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Haematological and physiological characteristics of diploid and triploid sea bass, Dicentrarchus labrax L.

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Abstract:

The purpose of this study was to determine whether diploid and triploid sea bass differed in terms of main haematological and physiological characteristics. Diploid and triploid fish were produced by sub-optimal pressure treatments and held in communal environments under standard rearing conditions. Total red blood cell count (RBCC), haemoglobin concentration (Hb), hematocrit (Hct), mean cell volume (MCV), mean cellular haemoglobin content (MCH), mean cell haemoglobin concentration (MCHC), plasma metabolites, osmotic pressure, gill Na^+/K^+-ATPase activity, electrolytes, cortisol, and 3,5,3′-triiodo-L-thyronine (T_3), were measured and compared. Triploidisation in sea bass led to an increase in erythrocyte size (32% in cytoplasm surface area, and 50% in nucleus) and a decrease in erythrocyte number (~34%). Haemoglobin and basal plasma cortisol levels were significantly lower in triploid sea bass than in diploids. There were also differences between ploidies in the plasma concentrations of some electrolytes, with triploids showing lower concentrations of K, Fe, Zn, S, and Cu than their diploid counterparts.

Keywords: Haematology; Physiology; Diploid; Triploid; Sea bass; *Dicentrarchus labrax*

1. Introduction

Effort in induced triploidy in fish is almost entirely due to its potential applications to commercial farming and fisheries management with the objective to generate sterility. Triploid fish show impaired gametogenesis and subsequent energy investment in somatic growth is not hindered by the metabolic cost of sexual maturation. Additionally, sterility makes triploids of interest as a means to prevent declines in flesh quality associated with sexual maturation and to address concerns regarding the environmental impact of domestic escapees (Peruzzi *et al*., 2004). The methods used to induced triploidy in a variety of fish species, the performances of triploid fish and the rationales for their practical use are largely documented and reviewed (Arai, 2001; Dunham, 1990; Felip et al., 2001; Mair, 1993; Pandian & Koteeswaran, 1998; Purdom, 1993). Despite the interest, results on the performance of triploid fish remain conflicting, seem to be species related and depend on rearing conditions. In particular, it has been suggested that triploid fish perform poorly under sub-optimal environments or when cultured in competition with diploids and maybe more sensitive to stress imposed by handling. Several studies are available on these and other aspects related to the physiology and behaviour of triploid fish compared to their diploid counterpart (see review by Benfey, 1999). This last author also put forward the potential advantage in the application of sub-optimal treatments that yield both triploid and diploid fish for use in comparative studies (Benfey, 1999).

In triploid European sea bass (*Dicentrarchus labrax* L.), growth performances are comparable or lower than those of diploids in juvenile or large fish, whereas some qualitative traits can be superior (Felip *et al*., 1999; Felip *et al*., 2001; Peruzzi *et al*., 2004). Overall, some interest in the use of triploid sea bass for aquaculture and environmental purposes still subsists.

Although time-course studies on several blood constituents of diploid sea bass are available in relation to environmental factors and/or feeding (Carrillo et al., 1982; Cerdá-Reverter et al., 1998; Gutierrez et al., 1984; Pavlidis et al., 1997; Planas et al., 1990; Roche et al., 1989), handling and stress (Hadj Kacem et al., 1986; Hadj Kacem et al., 1987; Marino et al., 2001; Roche & Bogé, 1996), no comparative assessment of the basic hematological and physiological characteristics of diploids and triploids is available yet.

The purpose of this work is to investigate the major blood and serum constituents in adult diploid and triploid sea bass in a view to provide some insights of their energy metabolism and possible adaptive behavioural interactions during rearing and husbandry procedures. For this purpose, the study examines and compares the main hematological parameters along with plasma metabolites, osmotic pressure, gill Na^+/K^+-ATPase activity, electrolytes, cortisol and thyroid hormone (T_3) in diploid and triploid fish produced by sub-optimal pressure treatments and held under communal environments.

2. Material and methods

2.1. Biological material and fish handling procedures

Sea bass of 500-1200g originated from a mixed diploid and triploid stock kept under natural conditions of photoperiod and temperature at IFREMER Palavas-les Flots. This consisted of n=56 diploid and n=45 triploid fish, individually PIT tagged and of certified ploidy. Fish were produced by sub-optimal pressure shock treatments (7,000 psi / 2 min duration) applied on newly fertilized eggs at 6 min post fertilization. Eggs that did not receive pressure treatment were used as diploid controls. Artificial insemination, pressure treatment procedures and flow cytometric analyses were performed following Peruzzi and Chatain

(2000). Phenotypic sex and weight of diploid and triploid fish were determined two weeks before sampling. Sex of triploid fish could only be ascertained at the end of experimentation.

2.2. Sampling

The experiment was conducted during summer 2003, otside the normal spawning season of local sea bass stocks (January-February). Fish were left undisturbed and fasting for 2 days prior to experimentation. On the day of sampling, the water level was reduced by slow siphoning to minimize stress. Fish were then lightly anaesthetized (Eugenol, Cooper, 15ppm), randomly sampled and their PIT-tag code identified. A total of n=16 diploids (mean weight ± CI = 1005.63 ± 93.10 g) and n=15 triploids (mean weight ± CI = 688.33 ± 65.37 g) were used for the experiment. Two milliliters of blood were drawn by caudal puncture using syringes containing 2mg of EDTA. Six operators performed the operation simultaneously and handling operations were completed within 15 minutes of first disturbing the fish. Two sub-samples of blood (approx. 1ml) were then transferred into disposable test tubes and kept on ice. Triploids fish were sacrificed by overdose of anesthetic, and their sex macroscopically determined (Chatain et al., 1999).

2.3. Haematology, plasma chemistry, metabolites and hormones

Blood smears and red blood cell count preparations were performed using standard hematological techniques (Dacie and Lewis, 2001). Two 50µl hematocrit tubes were filled with blood and kept refrigerated (4ºC) in an upright position until centrifugation (5mn at 12.000g). One blood sample was refrigerated for later hemoglobin (Hb) determinations. The remaining sample was centrifuged (5min at 9.000g), plasma drawn off and divided into three aliquots which were either refrigerated or frozen (-20ºC). Plasma glucose, total proteins and osmotic pressure were determined within two days from refrigerated plasma samples. All

other analyses were performed within two months from frozen samples. Osmotic pressure (mOsm) was measured on 100µl plasma samples by use of an automatic micro-osmometer (Autocal 13®Roebling). Red blood cell measurements (cytoplasm and nuclear area, width and length) were evaluated from blood smears by means of a Zeiss microscope equipped with video camera module and computer-assisted image analysis (Visilog 5.2, ©Noesis Vision Inc., Canada). A minimum of n=30 and a maximum of n=90 erythrocytes/sample from n=16 diploid and n=15 triploid fish were analysed in such way. Total red blood cell count (RBCC), hemoglobin concentration (Hb) and hematocrit (Hct) enabled the mean cell volume (MCV), mean cellular hemoglobin content (MCH), and mean cell hemoglobin concentration (MCHC) to be calculated according to the following formulas (Dacie and Lewis, 2001):

$MCV\ (fl) = Hct / RBCC\ (10^6\ \mu l^{-1})$

$MCH\ (pg) = [Hb\ (g\ dl^{-1})\ x10] / RBCC\ (10^6\ \mu l^{-1})$

and

$MCHC\ (g\ l^{-1}) = [Hb\ (g\ dl^{-1})\ x10] / Hct$

Total blood hemoglobin concentration was measured by colorimetric test, and plasma glucose by GOD-PAP enzymatic-colorimetric method using commercial kits and standards (Spinreact S.A., Spain). Plasma proteins were quantified according to Bradford (1976) with Comassie Brilliant Blue G-250 (Sigma) and calibrations prepared from known Bovine Serum Albumin (BSA) standards. All spectrophotometric measurements were performed using a Beckman DU®600 spectrophotometer.

Plasma concentrations of cortisol, expressed in ng/ml, were determined in duplicate by radioimmunoassay (RIA) according to Balm et al. (1994). The cortisol antibody has marginal cross-reactivity with 11-deoxycortisol (5.9%), cortisone acetate (0.16%), cortisone (2.6%) and 17α-OH-progesterone (0.4%). The intra- and inter-assay coefficients of variation were 3 and 5% respectively.

Plasma Na, K and Cl levels were measured in a flame photometric Auto Analyser. Ca, Mg, Mn, Fe, Si, Zn, Al, P, S, Cu, Cd and Pb were analysed with Inductively Coupled Plasma Atomic Emission Spectrometry (Plasma IL200, Thermo Electron, USA).

Plasma concentrations of lactate, expressed in mM, were determined with a pHOx Plus analyser (Nova Biomedical, The Netherlands).

Total triiodothyronine (T_3) concentrations were measured by RIA following the method by Boeuf and Prunet (1985) and were expressed in ng ml^{-1}.

2.4. Gill Na^+/K^+–ATPase

The first gill arch was removed from the right side of sacrificed fish. Dissected tissues were rinsed in a solution (pH 7.4) containing 300 mM sucrose, 20 mM Na_2EDTA et 100 mM imidazol (Zaugg, 1982), placed in tubes containing the same solution and stocked at -20˚C until use. During the whole procedure samples were kept on ice.

Stored samples were thawed at room temperature and briefly centrifuged. The conservation medium was then removed, and 2 mL of isotonic isolation medium (IIM: 250 mM sucrose, 5 mM of $MgCl_2$ and 5 mM Hepes; pH 7.4) added in each tube. Samples were then homogenized in a potter and subsequently centrifuged at 4000 rpm for 5 min to eliminate cellular debris. The supernatant containing the plasma membrane fragments was transferred into new tubes. During the extraction procedure samples were maintained at 4˚C on ice.

Enzyme activity was expressed as mg/protein. Protein content was determined by a colorimetric method (Biorad) using BSA as reference. Na^+/K^+-ATPase specific activity was assessed as the difference of total ATP hydrolysis (in presence of Na^+, K^+, Mg^{2+} and ATP) and that in absence of K^+ but in presence of an optimal concentration of the digitalis drug ouabain (1 $mg.mL^{-1}$) (Flik et al., 1983). The amount of phosphate released was assessed by comparison with commercial reference standards (Sigma). The enzyme specific activity was

expressed in µmole $Pi.h^{-1}$ per mg protein. The total activity was calculated as the product of the specific activity and the total protein of the sample and expressed in µmole $Pi.h^{-1}$. 10 measurements of the specific (V_{sp} in µmol $Pi.mg.h^{-1}.prot^{-1}$) of the enzyme were performed in diploid as well as in triploid sea bass.

2.5. Statistical analyses

Most of the measured traits were compared in a one-factor fix ANOVA (ploidy level) i.e. hematocrit proportion, total red blood cell count, osmolarity, glucose, hemoglobin and protein concentrations, electrolytes and T_3. The erythrocytes numbers, area, width and length were compared by two-factors ANOVA (mix nested model with ploidy as fix factor and counting repetition or counted slide as random nested factor). The normality and the variance homogeneity were tested with the tests of skewness, and kurtosis and Bartlett's respectively (Dagnelie, 1975). Differences were accepted as significant when $P<0.05$. Values are expressed as means ± 95% Confidence Interval (CI).

3. Results

The hatching rate was 35% within the pressure-shocked group, and 50% within the control-unshocked diploid. The application of sub-optimal pressure shocks produced 42% diploid (n=232), 31% triploid (n=174) and 27% aneuploid (n=151) fish as measured by flow cytometry at hatching and at the age of 7 months. Aneuploid fish included hypo/hyper-diploids (69%) and -triploids (31%). Aneuploid and control-unshocked diploid fish were not considered in this study.

At the time of sampling, there were significant differences ($F = 29.16$, $P<0.01$, $n = 1$ and 29) between diploid and triploid sea bass in body weight, with diploids (1005.63 ± 93.10g) being ~32% heavier than triploids (688.33 ± 65.37g).

Analysed diploid fish included 8 males and 7 females, whereas triploid fish included 13 males and 2 females only.

Comparisons of hematological measurements, plasma glucose, proteins, cortisol, lactate and T_3 in diploid and triploid fish are reported in Table 1. Hematocrit proportion (~0.25) was similar in both ploidies. Triploid fish displayed significantly lower RBCC (~35%) and total [Hb] (~13%), but higher MCV (30%) compared to diploids. No differences in MCHC were found between ploidy groups. Total proteins, glucose, lactate and T_3 concentrations were also similar in both ploidies, whereas cortisol levels were significant lower (~20%) in triploid fish.

Table 2 summarizes the results obtained from red blood cells measurements. Differences were observed in erythrocyte cellular and nuclear dimensions (area, width and length). Triploid *vs.* diploid ratios in erythrocyte cellular and nuclear areas were 1.32 and 1.50 respectively. As well, differences were recorded between diploid and triploid ratios of cytoplasmatic width to length. Triploid ratios were significantly smaller than diploid, giving to triploid erythrocytes a longer (~7%) appearance. Corresponding ratio values were 0.73 ± 0.01 in diploids, and 0.68 ± 0.01 in triploids.

Results on plasma osmolarity and ion concentrations in diploid and triploid sea bass are given in Table 3. There were no significant differences in osmotic pressure between ploidies. Na, Cl, Ca, Mg, Mn, Al, P, Cd and Pb plasma concentrations were also similar. The Na:Cl and (Na+K):Cl ratios were 0.98 and 0.99 respectively, and comparable in both groups too. Triploid fish had lower K (~20%), Fe (~45%), Zn (~20%), S (~18%), and Cu (~34%) concentrations than did diploid fish. On the contrary, plasma concentrations of Si were significantly higher (~32%) in triploids.

Finally, gill Na^+/K^+-ATPase specific activities (3.5 ± 1.08 µmol Pi.mg $prot^{-1}.h^{-1}$ in diploids and 3.85 ± 1.33 µmol Pi.mg $prot^{-1}.h^{-1}$ in triploids) were not statistically different between ploidies (F=2.570; P=0.126; n=1 and 18).

4. Discussion

To our knowledge, this study is the first to describe the hematological and physiological characteristics of diploid and triploid sea bass. Under our experimental conditions, diploid and triploid sea bass produced by sub-optimal treatments did not differ for a large majority of hematological parameters, whereas significant differences were found notably in red blood cells sizes, Hb contents, and plasma's cortisol levels. Gill Na+/K+-ATPase activities were similar in both ploidies.

Triploidisation in sea bass led to an increase of erythrocyte size (32% in cytoplasm surface area, and 50% in nucleus) with decreasing erythrocyte number (~34%). This hematological profile of fewer and larger erythrocytes observed in triploid sea bass is consistent with findings for other triploid species (Benfey, 1999). This homeostasis mechanism, which seems to compensate for the increase in cellular size by proportionately decreasing cell number, explains the lack of difference in hematocrit observed between diploid and triploid sea bass, as reported in other fish species too (Benfey, 1999). Similar results in terms of cell proportions and number were found in diploid and triploid sea bass by Felip and co-workers (Felip et al., 2001). These authors used erythrocyte measurement as a mean of verification of ploidy level in experimental groups. They reported the erythrocyte number, as calculated from haematocrit and cell volume, to be ~37% lower for an increase of ~50% of red blood cell volume in triploid fish (54% in cell and 50% in nuclear volume, respectively). Haematocrit values were also not significantly different between ploidies.

In our work, triploid erythrocytes increase in length was significantly greater than the proportionate increase in width (25% and 12% respectively), resulting in a more elliptical shape. This is in agreement with previous findings in diploid and triploid salmonids (Benfey and Sutterlin, 1984; Cogswell *et al*., 2002). According to Benfey (1999), the cause for this

unequal increase in triploid cell length and alteration of cell shape is likely to be a cytoplasmatic accommodation to the significant increase in nuclear length.

Overall, the size of diploid erythrocytes in the present study was consistent with that previously reported by Esteban et al. (2000), who measured sea bass blood cells using light and electron microscopy.

In this work, total Hb concentration was reduced in the blood of triploids containing significantly less erythrocytes. At cellular level, this deficiency was overcome by an increase in the average volume of red blood cells (MCV), such that the mean cellular hemoglobin content (MCH) in triploid erythrocytes was significant greater than in diploid cells. On the whole, the mean cellular hemoglobin concentration (MCHC) resulted equivalent between diploid and triploid erythrocytes. In the literature, values for total blood hemoglobin (Hb) and MCHC concentrations in diploid and triploid fish are not consistent and vary among species, whereas the mean cellular hemoglobin content (MCH) is commonly reported to be higher in polyploids (Benfey, 1999). Several reports are available on the relationship between the hematology of triploid fish and their respiratory physiology and aerobic capacity in standard or altered environments. In salmonids, despite some contrasting results on total blood hemoglobin levels and blood-oxygen carrying capacity, triploid fish were found to be similar to diploids in their overall oxygen-consumption rates and swimming performances under normal or stress conditions (Stillwell and Benfey, 1995).

Cortisol has both mineralo- and glucocorticoid actions in fish and thus is involved in both hydromineral balance and energy metabolism (Mommsen et al., 1999). It is also the major corticosteroid produced by teleosts during activation of the hypothalamo-pituitary–interrenal (HPI) axis, and is considered a principal component of the primary stress response. An elevation of cortisol is most widely used as indicator of stress in fish and its levels rise within few minutes following an acute stress, whereas return to steady state can take several

hours (review in Wendelaar Bonga, 1997). In our study, "rested" both diploid and triploid sea bass showed relatively high levels of plasma cortisol. Cortisol levels for unstressed specimens of sea bass reported in literature as "control" or "basal" range from 15 to 133 $ng.ml^{-1}$ (Roche et al., 1989; Roche and Bogé, 1996; Cedra-Reverter et al., 1998; Rotlant et al., 2003) suggesting that sampling and anaesthesia procedures can induce rapid changes in the hormone concentration in the blood. Eugenol in low concentration, as used in our study, is unable to block cortisol release in teleost fish (Iversen et al., 2003; Small, 2003) and has similar physiological effects to those generated by 2-phenoxyethanol (Tort et al., 2002). These findings can explain the high cortisol levels that we measured during our experiments. **Also, these levels might have been partly due to the increasing water temperature and photoperiod during the time of our sampling. In sea bass, significant annual variations of cortisol were in fact observed in coincidence with water temperatures and photoperiods, with maximum peaks towards the warmest months (Planas et al., 1990).** Very few studies –mainly carried out on salmonids- have focused on potential differences in the stress response between diploid and triploid specimens and the general view is that there is no difference in plasma cortisol profiles following acute stress (Biron and Benfey, 1994; Sadler et al., 2000). Interestingly, in the present work a significant difference was found in cortisol levels between diploid and triploid sea bass following a relatively short blood sampling procedure suggesting that the comparative physiology of the stress response in these two ploidies should be further addressed. Nevertheless, future studies on blood constituents and other indicators of fish functional state under homeostatic conditions will require selecting and rigorously executing alternative blood sampling procedures for this species.

Plasma profiles of glucose, proteins, and lactate were also similar for diploid and triploid sea bass under our experimental conditions. These blood metabolites represent important indicators of fish health and basic metabolic activity. Increases in plasma glucose

levels and hematocrit are secondary responses to stress, associated with corticosteroid and catecholamine release during the primary stress response (review in Wendelaar Bonga, 1997). Reduced capacity of hemoglobin to transport oxygen or reduced affinity for oxygen may result in anaerobic metabolism and accumulation of lactate in muscles, and possibly in blood. Reference values of serum glucose, total proteins, Na^+, K^+, Cl^-, Ca^{2+}, and osmolarity measured by various authors in captive sea bass have been reviewed by Marino et al. (2001). Despite the substantial variation in blood constituents observed in this species, and the difficulty in comparing different studies using different procedures, fish origins, and photoperiodic and feeding regimes, the values that we measured for both diploid and triploid sea bass are within the range of values compatible with normal physiological and metabolic functions in this species (Marino et al., 2001). **Moreover, some authors (Pavlidis et al., 1997; Planas et al., 1990) reported the presence of seasonal and daily variations of several blood parameters in sea bass, as observed in other fish species. The existence of such variations further complicates the possibility of performing accurate comparisons in the literature.**

T_3 is involved in the intermediary metabolism of fish and its production is commonly associated with anabolic states (Eales and MacLatchy, 1989)…(to be completed by Christian and/or IFREMER Brest)…

Gill Na^+/K^+-ATPase activity in diploid and triploid sea bass were not significantly different. Present in all animal cells, Na^+/K^+-ATPase participates in ion transport either directly through movement of Na^+ and K^+ across the cell membrane, or indirectly through generation of ionic and electrical gradients. This enzyme is considered to be the driving force for ionic exchanges in both fresh and seawater-adapted teleosts (review in McCormick, 1995). The euryhaline character of sea bass, which can live in salinities ranging from fresh water to hypersaline seawater, partially relies in adjustments in branchial Na^+/K^+-ATPase activity and

intracellular distribution (Varsamos et al., 2002). The importance of gills Na^+/K^+-ATPase in the maintenance of homeostasis through its involvement in osmoregulation, acid-base regulation and excretion at the gills level allows to consider this enzyme as a pertinent indicator of the physiological status of teleost species. The absence of differences in the enzyme's activity between diploid and triploid sea bass suggests that triploidy does not affect osmo- and ionoregulatory capacity in this species. However, experiments involving challenges are necessary to confirm this statement.

We have previously shown that the performances of triploid sea bass are comparable or lower than those of diploids in juvenile or large fish (Peruzzi *et al*., 2004). In this work we confirm that similar zootechnical performances rely, at least partially, on similar level of major haematological and physiological indicators. Further studies considering husbandry related experimental challenges should allow gaining a better understanding of the comparative adaptation capacities in diploid and triploid sea bass.

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Legends

Table 1

Hematology and plasma metabolites in diploid (n=16) and triploid (n=15) sea bass. Values represent means ± CI.

Table 2

Results obtained from red blood cells measurements of diploid and triploid sea bass. Values represent means ± CI.

Table 3

Plasma osmolarity and ions concentration in diploid and triploid sea bass. Values represent means ± CI.

Table 1

	Diploid (n=16)	Triploid (n=15)	*F*	*P*
RBCC (10^6 mm^{-3})	2.17 ± 0.10	1.43 ± 0.07	18.51	<0.001
Hb (g dl^{-1})	5.28±0.31	4.60±0.32	8.59	0.006
Hct	0.26±0.02	0.24±0.02	1.58	ns
Glucose (mg dl^{-1})	112.91±15.68	112.31±6.95	0.005	ns
Proteins (mg ml^{-1})	30.49±1.28	30.84±1.11	1.63	ns
MCV (fl)	120.85±7.41	172.20±15.78	34.747	<0.001
MCH (pg)	24.51±1.12	32.58±2.91	27.106	<0.001
MCHC (g l^{-1})	204.66±11.04	191.15±12.77	2.479	ns
Cortisol (ng ml^{-1})	152.00±17.44	122.27±17.72	5.320	0.029
Lactate (mM)	2.93±0.68	2.35±0.79	1.174	ns
T_3 (ng ml^{-1})	60.64±3.42	60.90±2.88	0.013	ns

Table 2

	Diploid (n=16)	Triploid (n=15)	*F*	*P*
Nuclear area (μm^2)	10.89 ± 0.06	16.38 ± 0.17	43.67	<0.001
Nuclear width (μm)	4.04 ± 0.02	4.52 ± 0.03	13.06	<0.001
Nuclear length (μm)	5.15 ± 0.02	6.46 ± 0.03	22.63	<0.001
Cytoplasm area (μm^2)	60.42 ± 0.58	79.89 ± 1.04	48.16	<0.001
Cytoplasm width (μm)	8.34 ± 0.05	9.18 ± 0.81	15.48	<0.001
Cytoplasm length (μm)	11.38 ± 0.06	13.51 ± 0.08	161.91	<0.001
Cytoplasm width/length	0.73± 0.01	0.68± 0.01	24.56	<0.001

Table 3

	Diploid (n=16)	Triploid (n=15)	*F*	*P*
Osmolarity (mOsm)	367.31±2.93	372.27±6.98	0.16	ns
Na (mM)	175.56 ± 7.43	177.17 ± 2.61	0.152	ns
K (mM)	3.51 ± 0.25	2.83 ± 0.13	20.993	<0.001
Cl (mM)	180.12 ± 7.76	179.31 ± 3.37	0.034	ns
Na/Cl	0.97 ± 0.01	0.99 ± 0.01	3.729	ns
(Na+K)/Cl	0.99 ± 0.01	1.00 ± 0.01	2.147	ns
Ca (mM)	2.51 ± 0.27	2.23 ± 0.18	2.739	ns
Mg (mM)	1.07 ± 0.10	1.22 ± 0.17	2.412	ns
Mn (μM)	5.08 ± 2.39	4.16 ± 2.40	0.280	ns
Fe (μM)	34.18 ± 10.48	18.93 ± 2.78	7.135	0.013
Si (mM)	3.83 ± 0.34	5.65 ± 0.11	92.433	<0.001
Zn (mM)	0.26 ± 0.03	0.21 ± 0.02	8.479	0.007
Al (μM)	28.10 ± 5.68	29.28 ± 7.91	0.058	ns
P (mM)	9.00 ± 0.91	7.92 ± 0.53	3.912	ns
S (mM)	15.91 ± 1.56	13.06 ± 0.71	10.172	0.004
Cu (μM)	18.95 ± 4.58	12.50 ± 3.92	4.338	0.047
Cd (μM)	8.43 ± 3.84	7.25 ± 2.78	0.230	ns
Pb (μM)	40.78 ± 28.67	35.95 ± 18.93	0.074	ns