enough, sensitive species or strains will be eliminated and tolerant ones will be selected, and the resulting restructured community will become more tolerant. Increased tolerance in a community can therefore be regarded as indicative of a direct impact of a pollutant on this community (for example for microalgal communities, see Bérard et al., 2003; Schmitt-Jansen & Altenburger, 2005). This pollution-induced community tolerance can be estimated by means of various different metabolic tests.

4. Molecular methods used to evaluate the impact of herbicides on microbial communities

Over the past 15 years, molecular tools have been increasingly used in ecotoxicological studies, mainly to assess the impact of pesticides on the structure and composition of microbial communities. Two approaches can be distinguished (Dorigo et al., 2005). The first group of tools uses an initial PCR to target one or several specific genes in the microbial communities, whereas the second group consists of non-PCR-approaches.

4.1 Methods based on an initial PCR amplification

The Polymerase Chain Reaction (PCR) is used to amplify target sequences in microbial communities. Depending on the choice of the primers, these sequences may be more or less specific, i.e. they target different taxonomic levels, ranging from a single species to the entire prokaryotic community. In most cases, rRNA genes, in particular 16S (prokaryotes) and 18S (eukaryotes) rRNA, are targeted, because they contain highly-conserved domains interspersed with more variable regions (Gutell et al., 1994). However other genes can also be used, for example those linked to a particular function, such as a herbicide degradation gene (Lee et al., 2005; de Lipthay et al., 2002).

After an initial PCR amplification, two main kinds of method are used to characterize the amplified sequences. The first is based on sequencing these amplicons and identifying the sequences obtained, for example by comparison with sequences available in databases (GenBank™). New sequencing technologies (454 sequencing) now make direct sequencing of the amplicons after PCR possible, but so far, sequencing is always preceded by a cloning step. Amplicon sequencing is used to compare the composition of different communities, for example with more or less exposure to a herbicide, and it provides a species identification

when rRNA genes are used, as has been successfully done in several ecotoxicological studies of herbicides (e.g. Dorigo et al., 2002).

The second kind of methods used after PCR amplification are known as fingerprinting methods, because they provide an overview of a microbial community (like a bar code), making it easy to compare several communities. These methods include SSCP (Single Strand Conformation Polymorphism), ARISA (Automated Ribosomal Intergenic Spacer Analysis), RAPD (Random Amplified Polymorphic DNA), T-RFLP (Terminal Fragment Length Polymorphism), and AFLP (Amplified fragment-Length Polymorphism), but DGGE (Denaturing Gradient Gel Electrophoresis) and TGGE (Temperature Gradient Gel Electrophoresis) are the ones that have been most frequently used in ecotoxicological studies involving microbial communities. These two methods were first introduced 15 years ago (Muyzer, 1999). They are based on the separation of the amplified fragments of DNA on a linear gradient of increasing chemical denaturants of urea and formamide (DGGE), or on a linear temperature gradient (TGGE). After the migration, a band pattern will characterize each amplified sample, and is used to compare them. Moreover, individual bands can be excised, reamplified and sequenced, and thus identified (Riemann and Winding, 2001). DGGE/TGGE approaches have been used in a huge number of studies to characterize the impact of herbicides on microbial communities (e.g. Lyautey et al., 2003; Schauer et al., 2003; Pesce et al., 2009a; Tadonléké et al., 2009). These methods provide a quick way of comparing several samples, but they have their limitations. In particular, it is very difficult to compare large numbers of complex band patterns across several migration gels (Dorigo et al. 2005). Other limitations are due to the fact that different DNA fragments often co-migrate, which limits the sensitivity of these methods. Finally, only dominant species are detected well, which makes it difficult to highlight changes in the presence/absence of rare species.

4.2 Non-PCR based molecular methods

These methods have so far been used less often than those based on the PCR, but the rapid development of "omic" approaches in microbial ecology suggests that they will probably soon be used in ecotoxicology.

In recent years, a few papers have been published reporting the use of the FISH (Fluorescence in situ hybridization) technique, which is based on the use of fluorescent probes to quantify different phylogenetic groups by fluorescence microscopy. This method was used, for example, by Pesce et al. (2006), in a study of the effect of the phenylurea herbicide diuron on river microbial communities. However, this method can also be used for the *in-situ* detection of bacterial strains that are able to degrade herbicides, as for example by Grenni et al. (2009).

The DNA reassociation kinetics method has been also used to compare the genetic diversity of bacterial communities in more or less polluted soils (Gans et al., 2005), but not so far applied to freshwater ecosystems. This method is unlikely to be used in the near future in ecotoxicological studies in natural environments, due to the technical difficulties of applying this method in this context, and also due to the difficulty of analyzing the results.

The use of microarrays is also a very promising method developed in the past. This method is based on the use of slides (microarrays) containing a very large number of microscopic spots of DNA oligonucleotides probes (from several hundred to several thousand). These microarrays can be used to detect and quantify the species in a community after extracting

the DNA and hybridizing it on the microarray. However, they are usually used to study the gene expression of a strain under various stressful conditions and in mixed microbial communities (Dennis et al., 2003). This method has also been used by Oldenburg et al. (2003) to monitor the diversity of the genes involved in the nitrogen cycle. However, the development of high throughput sequencing methods and the concomitant decrease in their cost will make probably the use of microarrays obsolete within a few years

4.3 Promising new molecular tools for ecotoxicological studies

All the methods described above have been in use for more than a decade, but there are many more recently developed methods, which look very promising for studying the impact of herbicides on freshwater microbial communities, and the responses of these communities.

Real Time PCR (RT PCR or qPCR) is not really a recent method, but its routine use in the laboratory has dramatically increased in the last year. This method can be used to determine the number of copies of target genes or the expression of target genes in a sample. For example, qPCR has been recently used by Bopp & Lettieri (2007) to study gene regulation in a marine diatom exposed to polycyclic aromatic hydrocarbons. Another example is the work of Gonod et al. (2006), who used qPCR to study the ability of bacterial communities in soil to degrade the 2,4-D (2,4-dichlorophenoxyacetic acid) herbicide. Although so far there have been no publications involving the use of this method in freshwater environments, it will probably soon be widely used in ecotoxicological studies of microbial communities.

So far, there has also been no paper reporting the use of isotope-labeling experiments (DNA-SIP, RNA-SIP…) to study, for example, the bacterial degradation of herbicides in aquatic environments. However in recent review, Neufeld et al. (2007) disclaimed that isotope-labeling experiments (DNA-SIP, RNA-SIP…) are coming of age, and are likely to be very useful for studying the degradation of pollutants by microbial communities. A paper by Park et al. (2006) also highlighted the potential of Stable Isotope Probing for enhancing the resolution of ecotoxicological assessment.

Finally, the introduction of "omic" methods opens a new area in the assessment of the impact of pesticides on microbial communities. The use of metagenomic, metatranscriptomic and metaproteomic methods in recent years has shown that environmental microbial ecology is already entering the "Omics" era (see for example Nelson et al., 2006; Vandenkoornhuyse et al., 2010). Despite some restrictions, there is no doubt that all these methods will soon be major tools for ecotoxicological studies.

5. Impact of herbicides on freshwater microbial communities

The impact of herbicide on freshwater microbial communities depends on a large variety of factors linked to the pollutant chemical, its mechanism of action, and the time and the level of exposure… However, the impact is also modulated by interactions with physical (flow, temperature…), chemical (nutrients, organic matter, salinity…), and biological factors and processes (the presence of grazers…).

The impact of herbicides on microbial communities can be estimated using structural and functional descriptors, which provide information about the short- and long-term effects of these compounds (Fig. 2).
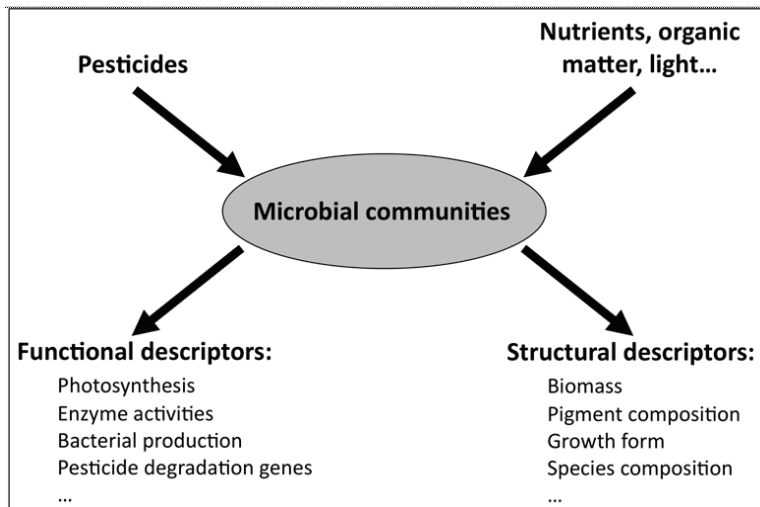
Fig. 2. Flow chart of the causes and effects of chemical and physical disturbances on microbial communities (adapted from Sabater et al., 2007)

5.1 Impact of herbicides on the composition and structure of freshwater microbial communities

Many authors have highlighted the fact that herbicides have significant effects on the composition and structure of freshwater microbial communities, by estimating the species richness and the diversity of autotrophic and heterotrophic microorganisms, and also by estimating their biomass and abundance.

Herbicides can have a direct impact on the composition of freshwater microbial communities by selecting those with greater herbicide resistance (Dahl & Blanck, 1996; Pérès et al., 1996; DeLorenzo et al., 1999). For example, Dorigo et al. (2007) found that there were changes in the composition of microbial communities in a small river displaying an upstream-downstream pollution gradient, and that species located in the polluted downstream area were more tolerant towards herbicides than those located in the upstream-unpolluted area. Recently, Vera et al. (2010) have shown that diatoms were more susceptible than cyanobacteria to glyphosate in periphyton communities, and consequently that over the long term a shift occurred in the composition of these communities. In the same way, Lürling & Roessink (2006) showed, by an experimental approach, that *Scenedesmus* (green algae) out competed *Microcystis* (Cyanobacteria) in the absence of metribuzin, a photosynthesis-inhibiting herbicide, whereas the reverse was true in the presence of this herbicide. However, herbicides can also have an indirect impact on the species composition of these communities by modifying the equilibria between species and also the interactions between them (as a result of effects on potential competitors or predators).

In addition to photosynthetic microorganisms, which are directly impacted by herbicides because they share some physiological metabolisms with terrestrial plants, these compounds can also have direct impacts on bacteria. For example, in a study of Foley et al. (2008), DGGE fingerprinting showed that freshwater bacterial communities exposed to acetochlor, an inhibitor of cell growth and division, displayed reduced diversity. However,

contradictory results have been obtained, and, for example Tadonleke et al. (2009) did not find any significant impact of diuron, a phenylurea herbicide, on bacteria, whereas previous studies had seemed to do so.

In addition to their effects on the composition of microbial communities, herbicides can also affect the viability and abundance of microorganisms and some other cellular parameters. For example, Ricart et al. (2009) found that there was a reduction of the biovolume of diatoms at low concentrations of diuron.

Herbicide exposure usually has a harmful effect, but some microalgae bioassays of various compounds have also revealed beneficial effects (Haglund, 1997; Franqueira et al., 1999; Wong, 2000; Rioboo et al., 2002; Yoshida et al., 2003). Stimulation of microalgal growth was, for example, found at the lowest paraquat concentrations tested in the study of Prado et al. (2009). Some papers have suggested that the growth stimulation observed in the presence of herbicides, such as glyphosate, may result from the use of degradation products of this compounds as sources of carbon, phosphorus or nitrogen (Wong, 2000). In the same way, bacterial species that are able to degrade herbicides are also stimulated by their presence, thus enabling them to become dominant in bacterial communities (Macur et al., 2007).

5.2 Impact of herbicides on the functional aspects of freshwater microbial communities

In addition to changes occurring in the composition and structure of microbial communities, their metabolic activities (respiration, photosynthesis, enzyme activities) can also be modified by herbicide application. These modifications may or may not be linked to changes in the composition of the community.

Ricart et al. (2009) observed a marked decrease in the photosynthetic efficiency of biofilms after a long–term exposure to low concentrations of diuron. There was also an increase in the proportion of dead bacteria and in the leucine-aminopeptidase bacterial activity with increasing concentrations of diuron. This increase in peptidase activity may be explained by the release of algal material as a result of cell lysis, which might provide bacteria with organic proteinaceous compounds. Similar results have been observed for other photosystem II inhibitor herbicides in freshwater microbial communities (Guash et al., 1997, Pesce et al., 2009; Villeneuve, 2008).

The microbial community could also acclimate to the new conditions by the induction of enzyme activity, i.e. gene expression. For example by Lipthay et al. (2002) observed the induction of *tfdA* transcription in aquatic microbial communities during acclimation to 2,4-D herbicide acid (a phenoxy herbicide, which affects protein synthesis and cell division). The *tfdA* gene is known to encode a 2,4-D/2-oxoglutarate dioxygenase involved in the degradation of 2,4-D to dichlorophenol.

Method dealing with changes in the composition and structure of microbial communities, and in some of their physiological capacities when they are exposed to herbicide pollution, provide complementary information. Indeed, an impact on some specific functions may not necessarily affect the structure of the community and *vice versa*. For example, Widenfalk et al. (2008) showed that no effect of glyphosate could be detected in microbial communities from sediments exposed to this herbicide using descriptors such as bacterial production or microbial biomass, but that changes in the composition of the bacterial community could be detected using T-RFLP.

5.3 Interactions between environmental selective pressures and herbicide contamination

Many studies have reported the effects of contaminants on natural freshwater microbial communities, but in all these studies, the main difficulty was distinguishing between the impact of contaminants and that of other environmental factors and processes. It is well known, for example, that the response of microbial communities to herbicides depends on the composition of the microbial community, which is itself influenced by (i) physico-chemical and other factors such as nutrient resource availability (DeLorenzo et al., 2001; Shabana et al., 2001), temperature (Bérard et al., 1998), light history (Guasch & Sabater, 1998), current velocities for periphyton communities (Villeneuve, 2008) and previous pollutant exposures (Dorigo et al., 2004), and (ii) biological interactions between microorganisms and biotic factors, such as predation by grazers (Muñoz et al., 2001). All these factors introduce seasonal variations in the structure and composition of the microbial communities of freshwater ecosystems, which influence their response to exposure to a contaminant. For example, Guash and Sabater (1998) showed that the differences in algal diversity linked to the light history of a biofilm can lead to differing responses to the presence of herbicides. Seasonal factors or spatial variability can also result in communities displaying differing diversity and differing susceptibilities to contaminants (Dorigo et al., 2009), including their ability to degrade pesticides (Pesce et al., 2009b). In a long-term laboratory experiment to test the effect of atrazine after grazing Muñoz et al. (2001) demonstrated that grazers increased the toxic effect of atrazine on river biofilms. In fact, in a context of heavy grazing, atrazine at 14 µg/L caused a greater decrease in periphyton carbon incorporation than either of these two factors individually. They also showed that grazing hindered adaptation to atrazine exposure. On the other hand, no significant interactions were found between diuron and grazers in a river biofilm community (López-Doval et al., 2010).

6. Special focus on the impact of glyphosate on freshwater microbial communities

Glyphosate (N-(phosphomethyl)glycine), the active ingredient in the commercial product Roundup, is a broad spectrum, non-selective and post-emergent herbicide, developed in 1971 by Monsanto (Franz et al., 1997). Since 1997, its agricultural use has increased considerably as a result of the introduction of genetically-engineered "Roundup Ready" glyphosate tolerant varieties of soybean, cotton and maize (Giesy et al., 2000; Woodburn, 2000). It has become one of the most commonly-used herbicides for agricultural weed control worldwide (Giesy et al., 2000; Kolpin et al., 2006), and also for domestic and industrial weed control in gardens or along rail tracks (Woodburn, 2000; Kolpin et al., 2006; Ghanem et al., 2007; Botta et al., 2009)**.**

6.1 The mechanism of action and different formulations of glyphosate

Glyphosate acts by inhibiting 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS), which is involved in a pathway common to all higher plants as well as to some microorganisms, the shikimate pathway (Amrhein et al., 1980; Carlisle & Trevors, 1988). As a result, it disrupts the biosynthesis of aromatic amino acids required by those organisms, for instance, to synthesize peptides, secondary metabolites or vitamins.

The initial glyphosate molecule is a free acid, and it is converted into a salt form, such as isopropylamine (IPA), monoammonium, diammonium, or potassium salts (in newer formulations), to make it water soluble and thus to able to move into the plant (Duke, 1988; WHO, 1994). There are numerous commercial formulations, most of which are based on the IPA salt (Table 1).

Product	Company	Formulation	
		g AE/L	Salt
Touchdown HiTech	Syngenta	500	Potassium
Roundup WeatherMax	Monsanto	540	Potassium
Roundup PowerMax	Monsanto	540	Potassium
Durango DMA/Duramax	Dow AgroSciences	/	Dimethylamine
Touchdown Total	Syngenta	500	Potassium
Roundup Ultra Max	Monsanto	450	IPA
Cornerstone	Agriliance	356	IPA
Credit	Nufarm	356	IPA
Credit Extra	Nufarm	360	IPA
Glyfos	Cheminova	360	IPA
Glyfos X-tra	Cheminova	356	IPA
Gly Star Original	Albaugh / AgriStar	356	IPA
Glyphomax	Dow AgroSciences	356	IPA
Glyphomax Plus	Dow AgroSciences	360	IPA
Honcho	Monsanto	360	IPA
Makaze	UAP	360	IPA
Roundup Original	Monsanto	356	IPA
Touchdown IQ	Syngenta	360	Diammonium

Table 1. Some of the commercial formulations of glyphosate. The acid equivalent (AE) is the corresponding weight of glyphosate acid, which is the active ingredient.

Those commercial formulations generally include a surfactant, which enhances the ability of glyphosate to penetrate through the cuticular waxes on target plants. With the exception of Roundup, in which the surfactant is known to be polyethyloxylated tallowamine (POEA), information about the surfactants involved are generally not clearly stated by the manufacturers. Furthermore, several studies have shown that the surfactant molecules also make the commercial product (in particular, Roundup) more toxic than the technical grade compound (salt or free acid forms) (Powell et al., 1991; Diamond & Durkin, 1997; Giesy et al., 2000; Tsui & Chu, 2003).

6.2 Glyphosate in the environment: degradation and occurrence in freshwaters

Glyphosate has long been assumed to be safe for the environment due to its supposed rapid biodegradation by soil microorganisms and/or the fact that it is tightly adsorbed by soil particles (Sprankle et al., 1975; Rueppel et al., 1977; Carlisle & Trevors, 1988; Pipke &

Amrhein, 1988; Kolpin et al., 2006). The main degradation pathway appears to involve splitting the C-N bond to produce aminomethyl phosphonic acid (AMPA) (Rueppel et al., 1977). However, despite being biodegraded in the soil, glyphosate is frequently found in freshwater ecosystems. In these ecosystems, the herbicide is dissipated by the same microbial biodegradation as in the soil, and additionally, through its adsorption onto sediments with the subsequent microbial breakdown of bound residues under anaerobic conditions (Tooby, 1985; Mallat & Barceló, 1998). This degradation generally occurs more slowly in water, because it contains fewer microorganisms than soil (Ghassemi et al., 1981). Glyphosate and its main degradation product AMPA are among the most common pesticides detected in water-pollution monitoring (Scribner et al., 2007). However, although glyphosate is the main source of AMPA, it is not its only source (Trass & Smit, 2003), since phosphonate compounds can also be degraded to form AMPA (Novack, 1997; Skark et al., 1998; Kolpin et al., 2006; Botta et al., 2009).

Most studies report fairly low concentrations in surface freshwater ecosystems in regions where glyphosate use is very high (Table 2). However, a few studies have reported very high glyphosate concentrations, such as 700 or 1700 µg/L in some ecosystems **(**Table 2**)**. These very large differences in concentrations may arise from differences in (i) the proximity of the site of application to water-bodies**,** (ii) terrain and soil types and also (iii) the contribution of overspray (WHO, 1994). In general, these differences suggest the possibility of short-term pulse phenomena**,** which are likely to occur in water contamination as previously found for other herbicides (e.g. Wauchope, 1978; Shipitalo & Owens, 2006; Gilliom, 2007).

6.3 Impact of glyphosate on aquatic microorganisms

There have been numerous studies of the toxicity of glyphosate on freshwater prokaryotic and eukaryotic photosynthetic microorganisms (Table 3), but most of them have been based on the use of isolated strains in laboratory experiments.

As Table 3 shows, a lot of data is available about the toxicity of glyphosate towards the *Chlorophyceae* and *Cyanobacteria,* and to a lesser extent towards diatoms. These data show that widely differing EC_{50} values have been found, depending on the species and formulations involved. For example, the *Chlorophyceae* exhibited EC_{50} values ranging between 2.1 and 1082 mg/L and *Chlorella pyrenoidosa*, EC_{50} values of between 3.5 and 590 mg/L. These wide differences could arise as a result of differences in study design, such as the duration of the experiments, the glyphosate formulation used (e.g. Sáenz et al., 1997), which is not always clearly identified, and the EC_{50} measurements, which may be based on the biomass, the chlorophyll concentration, or on the carbon uptake. In the same way, the *Cyanobacteria,* which are photosynthetic prokaryotes, exhibited a wide range of EC_{50} values varying from 2 to 1183 mg/L. However, despite this variability in EC_{50} values, *Cyanobacteria* in general appear to be less sensitive to glyphosate than eukaryotic photosynthetic microorganisms (Powell et al., 1991). This tolerance of glyphosate could be explained by various mechanisms, notably the ability of *Cyanobacteria* to metabolize phosphonate (Forlani et al., 2008). Little data is available about the toxicity of the main degradation product AMPA. As far as we are aware, the only values reported for its EC_{50} (72 h) are 72.9 mg/L and 79.7 mg/L for the *Chlorophycea* species *Scenedesmus subpicatus* (Trass & Smit, 2003; AFSSA, 2006). These values are consistent with the hypothesis that the toxicity of AMPA is equal to or less than that of glyphosate (Carlisle & Trevors, 1988; Giesy et al., (2000).

	Time period	Glyphosate (µg/L)		AMPA (µg/L)		Reference
		Min	Max	Min	Max	
Canada						
Rivers, streams & wetlands	2004-05	1	41	/	66	Struger et al., 2008
Urban & rural watersheds	2007	0.02	12	0.04	1.3	Byer et al., 2008
USA						
Stream	1987	35	1237	<1.0	10	Monsanto, 1990
Pond	1987	90	1700	2	35	Monsanto, 1990
Streams, rivers, lakes, wetlands & vernal pools	2001-06	0.02	427	0.02	41	Scribner et al., 2007
Stream	2002	0.1	8.7	0.4	3.6	Battaglin et al., 2005
Streams & wastewater effluents	2002	<0.1	2.2	<0.1	3.9	Kolpin et al., 2006
Vernal pools	2005-06	<0.02	328	<0.02	41	Battaglin et al., 2009
Argentina						
Streams	2003-04	100	700	/	/	Peruzzo et al., 2008
France						
NR	1999	/	6.06	/	5.05	Horth & Blackmore, 2009
NR	2000	/	35	/	2.99	Horth & Blackmore, 2009
Streams & rivers	2003-04	2	165	2.1	48.1	IFEN, 2006
River	2003	0.23	0.74	0.17	3.76	Pesce et al., 2008
NR	2004	/	50	/	17	Horth & Blackmore, 2009
Streams & rivers	2005	2.1	17	2.1	18.8	IFEN, 2007
NR	2005	/	9.6	/	30	Horth & Blackmore, 2009
Streams & rivers	2006	2.0	34.0	2.2	27.5	Horth & Blackmore, 2009; IFEN, 2009
Sweden						
NR	2000-08	0.06	13	0.07	4	Horth & Blackmore, 2009

NR = type of water not reported; / = no data.

Table 2. Data available on glyphosate and AMPA concentrations in surface water

	Glyphosate formulation	EC$_{50}$ (mg/L)	European toxicity classification	Reference
Bacillariophyceae				
Nitzschia sp.	IPA salt	<2.8	Toxic to very toxic	Peterson et al., 1994
Cyclotella meneghiana	IPA salt	<2.8	Toxic to very toxic	
Navicula pelliculosa	96.6%	39.9	Harmful	Hughes, 1987b [M]
	Acid	17	Harmful	Office of pesticides programs, 2000; Smyth et al., 1996[M]
		42	Harmful	Smyth et al., 1996[M]
	IPA salt	38.6	Harmful	Office of pesticides programs, 2000
Chlorophyceae				
Scenedesmus obliquus	95%	55.9	Harmful	Ma, 2002
	Knockdown	80	Harmful	Ermis & Demir, 2009
Scenedesmus quadricauda	95%	70.5	Harmful	Ma et al., 2003
	IPA salt	>>2.8		Peterson et al., 1994
		7.2	Toxic	Sáenz & Di Marzio, 2009
		9.02	Toxic	Sáenz et al., 1997
	Rondo	7.2	Toxic	Sáenz et al.1997
	Roundup	120	NC	Sáenz & Di Marzio, 2009
Scenedesmus acutus	97.5%	24.5	Harmful	Vendrell, et al., 2009
	IPA salt	10.2	Harmful	Sáenz, et al., 1997
		10.2	Harmful	Sáenz & Di Marzio, 2009
	Rondo	9.08	Toxic	Sáenz, et al., 1997
Scenedesmus subscapitatus	97.5%	26	Harmful	Vendrell et al., 2009
	IPA salt	72.9	Harmful	Tomlin, 1997
Chlorella saccharophila	97.5%	40.6	Harmful	Vendrell et al., 2009
Chlorella vulgaris	97.5%	41.7	Harmful	Vendrell et al., 2009
	95%	4.7	Toxic	Ma et al., 2002
	IPA salt	13.1	Harmful	Sáenz & Di Marzio, 2009
Chlorella pyrenoidosa	96.7%	590	NC	Maule & Wright, 1984

	Glyphosate formulation	EC$_{50}$ (mg/L)	European toxicity classification	Reference
	95%	3.5	Toxic	Ma & Wang, 2002
		3.5	Toxic	Ma, 2002
	Roundup	189	NC	Hernando et al., 1989
	NR	380	NC	Anton et al., 1993
	NR	1082	NC	Anton et al., 1993
Chlorella fusca	NR	377	NC	Faust et al., 1993
Chlorococcum hypnosporum	96.7%	68	Harmful	Maule & Wright, 1984
Pseudokirchneriella subcapitata [1]	96.6%	12.5	Harmful	Office of pesticide programs, 2000
		13.8	Harmful	Hughes, 1987c [M]
		460	NC	Smyth et al., 1995 [M]
		485	NC	Smyth et al., 1995 [M]
		270	NC	Cedergreen & Streibig, 2005
	95%	21.8	Harmful	Bozeman & Koopman, 1989
		129	NC	Pereira et al., 2009
	IPA salt	24.7	Harmful	Tsui & Chu, 2003
		41	Harmful	Tsui & Chu, 2003
	Roundup	5.8	Toxic	Tsui & Chu, 2003
		2.1	Toxic	LISEC, 1989a [M]
		8	Toxic	LISEC, 1989a [M]
		64.7	Harmful	Cedergreen & Streibig, 2005
	Spasor	71	Harmful	Pereira et al., 2009
	Sting	2.5	Toxic	LISEC, 1989b [M]
Cyanobacteria				
Microcystis aeruginosa	Acid	110	NC	López-Rodas et al., 2007
	NR	169**	NC	Forlani, 2008
Anabaena sp.	NR	338**	NC	Forlani, 2008
Anabaena flos aquae	96.7%	304	NC	Maule & Wright, 1984
	96.6%	11.7	Harmful	Hughes, 1987a [M]
	Acid	15	Harmful	Smyth et al., 1996 [M]
Anabaena varialis	Acid	2	Toxic	Hutber et al., 1979

	Glyphosate formulation	EC$_{50}$ (mg/L)	European toxicity classification	Reference
Aphanocapsa 6308	Acid	2	Toxic	Hutber et al., 1979
Aphanocapsa 6714	Acid	100	Harmful	Hutber et al., 1979
Nostoc sp.	Acid	2	Toxic	Hutber et al., 1979
Nostoc punctiform	NR	1183[*]	NC	Forlani, 2008
Aphanizomenon flos-aquae	IPA salt	<2.85	Toxic to very toxic	Peterson et al., 1994

[1] formerly, *Selenastrum capricornutum;* NR= not reported; >>= considerably more than; NC= no classification for this range; [*] estimation of EC$_{50}$ value based on graph; [M] = unpublished studies performed by the Manufacturers (Monsanto, Zeneca, Malcolm Pirnie)

Table 3. Overview of aquatic microorganism toxicity of various formulations of glyphosate

All these toxicity tests are simple ways of assessing the direct impacts of glyphosate on freshwater microorganisms, but they cannot be used to assess the real impact of this compound on natural populations and communities in freshwater ecosystems. For this reason, many experimental studies have attempted to use complex communities. Two papers by Goldsborough & Brown (1988) and Austin et al. (1991) found that glyphosate had no major impact on periphyton communities. However, Austin et al. (1991) suggested that the degradation of glyphosate could increase the concentration of soluble phosphorus, and consequently increase the biomasses of the communities. Subsequently, Vera et al. (2010) showed that the periphytic colonization of substrata was delayed in Roundup-contaminated large mesocosms, and that this phenomenon could be attributed to a direct effect of the contaminant. In addition they showed that in periphyton communities Roundup produced a long-term shift in the typology and functioning of contaminated mesocosms, which was consistent with data available from natural lakes in the Argentine Pampas. Another recent paper by Pérez et al. (2007) has also demonstrated an impact of Roundup on the structure of the phytoplankton and periphyton communities, and more particularly a decrease in the total micro- and nanophytoplankton and an increase in picocyanobacteria. Moreover, they have shown that these changes are more likely to be due to a direct toxic effect of glyphosate rather than to an indirect effect *via* a phosphorus enrichment of the streams linked to the degradation of this compound.

To conclude, herbicides containing glyphosate seem to have direct toxicological effects on non-target periphyton and phytoplankton communities in freshwater ecosystems, as well as indirect effects via the eutrophication potential of glyphosate degradation. These communities constitute the basis of food webs in these ecosystems, and so it would appear that glyphosate can potentially impact the overall functioning of freshwater ecosystems. This potential impact is reinforced by the fact that this compound is also known to be toxic for fish (e.g. Langiano & Martinez, 2008; Cavalcante et al., 2008), and could be involved in a trophic cascading process (Bengtsson et al., 2004).

7. Conclusions

As we have seen, a lot of data is available about the contamination of freshwater ecosystems by herbicides and also on the direct or indirect impact of these compounds on microbial

communities living in these ecosystems. These compounds appear to affect the structure and composition of these communities, and also the metabolism of the microorganisms involved. It is very difficult to evaluate the consequences of such changes on the whole functioning of freshwater ecosystems, but there can be no doubt that it is significantly affected by herbicide contamination, because microbial communities play a key role in these ecosystems. This impact is probably reinforced by the fact that freshwater ecosystems are simultaneously subjected to other selective pressures. For example, herbicide pollution is generally concomitant with pollution by mineral nutrients (phosphorus and nitrogen), which also influence the structure and the functioning of microbial communities. In the same way, even though only a small number of papers have been published on the topic, we know that climate change is already affecting aquatic microbial communities (e.g. Falkowski & Oliver, 2007; Jöhnk et al., 2008).

The situation concerning herbicide contamination of freshwater ecosystems will change in the next few years, as a result of changes in agricultural practice. In a developed country like France, a drastic reduction of herbicide (50%) use is planned within a short time scale (<10 years). The European community as a whole is also engaged in reducing the use of pesticides in agriculture. In contrast, demographic pressure and Biofuel production in some areas of the world, such as Asia and South America, are leading to increasingly intensive agricultural practices, and consequently to growing herbicide pollution of freshwater ecosystems. These divergent situations will probably result in sharply contrasting situations with regard to the herbicide pollution of aquatic ecosystems in these different regions.

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