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Effects of Atrazine and Nicosulfuron on Phytoplankton in Systems of Increasing Complexity

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Abstract. We have tested the sensitivity of phytoplankton to the herbicides atrazine and nicosulfuron in experiments conduced in increasingly complex systems, from single strain phytoplankton cultures (microplates) to mesocosms mimicking whole ecosystems. The endpoints used to assess sensitivity to atrazine and nicosulfuron were total biomass increase, photosynthetic efficiency, and community diversity, depending on the system considered. Nicosulfuron appeared to be very much less toxic to phytoplankton than atrazine, in accord with the planned changes in agricultural practices to reduce the effects of surface water contamination on aquatic biota. Nevertheless, nicosulfuron had significant effects in some systems (principally microcosms), whereas the single monocultures were almost insensitive to it. This points out the inaccuracy of standardized toxicity test on phytoplanktonic algae alone for predicting the effects of xenobiotics on natural communities and the need for tests in microcosms and mesocosms to obtain reliable evidence about the toxicity of a given chemical on freshwater aquatic ecosystems.

Intensive crop production has used more and more herbicides over the past several decades. Increased production crops, such as cereals, involves massive herbicide consumption that leads to the significant contamination of surface water. Some uses of atrazine can result in triazine content of such water exceeding 10 μg/L (Caux and Kent 1995; Solomon *et al.* 1996). As atrazine (chloro-2 ethylamino-4 isopropylamino-6 triazine-1,3,5) inhibits the photosystem II, it may damage nontarget aquatic primary producers (phytoplankton, periphyton, and macrophytes). Microalgae are potential targets of herbicides in aquatic ecosystems. Herbicides can alter the ecophysiology and algal population dynamics, so that the most sensitive species are killed, allowing more tolerant species to develop; contamination may thus cause a shift in community structure (Kasai *et al.* 1993). Atrazine may indirectly influence the whole trophic food web of aquatic ecosystems, with damage to phytoplankton, periphyton, and macrophytes that may reduce the capacity of the aquatic habitat to sustain invertebrates and fish (Solomon *et al.* 1996).

An agrienvironmental program was launched in France in 1993. This program promoted environmentally friendly agricultural practices (Dabène and Larguier 1994). One of these measures involves replacing atrazine with nicosulfuron [2-(4,6-dimethoxypyrimidin-2-ylcarbamoylsulphanoyl)-N,N-dimethyl nicotinamide], which is considered to be less harmful. This sulfonylurea herbicide prevents the synthesis of ILE, LEU, and VAL by inhibiting the enzyme ALS (Simpson *et al.* 1995). Unfortunately, there are few published data on the ecotoxicological effects of this compound. It is thus necessary to detect and compare the effects of atrazine and nicosulfuron on the aquatic biota in order to assess the contamination risk produced by changes in agricultural practices. This can be done in several ways. The most widely used experiments are monospecific toxicity tests because of their low cost, satisfactory reproducibility, and ease of execution. They are often standardized (*e.g.*, AFNOR 1998; EPA 1989). Though they are essential for understanding how toxic compounds act, they cannot yield reliable risk assessment (Pratt *et al.* 1988; Bérard and Pelte 1999). The most complex experiments involve aquatic mesocosms that have structural and functional characteristics similar to the ecosystems they are intended to model. The bioavailability and toxicity of pesticides can be assessed using a representative set of natural aquatic ecosystems (Crossland 1994; Touart 1994), and environmental realism is the main reason for using mesocosm instead of less complex experiments (Kraufvelin 1998). However, increased ecological realism often implies less accurate measurements and statistical analyses (Giesy and Allred 1985). This method is also costly and hard to implement. Indoor and outdoor microcosms, in which natural communities are isolated from the whole ecosystem, are intermediate between monospecific tests and a holistic approach (mesocosm). Toxicity can be assessed in a multispecies community, but not necessarily on an entire trophic web. These three experimental systems are thus complementary, and using them all provides a better understanding of the effects of toxic chemicals on aquatic species and their environment.

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We have performed a set of experiments to measure and compare the effects of atrazine and nicosulfuron on phytoplankton. The experiments were conducted with three experimental systems of increasing complexity (monospecific tests, indoor and outdoor microcosms, mesocosms). These allowed us not only to test the herbicide effects of these compounds but also to compare the relative advantages of increasing the scale and complexity of the experimental systems.

Materials and Methods

Single-Species Tests

Several species were cultivated in 96-well clear polystyrene microplates under 12:12 light:dark cycles (approx. 100–150 $\mu E/m^2/s$ light intensity at the lid). Growth was monitored daily by measuring OD_{650} with a Dynex MRX CE97 (Labsystems, Finland) spectrophotometer. Inoculates were from batch flask cultures in the exponential growth phase, diluted to give a final cell density of approximately 10^6 cells/ml, allowing the cultures to grow exponentially in the microplate for at least 1 week from the start of the experiment. The test solutions of atrazine (98%, Greyhound Service, UK) and nicosulfuron (95%, ISK Biosciences, UK) were prepared in pure acetone (final acetone concentration in the contaminated wells and controls 0.05%). A set of 60 wells contained a series of 10 concentrations of atrazine or nicosulfuron (0–256 μg/L for atrazine and 0−256 mg/L for nicosulfuron; six replicates per concentration). The cell density after 96 h of growth was used to calculate the EC50. Theoretical sigmoid dose-response curves were fitted to the data using the least-square method and the Solver function of Excel 98 software. Equation 1 was modified from Streibig *et al.* (1998):

$$OD_{mod} = OD_{con} + \left[\frac{OD_{min} - OD_{con}}{(1 + e_{bx(\log[\text{tox}] - \log \text{EC50}))}}\right] \quad (1)$$

where OD_{mod}, OD_{con}, and OD_{min} are the optical densities at 650 nm for the model, the control (no toxic), and the minimal value measured after 96 h of growth, respectively; [tox] is the toxic concentration, EC50 the theoretical toxic concentration reducing the total biomass by 50 %, and *b* is the slope of the modeled curve.

Indoor Microcosms

A set of 23 500-mL glass bottles were filled with 500 mL natural phytoplankton collected from the light-saturated layer of the Lake Geneva in December 1999. The systems were placed in controlled illumination (12:12 light:dark cycle, 200 $\mu E/m^2/s$) and temperature (12°C), corresponding to the environmental conditions in the upper water layer of the lake in early spring. Grazers and larger particles were removed by passing the water through a 250-μm net, and nitrogen was bubbled to kill the remaining heterotrophs. Samples were enriched with soluble P, N, and Si to obtain a nutrient medium with a composition similar to the natural spring water of the euphotic layer of the lake (soluble reactive phosphorus: 0.02 mg P/L; inorganic nitrogen: 0.60 mg N/L; silicates: 1.3 mg SiO_2/L). A precise description of the apparatus and protocol is given in Bérard (1996). The bottles were sampled at the beginning of the experiment to identify and count the cells. Herbicide was then added: one set of 18 bottles contained a series of nine concentrations of atrazine and nicosulfuron (0.1–100 μg/L atrazine and 0.1–100 mg/L nicosulfuron), and the remaining 5 bottles were kept as controls with only solvent blank. Total phytoplankton growth was monitored daily by measuring fluorescence *in vivo*, and the final composition (taxonomy and chl *a*) was determined at the end of the experiment.

Outdoor Microcosms

Six outdoor microcosm experiments with natural phytoplankton communities were carried out during the spring and early and late summer of 1997 and 1998. The sampling site was located in the center of Lake Geneva. Corvi *et al.* (1996) and Blanc *et al.* (1998) found atrazine concentrations of 0.03–0.04 μg/L in the euphotic layer of the whole lake, indicating a low, stable contamination with this herbicide during the 1995–1997 period. The nicosulfuron in the lake was not measured due to the lack of analytical method, but it is assumed to be very low.

A set of 15 5-L bottles were filled with 2.5 L of filtered (0.8 μm) lake water (as nutrient stock) and 2.5 L of natural phytoplankton sample and placed in outdoor natural variations in light and temperature at 1 m below the surface of the lake near the shore. Samples were treated as described for indoor microcosms. A precise description of the apparatus and protocol is given in Bérard *et al.* (1999a). The bottles were sampled at the beginning of the experiment to measure nutrients and chlorophyll *a*, cell types, and counts (Table 1; Figure 1). Herbicide was then added. One set of five bottles contained 10 μg/L atrazine, a second contained 10 μg/L nicosulfuron, and the remaining bottles were kept as control solvent blanks. Total phytoplankton growth was monitored daily by measuring fluorescence *in vivo*, and the final composition (taxonomy and biomass) was determined at the end of each experiment.

Mesocosms

Experimental Units: The experiment was carried out during a 2-month period, using 15 large outdoor circular tanks at the ENSAR campus (Ecole Nationale Supérieure d'Agronomie de Rennes, France). Each tank was 3.2 m in diameter, 1.2 m high, and held approximately 5,000 L. A crane and the funnel-shaped base of the mesocosm were used to drain the system. The tanks were sunk about 0.4 m into the ground to obtain stable temperature during the experiment (Neugebaur *et al.* 1990). The mesocosm physicochemical parameters are listed in Table 2.

The experimental units were first filled with 5 cm of natural sediment taken from an uncontaminated local pond. They were then filled with a mixture of tap and pond water from a nearby site to a depth of 0.7 m to stock them with phytoplankton and zooplankton. Aquatic macrophytes (*Glyceria maxima*) were introduced to obtain a structure similar to that of still water fish spawning areas (Bry 1996). Differences between mesocosms produced by the macrophyte covering rate were minimized by planting the same number of *Glyceria* in two concentric circles in each mesocosm.

Contamination: The mesocosms were left to develop for 3 weeks before being contaminated with atrazine and nicosulfuron (0, 2, and 30 μg/L for control, low, and high contamination, for each compound). Both herbicides where there dissolved in acetonitrile, which was proven to be less toxic than acetone for fish. Three mesocosms were used for each concentration, and the remaining three were kept as controls with only the solvent. The concentrations used (2 and 30 μg/L) are those found chronically or after application in superficial water in western France (Giovanni and Haury 1995).

Table 1. Biomass and diversity of the inocula used in the seven outdoor microcosms experiments, Nutrient concentrations in the natural water used as culture medium for each microcosms experiment, mean temperatures and average surface irradiance of microcosms cultures, and duration of each microcosms experiment

	June 97	Aug. 97	April 98	May 98	June 98	July 98	Sept. 98
Chlorophyll *a* (μg/L)	1.15	2.05	1.75	3.23	1.57	2.01	6.83
(H′: bits/individual)	2.20	1.80	1.40	1.14	2.11	2.94	1.90
SRP (mg P/L)	0.003	0.001	0.018	0.003	0.001	0.001	0.000
DIN (mg N/L)	0.38	0.207	0.55	0.42	0.45	0.037	0.156
Silica (mg SiO_2/L)	0.70	0.1	1.08	0.12	0.28	0.54	0.14
Mean temperature	19.3	23.9	9.3	14.2	17.5	22.0	17.4
Minimum	18.1	21.2	4.4	10.8	15.2	18.8	15.3
Maximum (°C)	20.5	25.2	12.4	20.7	20.6	24.1	20.7
Surface irradiance	1,038	883	616	1,242	866	751	546
min.	466	305	70	863	254	107	223
max. ($\mu mol\ m^{-2}\ s^{-1}$)	1,485	1,149	892	1,505	1,456	1,274	906
Duration	11	14	10	21	13	16	16

H′ Shannonn diversity index. SRP, soluble reactive phosphorus

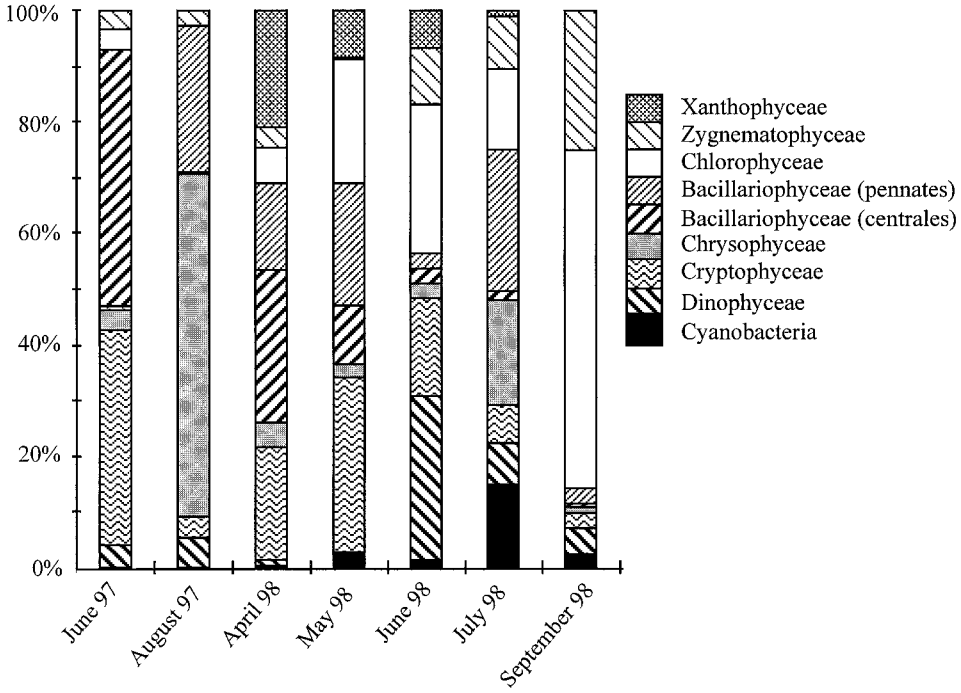

Fig. 1. Class-level taxonomic composition of algal communities in microcosms at the beginning of each outdoor experiment, expressed in % of the total phytoplankton identified

Sampling and Measurements: Phytoplankton was sampled with a home-made sampler (Jüttner *et al.* 1995). Samples were taken from several different points in each experimental unit and pooled (Kennedy *et al.* 1995). One liter was filtered through a Whatman GF/C filter for chlorophyll *a* determination. Chl *a* was extracted by incubation for 24 h at 4°C in 90% acetone and was measured spectrophotometrically according to Jeffrey and Humphrey (1975).

The effects of herbicides on photosynthetic efficiency were assessed in three mesocosms (control, atrazine 30 μg/L, and nicosulfuron 30 μg/L) by the method of Lewis and Smith (1980) using ^{14}C incorporation, α, β, and Pmax were calculated by counting radioactivity during experiments performed prior to and immediately after contamination and on day 11.

Species Diversity, Statistics, and Calculations

Samples were taken at the beginning, during, and at the end of the microcosm and mesocosm experiments. A 50-ml aliquot of algal suspension was removed from each experimental system, and lugol was added to kill and stain the cells. Cells were allowed to settle for 24 h, after which they were examined under a reverse phase microscope, identified, and enumerated. The abundance of the dominant species was estimated (± 10%) (Lünd *et al.* 1958). The density of each dominant species (> 100 individuals/ml) in the contaminated system was compared to the control, using the Mann-Whitney nonparametric test (Schwartz 1963). The differences between contaminated communities and the control were described using the Bray-Curtis dissimilarity index (Bray and Curtis 1957; Dalh and Blanck 1996). The

Table 2. Main water nutrient concentrations and temperature in the mesocosms

Orthophosphate (mg P/L)	0.0065
Inorganic nitrogen (mg N/L)	0.51
Mean temperature (°C)	18
minimum	14.4
maximum	24.8

Bray-Curtis index (BCI) was calculated for the mean density of replicate communities, and the significance of the difference between the control and treated samples was assessed (Mann-Whitney) by comparing the BCI for the controls (C-C) and the BCI between controls and tests (C-A and C-N) (Bérard *et al.* 1999a). A correspondence analysis with the ADE program (Thioulouse *et al.* 1997) between contaminated and control (density of five replicate community) was done at the end of the indoor microcosm experiment (the communities were described in the data matrix by the total density of the dominant species; Bérard *et al.* 1999a).

Results

Microplate Assays

Data from microplate growth assays for each strain are summarized in Table 3. EC50s were calculated using best-fitting for the equation given in Methods, whereas LOECs (lowest observable effect concentrations) were determined using Toxstat software, as the first experimental concentration giving an OD significantly different from the control (Dunnett's t-test). The wide range of EC50s for atrazine is similar to those reported elsewhere (Bérard and Pelte 1999); the diatoms were less sensitive than the chlorophytes, and the minimal and maximal calculated EC50s were 4.3 μg/L for *Chlorella vulgaris* (strain isolated from Lake Geneva) and 412 μg/L for *Nitszchia* sp. from the same location.

The toxicity of nicosulfuron in microplate assays was very low; the curves showed no evident effect or only LOEC (calculated as for atrazine assay after data normality and homogeneity test). EC50s were not attained in the experimental conditions, and the modeled data gave results much higher than the maximal solubility of the compound in water. Two diatoms and two chlorophytes were sparingly sensitive to nicosulfuron, whereas the two strains of *C. vulgaris* and the diatom *N. accomoda* were totally insensitive.

Indoor Microcosms

The microcosms contaminated with 50 μg/L atrazine 50 μg/L nicosulfuron (A8 and N8) were lost during the experiment. Neither contaminant showed any concentration effect with the *in vivo* fluorescence and chl *a* parameters at the end of experiment (data not shown). Anyway, the specific composition of the microcosms illustrated by the correspondence analysis (Fig. 2) showed an effect of atrazine concentration on the community structure with the development of chrysophytes, cryptophytes, then diatoms (axis 1 and 2). In contrast, the nicosulfuron-treated microcosm communities contained chlorophytes, like the communities in the control microcosms.

Outdoor Microcosms

The changes in phytoplankton biomass in the microcosms during the seven outdoor experiments were monitored by *in vivo* fluorescence. Though there were no great differences between treatments, the chlorophyll biomasses in the contaminated microcosms were significantly different during some experiments; the biomasses were sometimes higher or lower in the contaminated microcosms, depending on the experiment (Table 4).

Effect of Herbicides on Community Structure: The Bray-Curtis similarity index, used to compare the phytoplankton communities of the atrazine- and nicosulfuron-treated (10 μg/L) and control outdoor microcosms (Table 4), was lower in the atrazine-treated microcosms than in the nicosulfuron-treated microcosms ($p = 0.1$; $n = 6$). The BCI and the significance of difference between the control and treated (*) samples varied from experiment to experiment.

Effect of Herbicides on Species: Atrazine and nicosulfuron (10 μg/L) significantly affected the changes in the density of some species in each outdoor microcosm experiment. The Mann-Whitney tests for the seven experiments (Figure 3a) indicated that atrazine inhibited more algae than nicosulfuron. The Mann-Whitney tests also showed that nicosulfuron tended to stimulate the chlorophytes, while atrazine tended to stimulate the pennate diatoms (chlorophytes and pennate diatoms, Figure 3b).

Mesocosms

The gross phytoplankton biomass was generally low (maximum: 4.5 μg/L after seventeen days), with no statistically difference between the treated and control tanks (Figure 4).

There was a species succession during the experiment in all tanks (Figure 5). The phytoplankton community was dominated by the chlorophyte *C. vulgaris* for the first 10 days, while the diatom density was about 0.5% of the community. Then *C. vulgaris* slowly decreased to be replaced by the cryptophytes *Rhodomonas minuta* and *Cryptomonas ovata*, with an increase in total chl *a* (Figure 4). The relative abundance of *R. minuta* was high, and that of the chrysophyte *Ochromonas* sp. was increasing at the end of the experiment. The succession in all the tanks was similar to that of the controls, with some differences in time lags and proportions.

Effect of Herbicides on Community Structure: Bray-Curtis similarity indices were used to compare the structures of the phytoplankton communities in the control and contaminated mesocosms, as for microcosms. The indices were generally smaller for higher concentrations, indicating a dose effect, especially for atrazine. The indices were generally lower for atrazine during the second phase of succession (after day 10) than for nicosulfuron (Figure 6).

Table 3. Calculated EC50 and LOECs for atrazine and nicosulfuron from 96-h microplate assays for each strain tested

Herbicide Class	Strain	Atrazine (μg/L)		Nicosulfuron (mg/L)	
		EC50	LOEC	EC50	LOEC
Chlorophyceae	*C. vulgaris* Léman	4.3	3	NEO	NEO
	C. vulgaris Villaumur	172	128	ND	256
	P. subcapitata	118	64	ND	256
	S. acutus	45	32	ND	2
	A. formosa	261	10	ND	1
Bacillariophyceae	*N. accomoda*	164	50	NEO	NEO
	Nitszchia sp.	412	50	ND	62

NEO: no effect observed. ND: EC50 not determined, calculated concentrations far higher than solubility limits of nicosulfuron, given by the calculated dose-responses curve

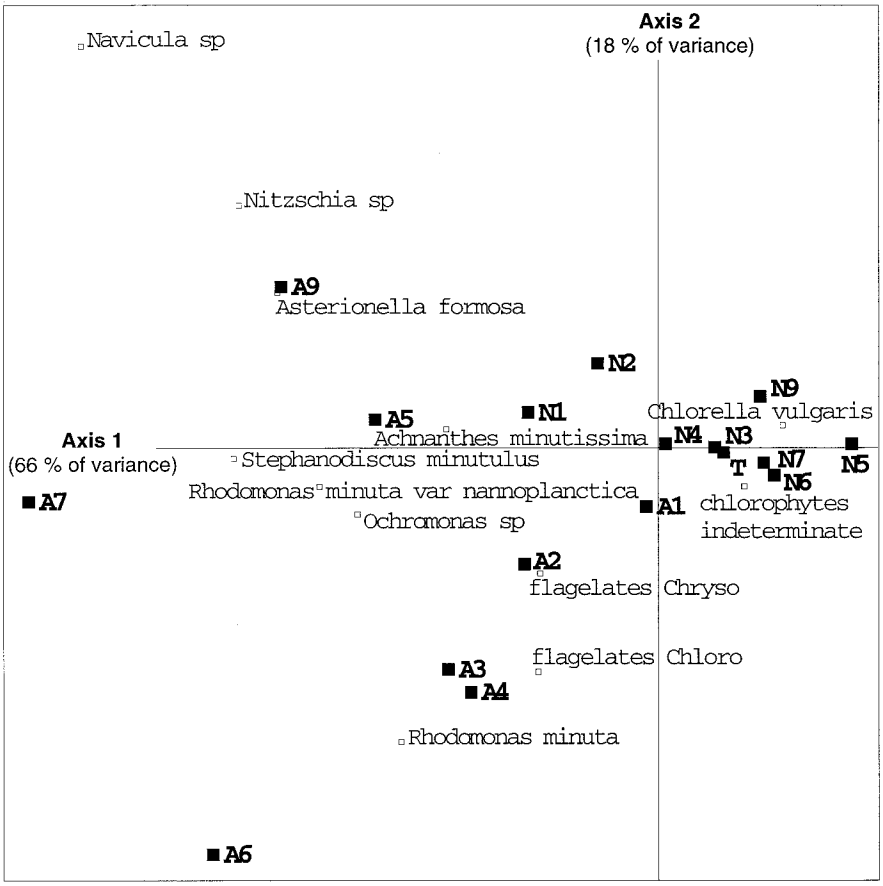

Fig. 2. Correspondence analysis of phytoplanktonic communities at the end of the indoor microcosms experiment (projection in the plane defined by axes 1 and 2). Ai: microcosms contaminated with an increasing concentration series of seven levels of atrazine (A1: 0.1 μg/L; A9: 100 μg/L). Ni: microcosms contaminated with an increasing concentration series of seven levels of nicosulfuron (N1: 0.1 μg/L; N9: 100 μg/L)

Effect of Herbicides on Species: Atrazine and nicosulfuron (2 and 30 μg/L) significantly affected the changes in density of the major taxonomic groups species (Table 5). Those effects were time-dependent. Diatoms were stimulated by a high dose of atrazine only 2 and 10 days after contamination, whereas the chlorophytes were sensitive to atrazine and nicosulfuron on days 17 and 23. Cryptophytes were stimulated by the higher atrazine (day 10) and nicosulfuron (day 27) concentrations. The chrysophyte densities were increased at the end of the experiment in all contaminated mesocosms.

P/I Curves: The parameters for each P/I curve obtained are listed in Table 6. Before contamination the P/I curve had high α and Pm and a slight photoinhibition at irradiances up to 800 $\mu E/m^2s$. There was a difference between the three treatments only on day 1; the lowest Pm (292 μmol C/mg chl *a*/h) was in the atrazine-contaminated mesocosm, and the higher photosynthetic efficiency (for the three parameters of the curves) was in the control mesocosm.

Discussion

Global Effects of Atrazine and Nicosulfuron on Algae

The sensitivity of algae to atrazine varies very widely (close to 100-fold) in the microplate bioassays, and the difference was

Table 4. Atrazine and nicosulfuron raw effects on chlorophyll biomass (*in vivo* fluorescence) during the seven outdoor microcosms experiments and Bray-Curtis indices

Experiment	Atrazine Effect	Nicosulfuron Effect	$BCI_{C/A}$	$BCI_{C/N}$
June 97	+	+	0.61*	0.76*
August 97	+	+	0.95	0.85*
April 98	−	−	0.86	0.89
May 98	0	0	0.93	0.94
June 98	0	0	0.86	0.86
July 98	0	0	0.89*	0.92
Sept. 98	−	−	0.81	0.85

+: Higher chlorophyll biomass in the treated microcosms compared to controls, during 2 or more days of the outdoor experiment (Student test, 95% confidence, five replicates)
−: Lower chlorophyll biomass in the treated microcosms compared to controls, during 2 or more days of the outdoor experiment (Student test, 95% confidence, five replicates)
The Bray-Curtis index (BCI) was calculated for the mean density of replicate communities, and the significance of difference between control and the treated microcosms (*) was tested with a Mann-Whitney test between indices calculated among controls (C-C) and indices calculated between controls and tests (C-A or C-N.)

very significant for a given species, certainly resulting from adaptation and genetic changes in that species (*C. vulgaris* from lake Geneva—unpolluted—and Villaumur—strongly polluted), under the selective pressure of chronic contamination of the ecosystem from which the strains were isolated. This has been observed with several herbicides (Hersh and Crumpton 1987; Kasai *et al.* 1993). The effect of nicosulfuron is difficult to assess because there is no strong evidence that this compound is toxic at 'environmentally attainable concentrations.' Our microplate bioassays results seem to be similar to those of others (PMRA 1996), showing very little toxicity during a 120-h growth assay.

Measurements of chlorophyll *a* biomass (by pigment analysis and *in vivo* daily fluorescence) in the mesocosm and microcosm experiments (indoor and outdoor) showed no drastic effect of the herbicides. The only effects were low stimulations of phytoplankton growth for some taxa. Similar cases have been reported with triazine herbicides (*e.g.*, DeNoyelles *et al.* 1982; Lampert *et al.* 1989; Shehata *et al.* 1993). These results could indicate that the toxic compound enhances growth of the more tolerant species by reducing competition between species (DeNoyelles *et al.* 1982; Bérard *et al.* 1999a).

The species compositions and the similarity indices at the end of the microcosms experiments (Table 4 and Figure 2) confirmed this, as both herbicides acted to disrupt the species composition of the contaminated microcosms. Nevertheless, atrazine seemed to have a greater effect than nicosulfuron, especially in the indoor microcosms (Table 4, Figures 2, 3a and 3b). Thompson *et al.* (1993) showed that chronic exposure to hexazinone (a triazine) resulted in a concentration-dependent inhibition of phytoplankton biomass in mesocosms, whereas a high concentration of metsulfuron methyl (a sulfonylurea) (1 mg/L) had only small, transient effects. Källqvist and Romstad (1994) showed that chlorsulfuron (a sulfonylurea) was generally less toxic to algae than atrazine and simazine (triazine) in microplate monocultures. Our mesocosm experiment gave BCIs showing a difference in the community structures of control and contaminated mesocosms. Those indices indicated that atrazine had a greater effect on population structure than nicosulfuron, as in microcosms. The effect may or may not be persistent in the slightly contaminated microcosms; the phytoplankton community structure sometimes recovered. Finally, the effects appeared to be definitive in the highly contaminated mesocosms.

Species-Specific Sensitivity to Herbicides Within Communities

Our microcosm and mesocosm experiments showed that the herbicides do not inhibit the same species. Atrazine inhibits chlorophytes more than diatoms, (Table 3 and 5; Figure 2). Many studies have shown that chlorophytes are very sensitive to PS II inhibitors like atrazine (Hamala and Kollig 1985; Herman *et al.* 1986; Hoagland *et al.* 1993; Kasai *et al.* 1993; DeNoyelles *et al.* 1982; Tang *et al.* 1998). Diatoms are known to tolerate PS II inhibitors (DeNoyelles *et al.* 1982; Bérard and Pelte 1999; Bérard *et al.* 1999a; Hoagland *et al.* 1993; Kasai *et al.* 1993; Tang *et al.* 1998). This tolerance is believed to be due to their heterotrophic behavior, which compensates for the inhibition of photosynthesis by PS II inhibitors (Rees and Syrett 1979). It could also be due to the specificity of the PS II structure and pigment composition; inhibition of photosynthesis by atrazine is comparable to limiting conditions of light (Plumley and Davis 1980), and PS II inhibitors are less toxic for algae that are adapted to low light conditions, as are diatoms (Guasch and Sabater 1998).

In contrast, nicosulfuron seems to inhibit diatoms more than chlorophytes. This could be due to species of phytoplankton having allozymes of ALS synthase that differ in their sensitivity to sulfonylurea in the different phyla, to differences in the capacity of algal groups to cope with a decrease in intracellular branched amino acids by taking them up from the pool of dissolved amino acids, or because diatoms need these amino acids more for the synthesis of the frustule matrix (Darley 1974). It is also true that our microplate bioassays and previous studies have not confirmed the greater sensitivity of diatoms to sulfonylurea (Källqvist *et al.* 1994; Källqvist and Romstad 1994; Nyström 1997; Peterson *et al.* 1994), and sensitivity of individual algae within each algal group to sulfonylurea varies greatly (Nyström 1997; Källqvist and Romstad 1994; Peterson *et al.* 1994; Sabater and Carrasco 1997).

Temporal Variations

The results of the outdoor microcosm experiments indicate that the responses to atrazine and nicosulfuron vary from experiment to experiment. The outdoor microcosm experiments were started and run at different times of the year, so that the characteristics of inocula (biomass, diversity, species composition) and experimental conditions (nutrients, light, temperature) in the seven experiments were quite different (Figure 1, Table 1). The sensitivity of the phytoplankton communities to the herbicides differed in the outdoor microcosms in terms of biomass and species composition (Table 4).

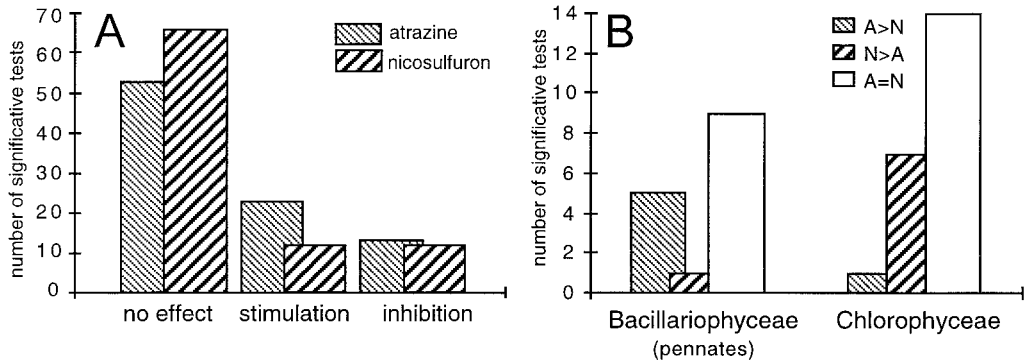

Fig. 3. A] Response of the dominant phytoplankton species to 10 μg/L atrazine and to 10 μg/L nicosulfuron (Mann-Whitney non parametric tests for the seven experiments) measured at the end of the seven outdoor microcosms experiments. B] Relative responses of the two dominant taxonomic groups to atrazine and to nicosulfuron (10 μg/L): the Mann-Whitney non parametric tests were recorded for the seven outdoor microcosms experiments. A>N: when the algal group is stimulated by atrazine relative to nicosulfuron. N>A when the algae is stimulated by nicosulfuron relative to atrazine. N=A: when the algae presents the same response to atrazine and nicosulfuron

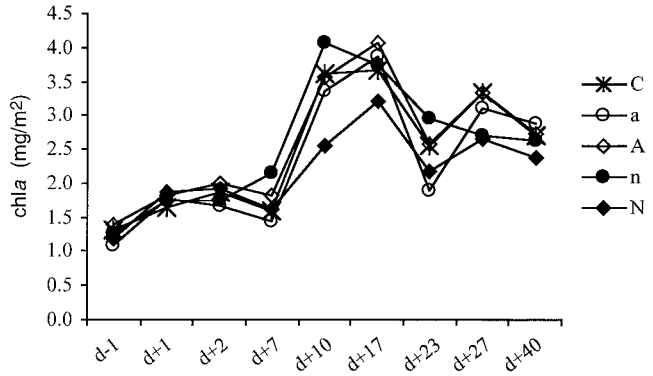

Fig. 4. Apparent growth of phytoplankton in the mesocosms, as chlorophyll *a* content (C: control; a: atrazine 2 μg/L; A: atrazine 30 μg/L; n: nicosulfuron 2 μg/L; N: nicosulfuron 30 μg/L)

The species composition and biomass of the inoculum, water quality, and physical and climatic conditions (depending on the season) may influence the direct impact of a toxic compound on algae (Bérard and Pelte 1999; Caux and Kent 1995; Fournadzhieva *et al.* 1995; Guasch and Sabater 1998; Guasch *et al.* 1997; Herman *et al.* 1986; Mayasich *et al.* 1986, 1987; Ramirez Torres and O'Flaherty 1976). Although it is impossible to determine the precise influence of one or more environmental factors on the sensitivity of algae to the herbicides atrazine and nicosulfuron in only seven experiments running for just 1 year, it is clear that the sensitivity to the herbicides of the plankton communities tested varies. Similar studies have shown that seasonal environmental factors like temperature and phytoplankton successions influence the toxicity of atrazine for algae (Bérard *et al.* 1999a, 1999b). Nyström *et al.* (1999) showed that the sensitivity of algae to sulfonylurea varies in periphyton microcosms. They suggested that variations in the amino acid concentrations in natural waters influenced the toxicity of sulfonylurea herbicides to algae that take up amino acids (Antia *et al.* 1991). The direct incorporation of amino acids by algae is species dependent (Neilson and Larsson 1980), which may also influence the sensitivity of algae to sulfonylurea herbicides.

The community structure and activity of phytoplankton varied with the treatment and from one sample to another during the mesocosm experiments (Figure 6, Table 5). For example, atrazine is believed to depress photosynthetic efficiency by inhibiting electron transfer at the PSII level; the resulting oxidative stress is thought to cause greater photoinhibition at high PAR than in the controls. This was only evident from the results obtained 1 day after the atrazine contamination, and the effect of atrazine could not be distinguished from the effect of nicosulfuron, since the sulfonylurea also depressed α and increased β and Pm (Table 6). Pm was sharply lower in the three microcosms after 10 days of running than at the beginning of the experiment. This could be linked to the plateau in fluorescence-based phytoplankton growth (Figure 4) as a sign of nutrient lack and general cell metabolism inhibition. Thus, we cannot conclude from these results that P/I curves are useful tools per se for assessing the overall sensitivity of phytoplankton to herbicide in seminatural systems, because other environmental parameters (like nutrient availability) can mask the main effects of xenobiotics. A better use of these P/I parameters, which nevertheless are ecologically relevant (often used in ecosystem-level models, *e.g.*, Walsby 1997), might be as the endpoint of short-term toxicity test. The P/I curves and toxic concentration could be used to construct dose-effect curves based on the theory of pollution-induced community tolerance (PICT, Blanck *et al.* 1988), which could be of great importance.

Conclusion

The quality and performance of a test are evaluated according to several criteria, such as accuracy, reproducibility, cost, sensitivity, and—last but not least—ecological relevance. A single test cannot fulfill all these requirements, so there is a need to use several assays.

Accuracy and Reproducibility

Organisms must be tested in totally controlled conditions (pure phytoplankton cultures in microplates) to determine the effects

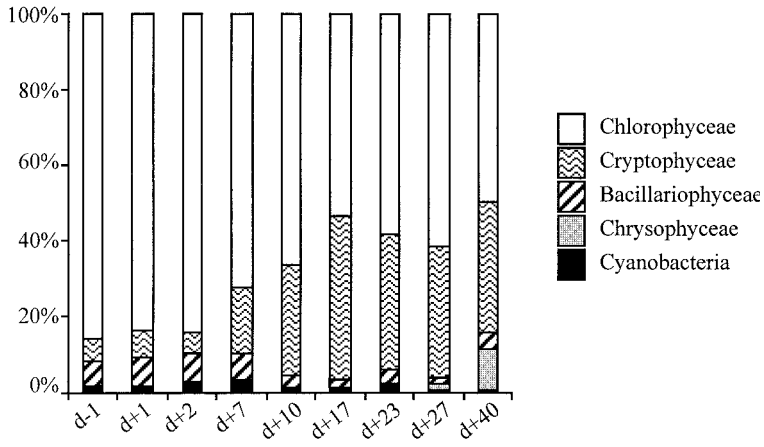

Fig. 5. Class-level taxonomic composition of algal communities in mesocosms at the different sampling periods (d−1 = day before the contamination; d+i = i days after the contamination date), expressed in % of the total phytoplankton identified

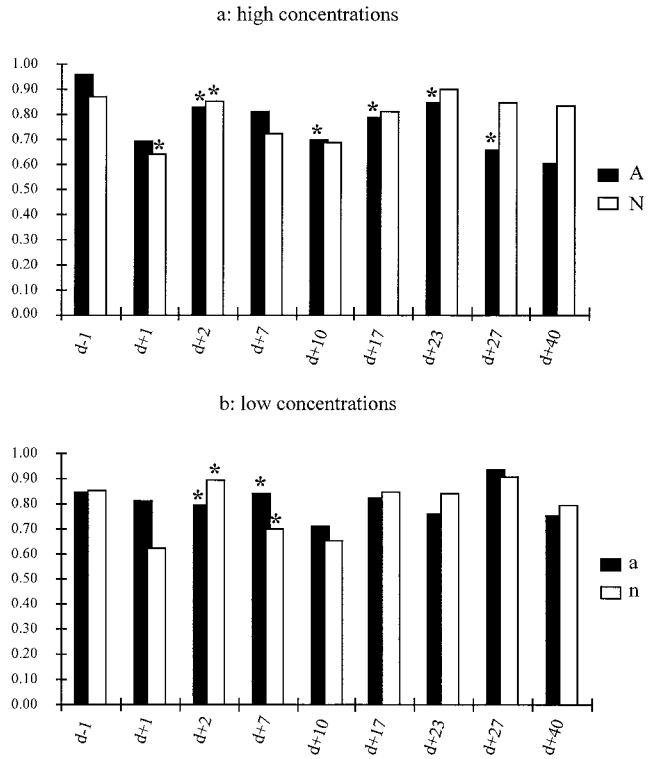

Fig. 6. Bray-Curtis indices of the different phytoplankton samples in the mesocosms. The BCI were calculated for the mean density of replicate communities, and the significance of difference between control and treated was tested with a Mann-Whitney test between indices calculated among controls (C-C) and indices calculated between controls and tests (C-A).*: (C-A/C-C) Mann-Whitney test (90 %)

Table 5. Significative response of dominant taxonomic groups to 2 μg/L and 30 μg/L atrazine and 2 μg/L and 30 μg/L nicosulfuron in mesocosms (Mann-Whitney nonparametric test at each sampling)

Class	Atrazine 2 μg/L	Atrazine 30 μg/L	Nicosulfuron 2 μg/L	Nicosulfuron 30 μg/L
Bacillariophyceae	0	> d2	0	0
	0	> d10	0	> d10
Cryptophyceae	0	0	0	> d27
Chlorophyceae	< d17	< d17	0	< d17
	0	< d23	< d23	0
	0	0	0	> d40
Chrysophyceae	> d40	> d40	> d40	> d40

<: fewer density of individuals than in controls. >: greater density of individuals than in controls
0: no significant effect
Significant effects appeared after several days: d2 means 'day 2,' d10 means 'day 10,' etc

Table 6. Parameters of the P/I curves obtained in the control, A (30 μg/L atrazine contamination), and N (30 μg/L nicosulfuron contamination) mesocosms. The parameters Pm (μmol CO_2/mg chl *a*/h), α (μmol C.mg chl $a^{-1} \cdot h^{-1} \cdot (\mu mol\ photons \cdot m^{-2} \cdot s^{-1})^{-1}$), and β (units as α) were calculated according to Lewis and Smith (1980) (see text for details)

	Sample	Pm	α	β
Day-1	Start	274	1.722	−0.081
Day 1	Control	392	2.201	0
	A	292	1.807	−0.028
	N	300	1.525	−0.105
Day 10	Control	184	2.193	−0.173
	A	194	2.901	−0.140
	N	224	1.84	−0.245

of xenobiotics on nontarget organisms, even if the results are not directly transposable to a whole ecosystem. The culture conditions used for microalgae, such as light intensity and temperature, may interfere with the actions of toxic compounds (Mayer *et al.* 1998). This can be overcome by using standardized methods for more species of algae from several classes, and also for several strains of a given species (*e.g.*, Behra *et al.* 1999; Kasai and Hanazato 1995; Källqvist and Romstad 1994; Nyström *et al.* 1999). These standardized methods could then be used in ecological studies, since they are of low cost and easy to perform. Controlled cultures are by far the most reproducible experiments. Replicates and experimental conditions are easily multiplied, giving large data sets and allowing statistical analysis.

The communities used in microcosms systems are more complex and have become widely used and validated tools

because they provide data under (mostly) replicated, controlled (depending on their indoor or outdoor use), repeatable conditions and at a relatively low cost. These systems are typically run indoors for short periods relative to the time scales of organisms and ecological processes. The natural origin of the tested communities (here the microbial compartment, mostly primary producers) means that the organisms inoculated at the beginning of each experiment vary both qualitatively and quantitatively. Confining them in a jar could alter their behavior more than the toxicant, the so-called bottle effect (Schelske 1984). Outdoor microcosms are more difficult to perform and provide less well-controlled experimental conditions, but these conditions are much closer to the original environment of the cells (Bérard *et al.* 1999a).

Statistic evaluation of effects is difficult in mesocosm experiments because of the great variation among replicated experimental units. This variation occurs among similar systems and has both spatial and temporal components. These must be taken into consideration in ecology and ecotoxicology studies (Kennedy *et al.* 1995). Studies on phytoplankton may be influenced by algal blooms appearing in some ponds at significantly different times. Because not every bloom may be detected because of the sampling interval, the results cannot reflect the details of the phytoplankton dynamics (Wellmann *et al.* 1998). Seasonal succession of phytoplankton also contributes to the variations because of major taxa changes due to temperature and availability of nutrients (Rosenzweig and Buikema 1994). This experimental framework may result in great variations between experimental units, which may compromise the conclusions drawn from the study. Significant perturbations of ecological processes can be difficult to detect against background variations, and the discriminatory power of such systems can be low (Barry and Logan 1998). Replication is also limited, due to logistical and cost constraints (Farmer *et al.* 1995).

Sensitivity of Organisms

Organisms can appear to be insensitive to toxic substances under laboratory conditions, especially when the endpoint is the overall growth of the organisms after 96 h. This is due to such factors as cells switching to alternate metabolic pathways, detoxification to bypass the inhibited pathways, nutrient 'overloading' that provides extreme favorable conditions, and the absence of competitors and predators.

Microcosms are more sensitive systems, in which the inoculated population is assumed to be in a 'dynamic equilibrium,' and hence more sensitive to external pressure. Exposure to a xenobiotic contaminant could enhance the competition for nutrient or light resources, and the absence of higher trophic levels ensures the development of such events. A small difference in the sensitivities of two cohabiting species to one or other herbicide may cause a switch between the two species. Thus, microcosms with complex or natural communities can show the selection pressure exerted by a toxin on the community tested.

Our mesocosm experiments focused on phytoplankton that responded to the stress in a multispecies/multicondition setting of the reconstituted ecosystem. The phytoplankton are controlled not only by the herbicide toxicity but also by other environmental factors (Abdel-Hamid *et al.* 1996). The phytoplankton, the target trophic level in our experiment, was not very sensitive to the herbicides tested, and homeostasis may have developed. But other mesocosm studies have shown that herbicide greatly affect nontarget communities like phytoplankton grazers (Lampert *et al.* 1989). Many interactions between trophic levels (algae, microbial loop, grazing pressure) and environmental factors that occur in mesocosms may either reduce or emphasize the consequences of such pollution in aquatic systems.

Ecological Realism

Microplate assays are still not able to predict the environmental impact of a xenobiotic on phytoplankton populations, despite numerous attempts to normalize the test procedure. This is due to the lack of representativity (either phylogenetically or ecologically) of the test organisms, which are mainly restricted to one species, one strain, *Pseudokirchnierella subcapitata* CCAP 278/4 (AFNOR 1998; Environment Canada 1992). Microplate assays should only be used for normalization and to test the overall specific sensitivity when needed from micro- or mesocosms studies, *e.g.*, to test the species that are believed to be very sensitive or tolerant.

The ecological realism of microcosm experiment have been extensively discussed (*e.g.*, Drenner and Mazumder 1999) and will continue to be. Nevertheless, microcosm experiments can indicate whether the tested community is or is not disturbed when exposed to a selected compound. This alone could be a caveat for the effects of the same compound on the whole ecosystem from which the community was harvested.

Mesocosms aim at being the most realistic experimental structure. Although many papers deal with this, mesocosms are at least representative of their own system. This points out the limitations of such systems for predicting changes in the real biota. But they are still useful tools for explaining and demonstrating the cascade effect produced by low-level contamination due to the trophic interactions. Microcosm and mesocosm studies using natural phytoplankton communities are also useful tools for demonstrating temporal variations in the responses of algae to toxins. Finally, simplified laboratory systems (monocultures) are very useful for demonstrating the impact of environmental parameters (associated with temporal events) influencing the sensitivity of algae to toxic compounds.

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