Readily available phosphorous and nitrogen counteract for arsenic uptake and distribution in wheat (Triticum aestivum L.)

Carsten Brackhage1, Jen-How Huang2, Jörg Schaller1, Evert J. Elzinga3 & E. Gert Dudel1

1Institute of General Ecology and Environmental Protection, Technische Universität Dresden, Tharandt, Germany, 2Environmental Geosciences, University of Basel, Basel, Switzerland, 3Department of Earth & Environmental Sciences, Rutgers University, Newark, New Jersey, USA.

Elevated arsenic content in food crops pose a serious human health risk. Apart from rice wheat being another main food crop is possibly cultivated on contaminated sites. But for wheat uptake mechanisms are not entirely understood especially with regard to nutrient fertilization and different moisture regimes taking into account heavy rainfall events due to climate change. Here we show that especially higher P-fertilization under changing redox conditions may enhance arsenic uptake. This counteracts with higher N-fertilization reducing arsenic transfer and translocation into aboveground plant parts for both higher P-fertilization and reducing soil conditions. Arsenic speciation did not change in grain but for leaves P-fertilization together with reducing conditions increased the As(V) content compared to other arsenic species. Our results indicate important dependencies of nutrient fertilization, moisture conditions and substrate type on As accumulation of wheat as one of the most important crop plants worldwide with implications for agricultural practices.

Arsenic (As) is an important environmental contaminant in many regions. Especially inorganic As species like arsenite and arsenate are highly carcinogenic posing a possible health risk to humans. Arsenic enters the human food chain mainly via drinking water or via food crops. Among these rice has been attributed a main source of As intake especially for populations with rice-based diet. Therefore rice has been the target cereal for investigating uptake and accumulation mechanisms in recent years1–3. Apart from rice wheat on a worldwide basis has a similar total production providing about 19% of global dietary energy4. But much less is known about uptake, transfer, accumulation and speciation of As. It was shown that transfer factors from soil to grain were on average 10fold higher for rice compared to wheat. This was in parts attributed to enhanced As bioavailability under flooded soil conditions with lowered redox potentials in rice paddy fields5. But reducing soil conditions may not only be found in rice growing regions. Recent models on climate change predict an increase in heavy rainfall events especially during vegetation periods even in temperate zones6. This leads to short pulses of reducing conditions different from longer flooding periods. At the same time contaminated soils are still used in agricultural production growing wheat. Total As concentrations in these soils can reach up to 1000 mg kg−1 coming from either contaminated groundwater or contaminated sediments caused by mining activities. Other sources of As on agricultural land would be emissions from coal combustion with subsequent dust immissions and As containing pesticides, which have been used to quite a large extent in the past7,8. Hence elevated As concentrations in agricultural soil is for some regions a large scale problem9. Since there is severe concern on how to meet the global food demand in the next decades10 growing wheat on these contaminated sites is still an option for farmers with an ongoing debate whether P-fertilization may sufficiently reduce As uptake11,12. So the effect of reducing conditions caused by heavy rainfall together with fertilization regimes on arsenic uptake by crop plants has a very important practical implication.

Reduced uptake of arsenate, the predominant species under aerobic soil conditions, may be achieved by suppression of the high-affinity phosphate/arsenate uptake system shown especially for resistant plant species13–15. This was evaluated for selected grass species but not for wheat so far. In reducing environments not arsenate but arsenite would be the predominant species in soil solution. Hence a reduced arsenate uptake by
P-fertilization is not indicated. Arsenite can readily be taken up by plant roots, comparable to that of arsenate in the absence of phosphate. At high concentrations arsenite influx was even faster than arsenate influx for rice roots and could not be inhibited by phosphate whereas an uptake suppression for arsenate was obvious. To evaluate whether this is not only a phenomenon for rice we investigated wheat for As uptake and translocation under periodically flooded conditions mimicking heavy rainfall events combined with different P-fertilization and N-fertilization regimes on different soil types.

**Results**

**Arsenic in soil solution.** The amount of As in the soil solution is mainly controlled by the soil type. As concentrations in the soil solution are higher in sandy soil (S1) compared to loamy soil (S2) throughout the experiment (Figure 1) although total As concentrations in the soil are about 6 times less than those in S2 (Table S1). During flooding periods of 24 h and 48 h the As concentrations in soil solution increase both for S1 and S2 with low N-fertilization (control). But the increase for S1 is proportionally higher compared to S2 (Figure 2). Higher P-fertilization (P1) leads to a doubled increase compared to the control treatment with low P-fertilization (P0). For the loamy soil (S2) such an influence of P-fertilization could not be observed although there is a similar tendency. After termination of flooding the As concentrations decrease again in all treatments reaching the initial level. With high N-fertilization no increase of As-concentrations in soil solution during flooding in any of the treatments even with high P-fertilization can be seen. Throughout the experiment phosphate concentrations in the soil solution did not exceed the limit of detection.

**Plant As distribution.** In the wheat plants at maturity on the loamy soil (S2) the total As concentration follows the order of roots > leaves > stem > grain (Figure 2b). The distribution is different on the sandy substrate (S1) (Figure 2a). With high P-fertilization and low N-fertilization highest As concentrations are found in leaves with translocation factors of up to 3 (ratio of As concentration in leaves to that in roots). A low P-fertilization here leads to roughly the same As concentrations in leaves and roots. With higher N-fertilization the distribution pattern reverts to what was mentioned for S2 with the exception of similar or even higher As concentrations in stems compared to leaves.

Although As concentration levels in the plants are very much different between soils the P-fertilization has a highly significant influence on As concentrations in aboveground plant parts on both soils (Table S2). For the sandy soil it even explains up to 83% of the total variation of As-concentrations in stems and still 62% and 39% in grains and leaves respectively. The treatments with high P-fertilization in general show higher As concentrations in stems and leaves.
leaves where for grain differences can be seen only on S1. In roots no such tendency is obvious.

Besides the influence of P-fertilization the additional variation in N-fertilization has a similar highly significant influence on As-concentrations especially in aboveground plant parts. For grain but especially for leaves N-fertilization leads to in parts very much lower As-concentrations both under different P-fertilization and moisture treatments. N-fertilization explains 44% of the total variation in As-concentrations in leaves and 15% in grain on S1 and still 33% in leaves on S2 (Table S2). There is a very strong interaction between N- and P-fertilization regarding As-concentrations in leaves on S1 showing that P-fertilization enhances concentration levels which are than lowered in the same manner in all treatments by N-fertilization. Stem and root As-concentrations are not as much affected by N-fertilization as the other plant parts.

The moisture treatment of the sandy soil (S1) is explaining only 8% of the total variation of As-concentrations in roots and 3% in leaves and has no significant influence on the As content in stem and grain. For the loamy soil (S2) the influence of the moisture treatment is more pronounced with a highly significant influence on root As-concentrations due to a decrease under periodic flooding conditions. The opposite effect can be seen for leaves in S2 where especially with high P-fertilization periodic flooding leads to higher As-concentrations (Figure 2b). Consequently the interaction between these factors is highly significant, explaining still 11% of the total variation in leaves.

More than 95% of the As in the plant was found to be in inorganic form at time of harvest (ripening and dieback of leaves and stem) (see Table S3). Methylated As could be detected only to a quite minor extent in leaves but not in grain. In grain on both soils As(III) is the dominant species counting for approx. 50% of total As. Comparing the different treatments As(III) as well as As(V) in grain is significantly influenced by the P-treatment (ANOVA; p < 0.001) with elevated concentrations under high P-fertilization in S1. This is in accordance with the findings for total As. For S2 a significant difference (p < 0.01) could be seen only for As(III) where speciation recoveries for grain samples are quite low hence cautious interpretation is indicated. Periodic flooding had no significant influence on As(III) or As(V) distribution in grain. Even the ratio between these arsenic species is not changed significantly in any of the treatments but for leaves a reduced As(III)/As(V) ratio with increased P-fertilization could be observed. This ratio is lower on the loamy soil, and on both soils lowered under reducing conditions (see Table S3).

The data from μ-XANES analysis show that the XANES spectra of the As species in fresh green leaves at the stage of early heading have similar edge positions as the realgar (\(\alpha-\text{As}_2\text{S}_3\)) and orpiment (\(\text{As}_2\text{S}_3\)) reference compounds (Figure 3a), suggesting that these are reduced As species complexed with S ligands. Spectra collected for leaves at the stage of ripening and leaf dieback are displayed in Figure 3b and indicate a mixture of As species with different oxidation states, as observed from the presence of two peaks in the main edge. Visible inspection of the peak positions (Figure 3b) and analysis of the first

### Figure 2 | Total As concentrations in different plant parts of *Triticum aestivum* on the sandy soil S1 (a) and on the loamy soil S2 (b) with moisture treatment (70; 100), P-treatment (P0; P1) and N-treatment (N0; N1); mean with ±sd; n = 5.

|               | S1 sand |               | S2 loam |
|---------------|---------|---------------|---------|
| grain         |         |               |         |
| leaf          |         |               |         |
| stem          |         |               |         |
| root          |         |               |         |
| grain         |         |               |         |
| leaf          |         |               |         |
| stem          |         |               |         |
| root          |         |               |         |
Figure 3 | μ-XRF and μ-XANES scans for As of fresh green leaf (a) and brown leaf after plant death (b) of winter wheat. Colours in the right pictures indicate the amount of As in the scanned leaf section with an increase from blue to yellow.
0.01 – 0.9 (median: 0.09)23. For methylated arsenic species translocation factors for inorganic As(V) ranged from at positions similar to those of the edges of the arsenite (NaAs(III)O2) and arsenate (Na3H2As(V)O8) reference compounds. These findings are consistent with a more oxidizing environment where sulfur ligands have been replaced with oxygen ligands and partial oxidation to As(V) has occurred. The μ-XRF maps of As distribution in the leaves (Figure 3) show that most As is located along the major longitudinal veins with only small amounts of As in the tissue in between. This correlates with the distribution pattern for copper and zinc (data not shown). Still As is more evenly found along major and minor longitudinal veins compared to other elements.

**Discussion**

In contrast to a common As distribution in wheat27,38 and other plants19,20 the very high As concentrations in leaves for the treatment with high P-fertilization on the sandy soil (S1) lead to translocation factors > 1 (Figure 4; Table S4). This distribution pattern has been shown so far only for hyperaccumulator plants like Pteris vittata21 with translocation factors of up to 25 and in a control treatment with very low As concentrations in solution for Helianthus annuus22. Most other studies report translocation factors < 1. In a survey with 46 plant species translocation factors for inorganic As(V) ranged from 0.01 – 0.9 (median: 0.09)23. For methylated arsenic species translocation factors were > 1 for a minority of plant species. But other studies using hydroponic culture techniques show translocation factors of 0.1 – 0.3 for rice47 or ~0.03 for Cytisus scoparius25 supplying inorganic As in different concentrations. However, most studies report translocation factors by calculating shoot to root ratios not differentiating aboveground plant parts. Recalculating our results on a shoot to root basis show highest translocation factors of 0.64 in the above mentioned treatment. Still with 83% of total plant content most of the As is found here in the shoot. This is due mainly to biomass partitioning between root and shoot but nonetheless indicates that most of the As taken up is readily transported into shoots and here especially into leaves where it accumulates.

We could show by μ-XANES analysis that in green leaves As(III) is the most prevalent As-species like it is in grain indicating a long distance translocation as As(III). This is supported by studies on transport characteristics for As-species in the xylem where xylem sap had been harvested. For seedlings of a strain of Brassica juncea inorganic arsenic was by far the dominant form of As in xylem sap with As(III) being the predominant As-species26. A similar pattern was found for Helianthus annuus22. Both studies confirm, that As in the xylem sap appears to be unbound from phytochelatins (PC). As-PC-complexes are described to even reduce translocation from roots to shoot and at the same time the efflux into the outer medium in Arabidopsis thaliana22. These complexes were therefore attributed essential for detoxification in non-hyperaccumulator plants24. The distribution pattern in the presented study could therefore be an indication for a low ability of wheat to form PCs in the roots in response to elevated bioavailable As in the soil. Considering As in the shoot another study on the translocation of As into rice grain and the role of phytochelatins shows that only some amount of grain As is derived from leaves by remobilization where another higher portion is directly derived from root uptake25. In our study grain As in wheat was not elevated above concentration levels reported elsewhere30, although in a survey of 26 sites in Europe concentration levels where found to be generally lower with the exception of one contaminated site having comparable concentration levels to our findings5. Hence in our study, like it was mentioned for rice25, As accumulating in leaves appears not to be remobilized considerably under the circumstances described. The results from μ-XRF mapping show that As in leaves is predominantly accumulated along longitudinal veins (see Figure 3) not reaching the interjacent tissue. Moreover the data from μ-XANES indicate a complexation of reduced As(III) with 5 ligands in green leaves. This in turn would support an As(III)-PC-complexing. As(III) accumulates until leaf dieback and is then oxidized to As(V). Whether this is a possible scenario for fixation and hence detoxification, may be accompanied by intracellular compartmentalisation in vacuoles, needs further investigation. Nonetheless wheat seems to have a high accumulation potential in leaves and may effectively avoid remobilization. Since inorganic As is seen to be more toxic than organic species with As(III) exceeding the toxicity of pentavalent species26 this might have implications on the use of wheat plants other than grain harvest in toxicity assessments.

The pattern of transfer factors (ratio of As concentrations in the soil to that in plants) in both soils is not different from the pattern of As concentrations in the plants (which could be expected). But on the sandy soil transfer factors especially for leaves and stems are much higher exceeding an order of magnitude compared to the loamy soil (Table S4). Although the sandy soil is much less contaminated the bioavailability is much higher reflected in a twice as high As concentration in the soil solution throughout the experiment (Figure 1). Obviously the soil characteristics generate a higher transfer and at the same time an even higher translocation within the plants (Figure 4). Considering root concentrations being higher on the loamy soil the form/species in which As is taken up seems to be responsible for the higher translocation in aboveground plant parts.

It is shown here like in other studies26 that reducing conditions mobilize As in soils with a considerable increase of As concentrations in the soil solution. This should reflect As bioavailability since roots take up As mainly from soil solution. Hence we would have expected an increased As transfer into plants under flooded conditions. This has been shown for rice in roots48 and aboveground plant parts49 with a more than 10fold concentration increase e.g. in grain. Our results for wheat reveal only a slight increase for leaves in most treatments, but for grain a slight increase can only be seen for the treatments with normal fertilization on S1 (Figure 2). For other plant parts no tendency is obvious. On the plant side this may be due to an ineffective uptake of As. Increased efflux of mainly As(III) but also As(V) almost balancing an enhanced uptake could be another explanation since efflux has been described for a variety of plant species36,37. But it has also been shown that this is likely to be significant only when providing As(V) in solution44. Whether As(III) efflux under reducing conditions may occur for wheat cultures has still to be elucidated.

Unlike an expected reduction of As uptake with higher P-fertilization As concentrations increased in almost all plant parts but most considerably in leaves which is more pronounced on the sandy compared to the loamy soil. Hence a suppression of the high-affinity uptake system for arsenate is not indicated. Instead our results show an increased As mobilization into the soil solution with increasing P-fertilization probably due to competing exchange/adsorption on soil mineral phases. This increase is especially pronounced under anoxic...
conditions on the sandy soil (see Figure 1) with lower organic matter content and lesser adsorption sites where on the loamy soil As seems to be more tightly bound with a lower mobilization potential. Hence As mobilization by phosphate might be followed by two processes. One is the reduction of mobilized As(V) to As(III) under anoxic conditions leading to an increased uptake and translocation into aboveground plant parts (especially leaves). The other process involves the fixation of probably As(V) on root surfaces leading to more or less unchanged root As concentrations. Although there are some results on enhanced As uptake following mobilization by phosphate the link of mobilized As and translocation within plants has not been shown so far. This is certainly overlain by ellux dynamics and has to be elucidated in more detail.

Accelerated N-fertilization in the form of NH₄NO₃ enhanced nitrate concentrations in the soil and lead to elevated nitrate in the soil solution (data not shown). Nitrate was shown to influence As cycling in aquatic systems by e.g. microbially mediated oxidation of Fe(II) even under anoxic conditions. This would produce particulate hydrous ferric oxides where especially As(V) may adsorb. Thus high nitrate concentrations effectively suppress the mobilization of As even with low redox potentials in contaminated soils and at the same time reduce the bioavailability of As which is reflected in lower As concentrations of leaves and grain throughout the treatments. This would be another indication for a direct link between uptake and transport into aboveground plant parts. Since As concentrations in roots and stems seem to be unaffected by higher N-fertilization it could be suspected that a possible site for hydrous ferric oxide precipitation with adsorbed As is the root surface although no visible Fe-plaque could be observed and the roots had been thoroughly washed with distilled water. An indication here is the strong correlation between Fe and As concentrations in roots both on S1 (r = 0.738; p < 0.001) and S2 (r = 0.995; p < 0.001).

In conclusion phosphate and nitrate may counteract for the mobilization of As in soil and subsequent uptake and translocation in wheat plants initiating different processes in soils and plants. Changing redox conditions in the soil by e.g. heavy rainfall events may accelerate arsenic uptake from the soil but the effect is highly dependent on soil characteristics which should be tested for a wider range of soils and fertilization regimes. Still, our results show first evidence that care should be taken with P-fertilization well above normal levels in agricultural practice, especially on contaminated sandy soils. Also the use of wheat straw from sites with the investigated characteristics might not be advisable since especially leaves may accumulate As.

**Methods**

**Experimental design.** Sandy and loamy soils were obtained from contaminated floodplain sites used for agriculture with forage and crop production in Colditz and Schopfheim (Germany). The sandy soil in Colditz (S1) is periodically flooded (on average once a year). The loamy soil in Schopfheim (S2) is influenced by near surface but changing groundwater levels (characterization of the soils see Table S1). The soils were sieved (<10 mm), homogenized and then placed in 13 L white polyethylene pots for a 1-year experiment. An initial 22 seeds of *Triticum aestivum* L. cv. Akteur (winter wheat; etched with Arena C, Bayer CropScience AG, Germany) were sown per pot in late autumn 2010. After germination pots were kept outside in a sand bed during winter. In early spring these were placed in greenhouses with filtered air and temperature control (mimicking outside values). At this time the number of seedlings was reduced to 16 (p = 0.738; p < 0.001).

For each soil two different moisture treatments were realized ("70%"; kept at 60–80% field capacity throughout the experiment; "100%"; kept at 80–100% field capacity and being periodically flooded in May and June 2011 in the stage of grain filling. 2 × 3 h = 2 × 24 h = 1 × 48 h in weekly intervals). Soils were flooded with deionized water maintaining a near soil surface water level. As second factor P-fertilization (added as K₂HPO₄ or KH₂PO₄) was altered at two levels ("P0"; 0.32 mM P kg⁻¹; "P1"; 3.2 mM P kg⁻¹). Finally the N-fertilization (added as NH₄NO₃) was adjusted (N₀; 5.6 mM N⁺; N₁; 56.0 mM N⁺ kg⁻¹) resulting either in a maintained N. P ratio of 17:7 or in a deflected ratio of 1.77 or 1.77 respectively. To provide for an equal K-fertilization KCl was added proportionally giving 3.1 mM K⁺ kg⁻¹ in all pots. Additionally to all pots Ca (0.37 mM kg⁻¹ added as CaCl₂), and Mg (0.61 mM kg⁻¹ added as MgSO₄) were added. Each pot was fertilized by irrigating an appropriate solution beginning of April (start of the growing season) and again beginning of May. Each treatment was replicated in five pots. The experiment was terminated in July.

**Sampling, sample preparation and analysis.** Soil solution samples were obtained using rhizon soil moisture sampler (0.1 μm pore diameter, 10 cm length) in three pots of each treatment. Samples were taken sporadically before the flooding period. Within the one day and two days flooding events samples were taken after 3 h, 24 h, and 7 days after each flooding. All samples were divided into one part being acidified for total element content analysis (see below) and one to be frozen below −20 °C immediately after harvest for analysis of anions (nitrate, phosphate, sulfate, chloride) by ion chromatography (Dionex DX 500, ThermoFisher, Bremen, Germany). Samples from leaves were taken in the early heading stages beginning of June and from roots, leaves, stems, and grains (husked) in July at ripening of grains and disbelief of the aboveground plant parts. Root samples were thoroughly excised and washed with destilled water. Samples were freeze dried and kept at −20 °C. For total element content analysis a CEM Mars5 microwave digestion system (Matthews, USA) was used for mineralization of the plant material using 3 ml HNO₃ and 2 ml H₂O₂. Calibration validity was confirmed with standard reference material GBW7604, poplar leaves (office of CRM’s, China), digested according to the methods for the other plant samples. For element analysis an ICPMS X-series instrument (Thermo Fisher Scientific GmbH, Bremen, Germany) was used according to 41. Calibration functions were recorded from mixed calibration samples, which were prepared from multi-element solutions (Bernd Kraft, Duisburg, Germany). LOD was calculated as threefold standard deviation of instrument blank (acidified water). All chemicals used in the experiment were of analytical grade.

For As speciation grain samples were extracted according to the method described in 41. Analysis was done using a high performance liquid chromatography (HPLC) apparatus (Aglent 1200 series, Tokyo, Japan) coupled to an ICP-MS (Agilent 7500 ce, Tokyo, Japan). The ion intensity at m/z 75 (³²As) was monitored using the "time-resolved analysis" mode. Speciation of As(III), As(V), MMA and DMA was performed using a PPR-X100 anion-exchange column (100 × 4.1 mm, 10 μm) from Hamilton Company (Reno, NV, USA) at 22 °C with a mobile phase (1.0 mL min⁻¹). Calibration validity was confirmed with previously mentioned mixed standard solutions 41. Detection limits for As speciation were estimated as three times the standard deviation of instrumental blank values.

Spatially resolved micro X-ray fluorescence (µ-XRF) maps were collected in combination with micro-focused As K-edge X-ray absorption near-edge spectroscopy (µ-XANES) spectra on beamline X27A of the National Synchrotron Light Source (NSLS) at Brookhaven National Laboratory, Upton, New York. Wheat leaf fragments (approximately 0.5 cm × 0.5 cm) were fixated on slide frames with Kapton tape, and mounted on an XYZ motorized stage sample positioned at 45° incidence to the beam. A S(N)I monochromator (resolution 0.45 eV) under a full phase sensitive modulation was used for element detection. The beam was focused with Kirkpatrick–Baez mirrors to produce a spot size of 10 μm × 15 μm on the sample. X-ray fluorescence was measured with a 13-element Canberra Ge array detector positioned 90° to the incident beam. Mapping was done at an energy of 12.5 keV over an area of approximately 2 mm × 5 mm by moving the samples through the beam in fly scanning mode using a pixel size of 0.01 mm and an integration time of 0.3 s.

Micro-XANES spectra of the As K-edge (11867 eV) were collected at various locations in each leaf sample, using steps and counting times of 5.0 eV and 3 s, 0.5 eV and 6 s, 1.0 eV and 6 s, and 5.0 eV and 3 s in the 11787–11857, 11857–11907, 11907–11957, and 11957–12167 eV energy ranges, respectively. Two or three scans were collected per spot, and averaged to improve signal noise. XANES spectra were also collected for the reference compounds Na₂HAS(V)O₄, NaAs(III)O₃, realgar (γ-As₂S₃), and orpiment (As₂S₅) to assist data interpretation of the As wheat data. For all XANES scans, the monochromator was calibrated by assigning the top of the white line of the spectrum of As(V) incorporated in toapaz to a value of 11875 eV. Both XANES and µ-XRF spectra were evaluated using WinXAS 3.1 software.

**Statistical analysis.** Data were examined using general linear modeling (GLM) and conducted using SPSS 21. Values were subjected to normal distribution and transformed logarithmically in case of non normal distribution. For element concentrations an ANOVA was used for examining the influence of factors.

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