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Screening of metallic pollution in complex environmental samples through a transcriptomic fingerprint method

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Abstract

Characterizing waste ecotoxicity is labourious because of both the undefined nature of environmental samples and the diversity of contaminants that can be present. With regard to these limitations, traditional approaches do not provide information about the nature of the pollution encountered. To improve such assessments, a fluorescent library of 1,870 transcriptomic reporters from *E. coli* K12 MG1655 was used to report the ecotoxic status of environmental samples. The reliability of the approach was evaluated with 6 metallic pollutants (As, Cu, Cd, Hg, Pb, Zn) used alone and in mixture in pure and complex matrices. A total of 18 synthetic samples were used to characterize the specificity of the resulting metallic contamination fingerprints. Metallic contamination impacted 4.5 to 10.2% of the whole transcriptomic fingerprint of *E. coli*. The analysis revealed that a subset of 175 transcriptomic reporters is sufficient to characterize metallic contamination, regardless of the nature of the sample. A statistical model distinguished patterns due to metallic contamination and provided information about the level of toxicity with 93–98% confidence. The use of the transcriptomic assessment was validated for 17 complex matrices with various toxicities and metal contaminants, such as activated sludge, wastewater effluent, soil, wood and river water. The presence of metals and their associated toxicity, which seems linked to their bioavailabilities, were thereby determined. This method constitutes a possible tool to screen unknown complex samples for their metallic status and identify those for which a deeper characterization must be achieved by the use of traditional biosensors and analytical methods.

Introduction

Annually, 76.8 million tons of hazardous waste is treated in Europe (Eurostat, 2020). Waste processing is mainly recycling (36.4%), landfilling (33.9%) and incineration (7.7% and 5.9% with and without energy recovery, respectively) (Eurostat, 2020). To choose a treatment for hazardous wastes, the EU2008/98/EC directive requires a global characterization including an assessment of the risk they pose to the environment (Directive (EC), 2008). Methods for environmental risk assessment were compiled in Hazard Property-14 (HP-14), which enables a classification of wastes in terms of environmental impact (Pandard and Römbke, 2013). This classification impacts waste treatment processes, such as incineration for dangerous wastes or recycling and reuse for safer wastes.

To characterize waste safety, 2 main strategies can be used. A mathematical modelling approach can be performed, but this strategy requires information about the compounds present in the waste (Directive EC, 1999; Hennebert et al., 2013). The second strategy is dedicated to undefined wastes of unknown composition, for which an experimental procedure employing bioassays must be undertaken to clarify their environmental impact (Council Regulation EC, 2008). For the latter approach, the recommended bioassays are those based on different trophic levels (producers, consumers and decomposers), and the use of many tests is proposed to report on acute and/or chronic toxicity, such as those based on fishes, microcrustaceans, microorganisms (bacteria and microalgae), earthworms or duckweed (Council Regulation EC, 2017). In France, the National Institute for Industrial Environment and Risks (Ineris)

Loading [MathJax]/jax/output/CommonHTML/fonts/TeX/fontdata.js and chronic tests, to establish the HP-14 property

in accordance with the literature (Pandard et al., 2006). These bioassays inform about the global toxicity but do not describe the nature of the toxicity, which can be crucial information in choosing a reprocessing strategy to reuse waste.

Identifying pollutant(s) responsible for a toxic effect is technically achievable in a simple liquid matrix through physico-chemical analyses. For wastes, which are generally complex mixtures of chemicals, this assessment is unfair, because the total amount of a pollutant can be radically disconnected from its bioavailable fraction (Kaag et al., 1998; Vig et al., 2003). Moreover, other constituents present in the matrix can alter its toxicity, notably due to synergistic or antagonistic effects (Cedergreen; 2014). In such matrices, bioavailable molecules such as metals or organic contaminants and their toxicity can be identified with the use of recombinant bioassays (Ivask et al., 2002; Jouanneau et al., 2011; Hua et al., 2015; Martin-Betancor et al., 2015). The bioassays are based on recombinant strains engineered to report the fraction of pollutants likely to cause a toxic effect, such as their bioavailable fractions. The presence of the pollutant induces the emission of a specific signal which can be bioluminescent, fluorescent, chemical or electric in nature (D'Souza 2001; Belkin 2003; van der Meer and Belkin 2010; Xu et al. 2013). Recombinant bioassays have been largely used to detect metallic compounds in the environment (Wong et al. 1995; Ivask et al. 2002; Trang et al. 2005; Gueuné et al. 2009). However, their development is limited because it requires the identification of resistance genes in response to the presence of a specific pollutant. Moreover, their use requires also complex statistical analyses to overcome the lack of specificity of reporting strains (Ben-Israel et al. 1998; Jouanneau et al. 2011).

The use of bioassays for ecotoxicological risk assessment remains arduous with complex mixtures such as industrial waste because of the diversity of pollutants encountered, their presence in mixtures and the possible interactions that can occur within the matrix. Ecotoxicological assessment tools identifying the types of pollutants present and their quantities would be of particular interest in waste management.

In the last few years, many studies have applied whole-genome transcriptomics analyses to assess the toxicity of chemicals (Zhang et al. 2011; Su et al. 2011; 2013). Such analyses are based on the assumption that a specific contaminant provokes a unique gene expression pattern in the cell. This concept was validated by Elad et al. (2015), who identified 5 classes of pollutants from 25 toxic compounds with a genome-wide bacterial bioreporter array. To date, the environmental reliability of the approach needs to be confirmed for complex matrices, such as waste and environmental samples, where the presence of many constituents can potentially alter gene expression profiles and decrease the usefulness of the approach.

Hence, this work aims to assess the possibility of exploiting gene expression profiling to identify pollution associated with metals in complex environmental matrices. For this task, the use of the whole-genome transcriptomic analysis of *E. coli* K12 MG1655 was first validated as a bioassay to report the presence of contaminants in pure matrices. Statistical analyses have allowed us to discriminate transcriptomic reporters responsible for specific metal fingerprints that were thereafter used to analyse complex samples to detect metallic contamination in environmental and waste samples. Validation of this screening

methodology to detect metallic pollution was performed with the use of analytical methods for the total fraction and the use of bioassays for the bioavailable fraction. The present study was conducted not only to scientifically complement the relevant previous works but also to provide a tool to industries involving complex matrix evaluations.

Material And Methods

Metal standards used in this study

The metals used in this study were arsenic (As_2O_3 , Sigma Aldrich, 311383; purity: > 99.95%), copper ($\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$, Sigma Aldrich, 209198; purity: > 98%), mercury (HgCl_2 , Honeywell Fluka, 215465; purity: \geq 99.5%), cadmium ($\text{CdCl}_2 \cdot 2.5 \text{H}_2\text{O}$, Sigma Aldrich, 529575; purity: > 99.99%), lead ($\text{Pb}(\text{CH}_3\text{COO})_2 \cdot 3 \text{H}_2\text{O}$, Panreac, 131466.1210; purity: > 99.95%) and zinc (ZnCl_2 , Sigma Aldrich, 229997; purity: > 99.99%).

Metals were solubilized in distilled water in accordance with good laboratory practices, with an adjusted pH enabling their complete solubilization.

Environmental and synthetic samples used to validate the approach

Description of the samples

A total of 35 samples was used in this study. Eighteen synthetic samples were produced with known concentrations of metal dissolved in deionized water. In addition, 17 complex samples from diverse origins, including environmental samples and wastes, were also used in this study. They originated from river, soil, sediment, wood and chemical industries (effluent and sludge) (Table 1).

Table 1: Description of samples

Type of sample	Nature	Number of samples	Description	Physical state	Origin
Synthetic	Distilled water	3 x 6	Single contamination of 6 metals (arsenic, copper, mercury, cadmium, zinc and lead) at 3 concentrations (C1, C2 and C3)	Liquid	Produced at the laboratory with water from a purification system (Elix 3, Millipore, Fr)
Complex	River	1 + 3	1 natural river water sample without metal addition and 3 river sample contaminated with copper and cadmium	Liquid	Sampled from the Yon river (Pays de la Loire; Fr) at GPS location: 46.676493, -1.408744
Complex	Sediment	2	commercial samples	Solid	Commercial origin. references NCS DC 73 022; NCD DC 73 317A (Techlab, Fr)
Complex	Sludge	2	1 commercial sample 1 industrial treatment	Solid	Commercial origin. References CRM009 (SigmaAldrich,Fr) Industrial waste collected from Solvay Tavaux (Fr)
Complex	Effluent	3	Wastewater treatment Plant	Liquid	Industrial effluent collected from Solvay Tavaux (Fr)
Complex	Soil	2	Unknown samples collected from a contaminated site	Solid	Environmental soils contaminated by anthropic activities given with the courtesy of the Greenation society (Fr)
Complex	Wood	4	Unknown samples wastes	Solid	Wood samples grinded at 500µm, containing 2 uncharacterized samples and 2 artificially contaminated samples (Fr)

Pretreatment for solid samples

Prior to the analysis, a leaching step was undertaken for solid matrices (i.e., soil, wood, sediment and sludge) as follows: 20 g of dried sample was shaken at 200 rpm in 200 mL of distilled water (pH = 6.5, at room temperature) in a 1 L polyethylene terephthalate bottle (Grosseron, Fr). After 24 hours of shaking (200 rpm), the leachate was centrifuged twice (6,000 g, 5 min) to withdraw suspended particles (Foucault et al., 2013).

Bacterial strains used in this study

In this study, two kinds of bacterial strains were used: bioluminescent strains, enabling biodetection of metals, and fluorescent strains, reporting changes in the transcriptomic profile of *E. coli* (Table 2).

Table 2
Bacterial strains used in this study

Name	Type of response	Plasmid	Antibiotic resistance	Reference
Bioluminescent strains				
<i>E. coli</i> DH1 pBtaclux	Constitutive	pBtaclux	amp ^R	Jouanneau et al., 2011
<i>E. coli</i> K12 MG1655 pBarslux	Inducible	pBarslux	amp ^R	Jouanneau et al., 2011
<i>E. coli</i> K12 MG1655 pBcoplux	Inducible	pBcoplux	amp ^R	Jouanneau et al., 2011
<i>E. coli</i> K12 MG1655 pBmerlux	Inducible	pBmerlux	amp ^R	Jouanneau et al., 2011
<i>E. coli</i> K12 MG1655 pBzntlux	Inducible	pBzntlux	amp ^R	Jouanneau et al., 2011
Fluorescent strains				
1,870 strains (host: <i>E. coli</i> K12 MG1655)	Inducible	pMSs201	kan ^R	Zaslaver et al., 2006

Preparation of bioluminescent strains to report bioavailable metals

The bioluminescent strains used in this study were *E. coli* DH1 pBtaclux, *E. coli* K12 MG1655 pBarslux, *E. coli* K12 MG1655 pBcoplux, *E. coli* K12 MG1655 pBzntlux and *E. coli* K12 MG1655 pBmerlux (Charrier et al., 2011; Jouanneau et al., 2011). The use of this panel enabled the identification and semi-

Loading [MathJax]/jax/output/CommonHTML/fonts/TeX/fontdata.js l) and copper (Cu) with 99% confidence

(Jouanneau et al., 2011). Bioluminescent strains were cultivated to prepare ready-to-use microplates to quantify the bioavailable fractions of metals. Bioluminescent strains were cultivated for 16 h in acetate medium at 30 °C at 250 rpm. Acetate medium was prepared in distilled water at $2.835 \text{ g.L}^{-1} \text{ CH}_3\text{COONa} \times 3 \text{ H}_2\text{O}$ (Sigma Aldrich, 32318-M), $0.1919 \text{ g.L}^{-1} \text{ NH}_4\text{Cl}$ (Sigma Aldrich, 09718), $0.028 \text{ g.L}^{-1} \text{ K}_2\text{HPO}_4$ (Dutscher, 471787-CER), $5 \text{ g.L}^{-1} \text{ NaCl}$ (Labogros, 9020401), 0.5 g.L^{-1} yeast extract (Biokar Diagnostics, A1202HA), 0.1 g.L^{-1} tryptone (Biokar Diagnostics, A1401HA) and $100 \text{ }\mu\text{g.mL}^{-1}$ ampicillin (Sigma Aldrich, A9518). The final pH of the medium was fixed at 6.8 (Charrier et al. 2011). Following a precultivation step, inducible cells were diluted in fresh acetate medium to an optical density of 0.2 at 620 nm ($0.2 \text{ OD}_{620\text{nm}}$). Strains were grown at 30 °C until $0.45 \text{ OD}_{620\text{nm}}$. The *E. coli* pBtaclux strain was diluted in fresh acetate medium to $0.075 \text{ OD}_{620\text{nm}}$ and cultivated until $0.18 \text{ OD}_{620\text{nm}}$. Thereafter, it was recovered by centrifugation (6000 g, 10 min, 4 °C) and diluted V/V with a saccharose solution (240 g.L^{-1}) to $0.075 \text{ OD}_{620\text{nm}}$. White 96-well microplates (Thermo Scientific Nunc, 136101, Fr) were filled with 100 μL of the diluted suspension and frozen for 3 h at -80 °C, afterwards the frozen preparation was lyophilized at -56 °C at 0.05 mbar with a Christ Alpha 1–2 instrument (Grosseron, Fr) for 36 h. At the end of the lyophilization process, the microplates were sealed with aluminium foil (Dutscher, 106570, Fr) and stored at -20 °C for months until use.

Preparation of a fluorescent library of 1,870 strains

A library of 1,870 fluorescent strains targeting all the promoters in *E. coli* K12 MG1655 was purchased from Dharmacon (<https://horizondiscovery.com/>). Each bacterial strain bears a recombinant plasmid pMSs201 that possesses a transcriptional fusion between one *E. coli* promoter inserted upstream of the fluorescent *gfpmut2* gene reporter (Zaslaver et al., 2006). In addition, two strains without a promoter upstream of the *gfpmut2* gene were used as controls in this study: *E. coli* K12 MG1655 pMSs201_U66 and *E. coli* K12 MG1655 pMSs201_U139. The fluorescent strains were cultivated to prepare long-term storage suspensions. For this, 100 μL of each fluorescent strain was cultivated for 16 h in 96-well microplates at 37 °C in 100 μL of HEPES medium, whose composition was 10 g.L^{-1} tryptone (Biokar Diagnostics, A1401HA, Fr), $5 \text{ g.L}^{-1} \text{ NaCl}$ (Labogros, 9020401, Fr), 2 g.L^{-1} glucose (Sigma Aldrich, 16301, Fr), 11.9 g.L^{-1} HEPES (Sigma Aldrich, H3375, Fr) and $25 \text{ }\mu\text{g.mL}^{-1}$ kanamycin in distilled water. The final pH of the medium was 6.8 (Elad et al., 2013). At the end of the incubation, 100 μL of 50% glycerol was added to the suspension, and the microplates were sealed with aluminium foil and kept at -80 °C until use (Dominique Dutscher, 106570, Fr).

Ecotoxicological assessment with bacterial bioassays

Growth inhibition bioassay to determine the intrinsic toxicity of the 6 metals used in this study

As a preliminary experiment, growth inhibition assays were performed in the presence of metals in synthetic medium to quantify their toxicity. This analysis was performed to define the effective concentrations of metals used in this study. For this task, the growth of *E. coli* K12 MG1655 pMSs201_U66 and *E. coli* K12 MG1655 pMSs201_U139 strains was measured in the presence of

increasing concentrations of a reference metal. The cell concentration was adjusted to 0.05 OD_{620nm} in HEPES base medium, and the cells were incubated at 37 °C for 20 h with 200 rpm agitation in transparent 96-well microplates (Greiner, 0220035, Fr). Bacterial growth was monitored using a SPECTROstar Nano reader (BMG Labtech, Fr). Dose response curves were established from the growth rate measured during the exponential growth phase. For each metal, the effective concentrations C1, C2 and C3 were determined using REGTOX Macro version 7.0.7 (Vindimian, REGTOX) (Figure S1, Supplemental Section). Concentration C1 corresponds to the highest tested concentration with no observed effect (NOEC for no observed effect concentration). Concentration C2 was defined as the lowest observed effect concentration (LOEC), and concentration C3 was the effective concentration for 25% effect (EC₂₅).

Bioassay of bioluminescent strains to assess the toxicities of complex samples

The overall toxicities of the samples were assessed using a *E. coli* DH1 strain carrying the pBtaclux plasmid using the method described by Charrier et al. (2011) and Jouanneau et al. (2011). The global toxicity of the sample was inversely proportional to the luminescence emitted by the strain after one hour of exposure. Briefly, 100 µL of reconstituted freeze-dried bacteria was treated with 25 µL of a toxicant for one hour at 30 °C. The bioluminescence was integrated for 1 sec at 30 °C with a Microlumat Plus LB96V luminometer (Berthold, Fr). The inhibition rate for bioluminescent strains (IR_B) was calculated (Eq. 1).

$$eq\ 1\ IR_B = 1 - \frac{RLU_{\text{toxicant}}}{RLU_{\text{control}}}$$

with IR_B as the inhibition rate and RLU as the level of bioluminescence in relative light units.

Bioassay of bioluminescent strains to quantify bioavailable metals

Metal semi-quantification was assessed with bioluminescent strains (Table 2). To perform metal semi-quantification, freeze-dried bioluminescent strains were hydrated 30 min with 100 µL of distilled water (30 °C). Thereafter, 25 µL of sample was added, and the mixture was incubated for one hour at 30 °C. The bioluminescence was integrated with a Microlumat Plus LB96V luminometer for 1 sec at 30 °C (Berthold, Fr). The induction ratio (IndR_B) for inducible strains was calculated (Eq. 2).

$$eq\ 2\ IndR_B = \frac{RLU_{\text{assay}}}{RLU_{\text{control}}}$$

with IndR_B as the induction ratio and RLU as the level of bioluminescence in the relative light units.

A control experiment was performed with distilled water. Metal quantification (As, Cu, Hg and Cd) was carried out using Metalsoft software, the bioluminescence emitted by the 4 metallic strains and that emitted by *E. coli* DH1 pBtaclux (Jouanneau et al., 2011).

Bioassay using the fluorescent strain library to characterize toxicity of complex samples

The response of the library was compared in the presence and absence of toxicants in both synthetic and environmental samples. Frozen 96-well microplates containing the fluorescent library were thawed and cultivated overnight at 37 °C in HEPES medium. Following cultivation, strain suspensions were diluted (1/10) to a final volume of 100 µL in HEPES medium in black 96-well microplates with transparent flat bottoms (Dutscher, 655090, Fr). The suspension was incubated for 2 h to allow bacterial growth up to 0.3 OD_{620nm} before the addition of 25 µL of toxicant. After 3 h of incubation, fluorescence (485 nm excitation/535 nm emission) and absorbance (OD_{620nm}), measurements were performed by a Spark 10M reader (Tecan, Fr). The induction ratio (IndR_F) for fluorescent strains was calculated as follows (Eq. 3): The activity of the tested transcript was defined as repressed when the IndR_F value was in [-∞, 0.5] and activated when the IndR_F value was in [2, +∞]. Finally, values were normalized in [-100, 0] and [0, +100] for repression and activation, respectively.

$$Eq. 3 \text{ IndR}_F = \frac{\left(\frac{RFU}{OD_{620nm}} \text{teststrain} - \frac{RFU}{OD_{620nm}} \text{promoterlessstrains} \right)_{\text{toxiccondition}}}{\left(\frac{RFU}{OD_{620nm}} \text{teststrain} - \frac{RFU}{OD_{620nm}} \text{promoterlessstrains} \right)_{\text{controlcondition}}}$$

with IndR_F as the induction ratio for fluorescent strains, RFU as the intensity of fluorescence in relative fluorescence units and OD_{620nm} as the optical density at 620 nm. The toxic condition was compared to a control condition, which was incubated in the presence of distilled water. The term test strain refers to a fluorescent reporter strain, and the promoterless strains are both *E. coli* MG1655 pMSs201_U66 and *E. coli* MG1655 pMSs201_U139.

Analytcs and statistical analyses

Concentrations of metals natively present in environmental samples were measured by ICP-MS by a certified laboratory (LEAV, Vendée, Fr).

Statistical analyses were performed on the results obtained from the fluorescent strain library with MATLAB software version 2012b using the Statistics toolbox and the SAISIR® package (Cordella and Bertrand 2014). Statistical analyses were based on principal component analysis (PCA), followed by factorial discriminant analysis (FDA) from SAISIR®. FDA was calculated using the first 10 principal component scores. A discriminant model was calculated using a cross-validation procedure. The model was calculated by random selection with 2/3 of the database, and the last 1/3 was used for the cross-validation procedure. The final classification rate corresponded to the average of 100 iterations.

Results

Toxicity of reference metals and effective concentrations

Determination of the effective concentrations

The growth inhibition produced by increasing the concentration of the 6 metals was modelled by logistic regression analyses to define the toxic concentrations referring to C1, C2 and C3 concentrations, which corresponded to the NOEC, LOEC and EC₂₅, respectively (Figure S1, Supplementary Information). Among the six tested metals, mercury was the most toxic, with an LOEC value of 2.5 µM. For this metal, EC₂₅ was reached quickly, at a concentration of 2.65 µM. Arsenic, lead and cadmium possessed similar levels of toxicity, with LOECs of 505 µM, 525 µM and 650 µM, respectively. Zinc showed intermediate toxicity between arsenic and mercury. Finally, copper was the least toxic, with an NOEC and LOEC of 250 µM and 1,405 µM, respectively (Table 3).

Table 3

Metal concentration thresholds inducing a toxic effect for *E.coli* MG1655 pMSs201_U139 and *E.coli* MG1655 pMSs201_U66. Metals concentrations are expressed in µM

Threshold	Acronym	Metal concentration (µM)					
		Arsenic	Zinc	Lead	Copper	Cadmium	Mercury
NOEC	C1	25	25	25	250	2.5	0.25
LOEC	C2	505	250	525	1,405	650	2.5
EC ₂₅	C3	1,015	300	1,320	1,450	905	2.65

Table 4

Number of regulated transcripts depending on the nature and the concentration of the metal (C1, C2 and C3)

Metal	C1	C2	C3	Total number of regulated transcripts per metal (*)	Number of overlapping transcripts between metallic concentrations
Arsenic	20 (1.1%)	32 (1.8%)	32 (1.8%)	82 (4.5%)	2
Copper	36 (2.0%)	72 (4.0%)	88 (4.9%)	184 (10.2%)	12
Mercury	38 (2.1%)	46 (2.5%)	38 (2.1%)	112 (6.2%)	10
Cadmium	18 (1.0%)	80 (4.4%)	43 (2.4%)	130 (7.2%)	11
Zinc	20 (1.1%)	52 (2.9%)	58 (3.2%)	128 (7.1%)	2
Lead	21 (1.2%)	44 (2.4%)	86 (4.8%)	140 (7.7%)	11
<i>The number of regulated transcripts in comparison to the total number of strains (1,870), its percentage is in parentheses.</i>					
<i>(*) The number of regulated transcripts per metal considers overlapping transcripts between two or more concentrations of metal that are regulated in the same way. Thus, these numbers do not equal the sum of transcripts for the C1, C2 and C3 concentrations.</i>					

Impacts of the different metals on transcript expression

Transcriptomic changes induced by the presence of metal were studied for the 18 synthetic samples with the 6 metals tested independently at C1, C2, and C3 concentrations. The transcriptomic changes provoked by metals at the C2 concentration are presented in Fig. 1. For the 1,870 strain library, more than 70,000 assays were conducted to characterize toxicity induced by metals. An assessment of transcript expression was performed to identify specific patterns related to metallic contamination. For this task, the expression of the 1,870 reporting strains was studied by PCA. The first, second and third principal components derived from the analysis represented 24.5% of the total variability (Fig. 2A). The PCA of the transcriptomic profiling showed that only 175 transcripts over the panel of 1,870 transcripts were implicated significantly in the response to metallic stress conditions (Fig. 2B; Table S1, Supplementary Information). Among them, the expression of 38 transcripts was modified by the presence of nontoxic concentrations of metal, i.e., C1, whereas for toxic metal concentrations, the expression of 80 and 88 transcripts was modified for C2 and C3, respectively.

The relevance of the transcriptomic changes was investigated from a physiological viewpoint for copper, whose toxicity is well described at the transcriptomic level. The transcripts whose expression was altered by copper were associated with 3 main metabolic functions: copper metabolism, the response to oxidative stress and secondary metabolism (Table 5). Among them, transcripts of the two copper homeostasis genes *cusC* and *cusR*, which code for an efflux pump and its regulatory protein, respectively, were both activated in a concentration-dependent manner. Transcripts implicated in iron import, such as *fes*, *cirA* and *fhuA*, were upregulated with increasing concentration of copper. An oxidative stress response was also found with the activation of transcripts belonging to the Cpx and Sox regulons, as evidenced by the activation of *htpX*, *ppiA* and *SoxS* (Table 5; details in Table S2 in the Supplementary Information).

Table 5
Metabolic pathways regulated by the presence of copper

Function	C1	C2	C3
Copper metabolism			
Copper export	<i>cusR</i> (6.2)	<i>cusR</i> (8.0)	<i>cusR</i> (28.5)
		<i>cusC</i> (21.5)	<i>cusC</i> (47.5)
Iron homeostasis	<i>fes</i> (16.8)	<i>fes</i> (4.5)	<i>fes</i> (13.0)
Iron import	<i>cirA</i> (3.8)	<i>yqjH</i> (9.17)	<i>fhuA</i> (2.2)
		<i>fecA</i> (4.0)	<i>fhuF</i> (3.3)
Oxidative stress			
<i>cpx</i> regulon		<i>htpX</i> (8.2)	
		<i>mraZ</i> (5.0)	
		<i>ppiA</i> (4.3)	
<i>sox</i> regulon			<i>fpr</i> (16.1)
			<i>sodA</i> (42.9)
			<i>soxS</i> (4.6)
Flagellar biosynthesis			<i>flgM</i> (2.4)
			<i>fliC</i> (8.4)
Secondary metabolism			
Polysaccharides	<i>glgB</i> (5.4)		
Fatty acids	<i>fadE</i> (6.0)	<i>fabB</i> (3.3)	
Amino acids		<i>argA</i> (3.0)	
Coenzymes			<i>metK</i> (0.24)

An FDA was carried out to model the metallic contamination in samples, with the subset of 175 transcripts considered relevant in the detection of metals (Fig. 2C). The FDA allows prediction of metallic contamination at C1, C2 and C3 concentrations in a sample according to its transcriptomic fingerprint with confidence levels of 98%, 98% and 93%, respectively. The results obtained by the FDA confirmed the relevance of the use of the transcriptomic pattern for the detection of metallic contamination in synthetic matrices.

Application of transcriptomic profiling to identify metal toxicity in complex samples

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The validity of the approach was investigated by assessing toxicity in complex samples with the use of bioassays. These bioassays were used to characterize the overall toxicity of the samples with *E. coli* DH1 pBtaclux and the toxicity related to metals by the transcriptomic fingerprint approach. A validation step was carried out by quantifying the overall content and the bioavailable fraction of metals by ICP-MS and the use of bioluminescent reporter strains. Assays were carried out directly on liquid matrices and on leachates for solid matrices. Toxicity was found in 11 matrices among the 17 tested and ranged from weak inhibition of approximately 20% to a high toxicity of 99%. In the cases of toxicity above 25%, samples were diluted for the analyses (Table 6). Transcriptomic fingerprints were used to identify metallic contamination and toxicity among the samples. For this purpose, the expression of the metal fingerprint composed of 175 transcripts was studied through dedicated statistical analysis, and transcript expression patterns were established as presented in Fig. 3.

Table 6: Characterization of the metallic contamination in complex samples and its related toxicity: Slu-1 corresponds to a contaminated sludge reference (Sigma Aldrich, Fr), and Slu-2 corresponds to sludge sampled from an industrial chemical waste treatment plant (courtesy of Solvay, Tavaux, Fr). Ieff-1, -2, and -3 are industrial effluents, specifically from the inlet, treatment and outlet areas of an industrial chemical waste treatment plant (courtesy of Solvay, Tavaux, Fr); WS-1 to WS-4 are 4 uncharacterized wood samples in which WS-1 and WS-2 have been artificially contaminated by an unknown cocktail of metals (courtesy of the Ecole Supérieure du Bois Nantes, Fr). So-1 and -2 are 2 environmental soil samples contaminated by anthropic activities (courtesy of Greenation society, Fr), while Riv-1 to Riv-4 correspond to water samples from the Yon river (Pays de la Loire, Fr). Riv-1 is the bulk sample, Riv-2 is the river sample spiked with a known concentration of copper, and Riv-3 and -4 are the river sample spiked with known mixes of copper and cadmium.

	Industrial origin										Environmental origin							
	Sludge		Effluent			Wood				Soil		Sediment		River				
Complex matrices	Slu-1	Slu-2	Ieff-1	Ieff-2	Ieff-3	WS-1	WS-2	WS-3	WS-4	So-1	So-2	Sed-1	Sed-2	Riv-1	Riv-2	Riv-3	Riv-4	
Whole toxicity	77%	0%	99%	0%	0%	23%	40%	52%	95%	0%	0%	40%	25%	0%	60%	78%	90%	
Metal presence	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	
Metal toxicity	C2													C2		C3	C3	
<i>Total fraction of metal</i>																		
Cu	5309.0	0.5	10.2	3.3	0.3	30.1*	108.6*	nd	nd	3.6	3.6	0.7	0.2	nd	950.0	151.5	303.0	
As	nd	nd	0.2	nd	nd	6.1*	nd	nd	nd	nd	nd	0.4	0.1	nd	nd	nd	nd	
Hg	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	
Cd	nd	nd	nd	nd	nd	nd	30.2*	nd	nd	nd	nd	nd	nd	nd	nd	66.3	132.5	
Zn	0.2	0.2	0.4	0.1	nd	nd	3195.2*	nd	nd	7.5	8.4	0.2	0.4	nd	nd	nd	nd	
Pb	171.3	0.2	nd	nd	nd	49.4*	nd	nd	nd	0.9	1.2	nd	0.1	nd	nd	nd	nd	
Cr	nd	nd	nd	nd	nd	43.3*	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	
<i>Bioavailable fraction of metal</i>																		
Cu	125-1250	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	250-25000	125-1250	250-2500	
As	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	
Hg	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	
Cd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	1.25-250	1.25-250	2.5-500	

*Asterisk corresponds to metallic concentration measured in the native sample, before the leaching step. Due to technical difficulties concentrations in the leachate was not measurable for these samples.

Toxicity assessment performed on sludge Slu-1, which corresponded to a reference sludge, showed an inhibition of 77% with the *E. coli* DH1 pBtaclux reporter, whereas sludge Slu-2, which corresponded to a sample freshly collected from an industrial treatment plant (Solvay Tavaux, Fr), did not show any toxicity by the bacterial bioassay (Table 6). To characterize the toxicity found in Slu-1, transcriptomic fingerprinting analysis was carried out, and the impact on the expression of transcripts was compared to

3). The analysis of the fingerprints indicated

that metallic contamination was present in the sample and that the metals exerted toxicity reaching the LOEC level (i.e., C2). To validate this result, the total fractions of metals were confirmed through analytical procedures, which showed that 5,309 μM copper was present, but only 125-1,250 μM was bioavailable according to the bioluminescent reporters. The bioavailable fraction found in the sample is in the range of C2 toxicity for copper, which is in accordance with the results given by the transcriptomic approach.

The industrial effluents came from an industrial treatment plant (Solvay Tavaux, Fr). They were collected at the inlet, treatment and outlet areas of the treatment plant and identified as leff-1 to leff-3, respectively. leff-1 presented high toxicity, whereas leff-2 and leff-3 were not toxic to *E. coli* DH1 pBtaclux (Table 6). Regarding metallic contamination, leff-1 was diluted until its toxicity reached EC_{25} for transcriptomic fingerprint analysis. The FDA model indicated the presence of metal in leff-1, but the concentrations of the metals characterized in this study was not, *a priori*, responsible for the toxicity found in this sample (Table 6). Chemical analyses revealed the presence of 10.2 μM copper as the total fraction, whereas its bioavailable fraction remained undetectable by bioluminescent bacteria. Consequently, the metals present did not appear to be the cause of the toxicity observed in leff-1.

Wood samples (WSs) were 2 wood waste samples contaminated by metals (WS-1, WS-2) and 2 wood samples in which no metal contaminants were reported (WS-3, WS-4). All the samples were lixiviated, and their leachates were assayed for toxicity prior to their analysis for metals. WS-1, WS-2, WS-3 and WS-4 showed toxicities of 23%, 40%, 52% and 95%, respectively (Table 6; Fig. 3). The fingerprint analysis indicated that toxicity found in the samples was not associated with metals for all the WS samples. These results were confirmed by bioluminescent strain-based bioassays reporting that no bioavailable metals were in the 4 WSs.

Soil matrices So-1 and So-2 showed no toxicity and no significant metallic contamination. Similarly, for Sed-1 and Sed-2, toxicity reported by *E. coli* DH1 pBtaclux was not linked to the presence of the relevant concentrations of metals, as indicated by the transcriptomic analysis and confirmed by the bioluminescent strains (Table 6).

River matrices corresponded to a unique environmental sample (Riv-1) to which different metal contaminants were added to generate Riv-2 to Riv-4 samples. No toxicity and no relevant bioavailable metals were found in the Riv-1 matrix for which no metallic contaminant was added. For Riv-2 to Riv-4, the samples were spiked with copper (Riv-2) or both copper and cadmium (Riv-3 and Riv-4). The toxicity of Riv-2, which was contaminated with 950 μM copper, as determined by *E. coli* DH1 pBtaclux resulted in an inhibition of 60% bioluminescence, which is in accordance with the results found previously in this study regarding the establishment of the C2 concentration for copper. Transcriptomic fingerprint analysis was performed on the sample after its dilution to 25% toxicity (Table 6). Prediction of metallic contamination using the FDA model indicated metallic contamination at C2, which is in accordance with the concentration of bioavailable copper found with the bioluminescent reporters (Fig. 2). The river samples contaminated by a mixture of 2 metals exhibited a higher toxicity. The Riv-3 and Riv-4 samples, which were contaminated with copper and cadmium at 151.5 μM and 66.3 μM for Riv-3 and 303 μM and

132.5 μM for Riv-4, respectively, showed overall toxicities of 78% and 90% (Fig. 3; Table 6). The samples were diluted until the effective toxicity reached 25% for the transcriptomic analysis. Prediction of the metallic contamination of Riv-3 and Riv-4 indicated C3 contamination, for which copper and cadmium were found to be bioavailable using bioluminescent reporters. Transcriptomic analysis makes it possible to consider the whole toxicity resulting from metallic contamination regardless of whether the source of the toxicity is a single contaminant or a mixture of metals.

Discussion

Assessing the ecotoxicological impact of contaminants is complex in natural samples because they are diverse and constantly changing and mixing. Traditional tools used to assess toxicity include physicochemical analyses and biological analyses that use reporter bacteria. Many bacterial reporters have been developed for metal analyses. However, although these techniques are specific and allow the quantification of a given metal, they are inefficient and costly to perform if there is no initial indication of the nature of the pollution present in the sample. To address this issue, this study developed a screening method that provides information about metal toxicity in unknown complex samples. Information given by this methodology aims to complement that obtained by the existing tools available to industry and ecotoxicologists to ensure safety management.

In this study, the toxicity of 6 metals was characterized with *E. coli* before transcriptomic studies were performed. The toxicity of the different metals aligned with literature results for *E. coli* K12 MG1655 (Jouanneau et al., 2011). The approach was validated by a comparison between the transcriptomic responses found for metal contamination and those in the literature; detailed information is presented in the Supplemental Section (Table S2). This analysis validates the biological relevance of using the transcriptomic method to detect toxicity.

The second part of our study developed a screening methodology for the analysis of complex samples to address a concept proposed in the literature for synthetic samples (Elad et al., 2013; 2015). This development used statistical tools to exploit the information given by the transcriptomic fingerprints. Statistical analysis allowed us to describe and explain the variations in transcript expression that were linked to metal contamination. In this way, a relevant core of 175 transcripts was found to be sufficient to characterize the presence or absence of a metal and to quantify its relative toxicity. Similar statistical approaches have already been successfully carried out to characterize the presence of contaminants in mussels (André and Gagné, 2020).

The panel of 175 transcriptomic reporters used in this study can identify the presence of metals and their associated toxicity in unknown samples. This fingerprinting method complements traditional analytical standard methods and bioassays reported in the literature (Charrier et al., 2011; Jouanneau et al., 2011). As its main advantage, the method enables the prioritization of samples according to their metallic status and the associated toxicity. In an industrial context, this type of screening can be employed since automation opportunities can be envisaged in the pipetting/incubation/fluorescence reading steps. In our

study, the use of screening microplates that had been stored frozen prior to their use illustrates the possibility of deploying the methodology in a ready-to-use bioassay directly on an industrial site. This strategy limits the associated labour due to the preparation of the transcriptomic library and offers an interesting way to reduce production cost. In summary, the transcriptomic profiling screening method is a realistic opportunity to save costs associated with ecotoxicity assessments. The screening method allows a characterization of the relevant matrices that were contaminated by metals and that require a complete metal quantification through regulatory analyses (from 35 to 100 euros per metal analysed) (Fig. 4).

Two main perspectives of development increase the relevance of this method. The first issue highlights the scope of the analysis, which provides information about different contaminants by the development of complementary fingerprints. Work performed in the literature opened the way with the identification of fingerprints targeting new contaminants. The next step in this development should be the identification of a relevant pattern of transcripts that exhibit specificity for a toxicant in complex matrices despite environmental disturbances. The second issue is the identification of specific contaminants. In this sense, the use of transcriptomic fingerprints should complement traditional bioassays, but this approach requires a dedicated experimental plan to ensure relevant significance with regard to several contaminants that are encountered in complex matrices.

Conclusion

The use of transcriptomic fingerprints to characterize toxicity elucidates the impact of contaminants on living organisms. The aim of this study was to adapt the use of a transcriptomic database to complex samples to identify metallic contamination. A set of 175 reporting strains was constituted to establish the metallic status of complex samples, including industrial wastes and environmental samples containing metals, whether at toxic concentrations or below. The statistical approach allows quantification of the toxicities associated with the metals in samples and provides an interesting screening tool for an environmental assessor to determine the role of metals in the toxicity of a matrix. This study partially overcomes the bottleneck to the transfer of methodologies established for the detection of a contaminant in a synthetic sample to their real applications in environmental samples and waste. According to the large variety of fingerprints offered by the transcriptomic assays, the development of this approach can be interesting for the detection of a wide variety of pollutants in complex matrices such as waste or the environment.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

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Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

CM and HA contributed equally to the preparation of the manuscript. The authors CM and HA analysed and interpreted transcriptomic data. The authors AA and CC contributed to the statistical analyses. The authors SJ, GT and MJDs contributed to the bioassay expertise. All authors read, expertise and approved the final manuscript.

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Figures

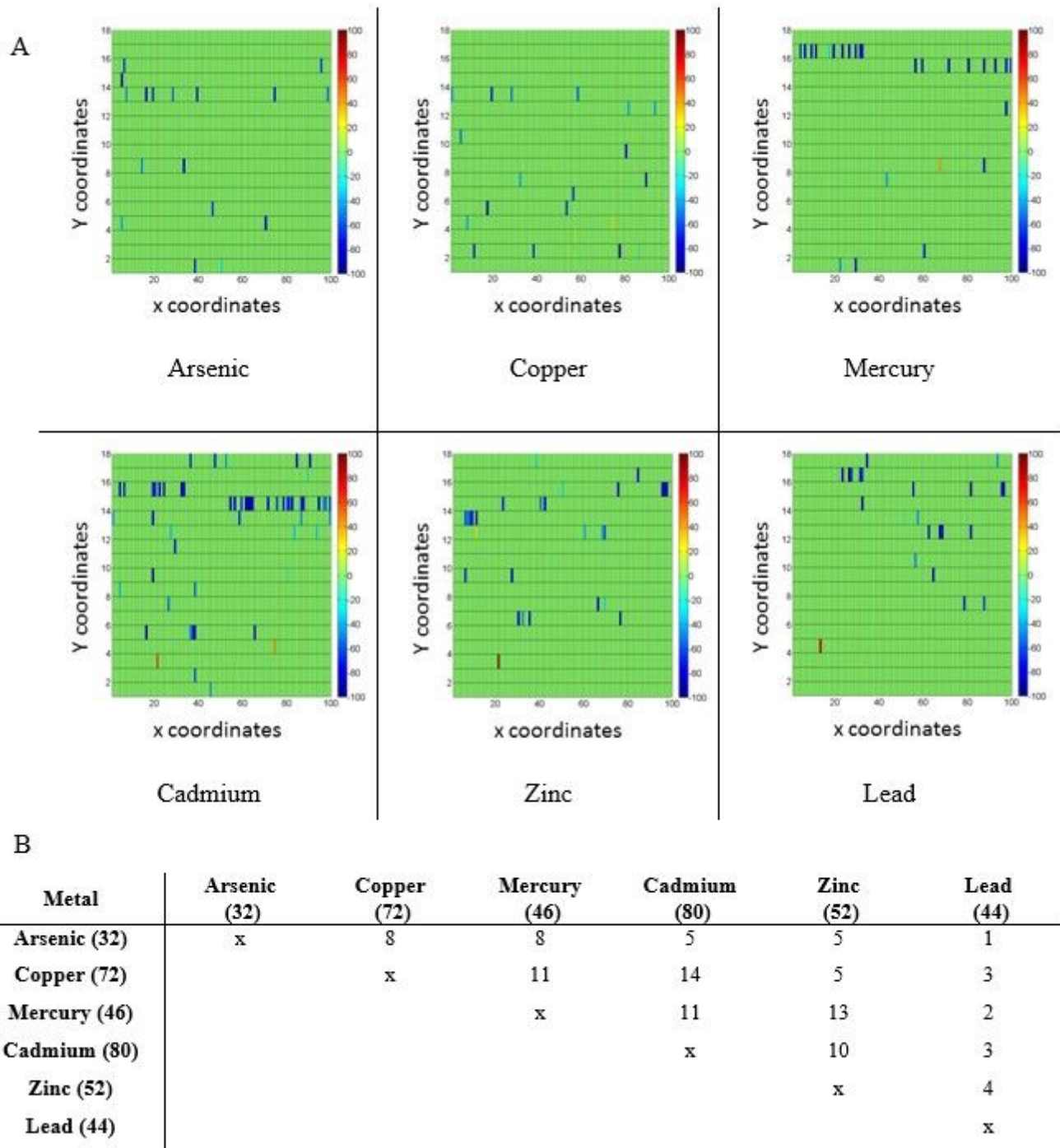


Figure 1

Transcriptomic regulation in response to metals at the C2 concentrations. (A) Transcriptomic fingerprints induced by the presence of the dedicated metal with IndRF values of $[-100, 0]$ for repressed transcripts, 0 for unregulated transcripts and $[0, 100]$ for activated transcripts. The results presented are the averages of two independent experiments. (B) Number of transcripts regulated in common between the tested metals at C2 concentrations. The number of transcripts regulated by the metal at C2 is shown in parentheses.

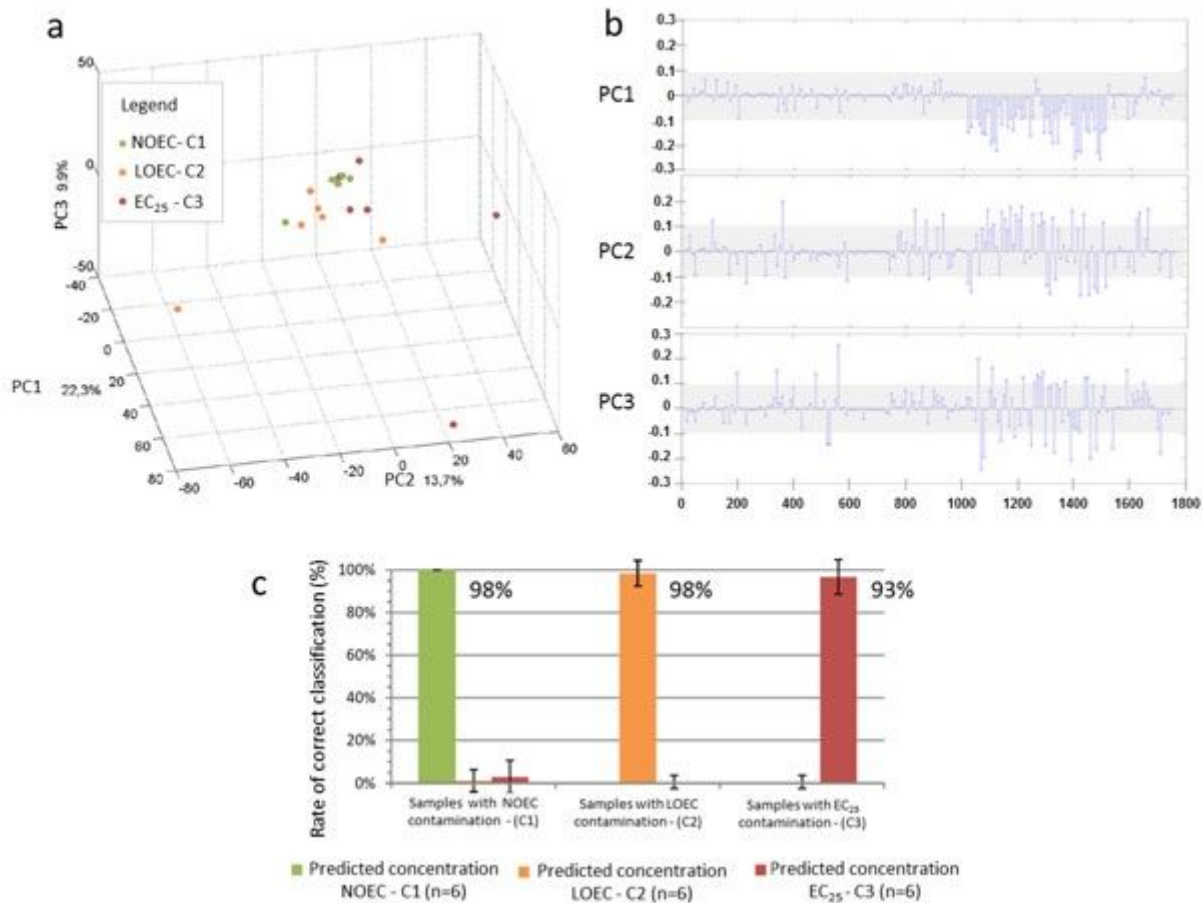


Figure 2

Principal component analysis of synthetic samples based on the global panel of 1870 transcriptomic reporters according to their metallic contamination (A) and the associated loadings presenting the contribution to the first 3 axes of the PCA. The shaded space corresponds to the pool of transcripts that are not relevant for metal detection (B). Factorial discriminant analysis performed on the restricted panel of 175 transcriptomic reporters that are relevant for the detection of metallic contamination (C). The confidence of the analysis ranged from 93% to 98%.

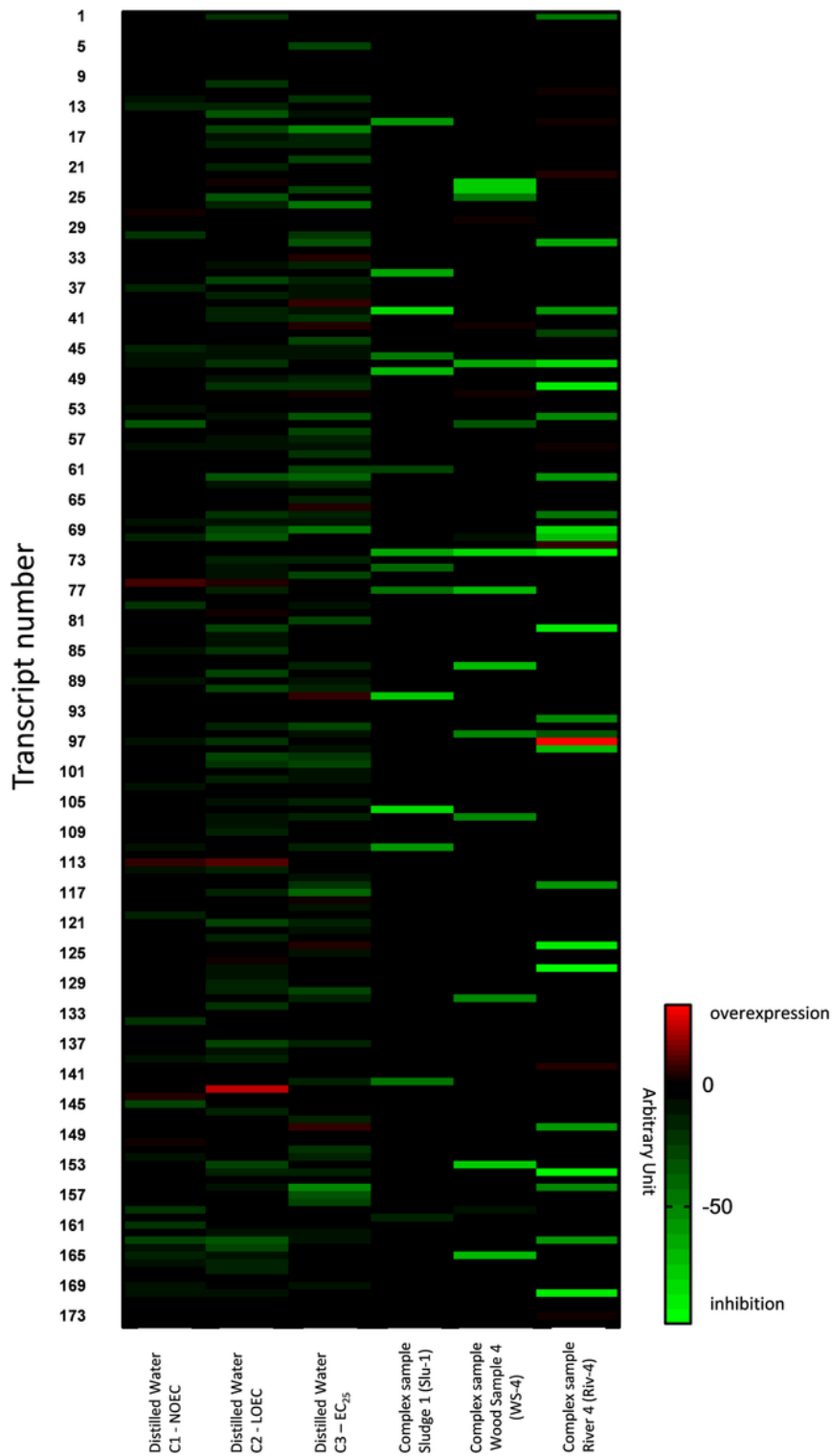


Figure 3

Response of the 175 transcripts implicated in the detection of metallic contamination, i.e., general fingerprints for C1, C2, and C3 from the synthetic matrix and 3 transcriptomic fingerprints given from complex samples (Slu-1, WS-4 and Riv-4). The names of the transcripts are reported in Table S1 in the Supplemental Section.

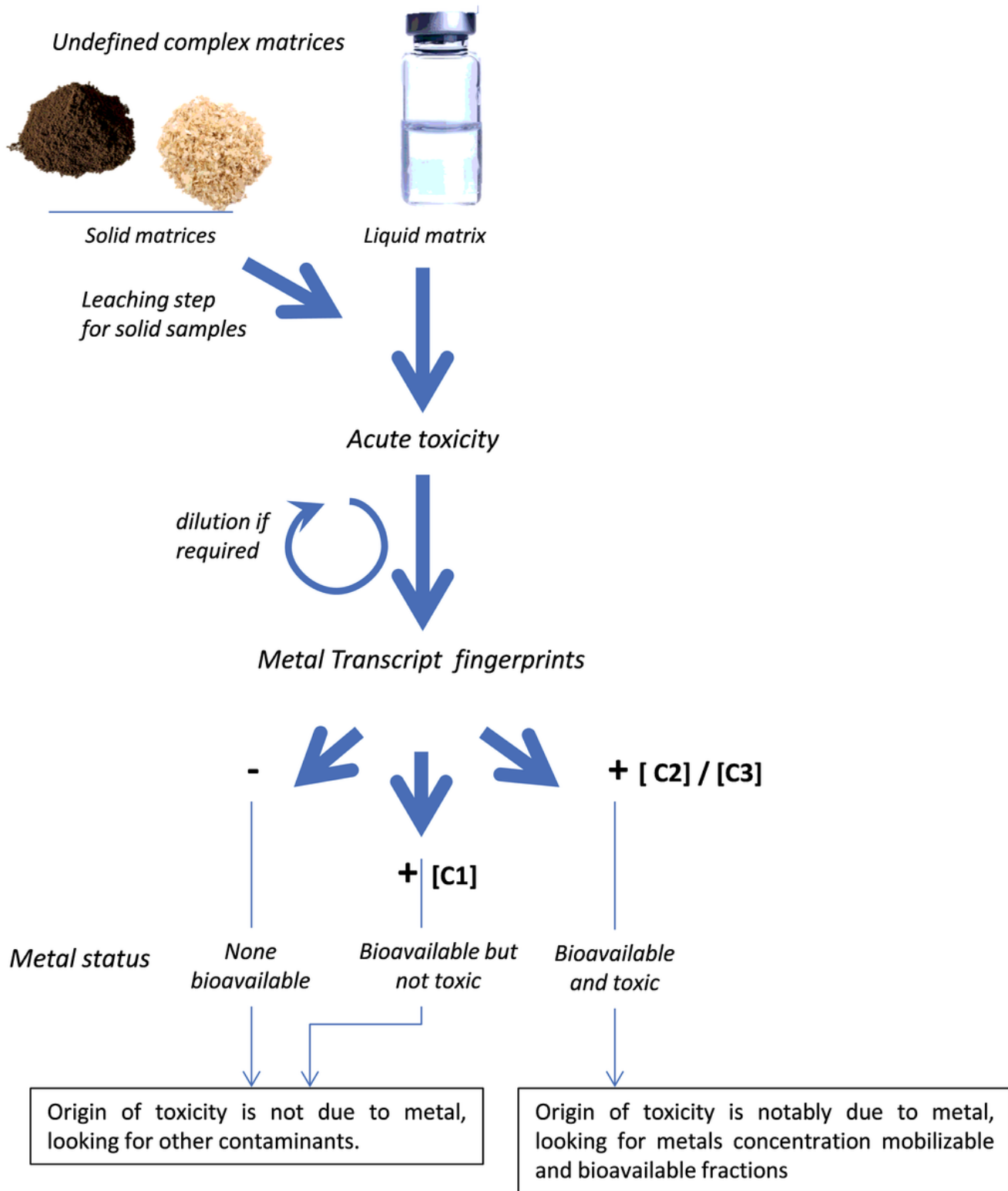


Figure 4

Schematic role of the testing procedure in the characterization of unknown complex samples

Supplementary Files

- [SupplementalInformationHuaetal2020avtAJE.docx](#)