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# Effect Assessment of Antimicrobial Pharmaceuticals on the Aquatic Plant *Lemna minor*

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Environmental risk assessment protocols require the use of ecotoxicity data on a set of taxonomic groups considered key elements for each environmental compartment. Fish, aquatic invertebrates (mostly daphnids) and unicellular algae are the key elements for aquatic systems (Bro-Rasmussen et al. 1994; EC 1996). However, unicellular algae and aquatic vascular plants possess large physiological differences which affect chemicals sensitivity. Differences are particularly significant for chemicals with specific mechanisms of action. Certain molecules, such as chlorate, are highly toxic to vascular plants but harmless for algae. This difference is recognised for herbicides and therefore, aquatic plants are specifically included in the assessment (EC 2001). Antimicrobial pharmaceuticals also possess specific mechanisms of action. Large sensitivity differences between algae from different taxa have been observed (Halling-Sørensen 2000), but no information on the toxicity to aquatic vascular plants is available. This paper studies the toxicity of antimicrobials to aquatic plants. Two model antibacterials, the sulfonamide sulfochlorpyridazine and the tetracycline oxytetracycline dihydrate, representing different modes of action, were used. The effects on the aquatic plant *Lemna minor* are compared to those observed for green algae, which are commonly selected as the only representation for aquatic vegetation.

## MATERIALS AND METHODS

*Lemna minor* was collected in the Jarama river (Madrid, Spain) and cultured in our laboratory for over 3 years. Culture and test conditions include: Duckweed Nutrient Solution 1% at pH 6.5 (APHA 1995); temperature 25° ± 2°C; photoperiod 18/6 hours light/darkness; light (Mazda JO TF 18W/LJ) intensity 7000 lux.

Sulfachlorpyridazine sodium salt and oxitetracycline dihydrate were kindly provided by Novartis and Huashu Pharmaceutical Corporation, respectively. Initially a range finding study was performed (from 0.01 to 100 mg/L in orders of magnitude, triplicated). On the basis of the results, the definitive test was designed (control plus five concentrations around the expected EC50, geometric distribution with a factor equal to or lower than 2, triplicate assays). Exposure was static for 7 days (168 hr) in 250 ml glass vessels, with initial density of 12 fronds

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per vessel, wrapped in aluminium foil to minimize algae growth, and individual pH adjustment for each concentration. As no other studies on the toxicity of these chemicals to *Lemna* have been reported, several toxicity endpoints were selected to cover different mechanisms: total fronds and colonies number, green fronds, chlorotic fronds, fresh and dry biomass, and daily growth rate.

The toxicity of both antimicrobials to the unicellular algae *Chlorella vulgaris* was also studied. Tests were conducted on 96 well microplates as previously described (Ramos et al. 1995). Growth inhibition was measured by cell counts and UV absorption at different times during 48 hr of exposure. Growth rate inhibition was estimated following the OECD guidance (OECD 1984).

## RESULTS AND DISCUSSION

Table 1 presents an outcome summary for the different selected endpoints. Quantitative assessment for intra-assay variability and qualitative estimations on cost/effectiveness and relevance are included.

**Table 1.** Summary comparison for the selected endpoints.

| Endpoints | Variability (%) Mean | Range | Relevance | Cost/ Efectiviness |
|---|---|---|---|---|
| Number of total fronds at 168 h. | 9.5 | 7.7-13.1 | ++ | - |
| Number of green fronds at 168 h. | 10.1 | 7.2-15.5 | +++ | +++ |
| Number of chlorotic fronds at 168 h. | 42.0 | 0-138.6 | ++ | - |
| Number of colonies at 168 h. | 11.7 | 8.2-16.2 | + | - |
| Fresh biomass at 168 h. (mg) | 23.0 | 18.4-31.0 | +++ | ++ |
| Dry biomass at 168 h. (mg) | 29.4 | 11.4-41.3 | +++ | - |
| Daily growyh rate | 16.8 | 6.3-28.1 | ++++ | + |

The best scores were obtained for the number of green fronds and fresh biomass. Linear correlations, with correlation coefficients between 0.84 and 0.98 were found between these parameters. For dry biomass, correlations were very good in some cases (>0.95) but very low in others, and inter-assay variability was very high. Daily growth rate was initially considered the more relevant endpoint but intra-assay variability was higher in some cases. Also, lag phases can interfere in the measurement, and requires extra cost during the assay. Therefore, the total number of green fronds was finally selected as the single endpoint, and EC50 values were calculated only for this endpoint.

Time and dose/response curves for the studied antimicrobial pharmaceuticals are included in Figures 1 and 2 respectively. Log-probit analysis estimated an EC50 value of 2.33 mg/L (95% confidence intervals of 1.4-3.9) and 4.92 mg/L (95%

confidence intervals of 3.6-6.8) for sulfachlorpyridazine (SCP) and oxytetracycline (OTC) respectively. Large differences were observed in the toxicity of both chemicals to the unicellular green algae *Chlorella vulgaris*.

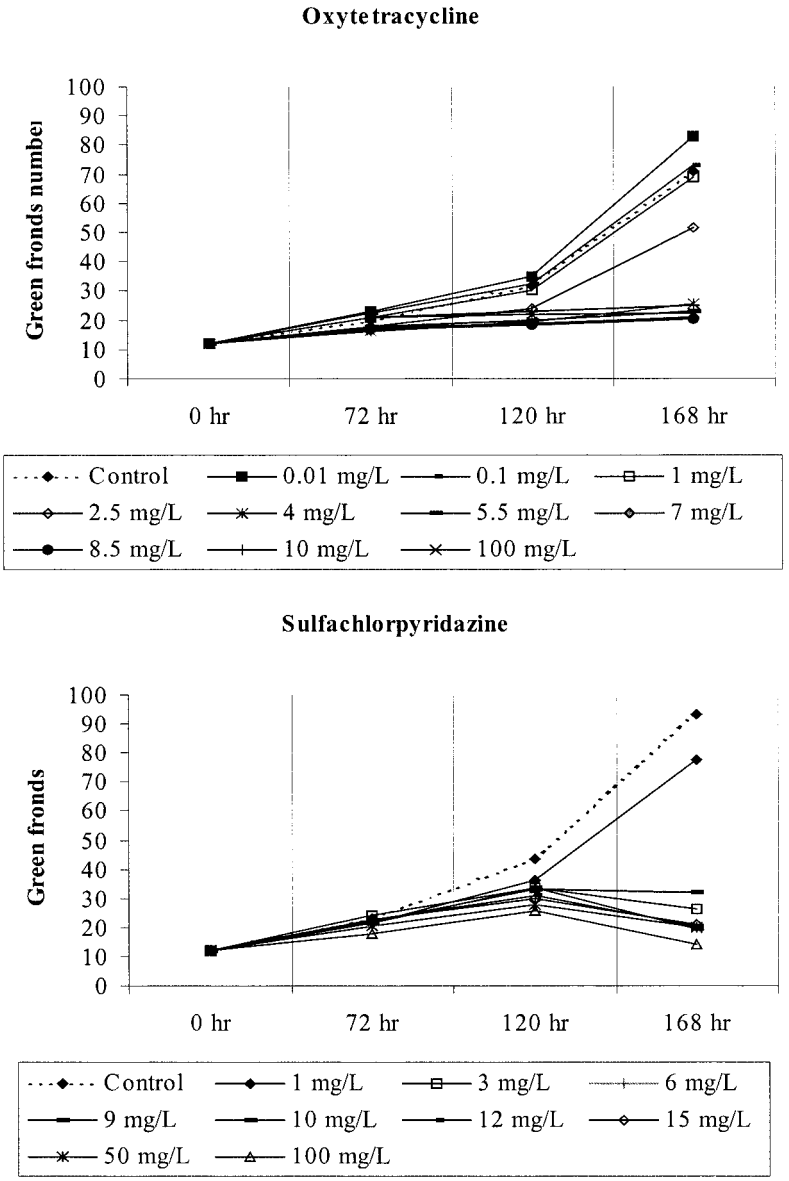


Figure 1. Time response curves for oxytetracycline and sulfachlorpyridazine on *Lemna minor*.

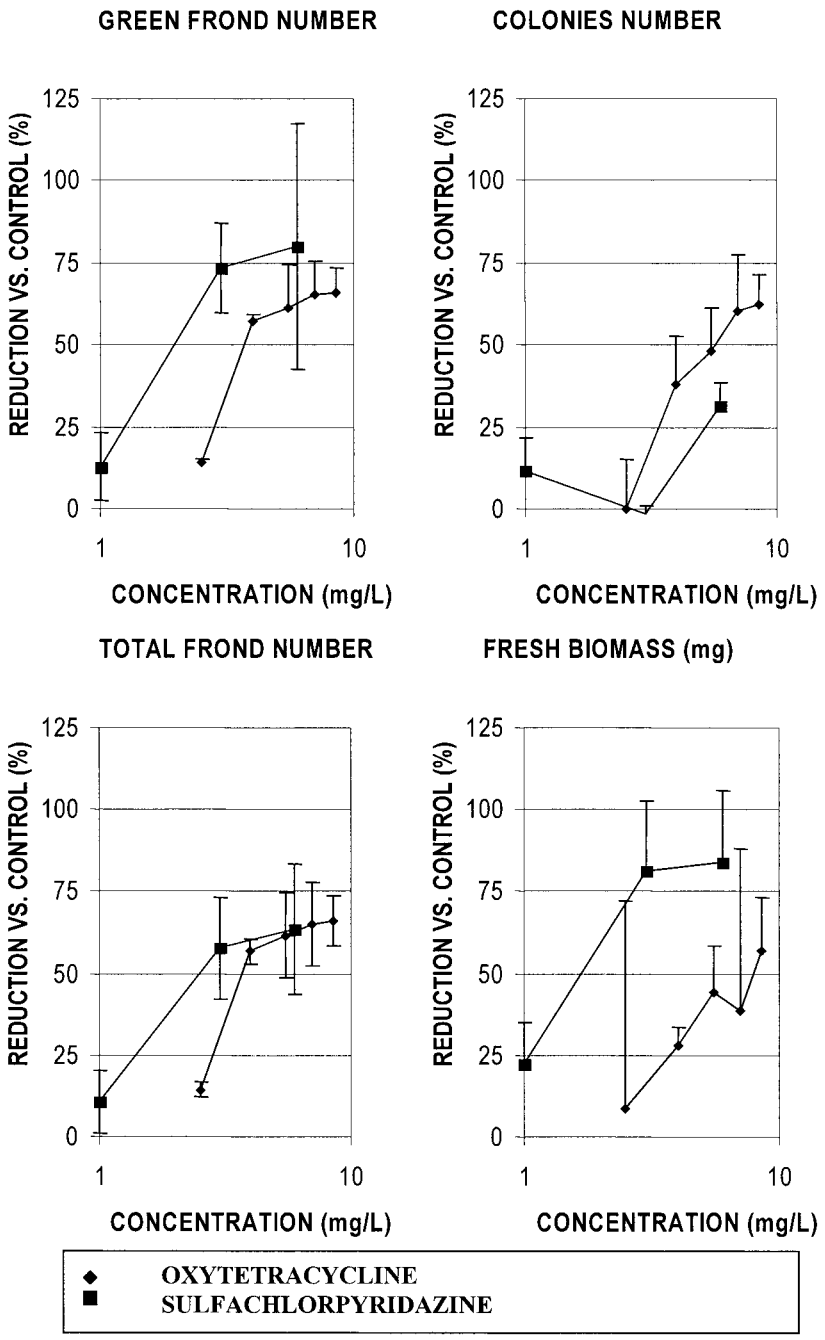


**Figure 2.** Dose-response curves for the different endpoints measured on *Lemna minor*. Values (mean ± variation coefficients) measured at the end of the study 168 hr.

The time-responses curves (Figure 1) indicate that the selected exposure period 168 hr allows measuring both growth inhibition and lethality. Growth inhibition was finally considered because it is the usual parameter for primary producers.

The *Chlorella vulgaris* 48 hr EC50 value for oxytetracycline was 6.4 mg/L (95% confidence intervals of 4.9-8.4), while no toxicity was observed at the highest tested concentration of sulfachlorpyridazine, 2000 mg/L which is assumed to be well above the solubility limit, suggesting that the chemical can be considered as harmless for this species.

Oxytetracycline showed a similar toxicity for *Lemna* than for *Chlorella*. Results are also in agreement with the EC50 of 4.5 mg/L reported for other green algae, *Selenastrum capricornatum* (Holten Lützhøft et al. 1999). However, sulfachlorpyridazine was particularly toxic for *Lemna*, being harmless to *Chlorella*.

Differences in sensitivity between unicellular algae and aquatic plants are commonly observed for chemicals with herbicidal activity. In fact, the ecological risk assessment of herbicides requires information on both groups for conducting a proper risk assessment. Antimicrobials are between one and two orders of magnitude more toxic for cyanobacteria than for green algae (Holten Lützhøft et al. 1999; Halling-Sørensen 2000), difference that are associated with mechanisms of action. Our results show a large difference, three orders of magnitude, between green algae and aquatic vascular plants. The use of the standard safety factors of 100-1000 for the derivation of acceptable concentrations in water (EC 1996; Tarazona 1998) would not cover this margin, and therefore the risk of sulfachlorpyridazine to aquatic vascular plants would not be covered by algal tests.

Several reviews on the available ecotoxicity data of pharmaceuticals have been recently published (Webb 2001; Servos et al. 2002). Effects on several algae species are reported, but no a single data on aquatic vascular plants is included.

The use of *Lemna* as a model for ecotoxicity evaluations on aquatic plants is receiving large support. Different organisations including APHA (1995); EPA (1996); OECD 1998; 1999; 2000) and ISO (2001) are involved in the test standardisation. The results presented in this study offer additional evidence on the capability of this assay, and their need for chemicals with specific mode of action. Data suggest that a single and practical parameter, number of green fronds, can be sufficient for summarising the outcome of this assay in a way compatible with other standard tests and the use of the results in environmental risk assessment.

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