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Proposal of a new ecotoxicity evaluation tool based on morphological responses of five helophytes to mixtures of pollutants: The Helophyte Development Index

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ABSTRACT

Industrial effluents discharged into the environment may have ecotoxic effects even if they come up to regulatory standards. Chemical evaluation of treatment performance by end-of-pipe treatment systems is thus not sufficient, especially when mixtures of metallic and organic contaminants are concerned. Given that contamination may alter biological characteristics of the environment, biomonitoring studies may provide information on integrated ecotoxical effects. However, there is a need for bioassays purpose-designed for direct use at industrial sites. Many biomonitoring tools already exist and have been proved to be efficient for evaluating the ecotoxicity of contaminated waters, but most of them require laboratory equipment. In this study, an experiment in microcosms under controlled conditions of pollution was carried out to assess the morphological responses of five helophytes exposed to mixtures of organic and/or metallic pollutants. The criteria of plant growth and development, i.e. aerial elongation and leaf senescence, that were the most relevant for reflecting the ecotoxicity of contaminant mixtures and that could be monitored on-site with a user-friendly method, were then selected. Focusing on these selected criteria, a new bioindicator tool, named the Helophyte Development Index (HDI), was created. Our results suggest that the HDI is a promising tool to use on-site for assessing the ecological state of waters released in aquatic environment by industrial factories, following the recommendations of the European Water Agency.

1. Introduction

Member states of the European Union have to achieve good chemical and ecological status of water bodies by 2015 (European Council, 2000). To this purpose, chemical regulation levels are defined for industrial wastewaters released in the environment (European Union, 1976). Nevertheless, industrial wastewaters are characterized as complex mixtures with varying concentrations of pollutants (Soupilas et al., 2008) and given that interactions between contaminants frequently occur (Chen et al., 2004; Millward et al., 2004), the ecotoxicity of purified industrial wastewaters may not always be equal to the sum of the ecotoxicity of each contaminant. Effluents discharged into the environment may thus have ecotoxical effects even if each chemical is present at a level below regulatory standards (Charles et al., 2011). This stresses the importance of complementing the chemical approach with the ecotoxical one to better assess the quality of industrial wastewaters (Hoshina and Marin-Morales, 2009; Mendonça et al., 2009; Zhou et al., 2008) before their release in aquatic bodies. While effective off-site tools do exist to assess the chronic and acute toxicity of wastewaters (e.g. whole effluent toxicity test

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methods, US EPA, 2000), user-friendly bioassays that could be used directly on-site are still needed (Guittonny-Philippe et al., 2014; Jones et al., 2010; Libralato et al., 2010) for a more widespread use.

To this end, several organisms could be used (Sims et al., 2013), e.g. macroinvertebrates (Mondy et al., 2012), bryophytes (Bleuel et al., 2005), mussels (Monirith et al., 2003), daphnia (Martins et al., 2007), lichens (Monnet et al., 2005), microalgae (Araújo and Souza-Santos, 2013), fishes (Yeom et al., 2007), bacteria (Soupilas et al., 2008) or aquatic plants (Bonanno, 2012; Lewis, 1995; Haury et al., 2006; Trémolières et al., 2007). Among these organisms, macrophytes integrate temporal, spatial, chemical, physical, and biological qualities of their ecosystem (Lacoul and Freedman, 2006; Rambaud et al., 2009) and simple measurements based on morphological observations may indicate harmful effects of exposure to contaminants (Zhou et al., 2008). Aquatic plant species exhibit multifaceted responses to industrial pollutant mixtures, that are dependent on the species exposed (Deng et al., 2006; Kearney and Zhu, 2012; Zhang et al., 2010) and on the characteristics of pollutants, including their concentrations, their chemical types and the potential interaction between them (Babu et al., 2001; Lin et al., 2008; Zhang et al., 2011). For these reasons, macrophytes could be appropriate bioindicators in industrial context, for an *in situ* use.

The aim of our study was to assess five helophytes' morphological responses towards mixtures of pollutants mimetic of industrial effluents (in a full factorial design) and to create a user-friendly index based on these responses. In this article, we report a new methodology – named the Helophyte Development Index (HDI) – which could have potential further applications for ecotoxicity assessment in European industrialised catchments.

2. Material and methods

2.1. Planted microcosm set up

Plantlets from five helophyte species commonly found in European water bodies and exhibiting different biological traits, i.e. *Alisma lanceolatum* With. (Alismataceae), *Carex cuprina* (Sandor ex Heuff.) Nendtv. ex A. Kern. (Cyperaceae), *Epilobium hirsutum* L. (Onagraceae), *Iris pseudacorus* L. (Iridaceae) and *Juncus inflexus* L. (Juncaceae), were collected from a polluted wetland (South of the Berre lagoon, South-East France; WGS 84 GPS coordinates: longitude: E 6,426519; latitude: N 43,359009, Guittonny-Philippe et al., 2015a) and maintained in the greenhouse for 4 months of vegetative reproduction before experiment. The experiment has been designed in order to distinguish metallic and organic pollutant effects on plants and the possible interactions between both types of contaminants in a full factorial design, as recommended by Lewis et al., 1999. Twenty microcosms consisting of rectangular plastic tanks (413 × 345 × 294 mm, length × width × depth) filled up with pozzolan were implemented, as previously described (Guittonny-Philippe et al., 2015a). The microcosms were planted with six plant individuals per species X condition. For each species, one microcosm was kept without contamination and served as control and three other microcosms were independently exposed to three different pollutant mixtures.

2.2. Chemicals and exposure phases

Three types of pollutant mixtures mimetic to industrial effluents were added in the microcosms, as previously described (Guittonny-Philippe et al., 2015a):

- A metallic pollutant mixture (MPM) consisting of an aqueous mixture of eleven metallic salts, i.e. $AlCl_3{\cdot}2H_2O$; AsO_3; $CdSO_4{\cdot}8H_2O$; $K_2Cr_2O_7$; $CuSO_4$; $Fe_2O_{12}S_3$; $MnSO_4{\cdot}4H_2O$; $NiSO_4{\cdot}7H_2O$; $Pb(NO_3)_2$; $SnCl_2$; $ZnCl_2$.
- An organic pollutant mixture (OPM) composed of total hydrocarbons (THC) (i.e. Blend Arabian Light petroleum topped at 250 °C (BAL 250)), as well as phenanthrene (PHE) and pyrene (PYR) obtained in reagent quality from Merck (Germany) and an anionic detergent linear alkylbenzene sulfonate (LAS) named CARPHEM®.
- An organic and metallic pollutant mixture (OMPM) containing both types of contaminants at concentration levels identical to the ones used for the MPM and the OPM.

Two main criteria were considered for the choice of contaminants: ubiquity of selected chemicals in industrial context (Haritash and Kaushik, 2009; Megharaj et al., 2011; Wasi et al., 2013) and their potential ecotoxicity in mixtures (Banat et al., 1974; Hernández-Soriano et al., 2011; Thavamani et al., 2012; Radić et al., 2010; Zhang et al., 2011).

After 40 days of plant acclimatisation in microcosms (Guittonny-Philippe et al., 2015a), exposure to the artificial effluents was conducted in the microcosms for 113 days in three successive pollution events. In the first phase, pollutant concentration levels in the artificial effluents were set at the European environmental quality standards (Table 1, European Union, 1976). In the second and third phases (that began on days 35 and 70, respectively), pollutant concentrations in the artificial effluents were ten times higher than the European environmental quality standards, except for the anionic detergent LAS whose concentration was equal for the three pollution phases. In all the microcosms, 25 mL of an organo-mineral fertilizer (NutriActiv®, NF U 42-001 produced by FLORENDI JARDIN SAS) containing 3% of total nitrogen, 3% of total P_2O_5 and 3% of water-soluble K_2O was also added at the beginning of the first test-phase.

2.3. Physico-chemical and plant parameter monitoring

In each microcosm, pH was monitored with a portable pH meter (Hanna Instruments®). Electrical conductivity (EC, µS/cm), dissolved oxygen level (DO, percentage of saturation % 0_2/L), and temperature (T, °C) were monitored with a WTW® device. These measurements were repeated at least once per month. Aerial height (height of the longest leaf or shoot) and number of leaves (green, senescent or dead) of each plant individual were monitored at least every two weeks during the experiment. A leaf was considered senescent when at least one third of its surface was yellow or brown (Holopainen et al., 2010). Heights were measured

Table 1
Metal and organic pollutant concentrations that industrials are authorised to release in aquatic bodies (European Union, 1976).

Chemicals	European environmental quality standard (authorized concentrations in released effluents) (mg/L)
Al	2.5
As	0.05
Cd	0.2
Cr	0.5
Cu	0.5
Fe	2.5
Mn	1
Ni	0.5
Pb	0.5
Sn	2
Zn	2
PHE	0.05
PYR	0.05
THC	10
Anionic detergent LAS	10

from the level of the pozzolan surface. The biomonitoring data were recorded in 13 times of measurements along the experiment.

2.4. Chemical analysis

Before enriching microcosms with the artificial effluents, three water aliquots were taken in the pollutant mixtures in order to determine the real value of initial concentrations of pollutants added in the microcosms (Guittonny-Philippe et al., 2015a). At the end of each test-phase, water samples were collected in the contaminated microcosms to determine the residual concentrations of pollutants. Rhizospheric pozzolan (pozzolan in contact with plant roots) samples were taken at the end of the third test-phase in each microcosm in order to analyse metals (in all microcosms) and organic pollutants (Guittonny-Philippe et al., 2015a). At this time, five plant individuals per microcosm were harvested for metal analysis in plant biomass (Guittonny-Philippe et al., 2015b).

Samples were analysed for Al, Cd, Cr, Cu, Fe, Mn, Ni, Pb, Zn content by inductively coupled plasma-atomic emission spectrometry (ICP-AES; Sn was not quantified because of analytical constraints linked to the presence of spectroscopic interferences with other elements and to the low sensitivity obtained with the available apparatus), as previously described (Guittonny-Philippe et al., 2015a).

The concentration of anionic detergent LAS was monitored by MBAS analysis according to EPA 425.1 method (Clesceri et al., 1998).

Briefly, for the analysis of phenanthrene (PHE), pyrene (PYR) and total hydrocarbons (THC), water samples were extracted by liquid–liquid extraction with *n*-hexane. Sample extracts were reduced to 1 mL and analysed using a gas chromatograph (7890A GC System, Agilent Technologies, USA) coupled to a 7000 Triple Quad mass spectrometer, equipped with an HP-5MS silica fused capillary column (30 m × 0.25 mm inner diameter × 0.25 μm film thickness). The quantification of PHE and PYR was performed by using chrysene-D12 as surrogate and phenanthrene-D10 as internal standard. The amount of THC was determined as the sum of resolved and unresolved components eluted from the GC capillary column between the retention times of *n*-decane and *n*-tetracontane. The specific conditions used for extractions together with the chromatographic and mass spectrometry parameters have been previously detailed (Guittonny-Philippe et al., 2015a).

2.5. Statistical analysis

For each species in control and OPM, MPM or OMPM conditions, growth traits (aerial height and proportion of green leaves) were analysed using an univariate analysis of repeated measures (rmANOVAs) since models were set up of independent orthogonal components (Von Ende, 2001). Two-tailed Student's *t*-tests (for comparison of two means with equal variances assessed by F-test) were performed to test significant differences between control and MPM, OPM or OMPM plants in growth traits, at each date of plant measurement. In case of unequal variances, two-tailed *t*-test with Welch's correction was carried out.

Data were analysed statistically using GraphPad Prism version 6.00 for Windows, GraphPad Software.

Table 2
Contaminant concentrations in water of control microcosms at the end of the experiment (mean of microcosms, $n = 5$). n.m.: not measured.

Chemicals	Concentrations in control microcosms (mg/L)
Al	0.24 ± 0.20
As	<0.03
Cd	<0.01
Cr	0.025 ± 0.001
Cu	0.02 ± 0.01
Fe	0.19 ± 0.29
Mn	0.15 ± 0.29
Ni	0.03 ± 0.03
Pb	<0.04
Sn	n.m.
Zn	0.04 ± 0.03
PHE	$0.27 \times 10^{-3} \pm 0.03 \times 10^{-3}$
PYR	$0.10 \times 10^{-3} \pm 0.003 \times 10^{-3}$
THC	0.23 ± 0.03
Anionic detergent LAS	n.m.

3. Results and discussion

3.1. Fate of pollutants in microcosms during the test-phases

In every microcosm and for all the contaminants, mean aqueous concentrations in water at the end of each test-phase (Tables 2–5) were below the water regulatory limits that industrial factories are authorized to release in natural environments (Table 1, European Union, 1976), except for Mn in OMPM microcosms that was slightly over 1 mg/L at the end of phases 2 and 3 (Table 3). In MPM microcosms, mean removals of metals varied from 52% for Mn to 98% for Cu in the first test-phase, while mean removals of metals were all over 88% in the second and third test-phases. In OPM microcosms, mean removals of organic pollutants were comprised between 64% for THC to 99% for PHE in the first test-phase, and were all over 87% in the second and third test-phases. In OMPM

Table 3
Contaminant concentrations in water of OMPM microcosms at the end of the test-phases (mean of microcosms, $n = 5$). n.m.: not measured.

Chemicals	Organic pollutant and metal concentrations (mg/L) in water of OMPM microcosms		
	Phase 1	Phase 2	Phase 3
Al	0.33 ± 0.34	0.34 ± 0.21	0.25 ± 0.27
As	<0.03	<0.03	<0.03
Cd	<0.01	0.017 ± 0.017	0.005 ± 0.02
Cr	0.013 ± 0.03	0.021 ± 0.007	0.018 ± 0.004
Cu	0.01 ± 0.01	0.02 ± 0.01	0.01 ± 0.01
Fe	0.28 ± 0.22	0.54 ± 0.32	0.17 ± 0.07
Mn	0.59 ± 0.23	1.70 ± 0.38	1.21 ± 1.11
Ni	0.02 ± 0.01	0.08 ± 0.05	0.28 ± 0.20
Pb	<0.04	<0.04	<0.04
Sn	n.m.	n.m.	n.m.
Zn	0.06 ± 0.04	0.23 ± 0.08	0.17 ± 0.11
PHE	$0.28 \times 10^{-3} \pm 0.06 \times 10^{-3}$	$3.53 \times 10^{-3} \pm 1.61 \times 10^{-3}$	$0.85 \times 10^{-3} \pm 0.24 \times 10^{-3}$
PYR	$1.66 \times 10^{-3} \pm 0.15 \times 10^{-3}$	$2.55 \times 10^{-3} \pm 0.45 \times 10^{-3}$	$0.76 \times 10^{-3} \pm 0.11 \times 10^{-3}$
THC	3.42 ± 2.76	0.28 ± 0.07	0.79 ± 0.30
Anionic detergent LAS	0.44 ± 0.14	0.61 ± 0.14	0.22 ± 0.06

Table 4
Organic pollutant concentrations in water of OPM microcosms at the end of the test-phases (mean of microcosms, $n = 5$).

Chemicals	Organic pollutant concentrations (mg/L) in water of OPM microcosms		
	Phase 1	Phase 2	Phase 3
PHE	$0.29 \times 10^{-3} \pm 0.03 \times 10^{-3}$	$3.53 \times 10^{-3} \pm 2.12 \times 10^{-3}$	$0.68 \times 10^{-3} \pm 0.23 \times 10^{-3}$
PYR	$1.64 \times 10^{-3} \pm 0.09 \times 10^{-3}$	$3.62 \times 10^{-3} \pm 1.05 \times 10^{-3}$	$1.18 \times 10^{-3} \pm 0.58 \times 10^{-3}$
THC	2.53 ± 1.83	0.73 ± 0.45	0.55 ± 0.29
Anionic detergent LAS	0.44 ± 0.11	0.59 ± 0.19	0.24 ± 0.05

microcosms, mean removals of metals were comprised between 53% for Mn to 98% for Cu, and mean removals of organic pollutants were between 52% for THC and 96% for PYR in the first test-phase. In the second and third test-phases, mean removals of metals were all over 85%, and mean removals of organic pollutants were all over 87%.

The strong decrease of aqueous metal concentrations is attributable to a set of geochemical reactions (e.g. metal precipitation as oxides, sulfides or carbonates, co-precipitation, complexation) depending on physico-chemical conditions, even if for some metals like Al, Fe or Mn, sorption in plants also significantly contributed in metal removal (Guittonny-Philippe et al., 2015b). The strong decrease of aqueous organic pollutant concentrations may have been caused by adsorption in plants (Simonich and Hites, 1995), biodegradation by plants and/or rhizospheric microorganisms (Atlas, 1981; Cerniglia, 1993; Gramss et al., 1999; Imfeld et al., 2009; Thoumelin, 1995), or adsorption in plastic tank sides or pozzolan (Dordio and Carvalho, 2013; Temmink and Klapwijk, 2004).

3.2. Morphological responses of helophytes exposed to the pollutant mixtures during the test-phases

The OMPM and the OPM limited the aerial elongation of two out of the five species, i.e. *I. pseudacorus*, *J. inflexus* (Figs. 1A and 2A) from the second test-phase. The OMPM and the MPM provoked an acceleration of the leaf senescence in three out of the five helophytes, i.e. *A. lanceolatum*, *E. hirsutum*, *J. inflexus* (Figs. 1B and 2B), from the second test-phase. These results are consistent with previous studies concerning impacts of organic pollutants (Adieze et al., 2012; Alkio et al., 2005; Chaîneau et al., 1997; Liu et al., 2004; Ma et al., 2010; Yu et al., 2006) and metals (Briat and Lebrun, 1999; Kabata-Pendias, 2011; Rascio and Navarri-Izzo, 2011) on plant growth and development. Consequently, for revealing the global health status of plants exposed simultaneously to metals and organic pollutants, we created the species development values "sdv" calculated on the basis of two morphological criteria: green leaves' proportion and relative size of plants.

Table 5
Metal concentrations in water of MPM microcosms at the end of the test-phases (mean of microcosms, $n = 5$). n.m.: not measured.

Chemicals	Metal concentrations (mg/L) in water of MPM microcosms		
	Phase 1	Phase 2	Phase 3
Al	0.55 ± 0.49	1.71 ± 2.66	2 ± 0.71
As	<0.03	<0.03	<0.03
Cd	<0.01	0.018 ± 0.02	0.10 ± 0.03
Cr	<0.02	0.024 ± 0.018	0.07 ± 0.05
Cu	0.01 ± 0.01	0.04 ± 0.04	0.11 ± 0.03
Fe	0.46 ± 0.26	0.66 ± 0.36	0.57 ± 0.12
Mn	0.61 ± 0.04	0.39 ± 0.29	0.09 ± 0.07
Ni	0.03 ± 0.00	0.27 ± 0.16	0.43 ± 0.16
Pb	<0.04	0.05 ± 0.02	0.05 ± 0.01
Sn	n.m.	n.m.	n.m.
Zn	0.09 ± 0.03	0.43 ± 0.35	0.93 ± 0.25

3.3. Development of the ecotoxical index on the basis of plant morphological responses

3.3.1. Calculation of the species development values "sdv"

Green leaves proportion of the plant individuals (Glp_i) is calculated as the number of green leaves of the plant individual (gl_i) divided by the total number of leaves (including senescing and dead leaves still attached to the stem) of the same plant individual (tl_i): $Glp_i = gl_i/tl_i$. On the basis of the results previously described, the Glp_i should traduce the alteration of plant development linked with the ecotoxicity of metals.

Relative size of the plant individuals (Rs_i) is calculated as the height of the plant individual (h_i; corresponding to the height of the longest shoot for *E. hirsutum* and of the longest leaf for the four other species) divided by the height of the plant individual of reference at the corresponding time of measurement (hr_i; corresponding to the height of the highest individual among control individuals at the time of measurement): $Rs_i = h_i/hr_i$. On the basis of the results previously described, the Rs_i should traduce the alteration of plant growth linked with the ecotoxicity of organic pollutants.

The species development values of each individual (sdv_i) are then calculated at each time of measurement, as follow: $sdv_i = (Glp_i + Rs_i) \times 10$.

The multiplication by a factor 10 enables obtaining scores of sdv_i on a scale of 20.

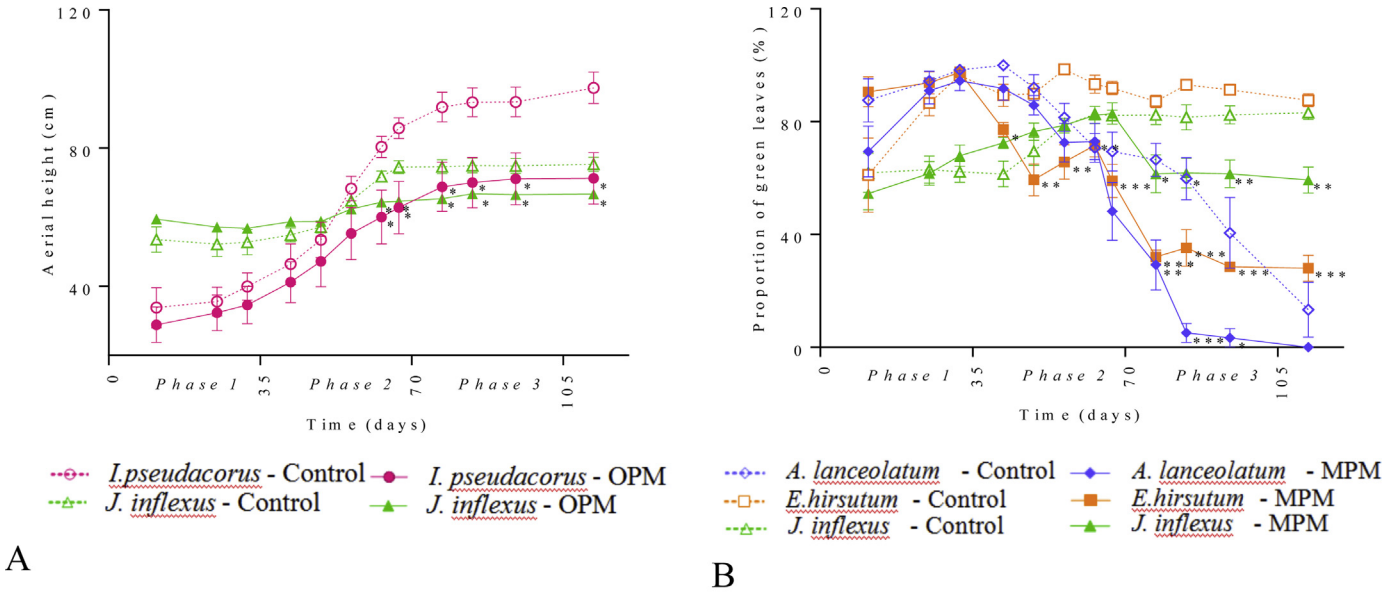
Then, the mean of sdv_i for each "species × condition" is calculated, to obtain the species development values (sdv) at each time of measurement which is expected to reflect the ecotoxicity of both metals and organic pollutants on the considered species.

3.3.2. Calculation of the HDI

In order to reveal ecotoxicity of pollutant mixtures, we created an index based on global responsive aerial traits and called it "Helophyte Development Index (HDI)". The HDI is calculated at each time of biometric measurement, by summing, for the n species considered ($n = 5$ in this study), the differences of sdv between control and contaminated conditions, when significant (non-parametric Mann–Whitney U-tests; P-value < 0.05):

$$HDI = \sum_{n \quad \text{species}} [sdv(control) - sdv(contaminated)]^{*}.$$

The theoretical maximum ecotoxical value of the HDI calculated with five species is +100 and is reached when all the plants in contaminated environment are dead while all the plants in control environment both reached the maximum height and do not have any dry or senescent leaves. In order to reveal the ecotoxicity of the OMPM, we calculated the HDI on the basis of the five species used in this study (Table 6).



C

	Proportion of green leaves						Aerial height			
	E. hirsutum		*A. lanceolatum*		*J. inflexus* (control vs MPM)		*I. pseudacorus*		*J. inflexus* (control vs OPM)	
Source of Variation	**F**	**P value**	**F**	**P value**	**F**	**P value**	**F**	**P value**	**F**	**P value**
Time X Treatment	20,57	< 0,0001	4,908	< 0,0001	9,185	< 0,0001	6,095	< 0,0001	4,48	< 0,0001
Time	15,2	< 0,0001	62,1	< 0,0001	13,51	< 0,0001	123,9	< 0,0001	21,47	< 0,0001
Treatment	224	< 0,0001	11,98	0,0072	1,76	0,2142	4,022	0,0727	0,7071	0,4201
Subjects (matching)	0,9445	0,4959	5,105	< 0,0001	13,87	< 0,0001	47,25	< 0,0001	14,74	< 0,0001

Fig. 1. (A) Impact of the OPM on aerial elongation of *I. pseudacorus* and *J. inflexus* (means ± SEM); (B) impact of the MPM on leaf senescence of *A. lanceolatum*,*C. cuprina*, and *E. hirsutum* (means ± SEM); and (C) results of the repeated measures ANOVA performed on the proportion of green leaves of *E. hirsutum*, *A. lanceolatum* and *J. inflexus* and on the plant aerial height of *I. pseudacorus* and *J. inflexus*. Asterisks associated with values at a given time indicate a significant difference (***$p \leq 0.001$; **$p \leq 0.01$; *$p \leq 0.05$) between the control and the contaminated plants (two-tailed Student's *t*-tests).

3.4. Use of the sdv and HDI for revealing the ecotoxicity of the OMPM

3.4.1. sdv of the five helophytes in control and OMPM microcosms

The sdv of *A. lanceolatum* in control and OMPM conditions became significantly different (Mann–Whitney *U*-tests, *P*-value ≤ 0.05) immediately after the second addition of the pollutant mixture (Fig. 3A). For this species, significant differences of sdv were observed on 6 out of 9 measurement times performed during the two last test-phases (Table 4).

No significant difference of sdv could be observed between control and OMPM conditions for *C. cuprina* on the 13 measurement times performed throughout the experiment, and sdv stayed quite constant, at a level over 15/20 (Fig. 3B).

The sdv of *E. hirsutum* in OMPM condition became significantly lower compared to the sdv in control condition, from the middle of the second test-phase, until the end of the experiment (Fig. 3C).

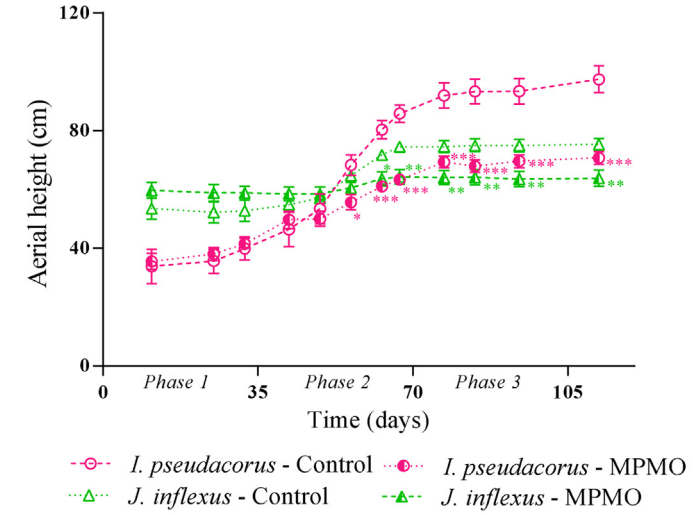
In control and OMPM conditions, *I. pseudacorus* had similar sdv from the beginning of the first test phase until the end of the second test-phase (Fig. 3D). Then sdv in control condition became significantly higher than that in OMPM condition (Table 4).

For *J. inflexus*, during the end of the first test-phase and the beginning of the second, sdv in the OMPM condition was significantly higher than that in the control condition (Fig. 3E). After the third addition of artificial industrial effluent, *J. inflexus* in the OMPM condition had a significantly lower sdv than in the control condition, until the end of the experiment.

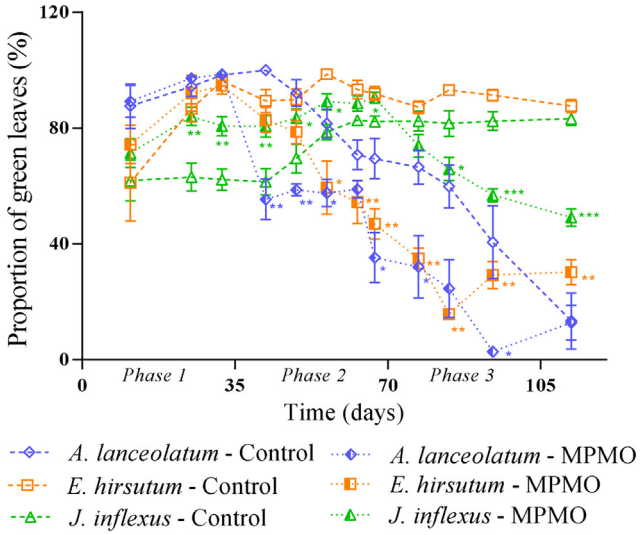
Significant sdv differences between the control and the OMPM conditions were always positive for *E. hirsutum*, *A. lanceolatum*, *I. pseudacorus* and *J. inflexus* after the 30th April, during the second and third test-phases. This means that the OMPM altered the development of these helophytes compared to tap water.

3.4.2. Use of HDI as ecotoxical index of OMPM water

The HDI, calculated on the basis of the significant sdv differences of the five plant species used in this study, reached a positive value from the moment pollutant concentrations in the OMPM went over regulation levels (after the beginning of the second test-phase, day 35), and it rose remarkably following a linear trend between the second phase and the beginning of the third phase (Fig. 4). Consequently, the HDI variation range revealed the harmful effects on the helophytes exposed to the OMPM, with pollution over the regulation levels. At the end of experiment, the HDI reached a plateau *ca.* +20 points. On one hand, the plateau may be due to the fact that we did not renew the feeding of the



A



B

	Proportion of green leaves						Aerial height			
	E. hirsutum		*A. lanceolatum*		*J. inflexus*		*I. pseudacorus*		*J. inflexus*	
Source of Variation	**F**	**P value**	**F**	**P value**	**F**	**P value**	**F**	**P value**	**F**	**P value**
Time X Treatment	17.11	< 0.0001	4.967	< 0.0001	23.49	< 0.0001	12.14	< 0.0001	13.23	< 0.0001
Time	14.08	< 0.0001	46.14	< 0.0001	13.60	< 0.0001	112.2	< 0.0001	35.46	< 0.0001
Treatment	311.6	< 0.0001	20.33	0.0015	0.2574	0.6229	11.85	0.0063	1.612	0.2329
Subjects (matching)	0.7220	0.7022	3.950	0.0003	11.44	< 0.0001	11.96	< 0.0001	21.25	< 0.0001

C

Fig. 2. (A) Impact of the MPMO on aerial elongation of *I. pseudacorus* and *J. inflexus* (means ± SEM); (B) impact of the MPMO on leaf senescence of *A. lanceolatum*, *C. cuprina*, and *E. hirsutum* (means ± SEM); and (C) results of the repeated measures ANOVA performed on the proportion of green leaves of *E. hirsutum*, *A. lanceolatum* and *J. inflexus* and on the plant aerial height of *I. pseudacorus* and *J. inflexus*. Asterisks associated with values at a given time indicate a significant difference (***$p \leq 0.001$; **$p \leq 0.01$; *$p \leq 0.05$) between the control and the contaminated plants (two-tailed Student's *t*-tests or Mann–Whitney *U*-test).

Table 6
Differences of sdv between control and OMPM conditions for the 5 helophyte species and HDI. Values in bold correspond to significant difference between the sdv ($p \leq 0.05$). n.r.: not relevant.

Date of measurement	Phase	Differences of sdv (sdv control – sdv OMPM)					HDI
		A. lanceolatum	*C. cuprina*	*E. hirsutum*	*I. pseudacorus*	*J. inflexus*	5 Species
19/03/2012	1	−5.2	0	−0.1	−0.6	−0.3	n.r.
30/03/2012		−4.0	0.3	1.4	−0.9	−1.9	n.r.
13/04/2012		−1.7	0.3	2.3	0.3	**−3.2**	−3.2
20/04/2012		−0.3	0.9	2.4	−0.3	**−2.8**	−2.8
30/04/2012	2	**4.5**	1.1	0.9	−0.6	**−2.5**	2
07/05/2012		**3.2**	1.0	1.7	0.8	−1.6	3.2
14/05/2012		**2.2**	1.2	**4.7**	0.5	−0.4	6.9
21/05/2012		1.1	1.1	**5.0**	**2.8**	0.5	7.8
25/05/2012		**3.5**	1.0	**5.5**	**3.0**	0.4	12
04/06/2012	3	**5.1**	1.1	**6.7**	**3.0**	**2.2**	17
11/06/2012		4.8	1.2	**9.4**	**2.8**	**2.9**	15.1
21/06/2012		**3.5**	0.9	**7.4**	**5.6**	**3.9**	20.4
09/07/2012		1.5	0.7	**5.8**	**8.4**	**4.8**	19

microcosms with artificial effluent. On the other hand, summer high temperatures may have induced the senescence of control plants, thereby, hiding the differences between control and OMPM conditions. All these results demonstrated that the HDI, as described here, provided a means of revealing the ecotoxicity of the artificial industrial effluent containing both organic and metallic contaminants. For a more polluted effluent, we could expect a supplementary increase of the HDI, given that some of the

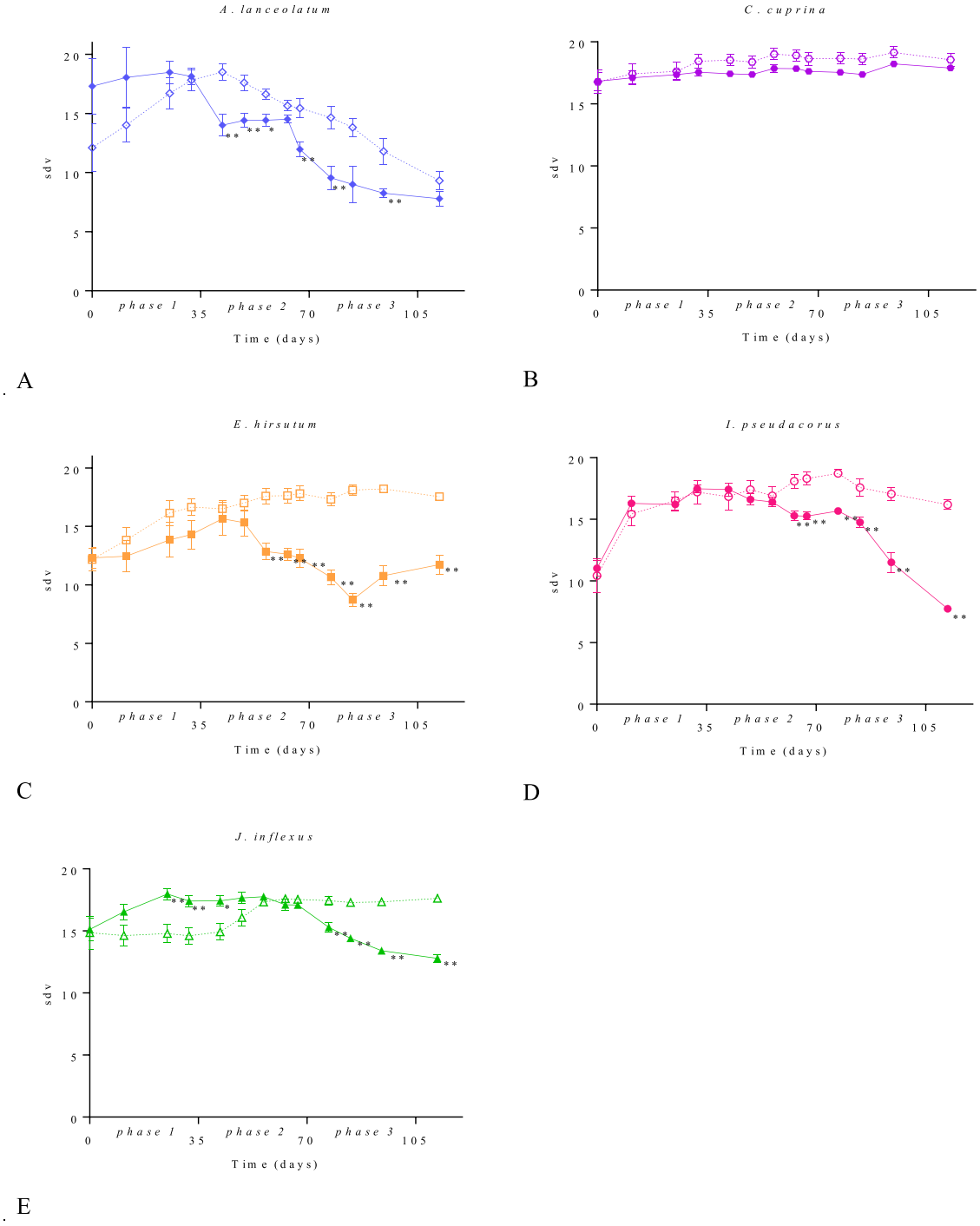


Fig. 3. sdv of control and OMPM conditions for (A) *A. lanceolatum*, (B) *C. cuprina*,(C) *E. hirsutum*, (D) *I. pseudacorus* and (E) *J. inflexus*. Control sdv are represented with empty symbols and dotted lines, OMPM sdv are represented with full symbols and continuous lines. Asterisks associated with values at a given time indicate a significant difference ($^{**}p \leq 0.01$; $^{*}p \leq 0.05$) between the sdv of control and OMPM conditions (Mann–Whitney *U*-tests).

used helophytes did not exhibit any change in their health status (no sdv differences between OMPM and control conditions) and none of the plants died in OMPM condition during the experiment.

3.4.3. Perspectives for using the HDI

Each species exhibits its own responses to a given contaminant, with various levels of sensitivity and reaction time. This highlights the benefit of using multiple species for assessing the ecological state of water (Bae and Park, 2014). The present study shows that the use of *A. lanceolatum*, *I. pseudacorus* and *E. hirsutum* species seems to be well suited for providing information on the ecotoxicity of industrial pollutant mixtures in ranges of concentrations up to 10 times higher than those authorised for industrial releases in aquatic environment. *J. inflexus* and *C. cuprina* species, that appeared to be more tolerant to pollutant mixtures, can also be used in the case of conditions of higher contamination.

Given that ecotoxicity of an industrial effluent may vary in time depending on the entrant effluent, performance of the treatment station or rainfall, it is appropriate to have at least five species in the HDI calculation to cover the various ranges of ecotoxicity. This

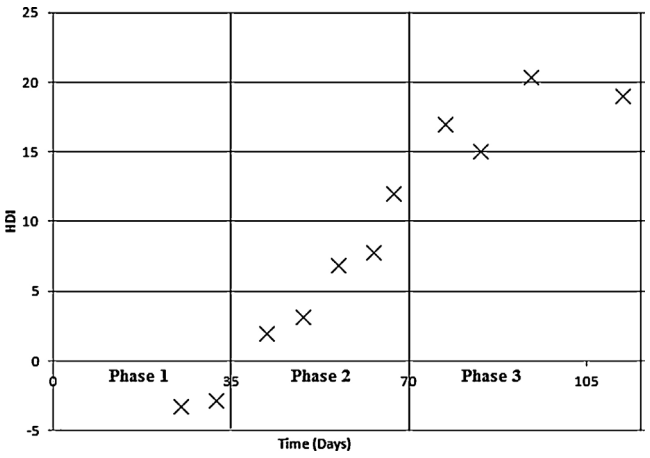


Fig. 4. Evolution of the HDI calculated on the basis of the 5 helophytes, during the three test-phases.

is one other benefit provided by this multi-specific bioindication tool in comparison with the majority of existing ecotoxical tests that are based on a single species (De Laender et al., 2009).

The HDI has been tested in microcosms with artificial effluents containing metals and organic pollutants and should now be tested on-site to estimate the ecotoxicity of several types of effluents (e.g. leachates from ultimate waste storage plants or effluents from recycling plants of used cars) in view of confirming its relevance and reliability.

4. Conclusion

We assessed the morphological responses of five helophytes of different biological types in order to create a new bioindicator tool, the Helophyte Development Index (HDI), which may provide relevant information on the ecotoxical potential of industrial wastewaters, following the recommendations of the European Water Agency. The HDI tool has the potential to be routinely used to check the ecotoxicity of industrial discharges, but it is first necessary to accumulate *in situ* data in order to validate this method under various real environmental and contamination conditions.

For the purposes of Water Framework Directive implementation, direct ecotoxicity assessment of wastewater treatment plant discharges is a way of attaining or maintaining ecological quality objectives in water masses. Calculation of the HDI is a promising tool to be used on-site for assessing the ecological state of waters released in aquatic environment by industrial factories and this new tool addresses strong expectations of engineering consulting firms for their environmental diagnoses. The HDI could be also well-suited to assess the ecotoxicity of other types of waters (e.g. containing biotoxins, pesticides). We advocate testing the HDI suitability in other contexts, taking care to adapt the choice and the number of species to the objectives.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ecoleng.2015.01.022.

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