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Effects of ozone (O_3) and ethylenediurea (EDU) on the ecological stoichiometry of a willow grown in a free-air exposure system

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ABSTRACT

Ground-level ozone (O_3) concentrations have been elevating in the last century. While there has been a notable progress in understanding O_3 effects on vegetation, O_3 effects on ecological stoichiometry remain unclear, especially early in the oxidative stress. Ethyelenediurea (EDU) is a chemical compound widely applied in research projects as protectant of plants against O_3 injury, however its mode of action remains unclear. To investigate O_3 and EDU effects early in the stress, we sprayed willow (*Salix sachalinensis*) plants with 0, 200 or 400 mg EDU L^{-1}, and exposed them to either low ambient O_3 (AOZ) or elevated O_3 (EOZ) levels during the daytime, for about one month, in a free air O_3 controlled exposure (FACE); EDU treatment was repeated every nine days. We collected samples for analyses from basal, top, and shed leaves, before leaves develop visible O_3 symptoms. We found that O_3 altered the ecological stoichiometry, including impacts in nutrient resorption efficiency, early in the stress. The relation between P content and Fe content seemed to have a critical role in maintaining homeostasis in an effort to prevent O_3-induced damage. Photosynthetic pigments and P content appeared to play an important role in EDU mode of action. This study provides novel insights on the stress biology which are of ecological and toxicological importance.

Keywords: iron homeostasis, nutrient cycling, nutrient resorption, ozone, re-translocation

Key message: Elevated O_3 alters ecological stoichiometry, and Fe homeostasis plays an important role early in O_3 stress. Photosynthetic pigments and phosphorus are important components in EDU mode of action.

1. INTRODUCTION

The levels of tropospheric ozone (O_3) are nowadays elevated at wide areas of the Northern Hemisphere (Feng et al., 2015; Hendriks et al., 2016; Kalabokas et al., 2017; Sicard et al., 2017; Tian et al., 2016; Trieu et al., 2017), and are potentially phytotoxic at least to O_3-sensitive vegetation (Feng et al., 2008; Hayes et al., 2007; Saitanis et al., 2015). When elevated O_3 doses are taken up by plants, plants undergo a responsive process which involves a series of physiological and biological adjustments ranging from single cell to whole plant level (Ashmore, 2005; Bussotti et al., 2007; Fiscus et al., 2005; Jolivet et al., 2016; Matyssek and Innes, 1999; Munne-Bosch et al., 2013). Exposure of vegetation to elevated O_3 doses poses a risk for food supplies, ecosystem health and biosphere sustainability (Andersen, 2003; Broberg et al., 2015; Felzer et al., 2004; Fuhrer, 2009; Lindroth, 2010; Lu et al., 2015; Peñuelas and Staudt, 2010; Tian et al., 2016; Wang et al., 2016; Wilkinson et al., 2012).

The protection of vegetation against O_3 adverse effects is thus an important matter. Plenty of potential agrochemicals have been tested as to their efficacy to protect plants against O_3 phytotoxicity (Agathokleous et al., 2016c; Manning et al., 1973a, 1973b; Runeckles and Resh, 1975; Saitanis et al., 2015; Taylor, 1974). The most effective phytoprotectant currently known is the synthetic chemical ethylenediurea (EDU, $C_4H_{10}N_4O_2$) (Agathokleous, 2017). EDU has been a matter of study in dozens of investigations over the past four decades (Carnahan et al., 1978; Oksanen et al., 2013; Paoletti et al., 2009; Salvatori et al., 2017; Singh et al., 2015), and it is now recognized that EDU applied repeatedly at low concentrations of up to 400 mg L^{-1} sufficiently protects against O_3 phytotoxicity across plant species (Agathokleous et al., 2015; Manning et al., 2011a; Oksanen et al., 2013; Paoletti et al., 2009; Singh et al., 2015). EDU can protect numerous biological or physiological endpoints; nonetheless, literature review studies suggest that the EDU

mode of action in protecting against O_3 phytotoxicity remains unclear (Agathokleous et al., 2015; Manning et al., 2011a; Oksanen et al., 2013; Paoletti et al., 2009; Singh et al., 2015). It was recently proposed that EDU may protect plants through hormesis, by activating plant defense at low doses via homeostatic disruptions, and the necessity for studying plant metabolism was highlighted (Agathokleous, 2017). Homeostatic re-adjustments after disruption may imply alterations in leaf metabolism and in quantitative and qualitative traits of leaf nutrients. However, there is hitherto no evidence on leaf stoichiometry under EDU treatments.

Ecological stoichiometry is critically important for predicting stress consequences in biochemical cycles and other ecological processes. Leaf stoichiometry and nutrient resorption efficiency (NRE) have been mainly studied in plants experiencing prolonged O_3 stress (Cao et al., 2016; Nakaji et al., 2004; Oksanen et al., 2005; Shang et al., 2018; Shi et al., 2016, 2017; Zhuang et al., 2017) but not at an early stage of the stress so as to be utilized as an early diagnostic tool. Leaf age is an important factor determining O_3 effects on stoichiometry. Younger or late leaves may be more resistant to O_3 than older, mature leaves (Li et al., 2017; Moreno Pina et al., 2017) as a result of adaptive responses which may take place at plant level. For example, relative to mature leaves, younger leaves may: emit greater concentrations of volatile organic compounds (Bison et al., 2018); take up less amount of O_3 (Craker and Starbuck, 1973); have greater carbon (C) assimilation rate and water use efficiency (Moreno Pina et al., 2017); and overall have greater efficiency in apoplastic O_3 detoxification as indicated by avoidance of accumulation of reactive oxygen species in the chloroplasts (Oksanen et al., 2005). Besides, shed leaves may be more affected than mature leaves as they experience O_3 pressure for longer time and the nutrient re-translocation may also be affected (Shang et al., 2018; Shi et al., 2017; Uddling et al., 2006). Despite NRE was studied at later stages of oxidative stress (Shang et al., 2018; Shi et al., 2017;

95 Uddling et al., 2006), leaf nutrient cycling across the crown has not been explicitly investigated
96 under O_3 pressure; none of the previous studies included all leaf classes (i.e. mature, young,
97 shed). Effects of EDU on ecological stoichiometry, including NRE and leaf nutrient cycling
98 across the crown, remain elusive too.
99 The principal aim of this study is to investigate the ecological stoichiometry in willow (*Salix*
100 *sachalinensis* F. Schmidt) plants sprayed with 0, 200 or 400 mg EDU L^{-1} and exposed to ambient
101 O_3 or an O_3-enriched atmosphere. The main research question is whether EDU and O_3 affect the
102 leaf stoichiometry and leaf nutrient cycling across the crown (basal, top and shed leaves) early in
103 oxidative stress, before leaves develop visible O_3 symptoms. Based on previous studies where
104 O_3 affected leaf stoichiometry and NRE at later stages of oxidative stress (Shang et al., 2018; Shi
105 et al., 2017; Uddling et al., 2006), it is hypothesized that leaf stoichiometry and nutrient cycling
106 across the crown may be altered at early stages of oxidative stress when plants are under
107 homeostatic disruption. An additional aim is to assess photosynthetic pigments and leaf mass per
108 area (LMA) in mature leaves, as a reference of the stress status, and to assess essential and non-
109 essential elements in an analysis of patterns in the dataset of the elements. Photosynthetic
110 pigments and LMA are known as simple and effective indices for assessing health status in
111 mature leaves (Agathokleous et al., 2017; Onoda et al., 2017; Ronen and Galun, 1984). Mature
112 leaves experience O_3 exposure for longer time and are less affected by ontogenic adjustments
113 than young leaves, and are widely assessed as to gas exchange and other traits under stress.

2. MATERIALS AND METHODS

2.1. Experimental site

The experiment was conducted at Sapporo Experimental Forest of Hokkaido University, Sapporo, Japan (43°.04′ N, 141°.20′ E, 15 m a.s.l.), in the year 2015. For the period June-September, mean monthly average of air temperature was 17.83 (±1.83 s.d.) °C; daily maximum temperature was 22.52 (±1.75 s.d.) °C; daily minimum temperature was 14.07 (±2.02 s.d.) °C; wind speed was 3.52 (±0.17 s.d.) m s^{-1}; relative humidity was 68.83 (±1.58 s.d.) %; mean monthly total sunshine duration was 186.25 (±9.98 s.d.) h; and mean monthly precipitation was 120.50 (±23.19 s.d.) mm, respectively (43°03.6'N 141°19.7'E; Japan Meteorological Agency, 2016; http://www.jma.go.jp/jma/indexe.html).

2.2. Plant material & Design of the Experiment

The plant materials were current-year cuttings of *S. sachalinensis* (=*S. udensis* Trautv. et C.A. Mey.), originated from the river basin of Ebetsu city, grown in 15-L pots filled with Akadama (well-weathered volcanic ash) and Kanuma (well-weathered pumice) soils at a ratio of 1:1 (DCM Homac CO., LTD., Sapporo, JP). This soil substrate is free from organic matter and poor in phosphorus (P) and nitrogen (N) (for further information please see Electronic Supplementary Material, Table 2S). This willow was selected as biological model due to prior knowledge with regards to its exposure to EDU and O_3 (Agathokleous et al., 2018, 2016a, 2016b). Eighty uniform plants were transplanted in the 15-L pots on June 9th, when they were well rooted. On August 14th, 72 pots were randomly allocated to six plots (12 pots per plot), of which three served as background O_3 (ambient) and three as elevated O_3 treatment. The pots in each plot were further randomly assigned three EDU treatments (four pots per EDU treatment). These plants were used for a different study and thus the full, detailed procedure has been previously

described (Agathokleous et al., 2016a). The eight remaining plants were harvested on August 6th, for an approximate image of the initial leaf traits. The length and width of each leaf was non-destructively measured, and the leaf size was calculated using a simple linear model as described earlier (Agathokleous et al., 2016b). Leaves (n = 549) were air-dried at 65 ^{o}C until a constant mass, and the dry matter of each leaf was measured using a digital balance. The specific leaf area (SLA, or its inverse, LMA) was calculated as the one-side area of a fresh leaf to the dry matter of the leaf. The average leaf size was 7.20 ± 0.26 cm^2, average leaf dry matter 0.09 ± 0.01 g, and SLA 134.27 ± 11.78 cm^2 g^{-1}.

2.3. EDU treatments

The EDU treatments were 0 (EDU0), 200 (EDU200), and 400 (EDU400) mg L^{-1} (Agathokleous et al., 2016a). EDU (100% a.i., source W.J. Manning, University of Massachusetts, USA) was prepared using an electric hotplate 30 min before each application. The required EDU amount was dissolved in partitions of 500 mL, so as the target concentration to be achieved in the final desired volume by adding cool pure water. The initial 500 mL stock for each EDU concentration was prepared using gently-warmed water (Manning et al., 2011) with continuous stirring. The initial stocks were prepared with pure water, and surfactant was not added. EDU was applied using an electric sprayer using Venturi effect, with two nozzles spraying simultaneously. Both abaxial and adaxial leaf surfaces were sprayed until run-off.

The first EDU application was carried out 50 days after transplanting (July 29), when the plants had 63 ± 2 leaves. EDU application was applied on a 9-day interval (Agathokleous, 2017). The last (5th) EDU treatment was applied on September 3rd. All the applications were conducted during the hours 10:00-11:00 am, JST.

2.4. Ozone treatments

O_3 treatments were carried out in the free air O_3 controlled exposure (FACE) system of Hokkaido University, Japan, located in the Sapporo Experimental Forest, Sapporo, Japan. This FACE system has been previously described (Agathokleous et al., 2017). The O_3 treatments were ambient O_3 (AOZ) and elevated O_3 (EOZ). O_3-enrichment lasted from August 15th to September 11th, 2015, during daylight hours, when the photosynthetic photon flux density (PPFD) was >70 μmol m^{-2} s^{-1}. This PPFD threshold is considered the light compensation point of photosynthesis of targeted plants (Koike, 1988). Background O_3 mixing ratios at the experimental site were continuously monitored (1-min interval) by an ultraviolet absorption O_3 analyzer (TUV-1100; Tokyo Industries Inc., Tokyo, Japan), from August 15th to September 11th, 2015. Data were averaged per hour, and the 10-hour (07:00 am – 17:00 pm, JST) means were calculated. The mean background O_3 mixing ratio for the period August 15th to September 11th was 14.52 ± 0.34 nmol mol^{-1}, whereas the index of Accumulated exposure to O_3 Over the Threshold of 40 nmol mol^{-1} (AOT40) (Mills et al., 2007) was practically 0 μmol mol^{-1} h. The mean O_3 mixing ratio in the three EOZ plots for the monitored period was 68.84 ±1.12 nmol mol^{-1}, which can be translated to AOT40 of 7.79 ± 0.30 μmol mol^{-1} h until September 10th and 6.34 ± 0.25 μmol mol^{-1} h until September 5th.

2.5. Samplings, sample analysis and data collection

2.5.1 Leaf mass per area (LMA)

On September 4th, 2 mature (basal), 2 young (top) and 2-4 fallen leaves (after gently shaking of shoots) were collected from each plant, and air-dried at 65 ^{o}C until a constant weight, under dark conditions. Prior to drying, randomly-selected mature leaves (*n*=89, 4-25 leaves per experimental

condition) were scanned (Canon LIDE 40, Tokyo), and the leaf area was measured using the LAI32 v.0.377 software (© Kazukiyo Yamamoto). LMA was calculated for each leaf.

2.5.2 Non-photosynthetic elements

The leaves collected on September 4th were pooled per maturity level per plant so as to give one more robust sample per plant to be used for elemental analyses. Leaf samples were manually grounded into powder. An amount of grounded powder was used for digestion, as previously described (Chu et al., 2015): Leaf powder was digested with 2 mL of 61% nitric acid (HNO_3) (EL grade; Kanto Chemical, Tokyo) at 110 °C, in a DigiPREP apparatus (SCP Science, Quebec, Canada) for 3 h. Then, 0.5 mL of hydrogen peroxide (H_2O_2) (semiconductor grade; Santoku Chemical, Tokyo, Japan) was added two times, at a 30-min interval, and heated at 110 °C until the solution became clear. Tubes were left to cool to room temperature and afterwards filled to 15 mL with 2% HNO_3. The analyses were conducted with an Inductively Coupled Plasma Mass Spectrometer (ICP-MS) (Elan, DRC-e; PerkinElmer, Waltham, MA, USA), with ultratrace-level detection limits. The ICP multielement standard solution IV (Merck, Tokyo, Japan) was used as standard for ionomic determination. The essential elements magnesium (Mg), manganese (Mn), iron (Fe), calcium (Ca), nickel (Ni), P, potassium (K), and the non-essential elements chromium (Cr) and aluminum (Al) were quantified, and the ratios K/Mg, K/Ca, Fe/Mn, Ca/Mg, P/Fe, and P/Mn were calculated. The quantified elements were selected in accordance with a previous study under similar environmental conditions and with a similar soil (Shi et al., 2017). NRE was calculated for each element as $NRE=((C_{basal}+C_{top})-(C_{shed}))/(C_{basal}+C_{top})$, where C is the content in the leaf. This was considered a more robust calculation of NRE than the classical one (Aerts, 1996) because it reflects the nutrient content throughout the crown, i.e. from base to top. Nitrogen and C content was analyzed in leaf powder using a third generation Vario EL III

Element Analyzer (Elementar Analysensysteme GmbH, Hanau-Germany). In total, 204 samples were analyzed for all the chemical analyses, except K for which only 91 samples were analyzed.

2.5.3 Photosynthetic pigments

On September 9th, two mature, basal leaves were randomly selected from each plant, and one leaf disk (area=2.26 cm^2) was sampled from each leaf and stored in deep freeze. Five days later, photosynthetic pigments were measured. The two leaf disks consisted a sample per plant, whose pigments were extracted in dimethyl sulfoxide (DMSO) and stored in dark condition at 65 ^{o}C until the appearance of a "ghost-like" thallus (Barnes et al., 1992). Measurements were taken for the optical densities (OD) 415, 435, 470, 648, 665 nm (GeneSpec III; Hitachi Genetic Systems; MiraiBio, Alameda, CA). The OD 470 was used to estimate total carotenoid content (TCar) following the classical Lichtenthaler formula (Lichtenthaler, 1987). This formula is given for acetone 100% extracts and thus the actual TCar values may differ. The ODs 648 and 665 were used to estimate the chlorophyll *a* content (Chl_a), chlorophyll *b* content (Chl_b) and total chlorophylls, *a+b*, content (TChl) (Barnes et al., 1992). The ratios of OD_{435}/OD_{415}, Chl_a/Chl_b and *TChl*/*TCar* were calculated as potential indices of stress. OD_{435}/OD_{415}, has been proposed as an effective index of chlorophyll degradation to phaeopigments (Ronen and Galun, 1984). Since OD_{435}/OD_{415} is underexplored in higher plants, phaeophytinization was also assessed by the classical method of acidification (Ronen and Galun, 1984). Overall, after extracts were measured (before acidification), 60 µl of 1 M HCl were added per 10 ml of extract, stored in 4 ^{o}C for 12 min, and measured again (after acidification). The OD value at 665 nm before the acidification (OD_{665}) was divided by the OD value at 665 nm after the acidification (OD_{665a}) (OD_{665}/OD_{665a}).

Leaf sampling was conducted at the onset of O_3-induced visible foliar injury, when only very few leaves showed a small number of tiny spots of discoloration visible to naked eye.

2.5. Data handling & Statistics

The threshold for statistical significance was predefined at a level of significant α=0.05. The data of all the response variables were averaged per EDU treatment per experimental unit, i.e. O_3 plot (n = 3).

Data of pigments, ICP elements, and elements ratios were transformed by a Box-Cox power transformation (Box and Cox, 1964), as described in an earlier report (Agathokleous et al., 2016c). Data transformation was unnecessary for N, C and N/C data.

Data of pigments and LMA were analyzed by simple contrasts, based on prior theoretical knowledge, to answer only the most biologically meaningful questions (Agathokleous et al., 2016a; Ruxton and Beauchamp, 2008). A contrast represents a linear combination of experimental conditions whose sum of coefficients equals to zero. The contrasts were conducted among the following six combinational groups: AOZ×EDU0 (AOZ0), AOZ×EDU200 (AOZ200), AOZ×EDU400 (AOZ400), EOZ×EDU0 (EOZ0), EOZ×EDU200 (EOZ200), EOZ×EDU400 (EOZ400). The five degrees of freedom were partitioned to the following contrasts:

C_1: (AOZ0) *vs*. (AOZ200+AOZ400), which tests the null hypothesis that EDU does not affect plants under the ambient O_3 condition

C_2: AOZ200 *vs*. AOZ400, which tests the hypothesis EDU400 plants do not perform better than EDU200 plants under the ambient O_3 condition

C_3: (EOZ0) *vs*. (EOZ200+EOZ400), which tests the hypothesis EDU-treated plants do not perform better than plants not treated with EDU under the elevated O_3 condition

C_4: EOZ200 *vs*. EOZ400, which tests the hypothesis that EDU400 plants do not perform better than EDU200 plants under the elevated O_3 condition

C_5: AOZ0 vs. EOZ0, which tests the hypothesis that elevated O_3 does not affect plants.

To test the question of whether EDU and O_3 affect the cycling of leaf chemical elements across the crown, data were analyzed by a General Linear Model (GLM) where leaf position (three levels: basal (mature), top (young), shed) was set as within effects dependent variable and EDU (three levels: EDU0, EDU200, EDU400) and O_3 (two levels: AOZ, EOZ) categorical predictors. Analysis was based on Type 6 Sums of Squares with σ-restricted coding of effects that provides unique effect estimates even for lower-order effects (straightforward computing method). For aposteriori comparisons, the post-hoc Bonferroni test was utilized (at α=0.05).

To emphasize variation and observe patterns in the dataset of the response variables assessed in mature leaves, a Principal Component Analysis (PCA) was utilized. Data of the main variables (Mn, Fe, Mg, Ca, Ni, P, Al, K, Cr, N, C, Chla, Chlb, CarT, OD_{665}/OD_{665a}) were submitted to PCA. The derivative variables (i.e. the sums or ratios of the main variables: K/Mg, K/Ca, Fe/Mn, Ca/Mg, P/Fe, P/Mn, Chla/Chlb, ChlT, ChlT/CarT, N/C, and OD_{435}/OD_{415}) were considered redundant and thus, along with EDU and Ozone, were passively only included as supplementary variables (not considered in the calculations). The Centroid of AOZ and EOZ ozone groups are also additionally illustrated. Prior to PCA analysis, data were standardized so all the variables to have equal units with s.d. = 1 (Saitanis et al., 2015).

MS EXCEL 2010 (Microsoft ©), XLSTAT 2017 (© Addinsoft), and STATISTICA v.10 (StatSoft Inc. ©) software were used for data processing and statistics.

3. RESULTS

3.1. Photosynthetic pigments and LMA in mature leaves

In AOZ, EDU200 and EDU400 together increased TChl compared to EDU0 (C1). This increase was mainly upon EDU400 which led to greater TChl than EDU200 (C2) (Table 1, Fig. 1). EDU400 also led to higher Chla, Chlb and TCar than EDU200 in AOZ (C1 and C2), indicating superiority of EDU400 over EDU0 and no difference between EDU0 and EDU200. EDU200 and EDU400 had no effect on Chla/Chlb, OD435/OD415, OD665/OD665a, and TChl/TCar, compared to EDU0, in AOZ (Table 1).

In EOZ, EDU200 and EDU400 together increased Chla, Chlb and TChl compared to EDU0 (C3). EDU200 and EDU400, when pooled, had no effect on TCar, Chla/Chlb, OD435/OD415, OD665/OD665a and TChl/TCar in EOZ (C3). However, EDU400 plants had greater Chla, Chla/Chlb, and TChl/TCar than EDU200 and EDU0 plants in EOZ (Table 1).

EOZ decreased Chla, Chlb, TChl and Chla/Chlb but did not significantly affect TCar, OD435/OD415, OD665/OD665a and TChl/TCar (C5).

The LMA means were 8.0±0.23, 9.9±0.54, 8.6±0.63, 8.8±0.39, 9.3±0.1 and 8.1±0.12 μg mm^{-2} for AOZ0, AOZ200, AOZ400, EOZ0, EOZ200 and EOZ400, respectively. Only EDU in AOZ significantly affected LMA, and this was only upon an increase induced by EDU200 (Table 1).

3.2. Chemical elements across leaf types

N and C

Nitrogen differed significantly (Bonferroni) among the leaves in the order basal>shed>top (Table 2, Fig. 2). C/N also differed significantly among the leaves, but in the opposite order (basal<shed<top). There were no other significant effects in N, C and C/N (Table 2, Fig 2).

Two-factorial GLM revealed that NRE of C and N was not affected by Ozone (F<0.01, P=0.966), EDU (F=0.17, P=0.845) or Ozone×EDU (F=0.27, P=0.765) (data not shown).

Mn, Fe, Mg, Ca, Ni, P, Al, K, and Cr

EOZ led to 119.3% greater P compared to AOZ (Fig 3*A*, Table 2).

EDU was a significant factor for only Cr which was increased by EDU200 (115%) compared to EDU0 and EDU400; means of EDU0 and EDU400 were same (Fig 4*D*).

Leaf position (i.e. maturity) was significant for all the elements. At an α=0.05, in Mn (Fig 3*B*), Mg (Fig 3*D*) and Ca (Fig 3*E*), leaf position ranked Top < Basal = Shed. In Fe (Fig 3*C*) and Al (Fig 4*B*), the rank was Top < Basal < Shed, and, in Ni (Fig 4*A*) and K (Fig 4*C*), the rank was Shed < Basal = Top. In P (Fig 3*A*), ranking was in the order Shed < Top < Basal, whereas, in Cr (Fig 4*D*), Top was lower than Shed but Mature was non-different from Top and Shed. In many of the elements, the means of Shed had much higher variance than Basal and Top.

Ozone × EDU was significant only for P (Table 2), with the only difference being a 140.7% greater P content in EOZ*EDU0 than in AOZ*EDU0 (Fig 3*A*).

Ozone × Leaf position was significant only for Fe, Ni, K and Cr (Table 2). In AOZ, Fe (Fig 3*C*), Ni (Fig 4*A*) and Cr (Fig 4*D*) were non-different among leaf types, and K (Fig 4*C*) was lower in Shed than Basal leaves. In EOZ, Fe (Fig 3*C*) varied among leaf types with the order Shed>Basal>Top. Ni was greater in Basal than in Shed and Top (Fig 4*A*), K was greater in Basal than Shed (Fig 4*C*), and Cr was lower in Basal than Shed in EOZ (Fig 4*D*). When comparing each leaf type between AOZ and EOZ, Fe (Fig 3*C*) was lower in Top leaves of EOZ than Top leaves of AOZ and Cr (Fig 4*D*) much lower in Shed leaves of AOZ than in Shed leaves in EOZ; Cr was also much greater in Basal and Top leaves of AOZ than the pairwise leaves in EOZ.

313 EDU × Leaf position was significant only for Ni and Cr (Table 2). The only significant
314 differences in Ni were lower means of Top than Basal leaves of EDU200 and EDU400 in both
315 AOZ and EOZ; however the variance was high, especially for EDU0 (Fig 4*A*).

316 Ozone × EDU × Leaf position interaction was significant for all the elements except Fe, Al and
317 Cr (Table 2). Regarding Mn (Fig 3*B*), EDU treatment of 0, 200 and 400 mg L^{-1} in basal leaves
318 was not significantly different from the pairwise EDU treatments in top leaves in AOZ; there
319 was a high variance in Shed leaves. However, Mn in EDU400 was 206.7 and 218.0% greater in
320 Shed and Basal than in Top leaves in EOZ. Mn differences within EDU400 were similar as
321 within EDU200 in AOZ and as within EDU0 and EDU200 in EOZ, however the variance was
322 higher (Fig 3*B*). In Ca, EDU0 was not different among leaf positions in both AOZ and EOZ (Fig
323 3*E*). EDU200 was 256.7 and 299.1% higher in Shed than in Top in AOZ and EOZ but not
324 different from Basal in both AOZ and EOZ. EDU400 was not different among leaf positions in
325 AOZ, however it was lower in Top than in Shed (2.6 times) and Basal (2.8 times) leaves and
326 non-different between Shed and Basal leaves in EOZ (Fig 3*E*). Ni displayed very large
327 variability in Shed in AOZ (Fig 4*A*). The only significant differences in Ni content worth
328 mentioning are a lower mean in Top than Basal of EDU400 in AOZ and lower means in Shed
329 than in Basal of EDU0 and EDU400 in EOZ (Fig 4*A*). Regarding P, the only significant
330 difference of interest in AOZ was a lower content in Shed than in Basal and Top leaves treated
331 with EDU400; these comparisons were non-different in EDU0 and EDU200 which had higher
332 variance (Fig 3*A*). In EOZ, P means of EDU0, EDU200 and EDU400 were lower in Shed than in
333 Basal and Top, and non-different between Basal and Top.

334 **NRE** was greater in AOZ than in EOZ for Fe (F=5.92, P<0.05) and Cr (F=9.63, P<0.01).
335 However, NRE was greater in EOZ than in AOZ for Ni (F=4.12, P<0.05). There were no other

Ozone, EDU or Ozone×EDU significant differences in NRE (two-factorial GLM, $P>0.05$, data not shown).

Ratios of chemical elements across leaf types

EOZ increased P/Fe by 115.0% (Fig 4*E*, Table 2).

EDU was non-significant factor for all the ratios (Table 2).

Leaf position was significant for all the ratios. At an $\alpha=0.05$, leaf position ranked Top > Basal > Shed in K/Ca (Fig 5*B*), K/Mg (Fig 5*A*), P/Fe (Fig 4*E*), and P/Mn (Fig 5*E*), and Shed > Basal > Top in Ca/Mg (Fig 5*D*). In Fe/Mn, mean of Shed was greater than means of Basal and Top, and mean of Basal was non-different from mean of Top (Fig 5*C*).

Ozone × EDU was significant only for P/Fe (Table 2), with the only difference being a 103.7% greater ratio in EOZ*EDU0 than in AOZ*EDU0 (Fig 4*E*).

EDU × Leaf position was significant only for Ca/Mg and P/Fe (Table 2). Ca/Mg was lower in Top than Basal and Shed leaves for all the three EDU treatments, however, in EDU400, it was also lower in Basal than in Shed leaves (Fig 5*D*). P/Fe was greater in Top and Basal than Shed leaves for all the three EDU treatments, and greater in Top than Basal leaves only for EDU200 (Fig 4*E*).

Ozone × EDU × Leaf position interaction was significant for K/Ca and P/Fe (Table 2).

Variation & patterns in chemical composition of mature leaves

Pairwise Pearson correlation analysis revealed several significant ($P<0.05$) correlations (Electronic Supplementary Material, Table 2S). Some of the significant positive correlations are those between the pairs Mn_Ca, P_K, Mg_P, Mn_Mg, Ca_C, Ca_Mn and Ca_Mg and between the pairs of pigments Chla_Chlb, Chla_TCar.

The dataset submitted to PCA was highly structured (Table 3, Fig. 6). The variability of the entire data set can be explained, with a high degree of satisfaction (68%), by six factors (F1-F6) with eigenvalues >1, each of which explain 21, 15, 10, 8.5, 6.6 and 7.2% respectively (Electronic Supplementary Material, Fig 1S). However, only the first three (F1-F3) explain more than 10% of the total variability. Fe, C, Ni, Pha, Al and LMA were totally separated, having high loadings on the factors F3, F4, F5, F6, F7 and F8 respectively (Table 3A). Only one response variable had a significant contribution to the variability explained by the extracted factors F4-F8; these autonomous response variables were C, Ni, OD665/OD665a, Al and LMA (Table 3B). Taken into account this observation as well as the fact that their eigenvalues were <1, the factors F4-F8 were considered non-important. The only significant contribution to F3 was upon Fe. The elements Mn, Mg, Ca, K, and P had high positive loadings whereas Cr had a high negative loading on the first PC. The pigments Chla, Chlb and TCar had high positive loadings with the second factor. Biplot analysis shows high correlation of Ca, Mg, P, K, Mn and Ni with the first factor and of N, Chla, Chlb, TCar with the second factor (Fig. 6). This analysis visually separates the samples treated with EOZ and those under AOZ, and confirms the high inter-correlation between pigments. EDU seems to be highly correlated with N, Chla, Chlb, TChl and TCar and anticorrelated with OD665/OD665a. The chlorophyll pigments are also anticorrelated with ozone.

4. DISCUSSION

In this study, a willow was used as a biological model with prior knowledge about its responses to EDU and ozone. EDU and O_3 effects were studied early in the stress, before O_3 symptoms widely appeared on the leaves. We demonstrated the single and interactive effects of O_3 and EDU on leaf stoichiometry and leaf nutrient cycling across the crown by analyzing for the first

time the chemical composition of leaves of all maturity stages (mature, young, shed) treated with EDU and under O_3 stress.

4.1. Status of EDU-induced stress

The fact that EDU did not significantly affect the ratios Chla/Chlb, OD435/OD415, OD665/OD665a and TChl/TCar suggests that EDU did not induce severe stress in plants in AOZ (Agathokleous et al., 2017). EDU-induced stimulation of the main and accessory pigments content may imply increase in energy absorption and photosynthesis efficiency and is in agreement with previous studies showing EDU-induced stimulation in photosynthetic pigments or other endpoints in O_3-clean air such as in closed chambers (Agathokleous, 2017; Agathokleous et al., 2016c, 2014; Eckardt and Pell, 1996; Whitaker et al., 1990). The present study, indeed, suggests a hormetic effect of EDU in LMA which was increased by EDU200 but not significantly affected by EDU400. Hormesis is a phenomenon where low levels of stress induce stimulatory effects whereas high levels of stress induce adverse effects (Calabrese, 2013, 2011; Calabrese and Baldwin, 2001).

4.2. Status of O_3-induced stress

EOZ decreased TChl but decreased more Chla than Chlb, thus resulting into a lower Chla/Chlb, in line with other studies dealing with O_3 stress (Agathokleous et al., 2017b; Caregnato et al., 2013; Feng et al., 2008; Knudson et al., 1977; Neufeld et al., 2006; Saitanis et al., 2001). In a stress-induced need for photoprotection and chlorophyll breakdown, photooxidation occurs with an imbalance in the relation between carotenoids and chlorophylls which is found also under O_3 stress (Agathokleous et al., 2016c, 2014; Pellegrini, 2014; Peñuelas et al., 1995). In the present study, EOZ did not affect TChl/TCar and phaeophytinization (neither Mg content) as O_3 stress

was in the early stages. Direct stimulation by EDU200 and EDU400 might have helped plants maintain greater content of photosynthetic pigments in EOZ.

4.3. EDU and O_3 effects on stoichiometry and cycling of N and C

In earlier research with willow, it was found that 800 and 1600 mg EDU L^{-1} given as soil drench increased the N content in mature leaves when soil was infertile (Agathokleous et al., 2016b) but did not increase it when soil was fertile (Agathokleous et al., 2018). Based on findings from such high EDU doses, it was suggested that EDU at low concentrations applied for phytoprotection against O_3 damage (up to 400 mg L^{-1}) is not expected to increase foliar N content of willow plants, even in infertile soil (Agathokleous et al., 2016b). The present study verifies this assumption by showing that despite EDU was associated with N, it had no direct effect on C and N contents or C/N ratio. In the present study, where EDU was applied as foliar spray, plant leaf area was not a factor influencing the EDU amount available to leaves (Agathokleous, 2017). Hence, the findings verify the assumption that the EDU mode of action in protecting plants against O_3 is not upon N contribution (Agathokleous, 2017; Manning et al., 2011b, see also charcoal-filter treatment in Ali and Abdel-Fattah (2006)). Ozone did not affect C and N content and cycling or C/N balance across leaves at the early stages of plant stress in this study with relatively low O_3 exposure. In a study where European aspen (*Populus tremul*a L.) was exposed to O_3-free air, 50 nmol O_3 mol^{-1}, or 100 nmol O_3 mol^{-1} for two growing seasons and leaf N content was analyzed in mid- or late summer of the second growing season, the only significant effect was an increase in N content of O_3 symptomatic leaves by 100 nmol O_3 mol^{-1}; leaves in 50 nmol O_3 mol^{-1} had no visible injury like in the present study (Matyssek et al., 1993). No changes in N content were also found when European silver birch (*Betula pendula* Roth) clones were exposed to twice-ambient O_3 concentration for three growing seasons (Oksanen et al., 2005) and when Japanese red pine (*Pinus densiflora*) was exposed to 60 nmol O_3 mol^{-1} for two

427 growing seasons (Nakaji et al., 2004). Some other studies showed O_3–induced alterations in C, N
428 or C/N in mature leaves of different species (Cao et al., 2016; Shang et al., 2018), however these
429 observations represent later stages of O_3-induced stress. In the present study, NRE of C and N
430 was not affected by EOZ. NRE of N remained also unaffected in three deciduous tree species
431 exposed to elevated O_3 for two growing seasons (Shi et al., 2017). It was also affected only in
432 one from two poplar clones exposed to elevated O_3 for about three months (Shang et al., 2018);
433 however it was impaired in birch, *Betula pendula* Roth, exposed to elevated O_3 for two growing
434 seasons (Uddling et al., 2006). The present study verified that N is not involved in plant response
435 mechanisms early in O_3-induced stress and that the mode of action of EDU in protecting plants
436 against O_3 injury is not upon its contribution with N. Overall, EDU and O_3 did not affect C and
437 N stoichiometry and cycling throughout the crown early in the stress, suggesting that C and N
438 are not primarily involved at the early stages of the detoxification process.

439 **4.4. EDU and O_3 effects on stoichiometry and cycling of other elements**

440 EOZ significantly increased P content and EDU increased Cr content. The fact that P was
441 increased by EOZ when plants were treated with EDU0 but were not affected by EOZ when
442 plants were treated with EDU200 and EDU400 indicates that EDU200 and EDU400 contributed
443 in increasing P content in AOZ in a similar way with EOZ. In previous studies with prolonged
444 O_3 stress, elevated O_3 had no effect on P content in evergreen or deciduous trees (Cao et al.,
445 2016; Karlsson et al., 2002; Nakaji et al., 2004; Shi et al., 2016, 2017), decreased it (Shi et al.,
446 2017) or increased it (Matyssek et al., 1993; Shang et al., 2018). More P in leaves relates to
447 chlorosis (Dekock et al., 1960), which occurs under O_3 stress. For example, increased P content
448 was found in yellowish leaves of European aspen exposed to 100 nmol O_3 mol^{-1} for two growing
449 seasons; no increase was observed in leaves with no visible injury or in bronzing leaves
450 (Matyssek et al., 1993). In the present study, chlorosis was not observed by naked eye; however

EOZ-induced decreases in the photosynthetic pigments were observed. This study suggests a role of P in homeostatic adjustments early in the stress, perhaps to prevent oxidative damage (Ganz, 2013; Hernández and Munné-Bosch, 2015). It remains unknown at present if EDU can increase P content by improving uptake or utilization efficiency.

Along with P, Fe and Cr seem to have an important role in homeostasis and metabolism early in stress as NRE of Fe and Cr was lower in EOZ than in AOZ, suggesting a higher need for reducing Fe and Cr retranslocation under EOZ; nonetheless, it should be mentioned that Cr dynamics in plant have hitherto uncertain physiological roles. The EOZ-induced need for reducing Fe was reflected into the increased P/Fe which shows stoichiometric modifications to adjust to the stress. It seems that Fe homeostasis was involved in the stress response of young, top, leaves which had lower Fe in EOZ than in AOZ, perhaps to adapt to the stress. While Fe was not different among leaf types in AOZ, it presented re-adjustments in EOZ where more Fe was maintained as leaves were aging. Decreased P content was also found in yellowish leaves, but not in non-injured or bronzing leaves, of European aspen exposed to 100 nmol O_3 mol^{-1} for two growing seasons (Matyssek et al., 1993). EOZ led also to alterations in the stoichiometry of Ni, Cr and K among leaf types, indicating effects of EOZ on nutrient cycling and suggesting implications of EOZ in the aging process. Considering that EOZ also led to lower Fe in top leaves, lower Cr in basal and top leaves, and greater Cr in shed leaves compared to the pairwise leaf types in AOZ, it is suggested that assessing elevated O_3 impacts in mature leaves only is not realistic and may lead to erroneous conclusions regarding O_3 impacts. This assumption is in line with a series of studies providing evidence for different O_3 effects on different leaf ages (Bison et al., 2018; Craker and Starbuck, 1973; Moreno Pina et al., 2017; Shang et al., 2018; Shi et al., 2017; Uddling et al., 2006). The regulation of Cr may be a mechanism for maintaining carbon

474 metabolism (Samantaray et al., 1998). Fe homeostasis holds an important role in plant nutrition
475 as an element necessary for biochemical processes (Pich et al., 2001; Vigani et al., 2013). In the
476 tobacco cultivars Bel-W3 (O_3-sensitive) and Virginia (O_3-insensitive), basal leaves of both
477 cultivars grown in ambient air had greater Fe and indifferent Mn and Zn than control plants
478 grown in growth chambers, even if Virginia plants had no O_3-induced visible symptoms
479 (Alcántara et al., 2006). In further assessments in Bel-W3 and Virginia, Fe accumulation was
480 correlated with ethylene production and associated with O_3 tolerance (Alcántara et al., 2006).
481 This indicates that the mechanisms regulating Fe homeostasis interfere with stress response
482 mechanisms, in agreement with the present findings which suggest a need for reducing stress-
483 induced Fe accumulation in the attached leaves. However, greater NRE of Ni in EOZ than in
484 AOZ suggests a higher need of attached leaves for more Ni, a phenomenon whose physiological
485 explanations need further investigations.

486 EDU affected the stoichiometry and nutrient cycling across the crown as indicated by the
487 significant interaction EDU × Leaf position for Ni, Ca/Mg and P/Fe. The significantly lower
488 Ca/Mg in Basal than in Shed leaves of EDU400 and the greater P/Fe in Top than Basal leaves of
489 EDU200 were not observed in the rest of EDU treatments for each case. These results suggest
490 that the EDU mode of action differed between EDU200 and EDU400. EDU400 might have
491 altered Ca and Mg homeostasis across leaves whereas EDU200 seem to have contributed to
492 maintain less Fe, relatively to P, in Top than Basal leaves. The greater P/Fe in Top than Basal
493 leaves only for EDU200 may be attributed to the hormetic stimulation of health by EDU200.
494 Since only EDU200 affected Cr and P/Fe (and LMA), it can be postulated that the EDU-
495 conferred protection against O_3 was not only upon stimulation in Cr (and LMA) or changes in

P/Fe because EDU400 had an equally or even more effective protection than EDU200 at the end of the experiment (Agathokleous et al., 2016a).

4.5. Potential connections to activation of defence enzymes and/or signalling pathways under O_3 stress

Many nutrients have important roles as co-factors for several physiological functions. Absence of significant EOZ-induced decreases in concentrations of elements such as K may indicate that plants did not undergo an O_3 avoidance mechanism by decreasing stomatal conductance and transpiration rate (Oksanen et al., 2005; Riikonen et al., 2005). Furthermore, no significant effects of EOZ on LMA may suggest absence of dilution effect (Oksanen et al., 2005). The fact that EOZ did not affect N may also suggest that the quantity and activity of RuBisCO enzyme was not involved in the early stress mechanisms (Riikonen et al., 2003). EOZ decreased chlorophyll contents, and, as O_3 primarily reduces the maximum rate of RuBisCO carboxylation (Vc_{max}) (Cotrozzi et al., 2016), maintaining the capacity of electron transport with adequate Fe would not be of much relevance to prevent photoinhibition.

Different essential elements have different roles in plants. Phosphorus, which was higher in EOZ than in AOZ, has numerous critical roles in plants (Vance et al., 2003). Here, it may be speculated that plants in EOZ maintained a greater content of P in an effort to prevent O_3-induced photo-oxidative stress. As we have discussed, young, top, leaves had lower Fe in EOZ than in AOZ. This might relate to a mechanism for activating jasmonate signaling (Kobayashi et al., 2016) and ethylene production, which are known as regulators of plant response to stress (Hindt and Guerinot, 2012; Iqbal et al., 2013) such as for modulating O_3-induced hypersensitive cell death (Craker, 1971; Rao et al., 2002, 2000). However, Fe deficiency may be more apparent in young leaves than in old leaves because Fe is not easily transferred within a plant (immobile). Therefore, if O_3 causes a reduction in photosynthesis and an impairment of phloem loading, a

520 reduction in photosynthates transferred into roots may decrease the activity of Fe absorption by
521 roots as well as the activity of siderophore production by rhizosphere microbes (Qiu et al., 1993;
522 Rasheed et al., 2017; Wang et al., 2011). Reduced release of phyotosynthetates (sugar, organic
523 acid, etc.) into rhizosphere would associate with reduced production of siderophore (Amin et al.,
524 2009). Such effects can lead to Fe deficiency especially in young leaves. At the same time, these
525 malfunctions may result in lower NRE of Fe, a relatively immobile element with upward
526 movement (from root to leaves) through xylem (Marschner, 2012; Shen et al., 2011). On the
527 other hand, Ni and K, whose cycling was altered by EOZ, are water soluble elements and mobile
528 in phloem translocated from old to younger leaves (Marschner, 2012; Shi et al., 2017).

529 These associations between nutrients, enzymes and physiological functions provide a
530 preliminary line of suggestions that should be further experimentally tested.

531 ## 5. CONCLUSIONS

532 EDU did not contribute significantly to plants with N at concentrations within the range applied
533 to plants against O_3 damage. Therefore, it is concluded that EDU-conferred protection of plants
534 against O_3 injury was not upon N contained in EDU. While EDU affected Cr, it had more
535 profound effects on P which seems to be affected by EDU similarly as it is affected by EOZ.
536 EDU200 and EDU400 displayed different effects on Ca/Mg and P/Fe across leaf types. It is
537 concluded that EDU may affect stoichiometry and element cycling. However, the EDU mode of
538 action may differ upon EDU concentration, even within the low-dose zone, thus, a need for more
539 dose-response studies is highlighted.

540 EOZ altered the cycling of Fe, Ni, Cr and K throughout crown leaves, decreased NRE of Fe and
541 Cr, and increased P/Fe and NRE of Ni. Interestingly, Fe homeostasis and regulations in Cr

seemed to play an important role early in O_3 stress, and this should be further investigated in future studies. It is concluded that EOZ may alter leaf stoichiometry and element cycling throughout the crown early in stress. This may affect decomposition process and nutrient input in the soil via alterations in the chemical composition of shed leaves, thus with potential ecological implications in the long-term.

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Table 1

Table 1 Results of statistical hypotheses testing with contrasts. Five contrasts (C_1, C_2, C_3, C_4, C_5) were applied to answer research questions, regarding comparisons, which were defined *a priori*. Chlorophyll *a* content (Chla), chlorophyll *b* content (Chlb), total chlorophyll content (TChl), total carotenoid content (TCar), chlorophyll a to chlorophyll b ratio (Chla/Chlb), optical density 435 reading to optical density 415 reading ratio (OD435/OD415), OD value at 665 nm before the acidification to OD value at 665 nm after acidification ratio (OD665/OD665a), TChl/TCar and leaf mass per area (LMA) were assessed in mature leaves of *Salix sachalinensis* plants treated five times, every nine days, with 0, 200 or 400 mg EDU L^{-1} and exposed to background ambient ozone (AOZ) or elevated ozone (EOZ) for about a month. Hence, there were six combinational groups: AOZ×EDU0 (AOZ0), AOZ×EDU200 (AOZ200), AOZ×EDU400 (AOZ400), EOZ×EDU0 (EOZ0), EOZ×EDU200 (EOZ200), EOZ×EDU400 (EOZ400). The means are presented in Fig 1.

	1) (AOZ0) *vs*. (AOZ200+AOZ400)	**2) AOZ200 *vs*. AOZ400**	**3) (EOZ0) *vs*. (EOZ200+EOZ400)**	**4) EOZ200 *vs*. EOZ400**	**5) AOZ0 *vs*. EOZ0**
***A*) Chla**	*T*=-2.09, *P*=0.059	*T*=-2.23, ***P*<0.05**	*T*=-4.05, ***P*<0.01**	*T*=-2.51, ***P*<0.05**	*T*=3.37, ***P*<0.01**
***B*) Chlb**	*T*=-2.16, *P*=0.05	*T*=-2.40, ***P*<0.05**	*T*=-4.35, ***P*<0.001**	*T*=-0.44, *P*=0.67	*T*=52.03, ***P*<0.001**
***C*) TChl**	*T*=-2.22, ***P*<0.05**	*T*=-2.38, ***P*<0.05**	*T*=-4.41, ***P*<0.001**	*T*=-2.05, *P*=0.063	*T*=3.13, ***P*<0.01**
***D*) TCar**	*T*=-0.74, *P*=0.473	*T*=-2.52, ***P*<0.05**	*T*=-2.08, *P*=0.059	*T*=-0.26, *P*=0.800	*T*=1.04, *P*=0.317
***E*) Chla/Chlb**	*T*=-0.86, *P*=0.405	*T*=-0.37, *P*=0.715	*T*=-1.30, *P*=0.217	*T*=-2.71, ***P*<0.05**	*T*=3.08, ***P*<0.01**
***F*) OD435/OD415**	*T*=0.67, *P*=0.518	*T*=0.21, *P*=0.839	*T*=-0.35, *P*=0.734	*T*=-0.97, *P*=0.339	*T*=1.71, *P*=0.113
***G*) OD665/OD665a**	*T*=-0.16, *P*=0.876	*T*=-0.442, *P*=0.666	*T*=-0.61, *P*=0.557	*T*=-0.49, *P*=0.634	*T*=1.44, *P*=0.176
***H*) TChl/TCar**	*T*=-1.80, *P*=0.097	*T*=1.26, *P*=0.232	*T*=-1.93, *P*=0.077	*T*=-2.13, ***P*=0.054**	*T*=1.76, *P*=0.104
***H*) LMA**	*T*=-2.54, ***P*=0.026**	*T*=2.52, ***P*=0.027**	*T*=0.17, *P*=0.869	*T*=2.04, *P*=0.064	*T*=-1.36, *P*=0.199

Table 2

Table 2 Results of statistical hypotheses testing with General Linear Model (GLM). N content, C content, and C/N ratio were assessed in shed, basal and top leaves of *Salix sachalinensis* plants. Plants were treated five times, every nine days, with 0, 200 or 400 mg EDU L^{-1} and exposed to background ambient ozone (AOZ) or elevated ozone (EOZ) for about a month. The means are presented in Figs 2-5.

	O3	EDU	Leaf position	O3 x EDU	O3 x Leaf position	EDU x Leaf position	O3 x EDU x Leaf position
N	*F*=1.54, *P*=0.238	*F*=0.81, *P*=0.466	*F*=45.61, ***P*<0.001**	*F*=0.48, *P*=0.632	*F*=0.60, *P*=0.559	*F*=1.02, *P*=0.417	*F*=0.52, *P*=0.724
C	*F*=0.13, *P*=0.723	*F*=0.07, *P*=0.931	*F*=2.88, *P*=0.076	*F*=0.28, *P*=0.762	*F*<0.01, *P*=0.999	*F*=0.44, *P*=0.779	*F*=0.66, *P*=0.627
C/N	*F*=2.65, *P*=0.130	*F*=0.60, *P*=0.566	*F*=53.99, ***P*<0.001**	*F*=0.25, *P*=0.784	*F*=0.05, *P*=0.947	*F*=1.18, *P*=0.343	*F*=0.62, *P*=0.652
Mn	*F*=2.36, *P*=0.151	*F*=0.44, *P*=0.655	*F*=36.38, ***P*<0.001**	*F*=1.69, *P*=0.227	*F*=1.79, *P*=0.189	*F*=1.29, *P*=0.303	*F*=3.22, ***P*<0.05**
Fe	*F*=0.59, *P*=0.458	*F*=1.19, *P*=0.337	*F*=25.57, ***P*<0.001**	*F*=1.17, *P*=0.343	*F*=6.54, ***P*<0.01**	*F*=1.80, *P*=0.163	*F*=0.51, *P*=0.732
Mg	*F*=2.49, *P*=0.140	*F*=0.16, *P*=0.853	*F*=16.27, ***P*<0.001**	*F*=0.43, *P*=0.659	*F*=1.67, *P*=0.209	*F*=1.57, *P*=0.215	*F*=3.49, ***P*<0.05**
Ca	*F*=3.13, *P*=0.102	*F*=1.02, *P*=0.389	*F*=58.35, ***P*<0.001**	*F*=0.36, *P*=0.704	*F*=2.52, *P*=0.102	*F*=1.67, *P*=0.189	*F*=2.83, ***P*<0.05**
Ni	*F*=0.76, *P*=0.402	*F*=0.57, *P*=0.580	*F*=21.28, ***P*<0.001**	*F*=0.19, *P*=0.833	*F*=8.00, ***P*<0.01**	*F*=2.87, ***P*<0.05**	*F*=3.30, ***P*<0.05**
P	*F*=9.71, ***P*<0.01**	*F*=0.31, *P*=0.739	*F*=76.72, ***P*<0.001**	*F*=4.18, ***P*<0.05**	*F*=1.83, *P*=0.183	*F*=2.48, *P*=0.072	*F*=10.94, ***P*<0.001**
Al	*F*=0.16, *P*=0.700	*F*=0.06, *P*=0.939	*F*=34.05, ***P*<0.001**	*F*=0.20, *P*=0.822	*F*=0.30, *P*=0.743	*F*=0.40, *P*=0.809	*F*=0.42, *P*=0.792
K	*F*=3.28, *P*=0.095	*F*=0.50, *P*=0.621	*F*=29.18, ***P*<0.001**	*F*=0.95, *P*=0.415	*F*=6.37, ***P*<0.01**	*F*=0.71, *P*=0.594	*F*=4.26, ***P*<0.01**
Cr	*F*=0.30, *P*=0.597	*F*=13.29, ***P*<0.001**	*F*=4.24, ***P*<0.05**	*F*=3.30, *P*=0.072	*F*=10.93, ***P*<0.001**	*F*=3.88, ***P*<0.05**	*F*=1.36, *P*=0.277
K/Mg	*F*=3.25, *P*=0.097	*F*=0.20, *P*=0.820	*F*=40.36, ***P*<0.001**	*F*=0.73, *P*=0.505	*F*=0.37, *P*=0.693	*F*=1.27, *P*=0.310	*F*=2.28, *P*=0.091
K/Ca	*F*=3.20, *P*=0.099	*F*=0.07, *P*=0.936	*F*=107.44, ***P*<0.001**	*F*=1.07, *P*=0.375	*F*=0.27, *P*=0.764	*F*=2.41, *P*=0.078	*F*=3.25, ***P*<0.05**
Fe/Mn	*F*=0.38, *P*=0.548	*F*=0.05, *P*=0.955	*F*=16.01, ***P*<0.001**	*F*=0.02, *P*=0.985	*F*=1.98, *P*=0.160	*F*=0.22, *P*=0.926	*F*=2.09, *P*=0.056
Ca/Mg	*F*=0.43, *P*=0.527	*F*=0.29, *P*=0.756	*F*=160.12, ***P*<0.001**	*F*=0.11, *P*=0.895	*F*=2.39, *P*=0.113	*F*=3.60, ***P*<0.05**	*F*=2.25, *P*=0.094
P/Fe	*F*=6.58, ***P*<0.05**	*F*=1.35, *P*=0.295	*F*=226.78, ***P*<0.001**	*F*=9.36, ***P*<0.01**	*F*=0.76, *P*=0.478	*F*=3.33, ***P*<0.05**	*F*=12.38, ***P*<0.001**
P/Mn	*F*=3.71, *P*=0.078	*F*=1.56, *P*=0.251	*F*=238.21, ***P*<0.001**	*F*=3.04, *P*=0.086	*F*=2.76, *P*=0.084	*F*=2.38, *P*=0.071	*F*=2.46, *P*=0.073

Table 3

Table 3 Factor loading (A) and % contribution (B) of measured response variables to the variability of the eight principal components (F1-F8), after PCA analysis.

(A)	F1	F2	F3	F4	F5	F6	F7	F8
Chla	-0.31	**0.70**	-0.04	-0.40	0.13	0.00	-0.05	0.14
Chlb	0.10	**0.63**	0.23	-0.30	0.35	0.26	0.20	0.10
Tcar	-0.41	**0.57**	-0.22	-0.02	-0.32	-0.15	-0.34	0.10
OD665/OD665a	0.10	-0.16	-0.47	0.32	0.24	**0.67**	-0.12	0.06
N	0.09	**0.73**	0.10	0.38	-0.05	0.10	-0.18	-0.14
C	-0.31	0.21	0.17	**0.70**	0.41	-0.10	-0.11	0.06
Mn	**0.56**	0.33	-0.11	-0.11	0.21	-0.30	-0.37	0.19
Fe	0.27	-0.07	**-0.54**	0.31	-0.09	-0.40	0.14	0.28
Mg	**0.78**	0.00	-0.13	-0.07	0.09	0.21	-0.22	0.10
Ca	**0.76**	0.01	-0.37	-0.25	-0.18	0.13	-0.06	0.08
K	**0.62**	0.25	0.43	0.18	-0.07	-0.04	0.17	-0.18
Ni	0.47	-0.26	0.09	-0.25	**0.51**	-0.28	-0.14	-0.33
P	**0.70**	-0.07	0.33	0.29	-0.09	-0.12	-0.07	0.13
Al	0.27	0.40	-0.30	0.08	0.15	-0.10	**0.65**	0.15
Cr	**-0.48**	-0.28	-0.27	-0.07	0.45	-0.20	-0.06	0.14
LMA	-0.04	-0.32	0.60	-0.08	0.00	0.10	-0.04	**0.67**

(B)	F1	F2	F3	F4	F5	F6	F7	F8
	2.93	**20.36**	0.09	11.64	1.53	0.00	0.29	2.35
	0.29	**16.26**	3.16	6.67	11.62	6.49	4.56	1.10
	5.10	**13.59**	2.95	0.04	9.51	2.31	13.06	1.07
	0.29	1.07	13.67	7.59	5.68	**44.32**	1.62	0.48
	0.22	**22.28**	0.62	10.56	0.23	0.99	3.54	2.22
	2.87	1.82	1.71	**36.04**	15.76	0.88	1.45	0.42
	9.22	4.64	0.76	0.85	4.29	8.79	14.76	4.21
	2.23	0.20	**17.74**	7.15	0.81	15.82	2.23	9.56
	18.04	0.00	1.08	0.36	0.72	4.25	5.29	1.28
	17.05	0.01	8.48	4.46	3.01	1.53	0.46	0.79
	11.58	2.63	11.43	2.51	0.42	0.19	3.27	3.70
	6.51	2.90	0.48	4.48	**24.31**	7.52	2.14	12.54
	14.44	0.22	6.45	6.36	0.77	1.37	0.61	1.96
	2.21	6.65	5.29	0.51	2.03	0.92	**46.16**	2.67
	6.95	3.19	4.26	0.34	19.32	3.72	0.38	2.30
	0.05	4.17	21.82	0.45	0.00	0.91	0.19	**53.35**

Fig 1

Fig 1 Mean values ± SE (n = 3) of chlorophyll *a* content (Chla), chlorophyll *b* content (Chlb), total chlorophyll content (TChl), total carotenoid content (TCar), chlorophyll a to chlorophyll b ratio (Chla/Chlb), optical density 435 reading to optical density 415 reading ratio (OD435/OD415), OD value at 665 nm before the acidification to OD value at 665 nm after acidification ratio (OD665/OD665a) and TChl/TCar in mature leaves of *Salix sachalinensis* plants. Plants were treated five times, every nine days, with 0, 200 or 400 mg EDU L^{-1} and exposed to background ambient ozone (AOZ) or elevated ozone (EOZ) for about a month. Results of statistical testing are presented in Table 1.

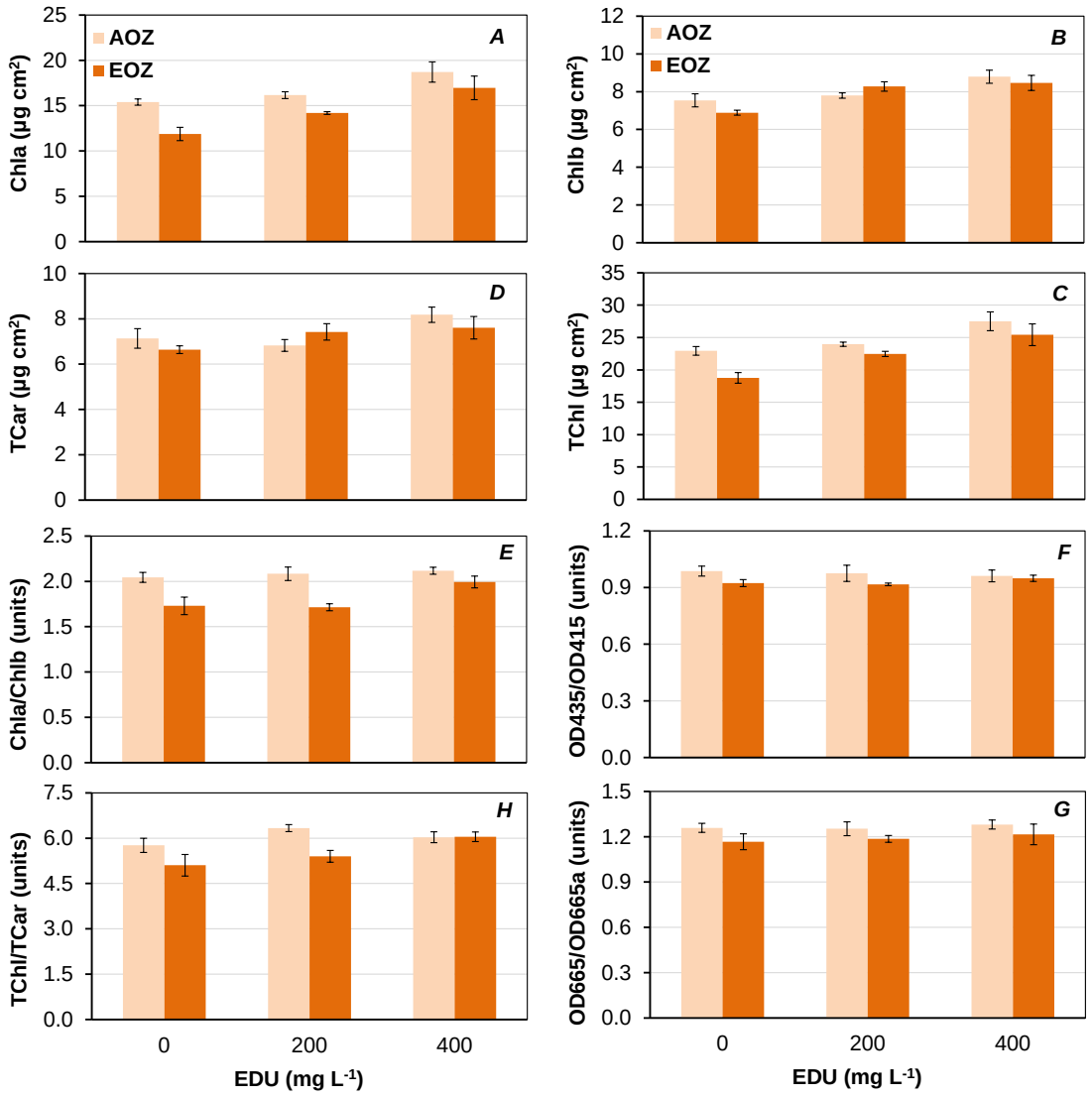

Fig 2

Fig 2 Mean values ± SE (n = 3) of N content, C content, and C/N ratio in shed, basal and top leaves of *Salix sachalinensis* plants. Plants were treated five times, every nine days, with 0, 200 or 400 mg EDU L^{-1} and exposed to background ambient ozone (AOZ) or elevated ozone (EOZ) for about a month. Statistical analysis was conducted with General Linear Model (GLM); the only significant factor was Leaf position for N (Basal>Shed>Top) and C/N (Top>Shed>Basal). Results of statistical testing are presented in Table 2.

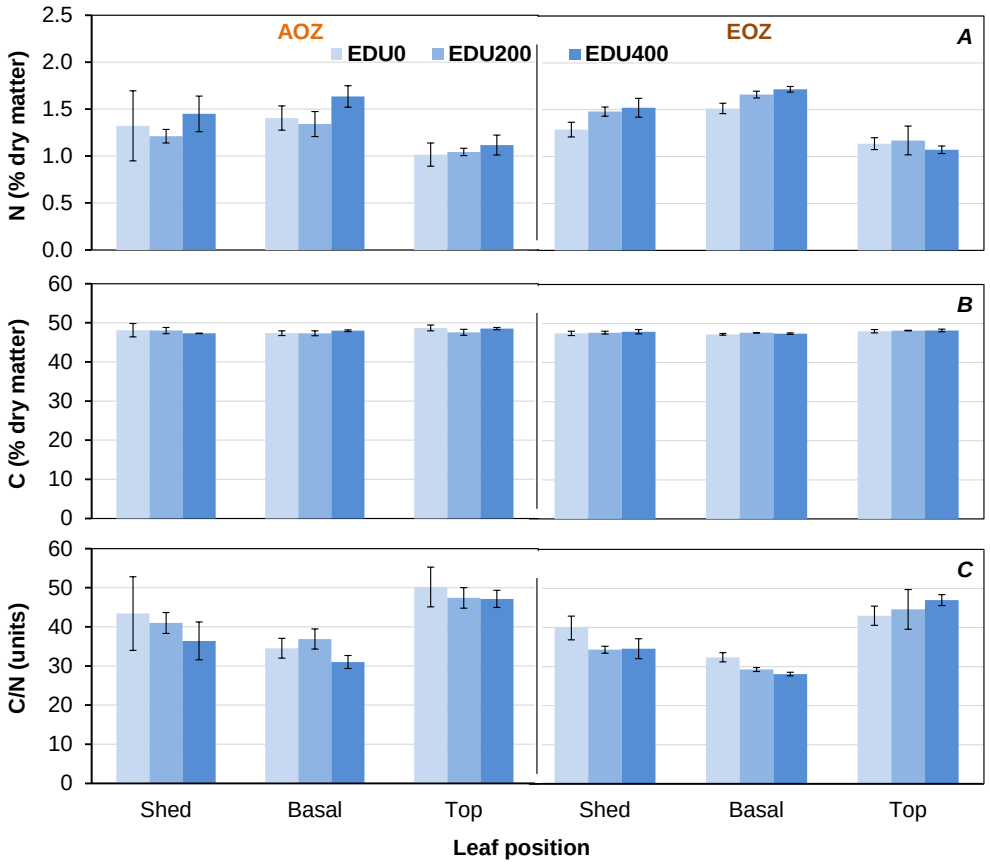

Fig 3

Fig 3 Mean values ± SE (n = 3) of P, Mn, Fe, Mg and Ca content in shed, basal and top leaves of *Salix sachalinensis* plants. Plants were treated five times, every nine days, with 0, 200 or 400 mg EDU L^{-1} and exposed to background ambient ozone (AOZ) or elevated ozone (EOZ) for about a month. Statistical analysis was conducted with General Linear Model (GLM), and the results are presented in Table 2. Different lowercase letters above means indicate statistically significant differences in the interaction Ozone×EDU×Leaf position according to Bonferroni test (α=0.05).

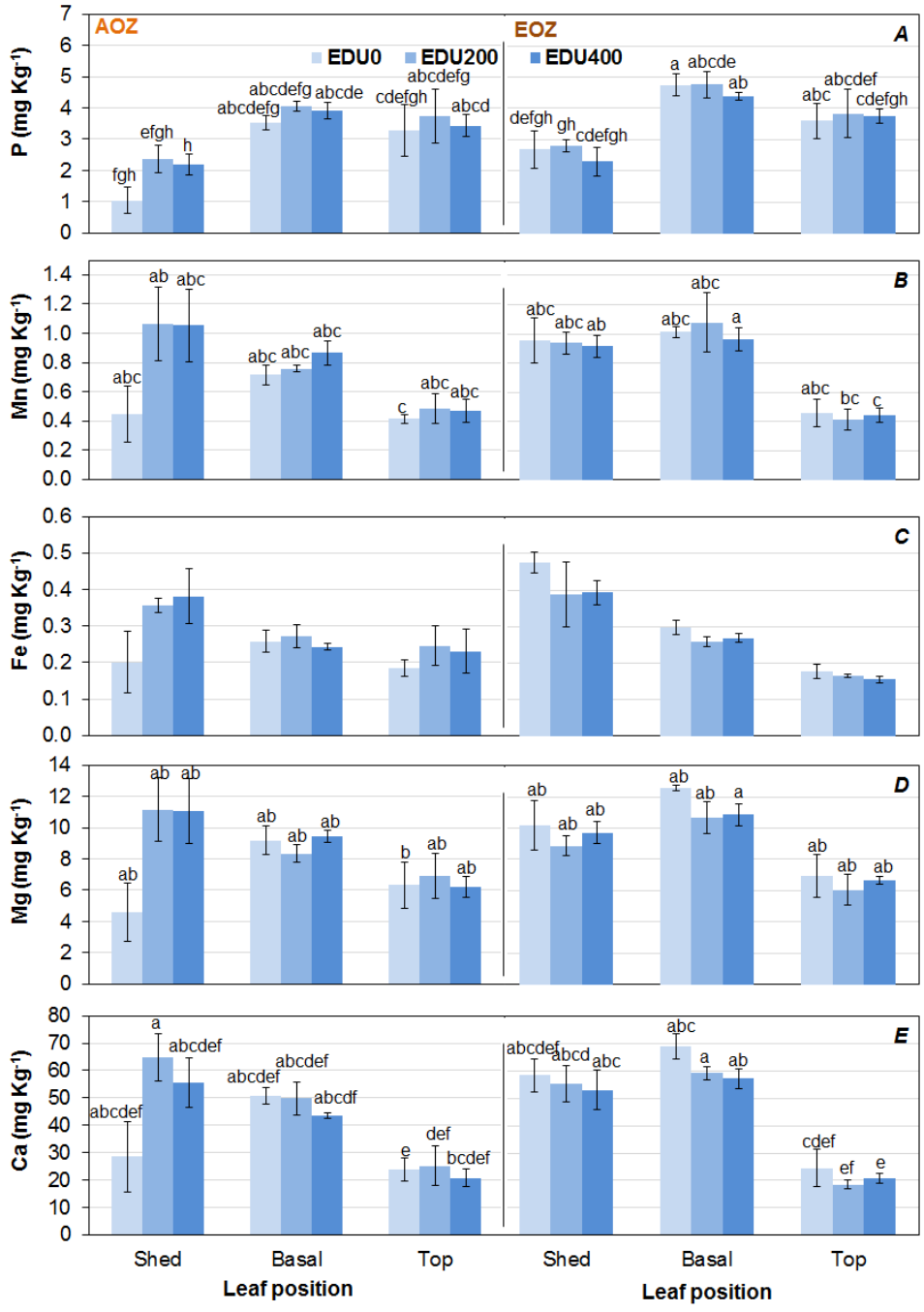

Fig 4

Fig 4 Mean values ± SE (n = 3) of Ni content, Al content, K content, Cr content and P/Fe ratio in shed, basal and top leaves of *Salix sachalinensis* plants. Plants were treated five times, every nine days, with 0, 200 or 400 mg EDU L^{-1} and exposed to background ambient ozone (AOZ) or elevated ozone (EOZ) for about a month. Statistical analysis was conducted with General Linear Model (GLM), and the results are presented in Table 2. Different lowercase letters above means indicate statistically significant differences in the interaction Ozone×EDU×Leaf position, whereas different lowercase below means indicate statistically significant differences in the interaction EDU×Leaf position according to Bonferroni test (α=0.05).

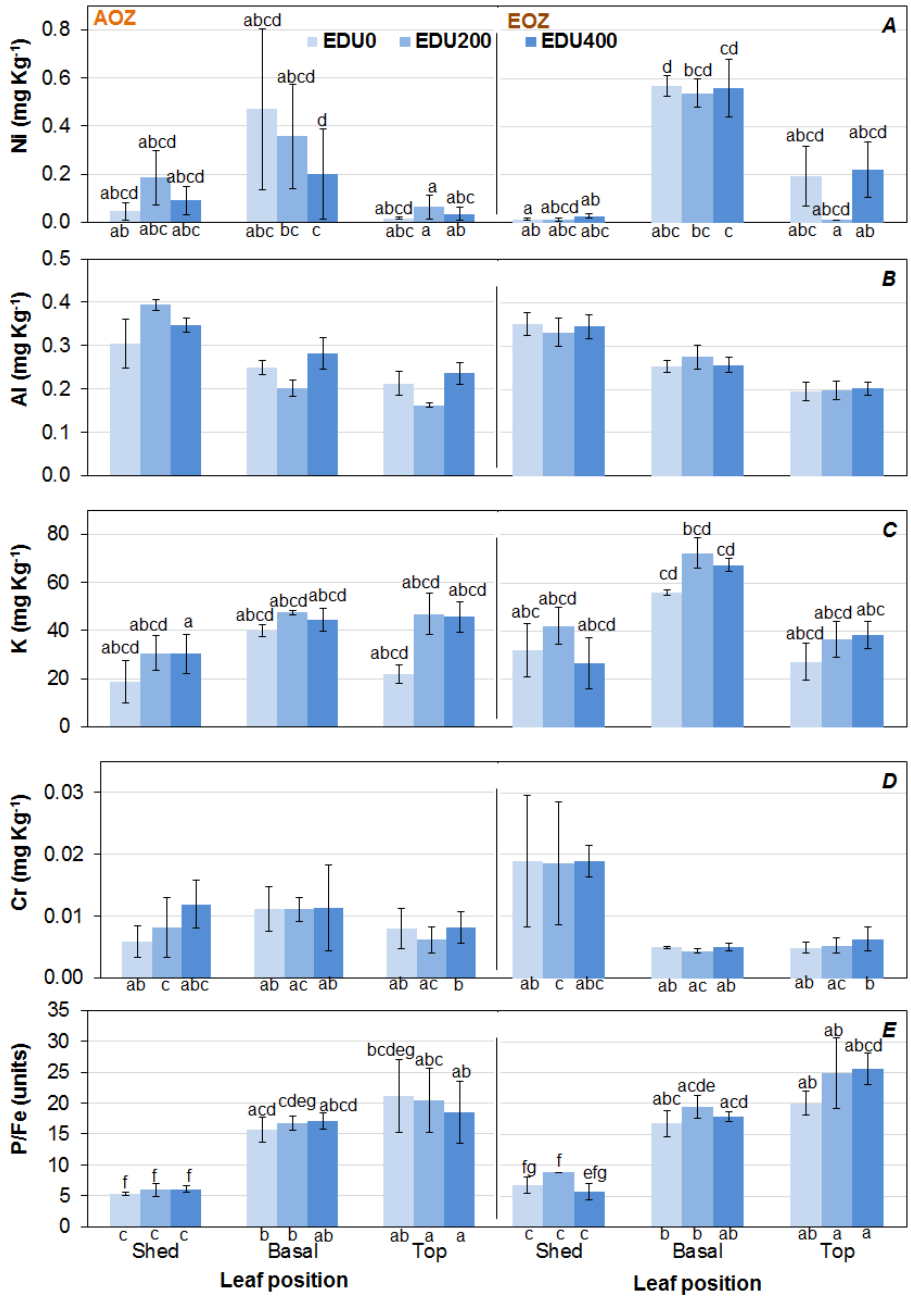

Fig 5

Fig 5 Mean values ± SE (n = 3) of Ni content, Al content, K content, Cr content and P/Fe ratio in shed, basal and top leaves of *Salix sachalinensis* plants. Plants were treated five times, every nine days, with 0, 200 or 400 mg EDU L^{-1} and exposed to background ambient ozone (AOZ) or elevated ozone (EOZ) for about a month. Statistical analysis was conducted with General Linear Model (GLM), and the results are presented in Table 2. Different lowercase letters above means indicate statistically significant differences in the interaction Ozone×EDU×Leaf position, whereas different lowercase below means indicate statistically significant differences in the interaction EDU×Leaf position according to Bonferroni test (α=0.05).

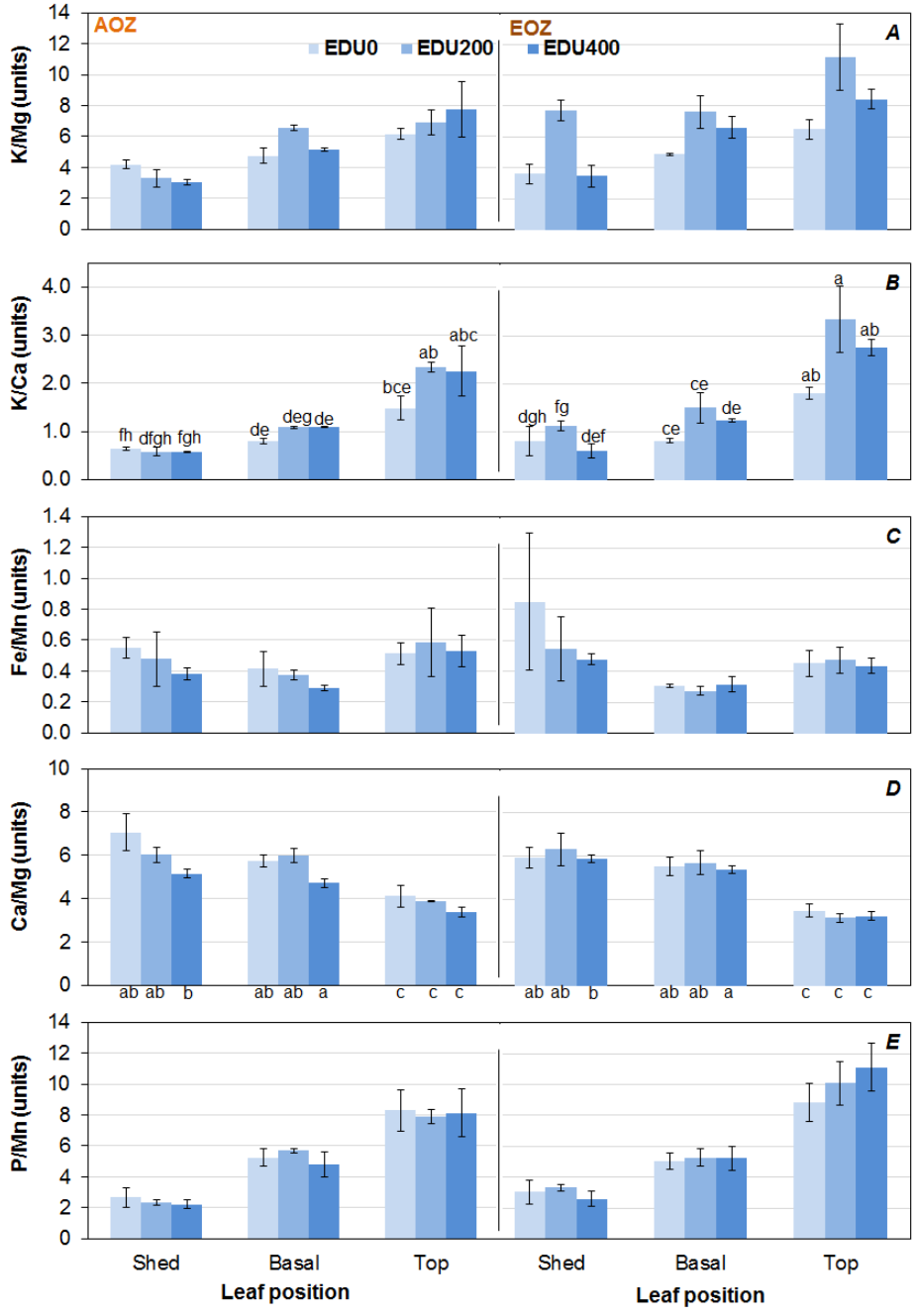

Fig 6

Fig 6 Biplot analysis of the main variables (shown as arrows): Mn, Fe, Mg, Ca, Ni, P, Al, K, Cr, N, C, Chla, Chlb, CarT, LMA and the OD665/OD665a. The variables EDU, Ozone, K/Mg, K/Ca, Fe/Mn, Ca/Mg, P/Fe, P/Mn, Chla/Chlb, ChlT, ChlT/CarT, N/C, and OD435/OD415 (shown as line segments) were passively only included as supplementary variables. The Centroid of the ozone treatments (Elevated Ozone, EOZ and Ambient Ozone, AOZ) as well as of the EDU treatments (0, 200 and 400 mg L^{-1}) groups of samples are also supplementary indicated by circles marked by italics fonts. Observations are shown as circle point. By the exception of EDU, Ozone and TChl, the rest of the supplementary variables, had very low contribution on the first two principal components and, although their line segments are shown on the biplot, they are not named for clarity of the figure.