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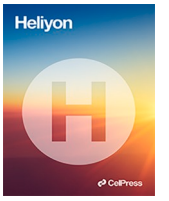

Research article

Season-dependent physiological behavior of *Miscanthus x giganteus* growing on heavy-metal contaminated areas in relation to soil properties

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ABSTRACT

Miscanthus x giganteus is often considered as a suitable plant species for phytomanagement of heavy metal polluted sites. Nevertheless, its physiological behavior in response to the level of metal toxicity throughout the growing season remains poorly documented. *Miscanthus x giganteus* was cultivated on three sites in Belgium (BSJ: non-polluted control; CAR: slightly contaminated; VM strongly polluted by Cd, Pb, Cu, Zn, Ni and As). The presence of Miscanthus improved soil biological parameters assessed by measurement of enzyme activity and basal soil respiration on the three considered sites, although to a lower level on VM site. Heavy metal accumulation in the shoot was already recorded in spring. It displayed a contrasting distribution in the summer leaves since heavy metals and As metalloid accumulated mainly in the older leaves of CAR plants while showing a uniform distribution among leaves of different ages in VM plants. Comparatively to plants growing on BSJ, net photosynthesis decreased in plants growing on CAR and VM sites. The recorded decrease was mainly related to stomatal factors in CAR plants (decrease in stomatal conductance and in C_i) but to non-stomatal factors such as decrease in carboxylation efficiency and non-photochemical quenching in VM plants. Stomata remained open in VM plants which presented lower instantaneous and intrinsic water use efficiencies than CAR and BSJ plants. High proportions of heavy metals accumulated in CAR plants were bound to the cell wall fraction while the soluble and organelle-rich fractions were proportionally higher in VM plants, leading to a decrease in cell viability and cell membrane damages. It is concluded that not only the intensity but also the nature of physiological responses in *Miscanthus x giganteus* may drastically differ depending on the pollution level.

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1. Introduction

During the last decades, the demand for energy related to industrial development and global economy induced an increase in fossil fuel consumption. Such an increase generates a wide range of ecological and political problems. To ensure energy independence, production of renewable energy is of paramount importance and the use of bioenergy crops appears as one interesting option [1–3]. Nevertheless, growing this type of crops on agricultural lands devoted to food production poses several deontological and social concerns.

An alternative would be to cultivate bioenergy crops on marginal lands unsuitable for food production. According to Grewin et al. [4], at least 380,000 km^2 of marginal lands are available for biomass production in Europe, among which 137,000 km^2 are contaminated by one or several toxic heavy metals or metalloids [5]. These potentially hazardous elements induce a strong ecotoxicological risk since they may endanger environmental and human health [6,7]. *Miscanthus x giganteus* J.M. Greef & Deuter is a sterile triploid issued from the diploid *Miscanthus sinensis* and the tetraploid *Miscanthus sacchariflorus* and is widely cultivated in Western Europe. After an initial phase of establishment of 3 years, Miscanthus is harvested each year for as long as 20 years. For energy production by combustion, Miscanthus is harvested at the end of the winter when the plant exhibits its lowest water content after the drop of senescing leaves [1]. During the senescing process, numerous compounds including inorganic ions are mobilized to the rhizome and stored as reserves to sustain subsequent growth during the next spring [8]. Alternatively, Miscanthus may be used for bioethanol production and is then harvested earlier in autumn when the plant lignocellulose is readily available to hydrolysis by enzymes [9].

Miscanthus x giganteus is well adapted to polluted soils and exhibit a high level of tolerance to several heavy metals [2,10,11]. According to Nsanganwimana et al. [11], Cd, Pb and Zn accumulations in roots may reach up to 120, 105 and 280 mg Kg^{-1} DW, respectively, while the highest values recorded in leaves were 3, 15 and 80 mg Kg^{-1} DW for Cd, Pb and Zn, respectively. Hence, the majority of absorbed heavy metals remain in the roots [12–14] but a consistent part of those pollutant may be translocated to the shoot [15,16]. The proportion of metals reaching aerial parts may vary according to the considered element, the phenological stage of the plant and agronomical practices [17]. However, data on pollutants distribution within the plants at distinct growth stages are surprisingly scarce. Similarly, the re-mobilisation of pollutants from the shoot to the rhizome during senescence is poorly documented [18]. Nevertheless, heavy metal distribution in the plant is of paramount importance since it will influence the quality and consequently the potential use of harvested biomass [19–22]. Moreover, the presence of heavy metals in senescing leaves will contribute to surface soil pollution through litter contamination [23] while the storage of putatively toxic elements in the rhizome could compromise the maintenance of the culture by hampering growing of new stems in early spring.

Miscanthus x giganteus displays a typical C4-type photosynthesis. Its high photosynthetic efficiency is mainly responsible of its fast-growing properties and partly explains why this perennial rhizomatous grass is able to produce up to 20 t DM ha^{-1} $year^{-1}$ under non-irrigated and non-polluted conditions [24]. As a consequence, any impairment of photosynthetic process induced by accumulated heavy metals may drastically affect plant final yield. However, studies devoted to the precise impact of heavy metal on photosynthesis in relation to leaf age are scanty [25]. As a promising plant for polluted sites restoration, *Miscanthus x giganteus* was also reported to improve soil biological activity [23,24], to increase organic carbon percentages and to influence metal availability [26]. Once again, the influence of plant phenological stage on soil biological properties remains unknown.

The tested hypothesis in the present study was that heavy metal distribution within the plant and the physiological consequences of this accumulation depend on the phenological stage of the plant and the level of contamination of the substrate. In the present study, an *in situ* experiment was conducted on three sites differing in the nature and the level of contamination. *Miscanthus x giganteus* was analyzed in spring (fast growing stage), in end of summer (before the start of senescence) and at the end of winter (full senescence). Photosynthetic properties and heavy metal distribution were analyzed in order to determine the impact of plant growing stage on physiological properties in relation to heavy metal toxicity.

2. Materials and methods

2.1. Sites and experimental plots description

This study was carried out in three distinct sites located in the South part of Belgium, close to the city of Liège. 1) Bois-Saint-Jean (BSJ; 50°35′37″ N, 5°33′09″ E) was located between Liège and Seraing. Although this area was previously devoted to industrial activities, it was completely rehabilitated with a deep layer of clean soil, and it will be hereafter considered as the non-polluted control; 2) Carcoke (Car; 50°28′28″ N, 3°48′16″ E) was located in the industrial area of Saint-Ghislain and was previously devoted to steel industry; 3) Vieille Montagne (VM; 50°37′44″ N, 5°28′48″) was located in Grâce-Hologne and was used during several decades for deposit of heavy-metal contaminated waste from metal industry.

At the three location, *Miscanthus x giganteus* was planted and the present study was initiated 5 years after the initial date of plantation in May 2015. Distance between the lines was set at 75 cm and rhizome density was 17,800 rhizomes.ha^{-1}. An initial fertilization using 26 kg ha^{-1} of diammonium phosphate and 360 kg ha^{-1} NPK (16-9-20) was applied during the first year, after what plots plants remained unfertilized. Three square plots (15 × 15 m; 225 m^2) were delimited in each site and plants within these plots were subsequently considered for physiological analysis. The experiment started at early spring (after biomass preliminar harvest at the end of winter) and ended at the end of winter of the following year after final harvest.

2.2. Soil sampling and analysis

For each plot, five samples of the topsoil (0–25 cm) were collected with a hand auger and crushed to pass through a 10 mm stainless steel. Soil pH was measured after adding samples (5 g) to 25 mL of distilled water and mechanically shaking for 5 min. Soil electrical conductivity (EC; $mS.cm^{-1}$) was determined on the same supernatant. Organic carbon and total nitrogen were determined by combustion according to the NF ISO 13878 standards after burning 50 mg of samples at 1000 °C in the presence of O_2. $CaCO_3$ determination was estimated by NaOH 0.5 N titration [27]. Major cation concentrations were determined by loss ignition at 950 °C of soil samples, addition of lithium metaborate and lithium tetraborate. Heavy metals concentrations were obtained after acid attack of soil samples with HNO_{3conc} and HF_{conc} as previously detailed [28]. Samples were prepared using ultrapure water with a resistivity of 18MΩ cm (Merck-Millipore, Germany). Measurements were performed with an inductively plasma emission spectrometry ICP-OES (Thermo Jarrel Ash Iris Advantage). Samples were injected through a Mainhard nebulizer and carried through an argon gas plasma conditions with reflected power of 7 W and forward power of 1300 W. Gas flow rates of plasma, auxiliary, and nebulizer were set at 16.0, 1.0 and 1.0 L min^{-1}, respectively. The internal standard multi-element stock solution obtained from Agilent (USA) allowed us to control stability of the instrument while certified reference material ERM-CA713 was obtained from Sigma-Aldrich (Germany). Exchangeable base concentration (Ca^{2+}, Mg^{2+}, K^{+}, Na^{+}) were determined using percolation columns and ammonium acetate 1.0 M pH 7.0 [27]. Cation exchangeable capacity was assessed using the same percolation columns and KCl 1.0 M pH 3.0.

Heavy metal bioavailability was determined after selective extraction using $CaCl_2$ 0.01 M [29]: 2.5 g of soil sample and 25 mM $CaCl_2$ were mixed during 24h and centrifuged at 3000 *g* during 15 min. The filtered supernatant was used for heavy metal measurements and corresponded to the total heavy metals available fraction, including heavy metal in soil solution and weakly adsorbed on soil surfaces [28]. Elements were quantified by ICP-OES.

Soil biological parameters were determined on 5 samples collected in each plot (3 plots per site) and on 5 samples collected in unvegetated places located close to the Miscanthus plots. Soil was collected in spring (15–17th May), at the end of summer (29 August -2nd September) and just before harvest at the end winter (9–13th March). Fresh collected samples were stored in the dark at 4 °C. The basal respiration was determined by the quantification of liberated CO_2 after 5 days of incubation at 20 °C using the colorimetric method of Rowell [30]. Fluorescein diacetate hydrolytic activity was determined after extraction with 60 mM Ba-phosphate solution pH 7.6 at 37 °C during 3h according to Green et al. [31]. Soil urease activity was assessed by colorimetric determination of ammonium through a modified indophenol reaction according to Kandeler and Gerber [32]. Laccase activity was determined on the basis of ABTS oxidation assay as described by Eichlerová et al. [33]. For measurement of acidic phosphatase activities, fresh soil was mixed with disodium phenylphosphate solution pH 5.0. After incubation (37 °C, 2h in the dark), suspension was filtered and treated with 4-amino-antipyrine and potassium ferricyanide: the phenol released was determined colorimetrically at 510 nm [34]. Microbial biomass carbon was quantified by the fumigation-extraction method using a KEC factor of 0.45 to convert the organic C content to microbial biomass C [35].

2.3. Plant photosynthesis

Photosynthetic-related parameters measurements were performed in spring (18–19th May) on the youngest fully expanded leaf on the main stem, and in summer (25–26th August) on two distinct leaves: the youngest fully expanded leaf on the main stem (hereafter named « young leaf » (YL)) and a mature leaf located just in the middle part of the main stem (hereafter named « old leaf » (OL))

Chlorophyll fluorescence-related parameters were measured on five randomly chosen plants within each plot and analyzed by a Fluorescence Monitoring System II (Hansatech Instruments, Norfolk UK). Leaf portions located in the middle part of the blade were acclimated to darkness for 30 min using pliers fitted with a sliding clip. After removal of the leaf clip, the minimal fluorescence level (F_0) was measured using modulated light (0.1 μmol m^{-2} s^{-1}). Maximal fluorescence with all PSII reaction centers closed (F_m) was assessed by applying a 0.8 s saturating pulse of 8000 μmol m^{-2} s^{-1}. The leaf was then illuminated with white actinic light (1200 μmol m^{-2} s^{-1}) during 4 min. F_s corresponding to the steady state fluorescence was recorded and a second saturating pulse of 8000 μmol m^{-2} s^{-1} was applied to determine the maximal fluorescence in the light-adapted state (F_m'). After removing the actinic light, minimal fluorescence level at the light adapted state was determined by illuminating the leaf with a 3 s pulse of far-red light. Maximal efficiency of PSII photochemistry in the dark-adapted state (F_v/F_m), photochemical quenching (qP), non-photochemical quenching (NPQ) and operational efficiency of PSII photochemistry (Φ_{PSII}) were calculated on the basis of the above-mentioned parameters according to Swoczyna et al. [36].

Gas exchange measurements were performed on the same leaves using an Infrared Gas Analyzer (LCA4 8.7., ADC Biocientific) with a Parkinson leaf cuvette, under an air flow of 300 mL min^{-1} during 1 min (20 records) at 23 ± 2 °C on a leaf segment of 6.25 cm^2. The net CO_2 assimilation rate (*A*) and instantaneous transpiration (*E*) were measured. The instantaneous water use efficiency (WUE = *A*/*E*) was then calculated. The stomatal conductance (g_s) was measured with an AP4 porometer system (Delta-T Devices), allowing the calculation of intrinsic water use efficiency (A/g_s). All measurements were performed between 11.00 a.m. and 2.00 p.m. in a sunny day with no clouds, and at an ambient light intensity comprised between 500 and 1000 μmol m^{-2} s^{-1}.

The leaves used for photosynthetic measurements were then collected and cut in small segments (3 × 2 cms). Photosynthetic pigments (*Chla* + *Chlb*) and total carotenoids (xanthophylls and β carotene) were quantified after extraction with cold acetone 80% and spectrophotometric analysis according to Lichenthlaer [37]. The remaining segments were used for ion content and cell viability measurements (see below).

2.4. Analysis of plant mineral content

Mineral concentrations were quantified in stems and in fully expanded leaves in spring, as well as stems, old leaves and young leaves in summer. At the beginning of winter, senescing leaves were also collected just before abscission. At the end of winter, stems were cut in segments of 5 cms long. Segments from the basal, middle, and upper part were pooled while fragments of rhizomes were harvested just below the main stem. Fresh matter was dried in an oven at 69 °C for at least 72 h until it reached a constant weight; *c.a.* 50 mg of dry matter were digested by incubation in a mix of 68% HNO_3 and 37% HCl at 80 °C on a sand bath. After complete evaporation, minerals were re-dissolved in HCl 0.1 N and filtered on a Whatman n°1 filter paper. Elements were quantified by ICP-OES (Varian, type MX). Quality control was based on the use of certified reference material CTA-VTL2 Virginica Tobacco Leaves.

Subcellular fractions of accumulated heavy metals were analyzed in leaves harvested in spring and at the end of summer and was assessed following the method of Zhou et al. [38]. Leaf tissues were quickly frozen in liquid nitrogen and then homogenized in pre-cooled (4 °C) extraction buffer (50 mM HEPES, 250 mM sucrose, 1.0 mM $C_4H_{10}O_2S_2$, 5.0 mM ascorbic acid and 1.0% w:v Polyclar AT PVPP, pH 7.5). Homogenate was sieved on a nylon cloth. After washing twice with homogenization buffer, the remaining residue corresponded to the cell wall fraction (cell wall + cell wall debris). The obtained filtrate was centrifuged at 10,000 *g* 4 °C during 40 min; the pellet contained the organelle-rich fraction and the supernatant was centrifuged again at 20,000 *g* for 30 min. The derived pellet and supernatant were referred as the membrane-containing and soluble fractions, respectively.

2.5. Cell viability and electrolyte leakage

Cell membrane stability and cell viability were estimated on leaf segments by the electrolyte leakage method, and by reduction of 2,3,5-triphenyl tetrazolium chloride (TTC) to red formazan, respectively. Electrolyte leakage was determined according to Bajji et al. [39] through measurements of electrical conductivity of PEG 10,000 (30%) solution containing leaf segments for 4 h, and a second time after autoclaving. For cell viability index, leaf segments were incubated at 30 °C in darkness in tubes containing 5 mL of 50 mM K_2HPO_4 at pH 7.0 for 15 h. After another incubation in ethanol 94% at 80 °C during 5 min, extracted formazan was quantified spectrophotometrically at 487 nm according to Lutts et al. [40].

Table 1
Physico-chemical properties of soil collected from the sites of Bois-Saint-Jean (BSJ), Carcocke (CAR) and Vieille Montagne (VM). Each value is the mean of 15 replicates ±SE. For a given parameter, means followed by the same letters are not significantly different at $P = 0.05$ according to the Tukey HSD test. Bioavailability of heavy metals and metalloids are indicated in italics into brackets below the concentration value and expressed as percentage of this value.

Soil parameters	BSJ	CAR	VW
pH H_2O	7.5 ± 0.2 a	7.0 ± 0.3 a	6.5 ± 0.1 b
Electrical conductivity (dS m^{-1})	1.3 ± 0.2 a	2.5 ± 0.3 b	2.8 ± 0.3 b
CEC (mol(+) DW)	10.9 ± 0.8 ab	13.5 ± 1.0 a	8.7 ± 0.5 b
Major elements (g.kg^{-1} DW)			
Ca^{2+}	26.7 ± 1.3 b	20.1 ± 2.7 a	35.3 ± 1.4 c
K^+	11.9 ± 0.9 a	8.2 ± 1.2 a	18.3 ± 2.1 b
Mg^{2+}	3.5 ± 0.6 a	3.6 ± 0.7 a	8.3 ± 0.9 b
Na^+	2.1 ± 0.2 a	7.2 ± 1.3 c	3.4 ± 0.1b
Fe^{2+}	15.4 ± 0.8 a	16.3 ± 0.3 a	84.6 ± 5.1b
Exchangeable basis (meq.100 g^{-1} DW)			
Ca^{2+}	22.3 ± 1.7 c	16.8 ± 1.2 b	2.4 ± 0.8 a
K^+	1.1 ± 0.2 b	0.8 ± 0.1 b	0.2 ± 0.0 a
Mg^{2+}	2.1 ± 0.3 b	0.6 ± 0.1 a	0.4 ± 0.1 a
Na^+	0.2 ± 0.1 b	0.1 ± 0.02 b	0.03 ± 0.01 a
Minor elements and heavy metals (mg.kg^{-1} DW)			
As	8.4 ± 1.3 a	28.4 ± 1.7 b	923 ± 84 c
	(0.47)	*(0.38)*	*(0.12)*
Cd	0.3 ± 0.1 a	3.2 ± 0.8 b	49 ± 3 c
	(2.12)	*(1.07)*	*(0.99)*
Cr	37 ± 2 a	35 ± 3 a	33 ± 4 a
	(1.45)	*(0.68)*	*(1.01)*
Cu	14 ± 1 a	42 ± 2 b	536 ± 41 c
	(0.45)	*(0.12)*	*(0.19)*
Ni	15 ± 1 a	97 ± 5 b	187 ± 21c
	(0.23)	*(0.17)*	*(0.11)*
Pb	23 ± 4 a	169 ± 11 b	9813 ± 111 c
	(0.14)	*(0.10)*	*(0.02)*
Zn	68 ± 9 a	225 ± 12 b	3012 ± 204 c
	(0.17)	*(0.15)*	*(0.32)*
Organic matter (%)	1.7 ± 0.2 a	17.8 ± 1.0 c	3.4 ± 0.4 b
Total N (%)	0.14 ± 0.02 a	1.27 ± 0.14 b	0.28 ± 0.19 a

2.6. Statistical treatment of the data

All statistical analyses were performed with R Software Version 3.6.1 (RDevelopment and C. TEM 2009). Prior to analyses, the normality of the variables was checked and the homogeneity of the variances was assessed on the basis of Levene's test. Then, ANOVAs were performed to measure the impact of site location and seasons on the measured variables. In the specific case of summer harvest, a two ways ANOVA was also performed to compare young- and old leaves in relation to site location. Post hoc test comparisons were performed using the Tukey HSD test. The data were expressed as means ± standard errors. Pearson correlation analysis between leaf pollutant concentration and leaf physiological properties were performed separately for each site, and for spring season on the one hand, and summer season on the other hand.

3. Results

3.1. Soil analysis

Tested soils were close to neutrality (Table 1) (BSJ and CAR) or slightly acid (VM). BSJ was not contaminated neither by heavy metals nor metalloid As. CAR was slightly contaminated by moderate concentrations of Cd, Ni and Zn. Although CAR contained higher levels than BSJ for As, Cu and Pb, the total concentrations in those three elements remained below the permissible limit imposed by the Belgian rules (65, 400 and 1840 mg Kg^{-1} DW for As, Cu and Pb, respectively). In contrast, VM was highly contaminated and exhibited quite high concentrations for all tested micro-elements and heavy metals (all above the permissible limits), with the noticeable exception of Cr which was present at similar concentrations for the three analyzed substrates. For all tested elements, bioavailability measured as a percentage of total concentration was the highest for the non-contaminated BSJ site. Bioavailability was higher in VM than in CAR for Zn ($P < 0.05$), Cu ($P < 0.01$), and Cr ($P < 0.05$) while an inverse trend was observed for all other elements. Contaminated sites contained higher organic matter than BSJ, and an extremely high concentration of organic matter was recorded for CAR which was 10 times higher than in BSJ and 5 times higher than in VM.

In most cases, soil enzyme activity (Table 2) was the highest in summer and the lowest in winter. The most polluted soil (VM) exhibited quite low values for microbial biomass and basal respiration while unpolluted BSJ presented the highest values for these parameters. Similarly, soil issued from BSJ had higher rate of fluorescein hydrolysis ($P < 0.01$), laccase ($P < 0.001$) and phosphatase ($P < 0.01$) activities than soil from the CAR area while an inverse trend was observed for urease activity ($P < 0.01$). Both CAR and VM soils had very low laccase activity which was even not detected, especially in winter. It is noteworthy that the presence of Miscanthus increased all recorded biological parameters and that this is valid for all sites ($P < 0.001$) at each season, although such a positive impact was not always significant in winter depending on the considered parameter.

Table 2
Soil biological properties recorded in spring, end of summer and end of winter on the sites of Bois-Saint-Jean (BSJ), Carcocke (CAR) and Vieille-Montagne (VM). Basal respiration, soil enzyme activities and microbial biomass carbon were recorded in plots cultivated with Miscanthus (+Misc) and on nearby unvegetated soils (0 Misc) located close to the cultivated plots. Each value is the mean of 15 replicates. For a given parameter, means followed by the same letter are not statistically different at $P = 0.05$ according to the Tukey HSD test. ND: not detected.

Parameter	BSJ		CAR		VM	
	0 Misc	+ Misc	0 Misc	+ Misc	0 Misc	+ Misc
Basal respiration (in mg CO_2.C Kg^{-1} h^{-1})						
Spring	2.07 e	2.36 f	0.98 c	1.28 d	0.17 a	0.35 b
Summer	2.38 f	2.73 g	0.95 c	1.19 d	0.23 ab	0.37 b
Winter	0.81 c	1.01 cd	0.37 b	0.41 b	0.10 a	0.18 a
Microbial Biomass Carbon (in mg.Kg^{-1})						
Spring	97.5 d	139.8 e	71.3 c	114.6 d	21.2 a	45.3 b
Summer	104.2 d	141.5 e	88.7 cd	145.9 e	19.3 a	23.6 a
Winter	50.3 BCE	64.9 c	28.3 ab	43.5 b	ND	19.7 a
Fluorescein hydrolysis (in mg.Kg^{-1} $3h^{-1}$)						
Spring	73.2 d	102.4 e	31.9 ab	40.8 BCE	35.3 b	37.6 b
Summer	98.7 e	141.6 f	43.2 c	69.6 d	33.4 b	35.5 b
Winter	51.4 c	70.8 d	21.6 a	28.4 ab	ND	ND
Urease activity (in µg N g^{-1} h^{-1})						
Spring	43.2 c	56.1 cd	91.7 e	121.8 g	11.2 a	31.7 b
Summer	63.8 d	79.9 e	103.4 f	147.9 g	17.3 a	40.8 c
Winter	21.6 ab	38.7 BCE	89.8 e	101.2 f	15.6 a	38.5 BCE
Laccase activity (µmol ABTS ox. g^{-1})						
Spring	5.2 de	10.1 f	ND	3.5 c	1.5 a	3.2 c
Summer	6.4 e	9.8 f	2.4 b	4.1 d	2.3 ab	3.7 c
Winter	6.0 e	7.3 e	ND	ND	ND	2.0 a
Acid phosphatase (in µg phenol.g^{-1} h^{-1})						
Spring	86.2 d	103.9 e	55.1 b	61.3 c	42.1 a	80.4 d
Summer	99.5 de	111.1 e	50.8 b	64.8 c	48.6 ab	78.5 d
Winter	40.3 a	60.4 c	47.9 ab	50.6 b	45.3 a	60.3 c

3.2. Plant growth and photosynthesis

Plant height, number of stems and plant dry weight (Table 3) were the highest for plants growing on BSJ. These parameters were significantly reduced in CAR and VM. Plants growing on the VM site exhibited the lowest values. Main stem diameter and number of leaves were similar in BSJ and CAR. Plants growing on VM exhibited a higher stem diameter and a lower number of leaves than those growing in BSJ and CAR.

Fluorescence-related parameters and pigment concentrations are provided by Fig. 1. In spring, leaves of plants growing on CAR were not affected for fluorescence-related parameters comparatively to plants growing on BSJ unpolluted control, while those growing on VM soil exhibited a reduced F_v/F_m ratio ($P < 0.001$) (Fig. 1a), a decrease in Φ_{PSII} ($P < 0.001$) (Fig. 1c) and a significant increase in NPQ ($P < 0.001$) (Fig. 1d). In summer, old leaves of plants growing on CAR presented a decrease in F_v/F_m and qP as well as an increase in NPQ. In contrast, young leaves chlorophyll fluorescence was not affected in those plants, except a small increase in NPQ values. As far as summer plants growing on VM soils are concerned, old leaves were seriously affected and all recorded parameters, including NPQ, strongly decreased. F_v/F_m, qP and Φ_{PSII}significantly decreased in young leaves, NPQ being unaffected comparatively to BSJ plants.

The total chlorophyll concentration (Fig. 1e) slightly increased in spring leaves of plants growing on CAR comparatively to plants growing on unpolluted BSJ ($P < 0.01$), while it decreased in leaves of VM ($P < 0.05$). In summer, total chlorophyll concentration decreased in old leaves on CAR site ($P < 0.05$) and remained unaffected in young ones. Total chlorophyll concentration decreased in all leaves of plants growing on VM site. The total carotenoid concentration (Fig. 1f) remained unaffected in leaves of plants growing on CAR site while it decreased in plants growing on VM site, especially in the old summer leaves ($P < 0.001$).

Plant growing on CAR soil presented a lower net photosynthesis (Fig. 2a) than those growing on unpolluted BSJ, although this was especially valid for old summer leaves ($P < 0.001$)). The recorded decrease in net photosynthesis was even more obvious for plants growing on VM soil since it was already recorded in spring leaves. The plants growing on CAR site exhibited a lower instantaneous transpiration rate (Fig. 2b) than those growing on BSJ ($P < 0.001$), while plants growing on VM had an opposite trend and displayed higher E values than plants growing in BSJ ($P < 0.01$). The intercellular CO_2 concentration (Fig. 2c) was lower in CAR than in BSJ ($P < 0.05$), while it was higher in VM than in BSJ, especially for leaves harvested on plants growing in summer ($P < 0.01$). In spring, the stomatal conductance (Fig. 2d) was decreased in plants growing on CAR comparatively to BSJ ($P < 0.05$), while g_s was increased in plants growing on VM site ($P < 0.001$). In summer, g_s values were higher in young than in old leaves, except for plants growing on VM site which exhibited a high g_s values in both leaves. As a consequence, instantaneous water use efficiency (A/E) ranged between 8.08 and 9.87 μmol CO_2.mmol^{-1} H_2O in plants growing on BSJ; it increased in plants growing on CAR (10.11–14.4 μmol CO_2.mmol^{-1} H_2O) while it decreased in plants growing on VM site (2.48–5.13 μmol CO_2.mmol^{-1} H_2O). The intrinsic water use efficiencies (A/g_s) were similar in BSJ (0.123 ± 0.006 μmol CO_2.mmol^{-1} H_2O) and in CAR (0.126 ± 0.017 μmol CO_2.mmol^{-1} H_2O) but was clearly lower for plants growing on VM site (0.053 ± 0.009 μmol CO_2.mmol^{-1} H_2O).

3.3. Plant mineral concentration

Leaves harvested from plants growing on the VM site accumulated higher concentrations for all considered trace elements, except Cr (Fig. 3). In plant growing on CAR soil, the recorded concentrations were similar in spring leaves on the one hand, and in young summer leaves on the other hand. In contrast, old leaves harvested in summer exhibited higher concentrations than young leaves. Such a preferential accumulation of trace elements in older leaves was never recorded for plants growing on the VM site, the concentration being statistically equivalent in the two types of leaves, whatever the considered organ. Concentrations recorded in winter leaves were significantly lower than in old summer leaves for Cd, Zn and metalloid As in both CAR and VM, but also for Ni and Pb for plants growing on VM soil, as well as Cu and Cr for plants growing on CAR.

Heavy metals and arsenic concentrations in stems and rhizomes are provided in Table 4. The stems were harvested in spring, end of summer and end of winter. Rhizome samples were collected in winter. As far as stems are concerned, plants growing on VM soil always exhibited higher concentration for trace elements than those growing on BSJ and CAR, whatever the considered element and the season. As far as plants growing on BSJ site are concerned, stem concentrations did not vary according to the growing season and remained constant throughout the growing cycle for all considered elements except Pb. For As, Cd and Ni, stems from plants growing on CAR exhibited a higher concentration in summer than in spring while a reverse trend was recorded for Pb. For plants growing on VM

Table 3
Plant growth parameters of Miscanthus growing on the sites of Bois-Saint-Jean (BSJ), Carcoke (CAR) and Vieille Montagne (VM). Plant height, main stem diameter, number of stems and single plant dry weight (mainly stems) were recorded at the time of harvest at the end of winter. Number of leaves was recorded during the time course of the experiment before leaf senescence and abscission. Each value is the mean of 10 replicates ± SE. For a given parameter, means followed by the same letters are not significantly different at $P = 0.05$ according to the Tukey HSD test.

Parameters	BSJ	CAR	VM
Height (cm)	196.2 ± 11.7 a	145.3 ± 9.7 b	80.3 ± 6.4 c
Main stem diameter (cm)	1.09 ± 0.21 a	0.89 ± 0.13 a	1.45 ± 0.09 b
Number of stems	15.4 ± 1.1 a	11.5 ± 1.2 b	6.3 ± 0.9 c
Number of leaves	21.9 ± 3.4 a	20.4 ± 2.9 a	10.7 ± 1.4 b
Single plant dry weight (g)	311.7 ± 21.6 a	225.8 ± 19.4 b	112.4 ± 8.9 c

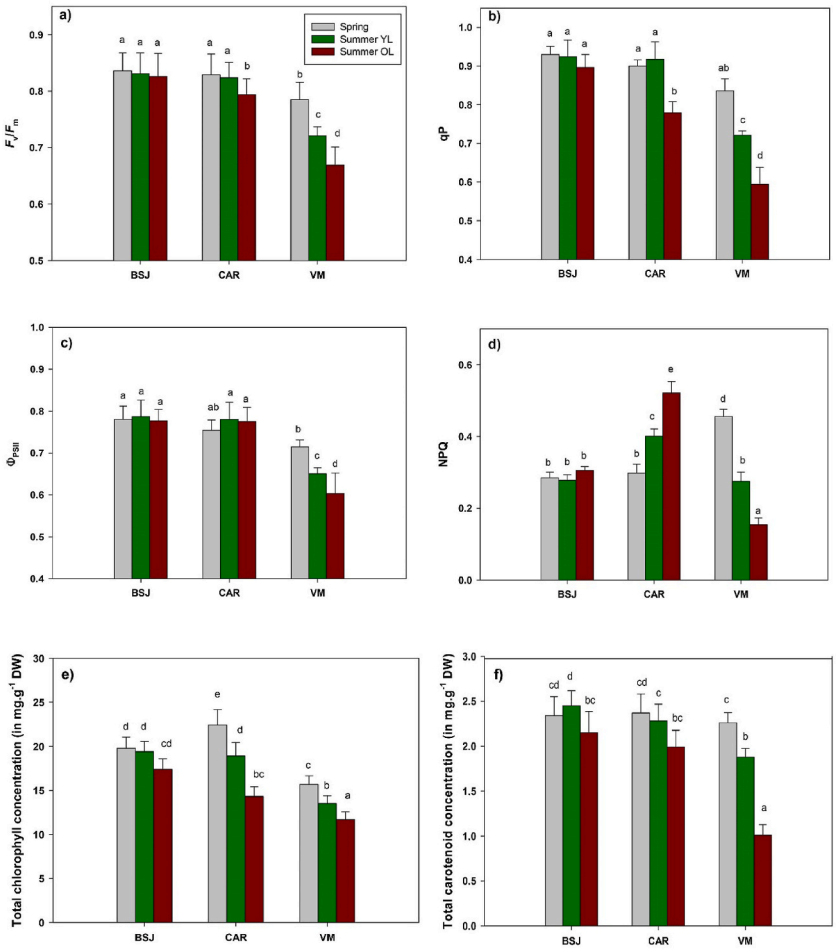

Fig. 1. Chlorophyll fluorescence-related parameters and pigment concentrations in leaves of Miscanthus growing on the sites of Bois-Saint-Jean (BSJ), Carcoke (CAR) and Vieille Montagne (VM). Maximal efficiency of PSII photochemistry (F_v/F_m; a), photochemical quenching (qP; b), operational efficiency of PSII photochemistry (Φ_{PSII}; c), non-photochemical quenching (NPQ; d), total chlorophyll concentration (Chl a + chl b; e) and total carotenoid (Car; f) were recorded on youngest fully expanded leaf of the main stem in spring, and separately on old leaf (OL) and young leaf (YL) at the end of summer. Each value is the mean of 15 replicates and vertical bars are standard errors. Values exhibiting the same letters are not significantly different at $P = 0.05$ according the Tukey HSD test.

soil, the highest concentrations of trace elements were always recorded in winter.

Rhizome samples collected on BSJ and CAR soils contained similar concentrations of trace elements, except for Zn and As whose concentrations were higher in the latter than in the former. In contrast, rhizomes collected on VM contained higher concentrations than those harvested on BSJ and CAR, except for Mn whose rhizome concentration was similar for the three studied sites.

Plants growing on polluted sites not only differed for the total trace elements concentrations, but also for their distribution within the leaf organs, as shown in Fig. 4 for Cd, Zn, Cu and As for the polluted sites CAR and VM. Cadmium (Fig. 4a) and zinc (Fig. 4b) displayed similar patterns: a highest percentage of these elements was found in the cell wall fraction of CAR plants comparatively to VM plants. In contrast, VM plants exhibited a high percentage of Cd and Zn in the soluble fractions than plants growing on VM site. The proportion of Cd and Zn in the organelle fraction was also higher in VM plants, especially for spring leaves than in CAR plants. Copper (Fig. 4c) also accumulated in high proportion in the cell wall fraction of plants growing on CAR soil, while *c.a.* 50% of Cu is present in the soluble fraction of VM plants. As far as metalloid As is concerned (Fig. 4d), the highest proportion was recorded in the soluble fraction and only young summer leaves of CAR plants presented an increase of As in the cell wall fraction. It is noteworthy that plants growing on VM presented a quite higher As proportion in the organelle fraction than plants growing on CAR site for all analyzed leaves.

3.4. Cell viability and electrolyte leakage

Cell viability and cell membrane stability are provided in Table 5. The highest cell viability and the lowest electrolyte leakage were observed for plants growing on BSJ. Additionally, values were similar for leaves collected in spring and young leaves collected in summer. Cell viability decreased and electrolyte leakage increased in leaves collected from plants growing in CAR. In summer, old

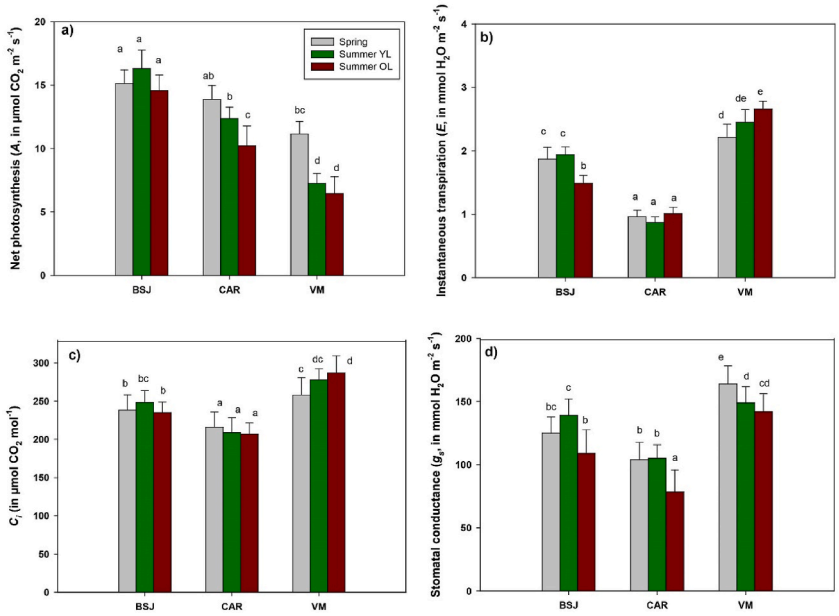

Fig. 2. Gas exchange-related parameters in leaves of Miscanthus growing on the sites of Bois-Saint-Jean (BSJ), Carcoke (CAR) and Vieille Montagne (VM). Net photosynthesis (*A*; a), instantaneous transpiration (*E*; b), intercellular CO_2 concentration (C_i; c) and stomatal conductance (g_s; c) were recorded on youngest fully expanded leaf of the main stem in spring, and separately on old leaf (OL) and young leaf (YL) at the end of summer. Each value is the mean of 15 replicates and vertical bars are standard errors. Values exhibiting the same letters are not significantly different at $P = 0.05$ according the Tukey HSD test.

leaves exhibited lower cell viability ($P < 0.01$) and higher electrolyte leakage ($P < 0.01$) than young leaves. The lowest cell viability and highest electrolyte leakage were observed for plants growing on VM; in this case all leaves exhibited similar cell viability index, and no difference in electrolyte leakages could be recorded between old and young leaves for samples collected in summer.

4. Discussion

4.1. *Miscanthus x giganteus is tolerant to pollutant and may accumulate arsenic and heavy metals*

The present study demonstrates that the fast-growing plant species *Miscanthus x giganteus* was able to grow on a moderately heavy metal contaminated site and, despite a strong decrease in biomass production was also able to survive on a highly polluted one, thus confirming the high level of heavy metal tolerance in this species already reported by other studies [1,2,10,13,24,41]. Nebeská et al. [42] recently demonstrated that *Miscanthus x giganteus* growing on marginal or abandoned sites may be strongly affected by nutrient deficiencies which may have stronger impacts on growth or plant physiology than heavy metals. It has however to be mentioned that CAR and VM substrates did not exhibit obvious lower amounts of essential elements than clean soil from BSJ (except for exchangeable Mg^{2+} (Table 1)) and that all plots were fertilized at the beginning of the trial (see Material and Methods).

It is frequently reported that *Miscanthus x giganteus* behaves as an excluder sequestering most absorbed heavy metals in the root system [11,12,14,18,41]. The present work, however, showed that heavy metals may also accumulate to some extent in the shoot part (mainly Cd, Zn, Ni and to a lower extent Cu) and may uptake As, supporting the view of Kocón and Matyka [43] and Barbosa et al. [10] who showed that *Miscanthus x giganteus* may be used for Cd and Zn phytoextraction purposes. We demonstrated that heavy metals distribution within the shoots differed according to the season but also according to the quantitative importance of heavy metal accumulation. Moreover, this study demonstrated that not only the intensity but also the nature of physiological modifications differed depending on the level of contamination.

4.2. *Miscanthus x giganteus increased soil microbial activity*

Numerous studies demonstrated that Miscanthus may contribute to soil remediation by acting on microbial community abundance and/or diversity [24,44,45]. It is therefore not surprising that for all the tested sites (including the non-polluted control BSJ) soil biological activity was higher for most recorded parameters for samples harvested within Miscanthus plots comparatively to those collected in close unvegetated places. Rakić et al. [46] identified several bacterial strains with high tolerance to Ni, Pb and Cu associated with Miscanthus growing on heavy metal-contaminated substrate. In our study, however, microbial biomass, basal respiration and enzyme activities were the lowest on the most polluted VM site.

Plant root exudates provide a variety of resource for microbial growth and stimulate their activity. The poor growth and

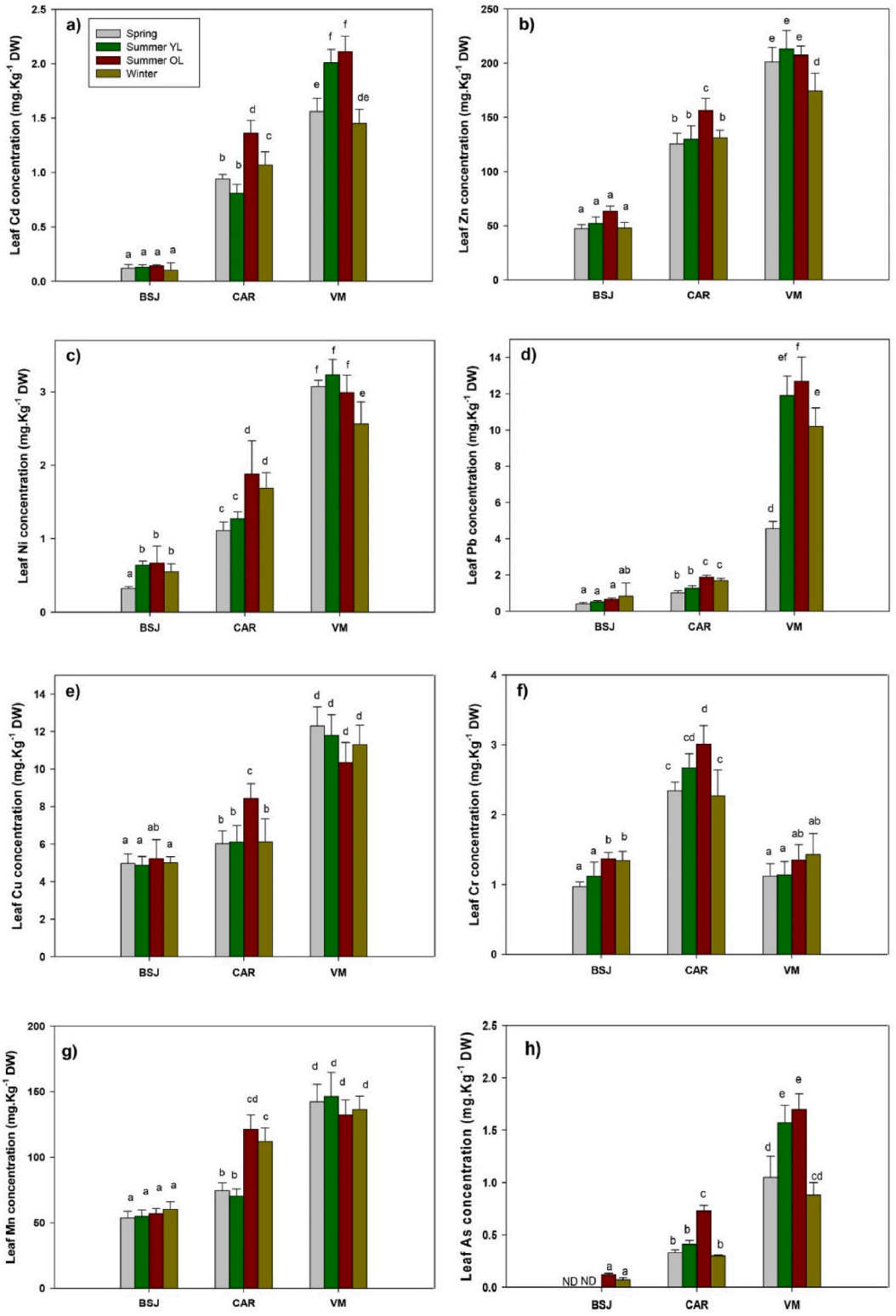

Fig. 3. Trace element concentration in leaves of Miscanthus growing on the sites of Bois-Saint-Jean (BSJ), Carcoke (CAR) and Vieille Montagne (VM). Values were recorded on youngest fully expanded leaf of the main stem in spring, separately on old leaf (OL) and young leaf (YL) at the end of summer, and on senescing leaves just before absicission in winter. Each value is the mean of 15 replicates and vertical bars are standard errors. Values exhibiting the same letters are not significantly different at $P = 0.05$ according the Tukey HSD test.

Table 4
Heavy metals and As concentrations (in mg.Kg^{-1} DW) in stem and rhizome of Miscanthus growing on the sites of Bois-Saint-Jean (BSJ), Carcoke (CAR) and Vieille Montagne (VM). Stems were analyzed in spring, at the end of summer, and at the end of winter. Rhizome samples were collected in winter, just before stem harvest. Each value is the mean of 10 replicates ± standard errors. For a given element and a given organ, means followed by the same letters are not significantly different at $P = 0.05$ according to the Tukey HSD test. ND: not detected.

Organ	Site	Season	Cd	Ni	Zn	Pb	Cu	Cr	Mn	As
Stem	BSJ	Spring	ND	0.23 ± 0.04 a	35 ± 2 a	0.8 ± 0.1 a	5.0 ± 0.4 a	1.0 ± 0.1 a	46 ± 4 a	0.65 ± 0.11 a
		Summer	0.15 ± 0.03 a	0.26 ± 0.03 a	36 ± 1 a	ND	4.9 ± 0.3 a	ND	44 ± 5 a	ND
		Winter	0.13 ± 0.02 a	0.25 ± 0.04 a	40 ± 2 b	1.2 ± 0.1b	5.0 ± 0.2 a	0.8 ± 0.2 a	48 ± 5 a	0.83 ± 0.12 a
	CAR	Spring	1.21 ± 0.07 b	2.18 ± 0.14 b	107 ± 9 c	3.7 ± 0.1 d	5.1 ± 0.3 ab	1.2 ± 0.1b	51 ± 3 a	1.26 ± 0.15 b
		Summer	1.45 ± 0.14 c	2.54 ± 0.17 c	112 ± 7 c	3.5 ± 0.1c	5.5 ± 0.4 b	1.1 ± 0.1 ab	52 ± 7 ab	3.23 ± 0.45 c
		Winter	1.17 ± 0.08 b	2.49 ± 0.15 c	104 ± 9 c	3.6 ± 0.2 cd	5.6 ± 0.3 b	1.5 ± 0.3 c	64 ± 8 b	3.07 ± 0.37 c
	VM	Spring	2.24 ± 0.05 e	3.13 ± 0.12 e	223 ± 17 d	4.7 ± 0.3 e	9.2 ± 0.4 d	5.0 ± 0.5 e	102 ± 9 cd	6.13 ± 0.59 d
		Summer	2.07 ± 0.07 d	2.89 ± 0.09 d	206 ± 15 d	4.9 ± 0.4 e	8.7 ± 0.3 c	4.1 ± 0.4 d	94 ± 8 c	6.27 ± 0.17 d
		Winter	2.15 ± 0.09 de	3.24 ± e	241 ± 14 e	4.9 ± 0.1 e	9.6 ± 0.1 e	4.8 ± 0.3 e	115 ± 6 d	8.11 ± 0.41 e
Rhizome	BSJ	Winter	0.11 ± 0.04 a	0.32 ± 0.05 a	28 ± 3 a	ND	8.1 ± 0.3 a	1.1 ± 0.3 a	23 ± 1 a	0.21 ± 0.05 a
	CAR	Winter	0.15 ± 0.03 a	0.28 ± 0.03 a	40 ± 5 b	ND	7.9 ± 0.4 a	1.0 ± 0.3 a	23 ± 2 a	1.02 ± 0.11 b
	VM	Winter	1.03 ± 0.06 b	2.07 ± 0.11b	60 ± 5c	2.8 ± 0.5	17.6 ± 1.4 b	5.2 ± 0.7 b	26 ± 2 b	3.03 ± 0.25 c

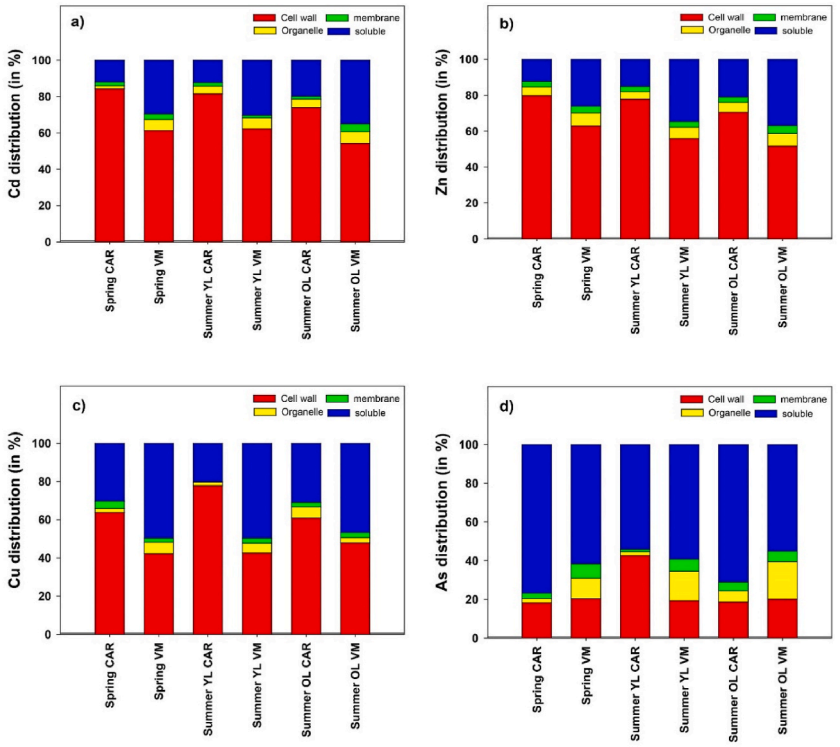

Fig. 4. Subcellular distribution of cadmium (a), zinc (b), nickel (c) and arsenic (d) in leaves harvested on Miscanthus growing on the sites of Carcoke (CAR) and Vieille Montagne (VM). Values were recorded on youngest fully expanded leaf of the main stem in spring, separately on old leaf (OL) and young leaf (YL) at the end of summer. Cell wall, soluble, organelle-rich and membrane-bound fractions are expressed as percentage of the total concentration for each element (see Fig. 3).

physiological performances of Miscanthus growing on VM soil may thus partly explain the low values recorded for soil biological parameters on this site. According to Lukowski and Dec [47], the recorded activities also depend on the season in relation to temperature, soil water content, and physiological activity of the rhizosphere, which could explain the highest values recorded for soil biological parameters at the end of summer in our study. At this period indeed, temperature allowed an optimal microbial development

Table 5
Cell viability index (in D.O. at 487 nm g^{-1} FW) and electrolyte leakage (in %) in leaves of Miscanthus growing on the sites of Bois-Saint-Jean (BSJ), Carcoke (CAR) and Vieille Montagne (VM). Leaves were analyzed in spring (considering the youngest fully expanded leaf) and in summer (separately for old leaves and young leaves). Each value is the mean of 10 replicates ± SE. For a given parameter, means followed by the same letters are not significantly different at $P = 0.05$ according to the Tukey HSD test.

Parameters	BSJ	CAR	VM
Cell viability index (D.O. at 487 nm g^{-1} FW)			
Spring leaf	0.67 ± 0.02 e	0.52 ± 0.03 c	0.39 ± 0.02 ab
Summer leaf – old	0.63 ± 0.03 d	0.45 ± 0.03 b	0.34 ± 0.03 a
Summer leaf – young	0.71 ± 0.02 e	0.53 ± 0.01c	0.33 ± 0.01 a
Electrolyte leakage (in %)			
Spring leaf	10.6 ± 0.8 ab	12.7 ± 0.2 b	23.2 ± 0.2 d
Summer leaf – old	11.7 ± 0.2 b	17.5 ± 0.1 c	28.9 ± 0.3 e
Summer leaf – young	9.5 ± 0.4 a	14.4 ± 0.6 BCE	29.4 ± 0.1 e

but also an active plant physiological activity leading to root exudation, while the low microbial biomass carbon and plant senescence occurring in winter may hamper microbial activity. Moreover, Nebeská et al. [48] reported a strong decrease in the proportion of gram-positive bacteria in heavy metal polluted soils comparatively to unpolluted ones, confirming that pollutants may have a direct toxicity on specific bacterial strains. It may also be argued that higher enzyme activity does not always imply a better soil health. Indeed, many extracellular enzymes (especially hydrolases) are secreted to provide available nutrients and higher activity can signalize more urgent microbial afford for insufficient nutrients and worse physiological state.

4.3. Pollutant distribution in leaves of different ages and their consequences on photosynthesis depend on the season and soil level of contamination

Although heavy metals bio-availability expressed as a percentage of total heavy metal concentration was lower in VM than in CAR soil, the total soil concentration on VM was so high that the amount of heavy metals which could be absorbed by the plants was by far higher on VM than on CAR soil. The higher leaf concentration in heavy metals for plants growing on VM was consequently expected and suggests that the root retention capacities were overwhelmed already in spring for plants growing on the most polluted substrate.

Correlation between leaf heavy metals or As and leaf physiological parameters are provided for BSJ, CA and VM sites inTables S1, S2 and S3, respectively (supplemental data). In plants growing on BSJ site, the leaf pollutant content was so low that almost no significant correlation was found (Table S1) but the situation was quite different for plants growing on the moderately polluted CAR site (Table S2) or the highly polluted VM site (Table S3).

In summer, heavy metals and metalloid As preferentially accumulated in old leaves of plants growing on CAR. This strategy could be regarded as an attempt to protect the most photosynthetic-active young leaves. Such a preferential accumulation of toxic elements in older tissues of *Miscanthus x giganteus* was already reported for Zn by Andrejić et al. [25]. In CAR site, photosynthesis protection in young leaves is supported by lower qP and higher NPQ values in old leaves comparatively to young leaves, as well as a lower chlorophyll concentration and net photosynthesis values. In contrast, such a process of leaf compartmentation was not efficient anymore in plants growing on VM soil and trace elements distribution between leaves of different ages was more uniform in this case. Nevertheless, the physiological consequences of heavy metal accumulation may still differ between old and young leaves of VM plants since all fluorescence-related parameters were lower in old than in young leaves despite similar heavy metal concentrations in these plants. This should not be regarded as an impact of leaf age since no similar trend was reported for BSJ plants. It is noteworthy that NPQ increased in summer old leaf of plants growing on CAR while it strongly decreased in plants growing on VM. On this VM site, the correlation between all pollutants concentrations in old summer leaves and NPQ was negative, while a positive correlation was found on the same site for spring leaves (Table S3).

As reported by Flores-Bastrello et al. [49], NPQ is directly related to various processes, the most important and rapid one being related to the interconversion of violaxanthine to zeaxanthine via the xanthophyll cycle. In old leaves of plants growing on VM site, we observed a strong decrease in carotenoid concentration which could hamper the dissipation of excess energy by the non-photochemical quenching [36]. Once again, a negative correlation was found between all pollutants and carotenoids concentrations in old summer leaves (Table S3). The lower value of NPQ in summer old leaves of VM site is reflecting the incapacity to regulate light capture or is indicating that the capacity of NPQ to regulate light capture is saturated. A toxic impact of heavy metals on PsbS proteins could however not be ruled out, especially if we consider that the organelle fractions contained a higher proportion of toxic elements in VM than in CAR (Fig. 4), although the maintenance of photosynthetic proteins is frequently considered as an integral component of photosynthesis robustness in *Miscanthus x giganteus* [50]. Net photosynthesis of old and young leaves was indeed similar for plants growing on VM site, suggesting that NPQ impairment in old leaves should not be regarded as the major factor limiting photosynthesis in old leaves.

Miscanthus x giganteus displays a typical NADP-ME-type pathway of C4 photosynthesis [50–52]. Beside NPQ values, other parameters were also affected in very contrasting ways in plants growing on CAR and VM sites. Although net photosynthesis was reduced in both cases, stomatal conductance was reduced in plants growing on CAR soil while it was increased in plants growing on VM site. As shown in Table S3 for summer young leaves from VM site, almost all pollutant concentration except Cr and Mn were positively correlated to g_s while negative correlation was found between g_s and Cd, Zn and Ni concentrations for the same leaves from plants

growing on the CAR site. Miscanthus is thought to behave as an anisohydric plant species [53]. In those species, plant stomata remain open at low water potential and the plants extensively use soil water resources through leaf transpiration. However, distinction between ishohydric and anisohydric plant species is commonly established in response to water stress. In the present study devoted to ionic toxicities, Miscanthus may adopt the two types of strategy depending on the level of contamination: it closes its stomata and behaved as a water-saving plant species in response to moderate pollution while it opened them in plants exposed to high levels of pollution. Heavy metals may interfere with ion channels involved in stomatal regulation at the guard cell levels and the nature and the level of toxic ions in these specific cells might differ for plants exposed to distinct levels of pollution. Although stomatal opening allowed plants growing on VM to regulate leaf temperature through transpiration when thermal dissipation of light energy was compromised as suggested by a decrease in NPQ, it also contributed to increase heavy metals and arsenic translocation to the shoot. Rusinowski al [54]. compared on a heavy metal-contaminated site, new hybrids issued from *M. sinensis* x *M. sacchariflorus* with commercial *Miscanthus x giganteus* and concluded that the improved behavior of hybrids was directly related to a more rapid stomatal closure allowing to reduce Cd and Pb accumulation.

It is interesting to notice that C_i decreased in plants growing on CAR soil, reinforcing the hypothesis that carboxylation efficiency was maintained in those plants and that photosynthesis decrease was mainly due to stomatal factors. In spring and summer young leaves from CAR site, a negative correlation ($P < 0.01$) was found between Ci and Cd or As concentration (Table S2). Once again, plants growing on VM displayed an opposite trend: a higher C_i concentration was noticed despite the maintenance of stomatal conductance leading us to hypothesize that photosynthesis limitation resulted from non-stomatal factors for VM plants. Although C4 plants require much less Rubisco than C3 plants, Rubisco activity still constitutes one of the major rate-limiting steps for photosynthesis in Miscanthus [25,51,52]. We may therefore not exclude that heavy metals and metalloid As have an impact on Rubisco activity or RbcS gene expression, as recently reported by Sun et al. [52] for salt stress. Rubisco alteration could lead to an increase in the bundle sheath leakiness of CO_2 [51]. Arsenic is easily absorbed by Miscanthus [55,56]. It was shown in the present study that the proportion of metalloid As in the organelle-rich fraction (Fig. 4) was quite higher for plants growing on VM comparatively to CAR soil. Arsenic is extremely toxic for chloroplasts where it links to dithiol compounds and enzyme containing closely spaced Cys residues [57]. Other elements such as Zn may also be toxic in chloroplasts [25]. Despite the higher values of E, C_i and g_s in plants on VM site, these plants showed the lowest CO_2 assimilation rate. This could be partly explained by the deficiency in the photochemical yield leading to the lack of ATP and NAPDH from the thylakoid membrane electron transport chain, this hypothesis being consistent with the recorded decrease in the fluorescence parameters.

4.4. Winter heavy metal remobilization in senescent plants in relation to subcellular distribution

The recorded decrease in trace elements in senescent winter leaves comparatively to summer old leaves (mainly for plants growing on VM site) suggests that some of the accumulated elements could be remobilized from the leaves to the rhizome at the end of the growing season. According to Amougou et al. [9], rhizomes and roots represent up to 50% of the total plant biomass, but our field approach did not allow us to reliably estimate the weight of below-ground organs. However, we noticed that rhizomes of plants growing on VM contained higher concentrations of metal(loids) than rhizomes collected on the other sites. Laval-Gilly et al. [18] also reported the presence of Cd, Cu, Pb and Zn in rhizomes of Miscanthus growing on contaminated brownfield. An easier mobilization of leaf elements to rhizomes for plants growing on VM might be related to a higher proportion of elements present in the cell soluble fraction (Fig. 4). In contrast, leaves harvested on CAR exhibit a high proportion of cell-wall bound trace elements which are hardly remobilized during senescence [38].

. Cell wall retention of toxic elements by strong linkage to negatively charged polymer of hemicellulose and pectin is a strategy of heavy metal tolerance which could partly contribute to the better behavior of plants growing on CAR comparatively to those growing on VM. This is supported by the higher cell viability index and lower electrolyte leakage in the former than in the latter (Table 5). In VM leaves, higher proportion of toxic elements in soluble and organelle fractions could have a deleterious impact on cell metabolism leading to a decrease in cell viability index. Similarly, the inability of the VM summer old leaves to perform thermal dissipation of light energy could lead to oxidative stress, leading to injuries in cell membranes and thus increasing electrolyte leakage [58,59].

Winter generates large amounts of leaf litter at the soil surface. Despite the marginal remobilization process occurring during senescence, dropped leaves still contain high concentration of trace elements and their decomposition may contribute to metal mobility in the plant-soil system [23]. For most elements except Cr, stems and leaf concentrations were in the same order of magnitude and stem concentrations were not above the critical threshold allowing combustion of ligno-cellulosic biomass [60]. Similarly, Bilandžija et al. [21] reported that *Miscanthus x giganteus* biomass collected on a Cd and Hg contaminated area was compatible with the ISO 17225–1:2014 norm for solid biofuel. The chemical composition, bulk density and flowability will be important parameters to quantify in order to determine the final fate of the obtained ashes [19]. Specific attention should be paid to avoid dissemination of metals to the atmosphere during the combustion process but also during ashes disposal considering the potential risk of heavy metal and metalloids for human health as clearly demonstrated by Yüksel et al. [6] and by Topaldemir et al. [7]. The quality of the harvested biomass should also be carefully assessed since heavy metals could modify hemicellulose, cellulose and lignin proportion and modify cellulose degree of polymerization and crystalline index which could have an influence on the combustion processes or bioethanol production from *Miscanthus x giganteus* [1,22,61].

5. Conclusion

In this study, we confirmed that heavy metal and As distribution within *Miscanthus x giganteus* and physiological consequences of

their accumulation depend on the phenological stage of the plant and the level of soil contamination. *Miscanthus x giganteus* is able to cope with moderate concentrations of heavy metals and could be recommended as a suitable plant species for phytomanagement of polluted sites. Plants cultivated on these soils improve soil biological parameter but heavy metal distribution within the plant and physiological consequences of accumulated pollutants depend on the level of contamination. In response to moderate concentration, heavy metals accumulate mainly in older leaves but not in young leaves and are sequestered in cell walls. Photosynthesis is reduced as a consequence of stomatal closure. At higher concentrations, heavy metals are evenly distributed among leaves of different ages and a consistent portion of heavy metals is present in the soluble and the organelle-rich fractions. These plants are unable to efficiently close their stomata and display a strong decrease in water use efficiency. In this case, the recorded decrease in net photosynthesis mainly results from photochemical yield deficiencies. Additional experiments are required 1) to precise the possible use of harvested biomass and 2) to identify molecular cues involved in plant response to pollutants.

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Data availability

Data will be made available on request.

CRediT authorship contribution statement

S. Lutts: Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **M.X. Zhou:** Writing – review & editing, Supervision, Project administration. **A. Flores-Bavestrello:** Writing – review & editing, Validation, Methodology, Formal analysis, Data curation. **P. Hainaut:** Writing – review & editing, Formal analysis, Data curation. **H. Dailly:** Writing – review & editing, Investigation, Formal analysis, Data curation. **G. Debouche:** Writing – review & editing, Software, Formal analysis, Data curation. **G. Foucart:** Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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