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Ecotoxicity Evaluation of Industrial Discharge Waters and Metallic Solutions using Two Organisms (*Lactuca sativa and Daphnia magna*)

Anne Priac, Amandine Poupeney, Coline Druart and Grégorio Crini*
Chrono-Environment, UMR 6249 usc INRA, University of Franche-Comté/CNRS, 16 route de Gray, 25000 Besançon, France

Abstract

Surface treatment industrial discharge water is a complex anthropogenic source of pollutants, including organic pollutants (PAHs, VOCs…) and numerous metal ions. We attempted to identify the main toxicants comparing impact assessment of real polycontaminated effluents and reconstituted polymetallic solutions via ecotoxicological bioassays performed with *Daphnia magna* immobilization test (24 h) and *Lactuca sativa* germination test (168 h). We focused first on 2 (Ni and Zn) then on 5 metals (Ni, Zn, Co, Cr, Al). Our results showed differences between metal toxicity order: Zn>Al>Ni>Cr~Co, for daphnids and Ni>Zn>Al~Co>Cr for lettuce. However, discharge waters remained more toxic than synthetic solutions: those 5 metals were not entirely responsible for the discharge water ecotoxicity. We also found *D. magna* to be more sensitive than *L. sativa*. This last assessment should be interpreted with care, knowing that immobilization and germination tests are respectively acute and chronic toxicity bioassays. Thus, battery tests are appropriate to evaluate industrial discharge water samples, and should be increasingly used as eco toxicological standards.

Keywords: Bioassay; Heavy metals; Reconstituted solutions; Waste water

Introduction

Industrial discharge waters, especially those from the surface treatment (ST) industry, released into the aquatic ecosystems have their own set of various environmental and sanitary issues, due to the fact that various loads of hazardous substances including: metallic trace elements (MTE; mostly Zn, Ni, Cu, Cr, Sn and Al), organic matter (oils, solvents, etc.) and diverse organics such as polycyclic aromatic hydrocarbons (PAHs) and volatile organic compounds (VOCs) [1]. ST industries are, like other industrial sectors, subject to specific release regulations, notably for metals. Although the discharge waters usually respect the regulatory standards, the present metals could be assimilated by fauna and flora and thus lead to long term toxic effects on the environment [2,3]. Nowadays, while pollutant mixtures present in discharge water after treatment are relatively easy to characterize chemically, assessing their impact on the environment is usually difficult and has rarely been reported [4]. Finally, the toxicity of treated ST waste remains poorly defined.

To assess the biological and chemical quality of water, 4 main kinds of approach can be used: (1) Chemical analysis to characterize the water mass studied qualitatively and quantitatively, (2) Comparing the analytical data to ecotoxicological information available in the literature to reach an *a priori* assessment of the hazard of substances (as in Draft Assessment Reports for pesticides), (3) Laboratory bioassays to assess the toxicity of substances and (4) *in situ* studies using native organisms or via active bio indication to assess the risk of natural populations exposed to substances released in the environment. Laboratory bioassays for water quality assessment are numerous and offer a large choice of indicators [5-7]. Three different types of standardized bioassays are the most commonly used, notably for the regulatory framework for chemicals management. They represent 3 trophic levels: primary producers with algae, primary consumers with crustaceans and secondary consumers with fish. Among them, the short-term bioassay based on the immobilization of a freshwater crustacean, *Daphnia magna*, is a test also used in the ecotoxicological assessment of industrial discharge waters. Nevertheless, it was pointed out that toxicity strongly relies on the choice of bio indicators and the endpoints used in the bioassays since sensitivity varies among taxonomic groups and species [8-10]. Consequently, it may be very useful to assess discharge water thanks to various bio-indicators in order to increase the ecological representativeness, to include a panel of sensitivity and to avoid a major risk of environmental effects and toxicity underestimation [11,12]. Recently, phytotoxicity tests using plants such as *Lactuca sativa* have been also proposed to assess the impact of industrial effluents by our group for the first time [3]. Our results demonstrated that these tests were simple, quick and reliable. Moreover, the use of these bioassays also presented the advantage of being inexpensive and not requiring major equipment as also reported in other works [8,10,13]. However, these tests were mainly used to assess the toxicity of single substances, such as metals (Table 1) [10,14-28] and there is a lack of studies concerning the impact of complex matrices such as discharge waters [29] or synthetic solutions of several metals.

The aim of this work was to assess the environmental impact of industrial discharge waters poly-contaminated with metals and to determine which metal(s) is (are) most responsible for the toxicity through the use of 2 bio-indicators *Daphnia magna* and *Lactuca sativa* via reconstituted solutions.

Materials and methods

Toxicity bioassays

Standardized germination tests [30] were performed following the method previously described in detail by Charles et al. [3]. The test assessed the germination of 30 plump lettuce seeds (*Lactuca sativa*

***Corresponding author:** Grégorio Crini, Chrono-Environment, UMR 6249 usc INRA, University of Franche-Comté/CNRS, 16 route de Gray, 25000 Besançon, France, Tel: +33381665701; E-mail: gregorio.crini@univ-fcomte.fr

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Element	Bioassay indicator	Index	Endpoint	Concentration [mg L^{-1}]	Reference
Zn	*L.sativa (var n.r.)*	96h EC_{50}	Root elongation	1	[14]
	P.subcapitata	72h EC_{50}	Growth rate	0.042	[15]
	D.magna	48h LC_{50}	Death	0.970	[16]
	G.pulex	48h LC_{50}	Death	4.920	[17]
Ni	*L.sativa (var Tro.)*	NOEC	Growth rate	1.8	[10]
	P.subcapitata	96h EC_{50}	Population	0.233	[18]
	D.magna	48h LC_{50}	*n.r.*	6.9	[19]
	G.sp	96h LC_{50}	*n.r.*	13	[20]
Cr	*L.sativa (var Rav.)*	72h EC_{50}	Growth rate	5.9	[21]
	P.subcapitata	72h EC_{50}	Population	0.030	[22]
	D.magna	48h LC_{50}	Immobilization	0.290	[23]
	G.pulex	48h LC_{50}	Death	0.809	[24]
Co	*D.magna*	48h LC_{50}	Death	4.4	[25]
Al	*D.magna*	48h LC_{50}	Immobilization	3.9	[26]
Cu	*L.sativa (var n.r.)*	96h EC_{50}	Root elongation	3	[14]
	P.subcapitata	72h EC_{50}	Growth rate	0.020	[15]
	D.magna	48h LC_{50}	Death	0.0111	[27]
	G.pulex	48h LC_{50}	Death	0.047	[28]

Specific lettuce varieties: *n.r.,* non reported; *Tro.,* Trocadero; *Rav.,* Ravel
End-points: LC_{50}, lethal concentration for 50% of the individuals tested; NOEC, no observed effect concentration.

Table 1: Toxicities of metallic trace elements (published data) on different organisms (*Lactuca sativa*, *Daphnia magna*, *Pseudoskirchneriella subcapitata* and *Gammarus pulex* or *sp.*).

Sample	Concentrations [mg L^{-1}]		EC_{50} [% of DW]	Germination (%)
	Ni	Zn	*D. magna*	*L. sativa*
DW1	0.34	1.91	17	54
DW2	0.28	1.51	5.2	58
DW3	0.54	2.46	21	42
DW4	0.35	1.84	11.3	48
DW5	0.51	2.06	32	44

Every control GR was higher than the required 90% of seed germination
All concentrations were above the quantification limits

Table 2: Ecotoxicity (EC_{50} and GR) of different discharge water samples (DW1 to DW5) on *Daphnia magna* and *Lactuca sativa* respectively in relation to Zn and Ni concentrations.

(L.) *var* Batavia Dorée de Printemps) watered with Reverse Osmosis Water ROW (controls; pH=6 ± 0.2), Discharge Water DW or Synthetic Solution SS, in triplicates, for 7 days, in the dark at 24 ± 1°C, on a filter paper substrate. As recommended by the standard, DW or SS pH must be between 5.5 and 9. To validate the test, germination rates (GR) of controls must be higher than or equal to 90%.

Bioassays using *Daphnia magna* were carried out by an accredited analysis laboratory (Carso, Lyon, France). The test was performed according to the "Inhibition Protocol Mobility" described in the standardized biomonitoring test ISO [31].

Industrial discharge waters & synthetic solutions

Five DW (denoted DW1 to DW5) were firstly collected in a ST company in Franche-Comté over a one-year period. Effluents were average sample characteristic of that day's activity. As the 2 main issues to be dealt with in DWs were Ni and Zn (specific company threshold emission values for these 2 MTE were 3.5 mg L^{-1}), DW5 Ni and Zn concentrations were mimicked in single and binary solutions S: S1 (Zn=2 mg L^{-1}), S2 (Ni=0.5 mg L^{-1}) and S3 (Zn=2 mg L^{-1} and Ni=0.5 mg L^{-1}). Each solution ecotoxicity was evaluated with germination test. We also determined Ni and Zn EC_{50} (concentrations range: 0 to 300 mg L^{-1}) for *L.sativa* and *D.magna*.

Four others DWs (DW6 to DW9) were then collected in the same company. Ni, Zn, Al, Cr and Co concentrations of these DWs were mimicked in mixture SS denoted SS6 to SS9. Each solution ecotoxicity was evaluated with germination and immobilization test (Table 2). We also determined Al, Co and Cr EC_{50} (concentrations range: 0 to 1000 mg L^{-1}) for *L.sativa* and *D.magna*.

For each of these 9 DWs, EC_{50} (expressed in percentage of DW) was determined through lettuce germination and daphnids immobilization tests. DWs samples were diluted with ROW. Every metallic synthetic solutions were prepared in ROW from sulfate salts of Al, Co, Cr, Ni and Zn (purchased from Fisher Scientific, France).

Chemical analyses

For each DW sample and synthetic solution, pH was determined (pH meter, model 3110, WTW, Alès, France). Metal concentrations were measured by spectrophotometry (cuvette test and/or reagent tests; portable Spectroflex 6100, WTW, Alès, France) or by ICP-AES (ThermoFisher, iCAP 6500 radial model, Courtaboeuf, France) after acid digestion for DWs, following a previously reported method [1]. All results are expressed in mg L^{-1}.

Statistical analysis

Germination rates of control, DW5, S1, S2 and S3 were compared using the Kruskal-Wallis test, with a significance threshold of $p<0.05$. All statistical analyses were performed with R (2.15.1) (R Development Core Team, 2013). Dose-dependent curves and EC_{50} values were calculated with Hill's model using the macro Excel Regtox free version EV 7.0.6.

Results and discussion

The toxicity of the first 5 DWs was studied through 2 bio-indicators (Table 2). The results showed deleterious effects on both bio-indicators since EC_{50} were low for daphnids (below 32%) and lettuce seed germination rates were significantly lower than those of controls (>90%). Due to activities of the industry focused on in our study, investigations of toxicity were firstly led on Ni and Zn. Concentrations

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Bio-indicator	EC_{50} [mg L^{-1}]				
	Al	Co	Cr	Ni	Zn
D.magna	8.45	11.69	10.36	9.8	6.35
L.sativa	237	247.7	265	58.3	154.3

Table 3: EC_{50} values for daphnia and lettuce for 5 MTE detected in DW samples ICP-AES analysis (Al, Co, Cr, Ni and Zn).

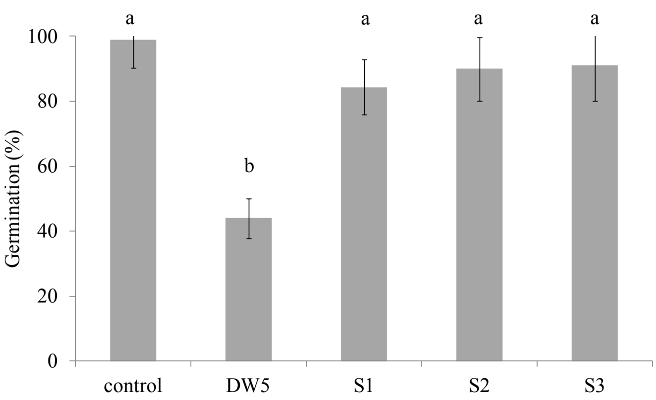

Figure 1: Germination (%) of *L. sativa* seeds for the DW5 sample and synthetic solutions S1, S2 and S3. Different letters indicated significant differences (p-value <0.01).

Sample	Metal ion [mg L^{-1}]					EC_{50} [% of DW or SS]	
	Al	Co	Cr	Ni	Zn	*D. magna*	*L. sativa*
DW6	5.09	2.75	0.15	0.62	2.67	6.1	45
SS6	5.06	2.72	0.15	0.63	2.69	56.8	86
DW7	5.70	4.05	0.35	0.74	1.97	5	68
SS7	5.58	4.06	0.24	0.70	1.96	52.1	84
DW8	2.66	1.69	0.24	0.31	1.45	ND	66
SS8	3.64	2.28	0.29	0.32	1.49	72.2	84
DW9	5.36	3.58	0.25	0.40	2.05	18.4	68
SS9	5.26	3.71	0.26	0.41	2.79	47.9	86

ND, not determined
All concentrations were above the quantification limits

Table 4: Concentrations of 5 metals (mg L^{-1}) in 4 discharge waters (DW6 to 9) and synthetic solutions (SS6 to 9) in relation to toxicity on *D. magna* and *L. sativa* (EC_{50} in % of DW or SS).

of both these suspected toxicants are presented in Table 2 and showed daily variability (as previously reported by Charles et al. [3]). From an analytical point of view, the chemical composition in Ni and Zn can be ranked as follows: DW3 > DW5 > DW1 ~ DW 4 > DW2. For the 2 bioassays, the sample toxicity range (decreasing order) was:

- DW2 > DW4 > DW1 > DW3 > DW5 for *D. magna* and
- DW3 > DW5 > DW4 > DW1 > DW2 for *L. sativa*.

The more toxic DWs for *D. magna* were the less toxic for *L. sativa*. This was also confirmed by Castillo et al. [32] studying the impact of final tannery industrial effluent (daphnids EC_{50} 24 h=77.9%) and lettuce ($EC_{50\text{-root growth}}$ 120 h>90%). Despite its low coefficient of variation CV (14%), it appeared that lettuce DW toxicity could be linked to Ni and Zn concentrations (except for DW4 and DW1 which were inverted). For daphnids, no correlation was shown (CV=59%). This variability was explained by the production activity, as suggested by Hitchcock et al. [33] who calculated a CV reaching 133.7% for the mortality of nematodes exposed to industrial effluents (pulp and paper industries).

To verify the hypothesis that Ni and Zn concentrations in the DW can be linked to lettuce ecotoxicological response, we ran germination tests on synthetic solutions S1, S2 and S3 containing Ni and Zn, alone or in a mixture, in the same concentrations as those found in DW5. We also performed ecotoxicological tests on both *D. magna* and *L. sativa* (Table 3), to assess individual EC_{50} of nickel and zinc. The results showed that GR of single (S1 and S2) and binary (S3) synthetic solutions were not significantly different from the control (Figure 1). This result was not surprising in regard to the values of EC_{50} determined in *L. sativa* for Ni and Zn (Table 3) which were far above the concentrations found in DWs. However, the EC_{50} results were not expected considering those found in the literature (Table 1). Indeed, toxicity values for other endpoints were much lower, of the order of 1 mg L^{-1}, both for Zn and Ni [10]. This major disparity could be explained by the variety of lettuce used for the assay, as criticized by Priac et al. (unpublished work) who demonstrated that among 4 varieties, Batavia (used in the present paper) was the least sensitive. Significant differences were found between the germination rates of lettuce exposed to synthetic solutions of Zn and Ni and those exposed to the DWs at same concentrations (Figure 1). Similar experiments and interpretations were reported by Yoo et al. [34] with Cu, Ag and cyanides, to reproduce an effluent from a lead frame manufacturing factory. Unlike our results, those of Yoo et al. [34] demonstrated that these 3 substances were responsible for the toxicity of the effluent on daphnids since they observed a similarity in the toxicity of the real and the synthetic effluents. In the present study, the DW toxicity observed on *L. sativa* was not explained only by the presence and the concentrations in Zn and Ni.

Investigations were conducted on a larger number of metals potentially responsible for the toxicity of DWs. Among 23 elements measured, 15 were present at quantifiable levels at least once, and 5 of them (Al, Co, Cr, Ni and Zn) were selected for the following experiments owing to their concentrations in DW6 to DW9 (higher than 1 mg L^{-1}; Table 4) and/or their known effects on the environment (Table 1). DWs 6, 7 and 9 appeared to be much more toxic (EC_{50} 6.1, 5, 18.4% of the sample) than their respective SS (56.8, 52.1, 47.9% of the solution tested) on *D. magna*. Results showed the same tendency for the GR of *L. sativa*, but not as dramatic: for instance daphnid EC_{50} values were 5 and 52.1% for DW7 and SS7, respectively, whereas lettuce EC_{50} values were 68 and 84%. Like for Ni and Zn, the presence of Al, Co and Cr did not explain all the DW toxicity on both *D. magna* and *L. sativa*, even though daphnid EC_{50} values showed these 5 metals to be toxic (Table 3).

To our knowledge, few studies have assessed the environmental impact of discharge water or synthetic solutions on more than one bio-indicator [7,12,14,35,36]. Bioassay batteries have already been shown to be a relevant way to evaluate toxicity, irrespective of the ecosystem studied [7,36,37]. Sensitivity differences observed between daphnids and lettuce (Tables 2-4) also occurred on comparison with data from the literature (Table 1). General differences can be explained by bioassay endpoint (acute or chronic toxicities) or protocol variability (bioindicator subspecies or cultivars, animal gender, lapse of exposure, number of individuals per Petri dish or tube, etc.; [38]). Yet, it appears that differences between bioindicator sensitivity remain in bibliographic data. For 3 metals for which we found comparative results (Zn, Ni, Cr), toxicity ranges were different for lettuce (Zn<Ni<<Cr) compared to algae, daphnids and gammarids (Cr<Zn<<Ni) as described in Table 1. Table 4 also shows single EC_{50} differences between indicators: toxicity range for daphnids being (from less to more toxic) Cr, Ni and Zn while the lettuce toxicity range was Cr, Zn and Ni. Another difference

between these 2 bioindicators was related to the order of magnitude of the EC_{50} (e.g. lettuce nickel EC_{50}: 58.3 mg L^{-1} vs daphnids: 9.8 mg L^{-1}).

Conclusions

In this study, the 2 bioindicators *Lactuca sativa* and *Daphnia magna* were proved to be pertinent to assess the ecotoxicity of polycontaminated discharge water from the surface treatment industry. The results showed that metal-based synthetic single and mixed solutions were less toxic than the discharge water, meaning that the ecotoxicity of these effluents could not be explained only by the 5 metals chosen in this work. Consequently, it would be interesting to lead future investigations not only towards a more exhaustive determination of the chemical composition of discharge water but also possible interactions (e.g. additivity, antagonism, synergy) between metals and/or trace organics and/or other minerals. Results also demonstrated that lettuce was more resistant than daphnids to the discharge waters and synthetic solutions. Ecotoxicological assessments complete chemical analyses as they integrate all chemical interactions. As reported in this study the use of a battery of tests was a relevant tool to include the whole variability of toxicity.

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Conflict of Interests

The authors declare that they have no conflict of interest.

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