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Phytotoxicity to and uptake of flumequine used in intensive aquaculture on the aquatic weed, *Lythrum salicaria* L.

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Abstract

Phytotoxicity of Flumequine on the aquatic weed *Lythrum salicaria* L. was determined by two laboratory models: a *single concentration test*, by which the effects of 100 mg l^{-1} were evaluated after 10, 20, 30 days and a *multiple concentration test*, by which the effects of 5000–1000–500–100–50 μg l^{-1} were evaluated after 35-day exposure. 100 mg l^{-1} are highly toxic and significantly decrease the growth of plants; this effect increases with time. Concentrations between 5000 and 50 μg l^{-1} induced hormesis in plants, by significantly increasing mean number and dimension of leaves and secondary roots. The effect is the highest at 50 μg l^{-1} and decreases with increase in concentration. Both toxic effect and hormesis can be related to plant drug uptake, quite high, in the order of μg g^{-1}. The ecological implication of Flumequine contamination in aquatic environments and the possible use of *Lythrum salicaria* for bioremediation and/or monitoring technique are discussed.

Keywords: Antibiotics; Aquaculture; Aquatic weed; Ecotoxicity test; Flumequine; *L. salicaria*; Phytotoxicity

1. Introduction

Intensive aquaculture imposes the use of antibacterial drugs. As a general rule drugs administered by oral route are slowly absorbed and excreted with deject (VV, 1981; Alderman and Michel, 1992; Lunestad et al., 1993). As a consequence, animal waste from intensive aquaculture contains antibiotics and other drugs in active forms: it has been demonstrated that 70–80% of drugs administered as medicated feed during a treatment period, ends up into the environment (Hektoen et al., 1995).

Flumequine is a Quinolone antibiotic, widely used in intensive aquaculture (Quevauviller and Maier, 1994). It is quite persistent (approximately 150 days half-life, in surface sediment, Hektoen et al., 1995), and has been found in the wastewater of a sea bass intensive aquaculture station, during a treatment period at levels of 50 μg l^{-1} (Migliore et al., 1995b).

Drug discharge can be a source of ecosystems alteration and the impact is suspected to act mainly at benthic level: the drug enters into the environment with particulate that deposit according to dimension of particles and water mass dynamics (Mann and Lazier, 1996). In benthic systems, the contamination of non-target species has been demonstrated by controlled transplantation in antibiotic-affected areas (Capone et al., 1994; Jones and Iwana, 1989; Tibbs et al., 1989) and by direct quantification of antibiotic residues in different organisms (i.e., crustaceans, shellfish, both pelagic and benthic fishes) living in the proximity of fish farm facilities (Samuelsen et al., 1992, 1993; Ervik et al., 1994). Some of these works referred to a first and a second generation of Quinolone antibiotics that were

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substituted by a third, the fluoroquinolones, e.g., Flumequine (Dixon, 1994). Biological effects of Flumequine were found on *Artemia* naupli in ecotoxicity tests (Brambilla et al., 1994; Migliore et al., 1995b, 1997a). Induction of resistance in the microbial community in the area close to a farm facility was determined (Migliore et al., 1996a). Plants are able to absorb and accumulate antibiotics under laboratory conditions: in those tests both crop plants and weeds demonstrated antimicrobial drug phytotoxicity and bioaccumulation (Migliore et al., 1995a, 1996b, 1997b, 1998).

Lythrum salicaria L. is a cosmopolitan aquatic weed, euryhaline, rooted into the sediment and, consequently, prone to be affected by Flumequine contaminated aquaculture wastewater.

The aim of this work has been to evaluate Flumequine toxicity and uptake in *L. salicaria* plants by two experimental models: (A) *single concentration test*, by which the effect of 100 mg l^{-1} Flumequine was evaluated at different times (10, 20 and 30 days) and (B) *multiple concentration test*, by which the effect after a fixed time (35 days) was evaluated at different concentrations (5000–1000–500–100–50 μg l^{-1}).

2. Material and methods

L. salicaria L. (Lythraceae), common name purple loosestrife, is an erect perennial herb or small shrub, 50–150 cm tall, with raceme inflorescences. Flowers are 6-merous, tubular, purple, solitary in the leaf-axils or in whorl-like cymes in the axils of small bracts, forming long, terminal spikes (Webb, 1968). Native of Eurasia, the species spread in all temperate regions (mainly North-America) due to its wide tolerance range (for example, *L. salicaria* L. is highly tolerant to salinity, Pignatti, 1982) and incredible seed production. Seeds dispersal seems to occur mainly by water movement although wind dispersal may move seeds several yards from the parent plant (Welling and Becker, 1990).

This weed grows in areas near water: the optimum are freshwater marshes, open stream margins, and alluvial flood plain. Plants generally grow in moist organic soils in full sun. Mature plants can persist for years on dry sites, seedlings become established only on moist soil sites. Establishment can occur on a variety of substrates (e.g., gravel, sand, clay, organic) and with soil pH levels ranging from acidic (4.0) to alkaline (9.1), although the optimal substrate seems to be organic and alluvial soils with a neutral to slightly acidic pH (Thompson et al., 1987).

Seeds of the plant were obtained from nature, in the Varriconi area, at the estuary of the Volturno River (near Naples). They were sterilised and transferred to Petri dishes for germination at 25°C in the dark. According to the already used method (Migliore et al., 1995a, 1996b, 1997b, 1998) after germination, seedlings were transferred to multipurpose jars on sterile solid Murashige and Skoog medium (Sigma, Milan, Italy), with or without Flumequine (Sigma, Milan, Italy) (nominal concentrations). The drug, completely soluble in alkaline medium, as happens in run-off water from intensive fish farming, was dissolved in NaOH 5N, filtered through a sterile Millipore 0.45 μm filter and added to the medium at 45°C; each control contained the same NaOH concentration of the referent treated jar. Sterility was maintained from the start to the end of experiment. Control results were pooled due to absence of any difference between them. The lowest level of drug applied has been determined on the basis of concentrations detected in the water output of a sea bass intensive aquaculture plant during a fish treatment period, about 50 μg l^{-1} (Migliore et al., 1995b). Both control and treated jars with seedlings were transferred to a growth room at 25°C under continuous light. The light intensity was 50 μE m^{-2} s^{-1}. In 35 days, all plant structures are completely produced and plant dimensions are compatible with jar dimension. At the end of each experiment (a) length of primary root, hypocotyle, cotyledons, (b) number of secondary roots and leaves and (c) length of leaves were measured. Plants were weighed at the end of the experiment and were frozen at −80°C until chemical analysis. A total of 596 + 507 plants were tested in the single and multiple concentration test, respectively. Length and weight data were statistically analysed by one way ANOVA, taking into account all the replicates.

Chemical analysis. Pooled plants of each test were dried at 50°C for 24 h and then weighed to obtain dry weight. Flumequine in plants was extracted according to the method of Haagsma et al. (1993), modified for the needs of our matrix. Samples were extracted in 10 ml *n*-hexane + 30 ml ethyl acetate by mincing (30 s). After centrifugation (3500 g, 15 min) the supernatants were cleaned up on 3 ml–500 mg SPE-NH_2 (amino solid phase extraction, J.T. Baker, Holland) previously conditioned with 10 ml methanol + 6 ml ethyl acetate/*n*-hexane 3:1. After washing columns with 5 ml ethyl acetate/*n*-hexane 3:1, Flumequine was eluted by 4 ml acetonitrile/oxalic acid 0.025M (pH 2) 8:2. This fraction was collected and dried under nitrogen stream, resuspended in 100 μl methanol and injected (50 μl) in the HPLC-Fluorimeter system. A Waters 600 pump equipped with a Symmetry column C18 (4.6 × 250 mm, i.d. 4.6 mm) and a Fluorimetric detector Waters 470 were used under the following conditions. Mobile phase: linear gradient acetonitrile/oxalic acid 0.035M (pH 2.2) from 20% to 100% acetonitrile in 15 min. Flow: 0.75 ml min^{-1}; Loop 50 μl; detector settled at λ excitation 327 nm and λ emission 369. Analyses were performed at room temperature. Recovery rates of the extraction procedure were calculated by spiking the control plants with

known amount of Flumequine standard in the range 25 μg–25 ng and applying 5 times this procedure for each concentration tested. Rates were higher than 90%. Flumequine content was determined by the external standard plot method (Calibration curve between the concentrations of 5 and 250 ng injected; $r = 0.998$), lower limit of determination 2 ng g^{-1} dry weight (ppm). Reagents were HPLC grade (Merck).

3. Results

3.1. Single concentration test

Growth test. The effect of 100 mg l^{-1} Flumequine on *L. salicaria* growth after 10, 20 and 30 days is shown in Fig. 1, as mean length of primary root, hypocotyle, cotyledon and mean number of secondary roots. In Fig. 2, as mean number of leaves per plant and mean length of first, second and third leaf, in both treated and control batches. Flumequine reduced the mean length of root, hypocotyle, cotyledon and the mean number of secondary roots. Also the mean number of leaves and the length of each leaf are reduced. The effect is increasingly evident with the increase of the exposure time.

Statistical analysis of the data. The differences between treated and control batches are reported in Table 1. At day 10 cotyledon, number of leaves and mean length of first leaf in treated plants showed significant differences with control. At day 20 all structures showed significant differences except secondary roots. At day 30 all structures showed highly significant differences. Also

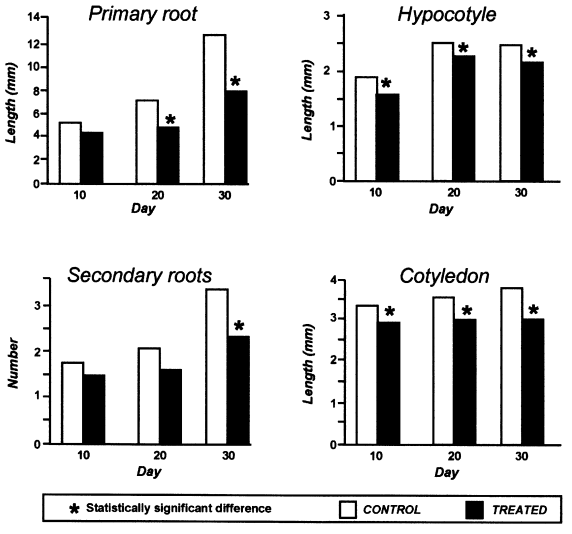

Fig. 1. Effect of 100 mg l^{-1} Flumequine on *L. salicaria* growth after 10, 20, and 30 days, as mean length of primary root hypocotyle, cotyledon and mean number of secondary roots.

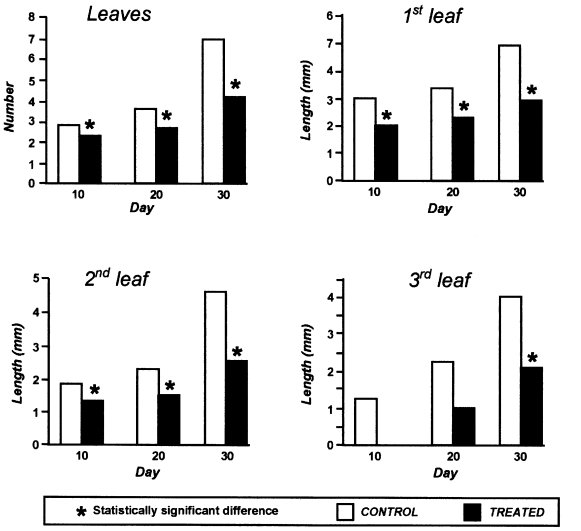

Fig. 2. Effect of 100 mg l^{-1} Flumequine on *L. salicaria* growth after 10, 20, and 30 days, as mean number of leaves per plant and mean length of 1st, 2nd, and 3rd leaf.

the percentage of plant producing secondary roots was reduced (Table 1).

Uptake. The final contents of Flumequine in pooled *L. salicaria* plants were 64.9, 31.6 and 15.7 μg g^{-1} dry weight (ppm), respectively after 10-, 20-, 30-day exposure.

3.2. Multiple concentration test

Growth test. The effect of five Flumequine concentrations on *L. salicaria* plants, after 35-day exposure is shown in Fig. 3, as mean length of primary root, hypocotyle, cotyledon and mean number of secondary roots, and in Fig. 4, as mean number of leaves/plant and mean length of first, second, third and fourth leaf, in both treated and control batches.

Flumequine, at all concentrations tested, increased the number of leaves produced per plant and, at concentrations between 500 and 50 μg l^{-1}, increased the number of secondary roots. Even the mean dimension of first leaves are increased; the effect is reduced or absent in the last leaves (Fig. 4). At 50 μg l^{-1} the mean dimension of hypocotyle is decreased (Fig. 3).

Statistical analysis of the data. The differences between treated and control batches are reported in Table 2A, as regards general plant structures, and 2B, as regards leaves dimensions. At 5000 and 1000 μg l^{-1} only the number of leaves per plant is significantly higher in treated batches, at 500–100 and 50 μg l^{-1} both number of leaves and secondary roots are significantly higher in treated batches. Significant differences are also found in the mean length of first leaf at all concentration tested.

Table 1
Difference between 100 mg l^{-1} Flumequine treated (T) and control (C) plants at different times

10 days							20 days							30 days						
Length (mm)		*N*	Mean	df	*F*	*p*	*Length* (mm)		*N*	Mean	df	*F*	*p*	*Length* (mm)		*N*	Mean	df	*F*	*p*
Primary root	C	98	5.32	1;194	9.30	n.s.	Primary root	C	80	7.10	1;182	40.04	<0.001	Primary root	C	60	12.67	1;214	64.41	<0.0001
	T	98	4.37					T	104	4.59					T	156	7.77			
Cotiledon	C	98	3.34	1;194	25.91	<0.001	Cotiledon	C	80	3.55	1;182	58.85	<0.001	Cotiledon	C	60	3.77	1;214	103.96	<0.0001
	T	98	2.93					T	104	2.99					T	156	3.02			
Ipocotyle	C	98	1.88	1;194	13.36	<0.05	Ipocotyle	C	80	2.51	1;182	10.12	<0.005	Ipocotyle	C	60	2.49	1;214	13.85	<0.0005
	T	98	1.57					T	104	2.25					T	156	2.19			
Number							*Number*							*Number*						
Secondary roots	C	98	1.75	1;194	2.88	n.s.	Secondary roots	C	80	2.05	1;182	3.66	n.s.	Secondary roots	C	60	3.33	1;214	17.00	<0.0001
	T	98	1.47					T	104	1.58					T	156	2.29			
Leaves	C	98	2.77	1;194	13.11	<0.001	Leaves	C	80	3.70	1;180	41.02	<0.001	Leaves	C	60	7.00	1;214	142.41	<0.0001
	T	98	2.22					T	102	2.65					T	156	4.24			
Plants producing secondary roots (%)					C	83.67	Plants producing secondary roots (%)					C	88.75	Plants producing secondary roots (%)					C	100
					T	72.45						T	79.81						T	100
Length (mm)							*Length* (mm)							*Length* (mm)						
First leaf	C	94	3.07	1;185	43.97	<0.001	First leaf	C	80	3.42	1;182	61.96	<0.0001	First leaf	C	61	5.01	1	273.08	<0.0001
	T	93	2.07					T	104	2.35					T	156	2.93	215		
Second leaf	C	25	1.86	1;27	5.39	<0.05	Second leaf	C	58	2.31	1;88	14.48	<0.0005	Second leaf	C	60	4.63	1	157.63	<0.0001
	T	4	1.31					T	32	1.51					T	125	2.53	183		
							Third leaf	C	10	2.25	–	–	–	Third leaf	C	53	4.01	1	49.62	<0.0001
								T	1	1.00					T	43	2.12	94		
														Fourth leaf	C	32	3.31	1	3.79	n.s.
															T	1	1.00	31		

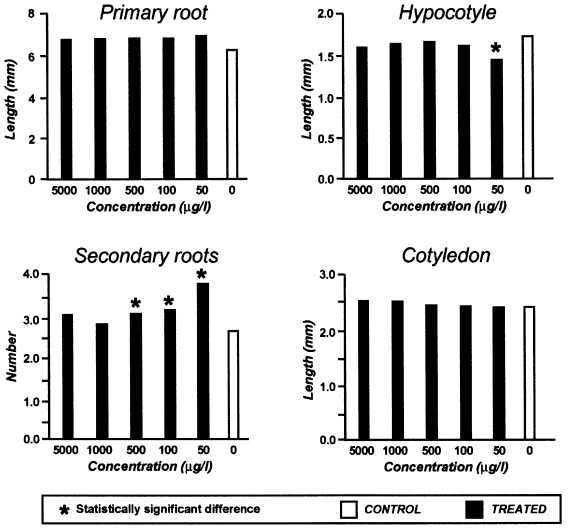

Fig. 3. Effect of five Flumequine concentration on *L. salicaria* plants, after 35 days exposure, as mean length of primary root, hypocotyle, cotyledon and mean number of secondary roots.

Second leaf is significantly different at all concentrations but 500 μg l^{-1}; at this concentration its mean length is higher than control but differences are not significant. Significant differences were found in the third leaf at 50 μg l^{-1}. The fourth, fifth and sixth leaves do not show significant differences in length between control and treated batches.

In Table 3 the percentage of plants producing each leaf is reported: at all concentrations treated plants produce the first–fifth leaf at higher percentage than control plants.

Uptake. After a 35-day period, the final contents of Flumequine in pooled *L. salicaria* plants were 13.3–8.7–0.7–0.3 and 0.2 μg g^{-1} dry weight (ppm), respectively at 5000, 1000, 500, 100 and 50 μg l^{-1}.

4. Discussion

Flumequine, at a concentration of 100 mg l^{-1}, is toxic to *L. salicaria* post-germinative development. The effect is already evident after 10-day exposure (Fig. 5), although not significant for all structures, confirming that plants are relatively independent from the external medium in the first days from seedling (Berlyn, 1972; Bewley and Black, 1978); after 30 days the reduction of post-germinative development is dramatic and highly significant in all structures. Drug uptake at concentrations of μg g^{-1} plant dry weight is the cause of toxicity; but concentration per weight unit regularly decreases with time, probably due to lower plants growth and/or drug biodegradation. It can be hypothesised that this decrease per weight unit can depend on the relative lower incidence of root weight with respect to the aerial portion of plants that happens during post-germinative development. In fact, after the very first phase, root growth lowers while leaves growth increase. Previous results demonstrated that roots are the main site of drug accumulation (Migliore et al., 1995a, 1996b).

Thirty-five days exposure to lower concentrations of Flumequine also causes alteration of post-germinative development but with different pathway: hormesis (*sensu*

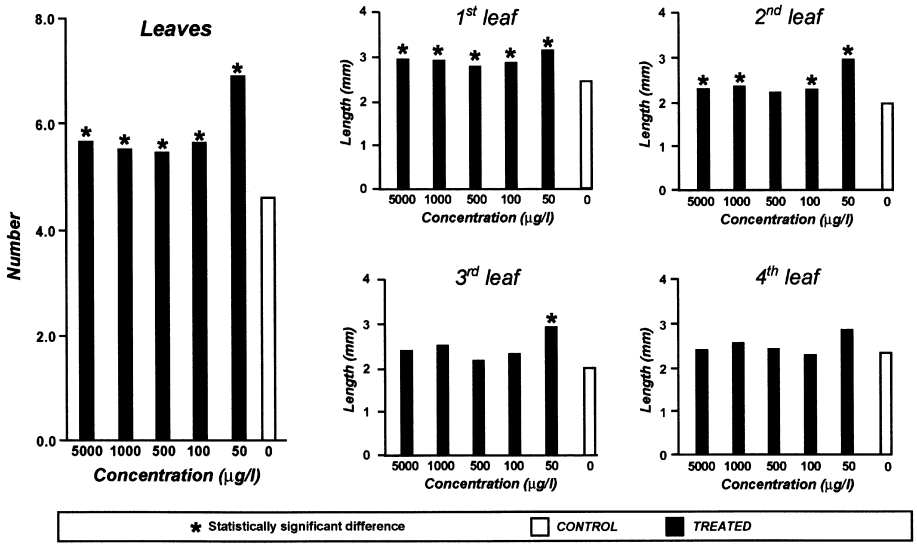

Fig. 4. The effect of five Flumequine concentration on *L. salicaria* plants, after 35 days, as mean number of leaves/plants and mean length of 1st, 2nd, 3rd and 4th leaf.

Table 2A
Differences between plant structures in Control and Treated plants

	Control		5000 μg/l vs Control					1000 μg/l vs Control					500 μg/l vs Control				
	N	Mean	*N*	Mean	df	*F*	*p*	*N*	Mean	df	*F*	*p*	*N*	Mean	df	*F*	*p*
Length (mm)																	
Primary root	94	6.29	77	6.78	1;169	1.23	n.s.	73	6.85	1;165	1.20	n.s.	91	6.90	1;183	2.60	n.s.
Cotiledon	94	2.40	77	2.56	1;169	2.81	n.s.	73	2.53	1;165	2.00	n.s.	91	2.46	1;183	0.30	n.s.
Ipocotyle	94	1.74	77	1.61	1;169	0.70	n.s.	73	1.66	1;165	0.92	n.s.	91	1.66	1;182	1.20	n.s.
Number																	
Secondary roots	92	2.63	76	3.04	1;166	3.59	n.s.	70	2.84	1;160	0.82	n.s.	87	3.09	1;182	4.08	<0.05
Leaves	94	4.59	77	5.64	1;169	11.32	<0.001	73	5.53	1;143	8.91	<0.005	91	5.43	1;183	7.18	<0.05

	100 μg/l vs Control					50 μg/l vs Control				
	N	Mean	df	*F*	*p*	*N*	Mean	df	*F*	*p*
Length (mm)										
Primary root	78	6.88	1;170	1.35	n.s.	94	7.03	1;186	2.78	n.s.
Cotiledon	78	2.45	1;170	0.26	n.s.	94	2.44	1;186	0.11	n.s.
Ipocotyle	78	1.62	1;170	1.82	n.s.	94	1.46	1;186	12.70	<0.001
Number										
Secondary roots	70	3.17	1;162	5.94	<0.05	94	3.77	1;182	24.84	<0.001
Leaves	78	5.64	1;170	11.94	<0.005	94	6.87	1;186	39.22	<0.001

Table 2B
Differences between leaves mean length in control and Treated plants

	Control		5000 μg/l vs Control					1000 μg/l vs Control					500 μg/l vs Control				
	N	Mean	*N*	Mean	df	*F*	*p*	*N*	Mean	df	*F*	*p*	*N*	Mean	df	*F*	*p*
Length (mm)																	
First leaf	94	2.43	77	2.87	1;169	11.8	<0.001	73	2.87	1;165	10.22	<0.005	91	2.75	1;183	7.06	<0.05
Second leaf	74	1.98	75	2.30	1;147	3.99	<0.05	66	2.35	1;120	4.11	<0.05	81	2.23	1;153	2.82	n.s.
Third leaf	36	1.99	41	2.36	1;112	1.81	n.s.	37	2.50	1;112	2.82	n.s.	48	2.16	1;82	0.49	n.s.
Fourth leaf	8	2.31	17	2.38	1;23	0.01	n.s.	18	2.83	1;21	0.65	n.s.	19	2.37	1;25	0.01	n.s.
Fifth leaf	3	2.67	7	1.57	1;8	1.63	n.s.	8	1.62	1; 9	3.26	n.s.	7	2.14	1;8	0.30	n.s.
Sixth leaf	1	2.5	–					–					–				

	100 μg/l vs Control					50 μg/l vs Control				
	N	Mean	df	*F*	*p*	*N*	Mean	df	*F*	*p*
Length (mm)										
First leaf	78	2.79	1;170	6.71	<0.05	94	3.12	1;186	22.25	<0.001
Second leaf	74	2.31	1;146	4.47	<0.05	90	2.94	1;162	22.07	<0.001
Third leaf	44	2.27	1;78	1.49	n.s.	64	2.91	1;98	11.85	<0.001
Fourth leaf	20	2.07	1;26	0.17	n.s.	42	2.82	1;48	0.72	n.s.
Fifth leaf	5	2.20	1;6	0.22	n.s.	20	2.50	1;21	0.04	n.s
Sixth leaf	–					9	2.33	1;8	0.02	n.s.

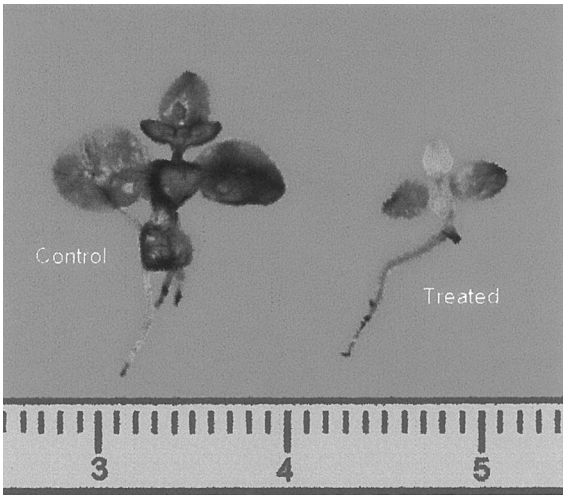

Fig. 5. The effect of 100 mg l^{-1} Flumequine on *L. salicaria* post-germinative development.

Stebbing: increased growth; Stebbing, 1998) is found at 50–100–500 μg l^{-1} for: (a) number of leaves and secondary roots; (b) length of first and second leaf. At 50 μg l^{-1} hormesis also affects the third leaf length. At 5000 and 1000 μg l^{-1} the hormesis remains restricted to the number and the length of first and second leaf. The hormesis decreases with drug uptake increase.

The two different experiments showed also how important is the uptake pattern on the response of plants. In the first test, after 30 days at 100 mg l^{-1}, plant uptake is 15.7 μg g^{-1}, in the second test, after 35 days at 5000 μg l^{-1}, plants uptake was 13.3 μg g^{-1}; quite a small difference for major toxic effect. This demonstrates that in the first test the very high – although decreasing – concentrations experienced by plants during the 30-day experiment are able to alter post-germinative development in a more effective way than the lower concentration experienced in the second test: i.e., it is not the absolute value of the drug into plants but the entire pattern of contamination that determines the development alterations. Lack of correspondence between drug concentrations in media and in plants could be addressed to the acute toxic effect induced in plants.

Flumequine introduction into plant metabolic arrays causes development alterations. This drug is an antibacterial active on bacterial DNA-girase. Eucariotic cells contain a DNA-topoisomerase II, conceptual and mechanical analogue of the bacterial girase; deleted mutants (*Δtop2*) are unable to duplicate DNA and perform cell division (Kornberg and Baker, 1992). Concentrations between 100 and 1000 μg ml^{-1} in eucariote causes inhibition of topoisomerase and negative effects on growth (Goodman and Gilman, 1992). Notwithstanding, low concentration (20 μg g^{-1} Flumequine) was able to promote eel lymphoid cell proliferation (Van der Heijden et al., 1995); this datum is somehow comparable to the 50 μg l^{-1} dramatic induction of plant growth we found in *L. salicaria*.

The acute toxicity of Flumequine on *L. salicaria* plants clearly agrees with our previous experimental data on the toxicity of Sulphadimethoxine on crop plants (*Hordeum disticum* L., *Panicum miliaceum* L., *Pisum sativum* L. and *Zea mays* L.) and weeds (*Amaranthus retroflexus* L., *Plantago major* L. and *Rumex acetosella* L.) (Migliore et al., 1995a, 1996b, 1997b, 1998; Brambilla et al., 1996); it further demonstrates the affinity of antibacterial drugs for the biota and confirms the methodological validity of our ecotoxicological tool, skill with different species and different drugs.

Flumequine is a common contaminant of aquaculture areas for both sediment and wild organisms and poses environmental concerns (Schneider, 1994; Halling-Sørensen et al., 1998). The environmental risk due to Flumequine contamination is highlighted: drug uptake and easy growing of *L. salicaria* plants in contaminated sites can reliably spread the drug via food nets.

In conclusion the introduction of Flumequine into environmental compartments, due to intensive aquaculture activities, can contaminate sediment and aquatic plants downstream, and produce toxic effects. *L. salicaria*, a good model to evaluate ecotoxicity of drugs, could be used: (a) as bioindicator of Flumequine contamination, due to its high uptake capacity and (b) to monitor drug presence in the environment, although concentrations in plants are not directly related to exposure. Furthermore, its possible role in bioremediation techniques is intriguing: its tilling for

Table 3
Percent plants producing each type of leaf

Leaf	Control (%)	5000 μg l^{-1} (%)	1000 μg l^{-1} (%)	500 μg l^{-1} (%)	100 μg l^{-1} (%)	50 μg l^{-1} (%)
First	100.0	100.0	100.0	100.0	100.0	100.0
Second	78.7	97.4	90.4	89.0	94.9	95.7
Third	38.3	53.2	50.7	52.7	56.4	68.1
Fourth	8.5	22.1	24.7	20.9	25.6	44.7
Fifth	3.2	9.1	10.9	7.7	6.4	21.2
Sixth	1.1	0	0	0	0	9.6

phytodepuration of aquaculture wastewater could be a remedy to limit the spreading of veterinary drugs into the environment.

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