Impact of alternate wetting and drying on rice physiology, grain production, and grain quality

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Abstract

As the world's population increases, demands on staple crops like rice (*Oryza sativa* L.) will also increase, requiring additional fresh water supplies for irrigation of rice fields. Safe alternate wetting and drying (AWD) is a water management technique that is being adopted across a number of countries to reduce the water input for rice cultivation. The impact of AWD on plant growth, yield and grain quality is not well understood. A field trial of AWD was conducted at Mymensingh, Bangladesh over two boro (dry) seasons using eight field plots, four under AWD and four continuously flooded (CF). This manuscript describes the results of check cultivar BRRI dhan28 which was replicated in 35-40 rows per plot giving a total of 140-160 replicates per treatment. A study on the soil solution concentration of many elements indicated that manganese, iron, zinc, and arsenic were different under AWD conditions compared to CF on a number of sampling time points, but did not show a pattern related to the AWD treatment. A survey of soil strength using a penetrometer detected a small, but significant, hardening of the surface soil of the AWD plots. At harvest the shoot and grain mass was significantly greater for the plants grown under AWD (9.0-9.4% and 12.0-15.4%, respectively) with the plants grown under AWD having a greater number of productive tillers. Physiological examination in the first year showed that although AWD decreased (~21%) leaf elongation rate (LER) of recently transplanted seedlings during the first drying cycle, subsequent drying cycles did not affect LER, while tillering was slightly increased by AWD and there was evidence of higher leaf abscisic acid (ABA) in AWD plants. In the second year analysis of six phytohormones revealed that AWD increased plant foliar iso-pentenyladenine (iP) concentrations by 37% while leaf trans-zeatin concentrations decreased (36%) compared to CF plants. The elemental composition of the shoots and grains was also examined. In both years AWD decreased grain concentration of sulphur (by 4% and 15%), calcium (by 6% and 9%), iron (by 11% and 16%), and arsenic (by 14% and 26%), while it increased the grain concentration of manganese (by 19% and 28%), copper (by 81% and 37%), and cadmium (by 28% and 67%). These results indicate that plants grown under safe AWD conditions at this site have an increased grain mass compared to plants grown under CF, and this may be partly due to a high number of productive tillers. AWD decreases the concentration of arsenic in the grains in this site, but it elevates the concentration of cadmium.

Key words: Rice, alternate wetting and drying, arsenic, cadmium, pore water

1 Introduction

Rice is one of the most important food crops in the world. For 3 billion people, rice contributes between 35-60% of their dietary calorie intake (Fageria, 2007). Irrigated lowland rice systems produce ~75% of global rice (Fageria, 2007). Producing high yield under irrigated systems requires large quantities of water (Bouman, 2009). It is estimated that to produce 1 kg of rice grain, 2500 L of water is needed (Bouman, 2009). Globally this equates to one third of the world's available fresh water being used for rice irrigation (Bouman, 2009). Within Asia, the proportion of fresh water being used for rice irrigation is greater, with approximately 50% of fresh water being used for rice irrigation (Kukal et al., 2004). With global rice production needing to increase by 70% by 2030 to feed an ever growing world population (Maclean et al., 2002), demands on fresh water for irrigation of rice will only increase unless water management techniques that reduce water use are developed and implemented. These water management techniques, while decreasing total water loss, should maintain or increase yield.

One technique that has been developed to reduce total water for irrigation in rice is alternate wetting and drying (AWD). In AWD the field is not continuously flooded (CF), instead the soil is allowed to dry out for one or more days after the disappearance of ponded water, and after this drying phase the field is re-flooded (Lampayan et al., 2015). While techniques that use this intermittently flooded system have been around for a number of decades, formalised guidelines on the implementation of AWD were outlined in 2002 by the International Rice Research Institute (IRRI) (Lampayan et al., 2015). Initially it is recommend that farmers use what is termed "safe AWD" to start with, where the water in the fields is left to drain to a depth of 15 cm during each cycle, but importantly, when the crop starts to flower, flooding is restored. Once farmers are confident in using safe AWD they can progress on to allowing the water to drain to depths of 20-30 cm (or deeper) and to allow the cycles to continue into flowering when the plants are more sensitive to water stress.

A growing body of evidence is being collected on the impacts of AWD on both water use and rice yield, compared to either CF conditions or standard farmer practises (FP). For example, in a meta-analysis across a number of different field trials, when AWD was compared to FP, Lampayan et al. (2015) indicated that there was no overall significant decrease in yield, and in 16 out of 24 farmer participatory demonstration sites (across multiple countries) there was a significant increase in yield. This increase in yield ranged from 0.2-1.0 t ha^{-1}. In the same analysis in the trials where water input was measured, all the AWD irrigated trials had lower water input compared to the FP trials. The percentage difference between the water management practices ranged from 17-38% less water used in the AWD trials (Lampayan et al., 2015). A number of other studies have also shown that AWD increases grain yield when compared to either CF or FP (Yang et al., 2009; Zhang, 2009; Wang et al., 2014). However, in some studies, AWD either does not alter (Yao et al., 2012; Linquist et al., 2015; Shaibu et al., 2015; Howell et al., 2015) or slightly lowers yield (Sudhir-Yadav et al., 2012; Linquist et al., 2015; Shaibu et al., 2015). AWD has now been implemented and is recommended practise in a number of countries including Bangladesh, the Philippines, Myanmar, and Vietnam (Lampayan et al., 2015).

It has been shown that AWD can affect the concentration of arsenic in rice grains. Arsenic in rice grains is a major concern in some parts of the world, especially South Asia and South-East Asia, where large quantities of rice are consumed (Zhao et al., 2010). Inorganic arsenic is a class I human carcinogen (NRC, 2001). Under anaerobic conditions inorganic arsenic is present as arsenite (Xu et al., 2008). Arsenite is more mobile in the soil than arsenate, the species of arsenic predominantly present under aerobic conditions (Xu et al., 2008). In a study exploring grain arsenic accumulation under AWD, CF, and aerobic conditions it was found that the concentrations of arsenic in the grains of plants grown under AWD were comparable to those grown under aerobic irrigation and significantly less than those grown under CF conditions (Chou et al., 2016). Linquist et al. (2015) observed that under AWD conditions where the plants were re-flooded at the reproductive stage (like safe AWD) the concentration of arsenic in the grain was either not significantly different or increased in comparison to the plants grown under CF. However, under an AWD treatment where the AWD is continued during the reproductive stage, grain arsenic was reduced by up to 64% compared to the plants grown under CF. Similar results have been seen under intermittently flooded conditions, where a 41% decrease in grain arsenic was observed in comparison to CF (Somenahally et al., 2011). Elements other than arsenic have been shown to be affected by AWD. For example, in a pot experiment the concentration of zinc was significantly greater in brown rice when the plants were grown under AWD compared to CF (Wang et al., 2014). The accumulation of elements by plants is affected by the availability of these elements within the soil. Changing from anaerobic to aerobic conditions and *vice versa*, will alter the redox within the soil and therefore the phytoavailability of elements. For example, dissolved arsenic, iron, and manganese concentrations increase under reducing conditions when compared to oxidising conditions, whereas the release of cadmium, copper, and strontium to soil solution increases under oxidising conditions when compared to reducing conditions (Rinklebe et al., 2016).

One of the impacts of soil drying is to make soils harder (Bengough et al., 2011). Hard soils impact on root growth (Bengough et al. 2011), and it has been established that soil hardening due to soil drying is likely to limit new root growth in droughted rice plants as much as reduced water availability (Cairns et al. 2004). It is important, therefore, to establish if AWD is likely to alter soil strength in a way that might impact new root growth. Drier, harder soil is also likely to alter vegetative growth such as leaf elongation rate and tillering. Despite expectations that soil drying (Bouman & Tuong, 2001) would decrease tiller initiation and cause more frequent tiller death under AWD (Yang & Zhang, 2010), tiller number was significantly higher under AWD than CF throughout development (Howell et al., 2015), and AWD plants had a greater number of productive tillers independent of whether tiller number during development was higher or lower (Chu et al., 2015). Increased tillering likely accelerated canopy development of AWD plants, unlike leaf elongation rate on the main tiller, which did not differ between AWD and CF plants (Howell et al., 2015). Vegetative growth processes such as leaf elongation and tillering have been correlated with differences in phytohormone concentrations (Liu et al. 2011; Yeh et al. 2015).

To date, while a large number of studies have explored the impact of AWD on yield, the reason why studies have shown a diversity of effects that AWD has on yield compared to other practices is unknown. Additionally the reason as to why AWD has been shown to increase yield is yet unknown. It could be down to a wide range of factors, a number of which are explored in this manuscript. Furthermore, for a few grain elements the impact AWD has been assessed, however this is for a limited number of elements and the known impacts that AWD has on soil chemistry is limited.

The aim of this study was to evaluate the impact of safe AWD practise on grain production and grain quality and to explore of this is related to plant physiological responses or changes in soil (pore water) chemistry and hardness. To explore this, a field experiment was conducted at the Bangladesh Agricultural University, Mymensingh, Bangladesh over two years (2013 and 2014), during the dry season, under AWD and CF. This paper reports the findings of the improved cultivar, BRRI dhan28, under AWD conditions. The effect AWD had on elemental concentrations in the soil pore water and the physical effects that AWD had on the soil properties compared to CF was determined, as well as the impact on vegetative growth, leaf phytohormone concentrations, grain production, and grain elemental composition.

2 Methods

A field trial was conducted at the Bangladesh Agricultural University, Mymensingh over two years (2013 and 2014). Two different irrigation treatment were tested; for each treatment four replicate plots were used with cultivar randomly distributed in each plot. The water irrigation treatment used were continuously flooded (CF) and alternate wetting and drying (AWD), as described below. The AWD and CF areas containing the AWD and CF plots were next to each other within a field that for the last 40 years has been treated as one area. Importantly, this field has not been used for experiments for the last 15 years, during which time it has been used for general cultivation. Furthermore, when deciding on the chosen area importance was placed on the observation that no differences in plant performance had been perceived in that area. The selected field had a natural gentle slope going East to West of < 0.03%. The AWD and CF plots were 14 m apart with the AWD plots placed on the Eastern side of the field while the CF plots were on the Western side, therefore if the CF plots leaked the water would naturally move down the field away from the AWD plots. To minimise seepage from the CF area in to the AWD area an additional precaution was taken. Drainage ditches were put around the AWD area. These drainage ditches were approximately 1 m away from the outer bund of the AWD area. Soil was collected from each of the plots prior to the start of the field experiment in 2013 and analysed for elemental composition.

2.1 Field experiment 2013

Rice seeds were sown in a nursery bed on 31st December 2012. The field site was ploughed on 8th February 2013, and then levelled. The day before transplanting (12th of February) the seedlings into the AWD and CF plots, the plots were fertilised with 40 kg ha^{-1} nitrogen, 20 kg ha^{-1} phosphorus, 70 kg ha^{-1} potassium, 15 kg ha^{-1} sulphur, and 3 kg ha^{-1} zinc. A further 40 kg ha^{-1} nitrogen was supplied during the tillering stage (26th March, 41 days after transplanting (DAT)), and another 40 kg ha^{-1} nitrogen at the flowering stage (6th April, 52 DAT). The seedlings were transplanted into the eight plots on the 13th of February 2013. Each plot was 10 m x 24 m, and subdivided into 5 columns each 2 m x 24 m. Plants were planted as two plants per hill in 2 m long rows with a distance of 20 cm between each plant in a row and a 20 cm distance between rows. Almost 300 rice accessions were planted in single rows within each plot, with the check cultivar BRRI dhan28 transplanted into every second row (a BRRI dhan28 row separated each of the 300 accessions). These 300 accessions make up a genome wide association mapping panel and will be described elsewhere. After the plants were transplanted the plots were flooded. For the four CF plots the surface water was kept at a depth of between 2 cm and 5 cm above the soil surface from the time of transplanting to shortly before physiological maturity (13th April 2013, 59 DAT). For the four AWD plots plastic perforated tubes (pani pipe) were placed across the blocks to monitor the water depth. The aim was to allow the perched water table to drop to 15 cm below the soil surface. At that point the plots were irrigated to bring the water depth to between 2 cm and 5 cm above the soil surface. The AWD plots went through 4 cycles of soil drying (Figure 1A). Both the AWD and CF plots were kept under the same flooded conditions up until 18 DAT (3rd March) when water was withheld from the AWD plots (start of the first AWD cycle). The water depth in the AWD plots was allowed to drop to ~15 cm below the soil surface; for the first cycle the plots were re-flooded 29 DAT (14th of March). This cycling was conducted 3 more times with the AWD plots reflooded 40 DAT (25th March), 50 DAT (4th April), and 57 DAT (11th April). At this point the rice plants had started flowering and the AWD plots were kept flooded and maintained the same as the CF plots until harvest.

Throughout these drying and re-wetting cycles, volumetric soil water content was continuously measured at four soil depths using a single profile probe (Model PR2/4, Delta-T Devices, Burwell, UK) in each replicate plot (8 in total), which was connected to a data-logger. The soil depths in the first cycle were 2.5, 12.5, 22.5, and 32.5 cm below the soil surface but during the subsequent cycles the probes were altered to measure at depths of 5, 15, 25 and 35 cm below the soil surface. Daily manual measurements of the growing leaf of the main tiller of sample plants were carried out on the first hill of nine randomly selected rows in one plot of each treatment (AWD and CF). The first plant was chosen for practical reasons, to avoid substantial trampling of the soil between rows that would occur if central plants were measured frequently. The end plants can be expected to experience a slightly different environment to the central plants, yet non-the-less they experienced an AWD or CF treatment and it would have been very similar to the central plants. For each leaf, its elongation rate was calculated as the difference in its length on subsequent days. Leaf elongation was determined to have finished when its daily elongation rate fell below 10% of its maximum, whereupon a new leaf was selected. At periodic intervals, the youngest fully expanded leaves were also collected for abscisic acid (ABA) analysis. On each day, samples were taken every two hours, starting at 10:30 and ending at 16:30, from a single hill from six plants randomly selected in one plot of each of AWD and CF treatments. Samples were immediately frozen in liquid nitrogen, freeze-dried, then ground to a fine powder before adding deionised water (1:50 ratio) and shaken overnight at 4°C. ABA concentration of the supernatant was determined with a radioimmunoassay as previously described (Quarrie et al., 1988).

Once the cultivars had flowered and the grain matured (as determined by 80% of the grains on the panicles developing a golden brown colouration), the grain and shoots from every 10th row of BRRI dhan28 was hand harvested from the six central hills of each row. The grain was then hand threshed and weighed to determine the grain mass. Grain mass is determined as the combined grain mass of the 6 hills. The shoots were harvested approximately 5 cm above the soil, dried, and then weighed to determine the shoot weight. Shoot biomass is determined as the combined shoot biomass of the 6 hills. Once dried the shoots were then cut into small pieces ~1-2 cm long. A sub sample of the grains and shoots was then sent to the University of Aberdeen, UK for chemical analysis.

Pore water samples were collected from each of the eight plots using 10 cm Rhizon samplers. One sampler was randomly placed in each of the plots. Pore water was collected on 11 separate occasions during the four AWD cycles both from the AWD and CF plots. Once pore water was collected it was acidified with nitric acid to a final concentration of 1%.

Soil hardness was recorded at 15 mm depth intervals from the soil surface to a depth of 600 mm with a CP20 cone penetrometer (AgridryRimik PTY Ltd, Australia), with a 30^{o} angle, 12 mm diameter cone, and a penetration rate of approximately 8 cm s^{-1}. Two transects were conducted across the plots measuring at 5 m intervals, providing 7-8 measurements per plot, and 30 measurements per treatment area. These were conducted on 9 DAT (22nd February, when all plots were flooded and before the first AWD cycle) and 28 DAT (13th March, at the end of the first AWD cycle, before the AWD plots were re-flooded).

2.2 Field experiment 2014

Rice seeds were sown in a nursery bed on 17th December 2013. The same field site was used in 2014 as in 2013 with slight modifications to the size of the plots. The field site was prepared as described for 2013, with the rice plants transplanted on 6th February into the eight plots (each plot was 22.7 m x 11.8 m). The fertiliser regime was as for 2013, with the split application of nitrogen fertiliser applied 21 DAT (27th February) and 49 DAT (27th March). The AWD cycles for the four AWD plots started on 5 DAT (11th of February), with the first cycle finishing 22 DAT (28th February), the second cycle finishing 39 DAT (17th March), and the third cycle finishing 54 DAT (1st April) (Figure 1B). The fourth cycle ended prematurely 63 DAT (10th April), due to heavy rainfall flooding the field. Once the fourth cycle had finished, the AWD and CF plots were maintained under flooded conditions during the flowering stage, shortly before physiological maturity the plots were no longer kept flooded. BRRI dhan28 plants were harvested as described above.

At periodic intervals, the youngest fully expanded leaves were collected for multi-analyte phytohormone analysis (Albacete et al., 2008). At midday, samples were taken from a single hill from six randomly selected hills in one plot each of AWD and CF. Samples were immediately frozen in liquid nitrogen, freeze-dried, then ground to a fine powder before measurement. Cytokinins (*trans*-zeatin, *t*Z; zeatin riboside, ZR; and isopentenyl adenine, iP), indole-3-acetic acid (IAA), ABA, and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) were analysed according to Albacete et al. (2008) with some modifications. Briefly, 20 mg of homogenized dry plant material was dropped in 1 mL of cold (-20^{o}C) extraction mixture of methanol / water (80/20, v/v). Solids were separated by centrifugation (20,000 x*g*, 15 min) and re-extracted for 30 min at 4^{o}C in additional 0.5 mL of the same extraction solution. Pooled supernatants were passed through Sep-Pak Plus †C_{18} cartridges (SepPak Plus, Waters, USA) to remove interfering lipids and plant pigments, and evaporated at 40^{o}C under vacuum either to near dryness or until organic solvent was removed. The residue was dissolved in 1 mL methanol / water (20/80, v/v) solution using an ultrasonic bath. The dissolved samples were filtered through 13 mm diameter Millex filters with 0.22 µm pore size nylon membrane (Millipore, Bedford, MA, USA).

Ten µl of filtrated extract were injected in a U-HPLC-MS system consisting of an Accela Series U-HPLC (ThermoFisher Scientific, Waltham, MA, USA) coupled to an Exactive mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA) using a heated electrospray ionization (HESI) interface. Mass spectra were obtained using the Xcalibur software version 2.2 (ThermoFisher Scientific, Waltham, MA, USA). For quantification of the plant hormones, calibration curves were constructed for each analysed component (1, 10, 50, and 100 µg L^{-1}) and corrected for 10 µg L^{-1} deuterated internal standards. Recovery percentages ranged between 92 and 95%.

Pore water samples were collected from each of the eight plots using 10 cm Rhizon samplers. Two samplers were randomly placed in each of the plots. Pore water was collected on seven separate occasions during the four AWD cycles from both the AWD and CF plots. After pore water samples were collected they were acidified with nitric acid to a final concentration of 1%.

Soil hardness was determined using a penetrometer as described above, except that only five measurements were taken per plot, providing 20 measurements per treatment area. The survey was conducted 74 DAT (21st April, 11 days after the AWD cycles had finished, when both AWD and CF plots were flooded).

2.2 Pore water analysis

Prior to elemental analysis of the pore water, the field-collected pore water was diluted 1:50 (in 1% nitric acid) for iron and manganese analysis, and 1:5 for all other elements. Elemental analysis was performed by inductively coupled plasma-mass spectrometry (ICP-MS, Agilent Technologies 7500) using hydrogen as the reaction gas at a rate of 1.4 mL min^{-1}. Standards with the appropriate ranges were made from 1000 mg L^{-1} ICP-MS grade multi-element stock solution. For quality control, blank samples were included, as well as water certified reference material (CRM, BCR 610). An external line of 10 µg L^{-1} rhodium was used as an internal control.

2.3 Soil chemical analysis

A transect was conducted across each of the eight plots (4 AWD and 4 CF), and five soil samples (~50 g, from the top 15 cm) were collected along each transect prior to the start of the experiment in 2013. The soil from each transect was then bulked to give a total of eight soil samples, one for each plot. The samples were air dried and sieved (2 mm); once sieved they were then oven dried at 105^{o}C until they were at a consistent weight. A total of 0.1 g of soil was then used for digestion following the block digestion methodology of Adomako et al. (2009), using NCS ZC73007 as a quality control reference material. Once digested the samples were analysed for a range of elements using ICP-MS as described above. Soil pH was determined by shaking 1 g of dried soil with 10 mL of Milli-Q water and allowing the samples to stand for 30 minutes, then the pH measurement was made.

2.4 Soil texture analysis

Transects were conducted across both the AWD and CF area after the 2014 field experiment. For each transect a total of nine soil samples were taken (~100 g from the top 15 cm). Each sample was then air-dried and particle size analysis conducted (Gee and Bauder, 1986).

2.5 Rice shoot and grain analysis

Rice grains were dehusked and oven dried (80^{o}C). For digestion, 0.2 g of dehusked grains were accurately weighed out into 50 mL polyethylene centrifuge tubes. Shoot samples were oven dried (80^{o}C) and then powderised using a ball mill. Shoot samples were accurately weighed (0.2 g) into 50 mL polyethylene centrifuge tubes. Grain samples were digested with concentrated nitric acid and hydrogen peroxide as described in Norton et al., (2012). Shoot samples were digested by the following method: rice shoot samples (powdered) were transferred into Pyrex test tubes (16 x 100 mm) and weighed (0.01g). Next, trace metal grade nitric acid spiked with indium internal standard was added to the tubes (1.16 mL per tube), and 1.2 mL hydrogen peroxide added. The samples were left overnight to pre-digest. They were then digested in dry block heaters at 115^{o}C for 4 hours. The digested samples were diluted to 11.5 mL with 18.2 MΩcm Milli-Q Direct water. Total elemental analysis (sodium, magnesium, phosphorus, potassium, calcium, manganese, iron, copper, zinc, arsenic, molybdenum, and cadmium) was performed by ICP-MS. Trace element grade reagents were used for all digests, and for quality control replicates of certified reference material (CRM) (Oriental basma tobacco leaves [INCT-OBTL-5], and rice flour [NIST 1568b]) were used; blanks were also included. All samples and standards contained 10 µg L^{-1} indium as the internal standard.

2.6 Statistical analysis

To compare treatments, analysis of variance (ANOVA) has been considered justified as site history, close proximity of the treatment areas, and relevant measures of soil properties (see results) indicates equivalence between the two areas. A similar approach has been used by Devkota et al. (2013). For data analysis, ANOVA were performed using Minitab 17 Statistical Software. For the soil chemical analysis one-way ANOVA was conducted with the locations of the plots (AWD and CF) as the explanatory variable. For the soil particle size analysis one-way ANOVA was conducted with the locations of the plots (AWD and CF) as the explanatory variable for each of the three particle size categories. For the plant mass traits and the plant elemental concentration traits one-way ANOVA was conducted with AWD and CF as the explanatory variable. For the hormone analysis and pore water analysis two-way ANOVA were used with AWD and CF, and sampling point (occasion / date) as the explanatory variables. For the two-way ANOVA the presence of an interaction between the two explanatory variables was also determined.

3 Results

3.1 Soil analysis

There were no significant differences between the soil elemental concentrations in the AWD and CF plots, therefore the data are presented as the average across both treatments (Table 1). The pH of the soil was determined to be pH 6.6. There was no significant difference in the percentage of different size particles in the soils collected from the transects across the AWD plot and the transect across the CF plots. The particle size composition (±SD) of the soil was 10.4% (±1.5) sand, 29.2% (±2.1) silt, and 60.4% (±2.2) clay. The soil is classified as a clay soil. Further soil properties can be found in Hossain et al. (2009).

3.2 AWD cycling

In 2013 the AWD plots underwent 4 AWD cycles. The first cycle lasted 16 days, while the second, third, and fourth cycles lasted 11, 10, and 7 days each respectively. In 2014 the AWD plots again underwent 4 AWD cycles; the first and second cycles lasted 17 days, while the third and fourth cycles lasted 15 days and 8 days respectively. The final cycle was cut short due to heavy rain fall that flooded the plots. As can be seen from the length of the cycles in 2013 and 2014, the number of days that the plots were under each cycle decreased in length in subsequent cycles (Figures 1A and 1B). This was likely due to crop water requirements increasing with plant size, and the temperature (and evaporative demand) increase from February to April.

At 22.5/25 and 32.5/35 cm depth in the AWD plots, soil moisture content (θ_v) was stable throughout the experiment (Figure 2). At 12.5/15 cm depth (the maximum depth at which the water table was allowed to drop in the pani-pipes), soil θ_v decreased to 0.43, 0.40, and 0.38 $m^3\ m^{-3}$ at the end of the sequential drying cycles. At the beginning of each drying cycle (following re-flooding), θ_v was similar at 12.5/15 and 22.5/25 cm, but these values diverged progressively earlier in each sequential drying cycle as the plants grew. The θ_v at 2.5/5 cm depth decreased considerably, sometimes from the beginning of the drying cycle and to the point of complete moisture depletion (Figure 2B).

3.3 Penetrometer results

In the field trial in 2013 the penetration resistance of the AWD and CF plots was measured on two occasions. The first was 9 DAT (22nd of February); at this point both the AWD and CF plots were under flooded conditions and before any AWD cycling had been conducted. At this time there was no significant difference between the CF and AWD plots (Figure 3A). The penetration resistance was between 40-100 kPa for the first 135 mm; this penetration resistance increased sharply to approximately 1500 kPa by a depth of 225 mm. When the plots were tested for penetration resistance at the end of the first AWD cycle, 28 DAT (13th March), there was a significant difference in the penetration resistance between the AWD and CF plots between 15-120 mm, with the AWD plots having increased penetration resistance (Figure 3B). The largest difference between the two different treatments was at a depth of 60 mm, where the soil under AWD had an average penetration resistance of 94 kPa compared to the CF soil which had an average of 61 kPa. After a depth of 135 mm there was no difference between the two treatments.

In year 2 a single measurement of penetration resistance was made after all the AWD cycles had taken place, and was at a point where both the AWD and CF plots had been under flooded conditions for 11 days, 74 DAT. There was a significant difference between the penetration resistances for the soils that had undergone AWD treatment compared to the soils that were under CF. The penetration resistance was different between 15-105 mm, with the greatest difference being at 45 mm, with the soil that had undergone AWD having a penetration resistance of 126 kPa while the soil that had been under CF had a penetration resistance of 69 kPa (Figure 3C).

3.4 Pore water

In 2013 the pore water concentrations of manganese, iron, zinc, and arsenic were determined. The concentrations of these four elements were not significantly different between the treatments prior to the first AWD cycle (12 DAT; Figure 4), however for a number of sampling time points the concentration of the elements did vary between the AWD and the CF plots (Figure 4A-D). The manganese concentration in the pore water collected from the CF plots was significantly higher at 30 and 45 DAT compared to the AWD plots (Figure 4A). For pore water iron there was a significant difference between the AWD and the CF samples at 30 and 55 DAT, with the concentration being greater in the CF plots (Figure 4B). The zinc concentration in the pore water collected from the AWD plots was significantly higher at 22 DAT compared to the CF plots (Figure 4C). The concentration of arsenic was significantly higher in the pore water collected from the CF plots at 45 and 55 DAT compared to the AWD plots (Figure 4D).

In 2014 the concentrations of the same elements (manganese, iron, zinc, and arsenic) were determined in the pore water (Figure 5A-D). The sampling was performed from the second AWD cycle onwards. There was no significant difference between the AWD and CF plots for pore water manganese and iron concentrations across all the time points (Figure 5A and 4B). There was a significant difference in the concentration of zinc in the pore water at 35, 40, 55, and 75 DAT, with the plots under AWD having a higher concentration of zinc (Figure 5C). There was a significant difference in the concentration of arsenic in the pore water at 37 and 75 DAT, with the plots under CF having a higher concentration of arsenic (Figure 5D). For both years the concentrations of cadmium were below the analytical limit of detection in the pore water samples (0.28 µg L^{-1} for year 1 and 0.38 µg L^{-1} for year 2), therefore these data are not presented. However, a new *in situ* sampling technique, DGT (diffusive gradients in thin-films), was used to measure the flux of cadmium from the soil solid phase to solution. The fluxes (not shown here) obtained in the plots of AWD were consistently higher than the results from CF plots and will be reported elsewhere.

3.5 Physiological and phytohormonal measurements during vegetative growth

Throughout the first drying cycle in 2013, daily leaf elongation rate (LER) of plants exposed to AWD was significantly less (by up to 33%) than that of plants exposed to CF, an effect that persisted on the first day after re-flooding the plot. Thereafter, LER did not differ between treatments, until the last day of measurements, when the LER of AWD plants was significantly greater (by 46%) than CF plants (Figure 2). At the end of the AWD cycles, AWD plants had two more tillers than CF plants, even if their height was 10% lower than CF plants (Table 2).

Throughout the first two drying cycles in 2013, there was no substantial variation in leaf ABA concentrations of AWD plants. However, during the third drying cycle, leaf ABA concentrations of CF plants declined from 250 ng g^{-1} dry weight (DW) to 150 ng g^{-1} DW, such that ABA concentrations of AWD plants were higher by 19% and 56% respectively on 45 and 47 DAT (which was 27 and 29 days after imposing AWD). On the last occasion that measurements were made (during the fourth drying cycle immediately after re-flooding the plants), there was no significant difference in leaf ABA concentrations between treatments.

Since there were minimal differences in leaf ABA concentrations in 2013, in the following year a larger range of phytohormones were measured. Again, measurements were taken at the end of a drying cycle (Measurement Occasions 2 and 4), and immediately after re-flooding the AWD plots (Measurement Occasions 3 and 5). Of the phytohormones measured, irrigation treatment had significant effect only on the cytokinins iso-pentenyladenine (iP) and trans-zeatin (tZ), with AWD increasing iP concentrations by 37% (averaged across Measurement Occasions 2-5) and decreasing tZ concentrations by 36% (averaged across Measurement Occasions 2-5). There was no consistent effect of re-flooding the soil on the concentrations of these, or other, phytohormones. Nevertheless, the measurement occasion was highly significant ($P < 0.001$) for the concentrations of all phytohormones measured, with significant increases in tZ, zeatin riboside (ZR), and ABA as the experiment progressed, and significant decreases in iP as the experiment progressed (Figure 6; Table 3).

3.6 Rice mass

In both years, the shoot mass and the grain mass were significantly greater in the rice plants grown in the AWD plots compared to the CF plots. There was a 15.4% and 12.0% increase in shoot mass and a 9.8% and 9.0% increase in grain mass in 2013 and 2014 respectively (Table 4). Despite early differences in tillering, there was no significant difference in the total number of tillers for plants grown in the AWD plots compared to the CF plots at harvest in both years. However, there was a small, but significant increase in the number of productive tillers, with the plants grown under AWD having 6% more productive tillers than the plants grown under CF (only measured in 2014).

3.7 Rice plant elemental concentration

The AWD treatment had a significant effect on the concentration of a number of elements in the rice shoots compared to the CF treatment (Table 5). The AWD treatment caused a significant decrease in the concentration of shoot sodium, magnesium, calcium, iron, arsenic, and molybdenum. The largest decrease in shoot concentration between AWD and CF was observed for shoot molybdenum, which decreased by 28.4%. The AWD treatment significantly increased shoot concentrations of manganese, copper, and zinc. The highest increase between the two treatments was in shoot copper, which increased by 38% in the AWD treatment.

The AWD treatment also had a significant effect on the accumulation of grain elements compared to the CF treatment (Table 6). Concentrations of sulphur, calcium, iron, and arsenic were all significantly lower in the grains of rice plants grown in the AWD plots compared to the CF plots in both years. In contrast, the concentrations of manganese, copper, and cadmium were significantly higher in the grains of plants grown in the AWD plots compared to the CF plots in both years. A number of elements (sodium, magnesium, potassium, and molybdenum) were either only significantly different between treatments in a single year or were significantly different in both years but the effect of the treatment was in opposite directions (molybdenum). Only phosphorus and zinc were not significantly different between the two treatments in either year (Table 6).

4 Discussion

The reported effects that AWD has on rice grain yield varies between different studies. In this study, grain mass significantly increased in both years of this study (9.8% & 9.0%) for plants grown under AWD compared to CF. In this study the grain production was determined as the mass of grain produced by the 6 central plants of each row. Using this information an approximation of grain yield can be made, by scaling up the value based on the planting density, which must be used cautiously. This would result in a yield of 7.7 t ha^{-1} for the plants grown under AWD and 7.0 t ha^{-1} for plants grown under CF in 2013, and 9.1 t ha^{-1} for the plants grown under AWD and 8.3 t ha^{-1} for plants grown under CF in 2014. One of the factors that has been proposed to be responsible for an increase in grain yield is an increase in the proportion of productive tillers (Yang and Zhang, 2010). In this study, while an overall increase in plant biomass was observed in the plants grown under AWD, there was no significant difference in the total number of tillers between the plants grown under AWD and CF. This is in contrast to previous experiments where total tiller number decreased under AWD (Yang and Zhang, 2010; Chu et al., 2015). Although Howell et al. (2015) observed an increase (14%) in the number of productive tillers in one of the two rice varieties they tested under AWD compared to CF, there was also a decrease (11%) in the number of filled grains per panicle for that same variety. In the second year of the field trial, both the number of productive tillers (those that produced grain) and total tiller number were measured, and plants that were grown under AWD had significantly more productive tillers compared to the CF plants (Table 4). Again this is in contrast to the study by Yang and Zhang (2010), where they observed under moderate AWD there was no significant difference in the number of productive tillers when compared to CF. This increase in productive tiller number could be the main driver for the increase in the observed grain mass in the present study.

Considering the possible importance of tillering in regulating grain mass under different environmental stresses (including AWD), relatively few studies have attempted to understand its regulation. Phytohormones seem important since tillering mutants show altered hormone signalling (Lu et al., 2015), and applying chemical inhibitors of hormone action affects tillering (Seneweera et al., 2001). Although measuring phytohormone concentrations in tiller buds is technically difficult, previous studies show similar phytohormonal responses in rice roots, xylem sap, and leaves (Zhang et al., 2011). AWD decreased foliar cytokinin (both Z + ZR and iP + iPR) levels at the end of the drying cycle, but re-wetting increased cytokinin levels as long as soil drying was not too severe (Zhang et al., 2011). Severe soil drying also decreased foliar IAA levels and these changes were not responsive to drying and re-wetting cycles (Zhang et al., 2010). In our studies, tZ and iP showed opposite responses to AWD (Figure 6), which again were insensitive to drying and re-wetting cycles, as were the “stress hormones” ABA and ACC. The relatively small impact of drying and re-wetting cycles on phytohormone concentrations is likely because only a small fraction of the root system (the upper 5-10 cm) is exposed to an appreciable soil drying given that at a depth of 15 cm the water context of the soil was never far below the water content of the deeper, still flooded soil (Figure 2). Indeed, split-root experiments with barley where half of the root zone was dried demonstrated that foliar ABA concentration significantly increased only if more than 30% of the root biomass was exposed to drying soil (Martin-Vertedor and Dodd, 2011). Thus, rice varieties which show a greater proportion of their root mass deeper in the soil profile might be expected to show more stable phytohormonal (and physiological) responses to AWD. BRRI dhan28 is not one of these varieties as it has been developed for flooded conditions.

The main factor that appears to impact on grain yield production under AWD (in comparison to CF) is the severity of the soil drying phase of the AWD cycle. Studies which have imposed varying degrees of soil drying indicate that when more severe soil drying conditions are imposed during AWD, there is a reduction in grain yield (Yang et al., 2009; Sudhir-Yadav et al. 2011 Linquist et al., 2015). Although the severity of soil drying did not alter grain-filling rate and duration of grain filling of superior spikelets, both were decreased in inferior spikelets (Zhang et al., 2010). The severity of soil drying will also affect leaf growth and photosynthesis, but transient limitation of LER by AWD observed in this study (Figure 2) did not compromise final grain yield, suggesting a more important role of physiological processes occurring during grain filling. To date, the precise mechanism(s) by which AWD increases yield under moderate soil drying is unknown, but future studies should try to distinguish the relative importance of AWD effects on vegetative development and grain-filling, especially when AWD is only applied until anthesis, as occurred here.

A penetration resistance of 1.5 MPa can slow root elongation by 20% to 75% (depending on the crop and soil type) (Bengough, 1997). Since the penetration resistances observed in the top 12 cm of soil under AWD were well under this (maximum penetration resistance: 172 kPa), soil strength is unlikely to have inhibited root elongation, however it does indicate that AWD alters the physical properties of the first 12 cm of soil. Interestingly, a penetration resistance that would inhibit root elongation is only observed at depths between 25 cm and 30 cm where there is no significant difference between treatments, presumably because the soil water content below 15 cm does not differ between treatments.

The AWD treatment affected soil solution concentration of a number of elements, as well as the concentration of elements within the shoots and grain of the rice plants. Of particular note is the effect that AWD had on two toxic elements, arsenic and cadmium. Both these elements have been identified as accumulating in rice, making rice an important pathway of human ingestion (Zhao et al., 2010; Meharg et al., 2013). In addition to the alterations in toxic elements, nutritionally important elements (such as iron) were affected by AWD.

The concentration of arsenic in the pore water was significantly higher when sampled from the CF plots compared to the AWD plots on a number of occasions across both years (Figure 4D and 5D). It has been demonstrated that iron (hydr)oxide hosts arsenic in soil, and if arsenic is entering the paddy field by applying arsenic-rich irrigation water, it is rapidly incorporated in iron (hydr)oxide during non-flooded periods (Takahashi et al., 2004). When the soil becomes flooded, arsenic is quickly released from the soil to the water due to the reductive dissolution of the iron (hydr)oxide and the reduction of arsenate to arsenite (Takahashi et al., 2004). While inorganic arsenic speciation was not determined in the collected porewater samples, it can be predicted that under CF conditions the dominant arsenic species would be arsenite, while under the dry phases of AWD the dominant inorganic arsenic species would be arsenate (Takahashi et al., 2004; Xu et al., 2008).

When the plants were grown under AWD, there was a significant decrease in both shoot arsenic and grain arsenic in the rice plants compared to the plants grown under CF, although it was more marked in shoots in year 1 where both shoot and grain were measured. Rice plants have different uptake mechanisms for arsenite and arsenate. Ma et al. (2008) showed that arsenite is taken up through the Lsi1 silicon transporter while arsenate is accumulated via phosphate transporters (Meharg and Hartley-Whitaker, 2002). This is important in rice as it can accumulate up to 10% of its dry mass as silicon, reflecting the fact that the silicon uptake mechanism is very efficient (Ma et al., 2006). Growing rice plants in flooded conditions compared to non-flooded conditions results in a 10-fold greater arsenic accumulation in rice grains (Xu et al., 2008; Norton et al., 2012; Norton et al., 2013). When grown under AWD it was observed that the reduction in grain arsenic was only 9% and 25%. These reductions in grain arsenic are less than previously observed for AWD when compared to CF in a number of studies (Linquist et al., 2015; Somenahally et al., 2011; Chou et al., 2016), but a greater reduction in grain arsenic than the mildest of the three AWD treatment imposed by Linquist et al. (2015). The final concentration of inorganic arsenic in the grain is likely due to direct uptake from the soil rather than remobilisation of inorganic species from the rest of the plant, as inorganic arsenic in rice leaves is poorly remobilized (Carey et al., 2011). Therefore, key to reducing grain arsenic will be the degree of flooding at grain filling. If the soil is aerobic at grain filling, inorganic arsenic will be predominantly present as arsenate, which has a reduced mobility and uptake by rice plants, while if the soil is flooded arsenite will be dominant, which is more mobile and rapidly accumulated by rice plants. The method of AWD used in this study is referred to as safe AWD (Lampayan et al., 2015), where the AWD plots were re-flooded at the start of the reproductive stage (however, AWD was not implemented during grain filling which is an option for safe AWD). The study by Linquist et al., (2015) directly addressed the issue of the effect of flooding during the reproductive stage by either extending the AWD cycling into the reproductive stage or by flooding at that stage. They observed that the AWD treatment with flooding at the reproductive phase had no effect on grain arsenic (or increased grain arsenic) in comparison to the CF treatment. However, when AWD was extended through the reproductive phase, a 64% reduction in grain arsenic compared to the plants grown under CF was observed (Linquist et al., 2015), but this more severe AWD treatment decreased grain yield by 12.6% (Linquist et al., 2015), clearly demonstrating a potential trade-off between large reductions in grain arsenic and yield.

The concentration of cadmium in the rice plants under AWD was not significantly different in the shoots compared to CF, however under AWD the concentration of cadmium was greater (up to 67.3%) in the grain of the rice plants compared to CF. In contrast, Yang et al. (2009) observed a decrease in grain cadmium under a mild AWD treatment but increased grain cadmium under a severe AWD treatment. Cadmium can be present in soil naturally (0.1-1 mg kg^{-1}) or soil can be contaminated with cadmium from anthropogenic sources (Smolders and Mertens, 2013). One source of anthropogenic cadmium to agricultural soils is P-fertilisers (Smolders and Mertens, 2013). Cadmium in the soil solution increased with increasing soil redox under oxidising conditions (Rinklebe et al., 2016). As soils become waterlogged, the increase in soil pH may contribute towards the immobilisation of cadmium in anaerobic soils (Smolders and Mertens, 2013). Under anaerobic conditions cadmium ions (Cd^{2+}) may precipitate as cadmium sulphate, reducing the soil solution concentration of cadmium (Barrett and McBride, 2007). On the other hand, during AWD Fe^{2+} is oxidised to Fe^{3+}. Protons are released in the Fe^{2+} oxidation process (eq 1), locally lowering the pH.

$$4Fe^{2+} + O_2 + 6H_2O = 4FeOOH + 8H^+ \text{ (eq 1)}$$

Cadmium is pH-sensitive and easily desorbed with decreasing pH. Therefore, it would be expected as the soil becomes more oxic during the AWD cycle, that the cadmium concentration increases in the soil solution (too low concentration to measure directly, but confirmed by DGT measurements) and this would lead to more cadmium being available to the plant to accumulate. However, it is interesting to note that the cadmium concentration in the shoots of the rice plants grown under AWD and CF are not different and it is only the grain cadmium concentration that is elevated. The concentrations of grain cadmium in this field experiment are low compared to other studies (Meharg et al., 2013). The highest average concentration of cadmium (year 2, plants grown under AWD) of 0.019 mg kg^{-1}, is below that of a survey of Bangladeshi rice grains where the average cadmium concentration was 0.099 mg kg^{-1}. With a rice grain cadmium concentration of 0.099 mg kg^{-1} it has been estimated that the weekly intake of cadmium from rice would lead to intakes deemed unsafe by international and national regulators (Meharg et al., 2013). Therefore, if AWD increased grain cadmium further, this could have impacts on human health, suggesting either AWD might be best avoided in areas with high grain cadmium, and/or breeding for low cadmium should be pursued for AWD.

Both iron and zinc are important nutritional mineral elements, and are key targets to increase the nutritional quality of edible crops (White and Broadley, 2009). In this study, zinc concentration in the grains was not affected by AWD, in contrast to a previous study which showed that grain zinc concentrations increased by approximately 4% under AWD treatment (Wang et al., 2014). However, AWD does decrease grain iron concentration in this study. On a small number of sampling points the soil solution concentration of iron was greater in the CF plots than in the AWD plots, and the concentration of iron in the shoots was greater in the CF-grown plants as was the grain concentration of iron. This is explained by the impact that altering the water conditions has on soil iron availability. Under anaerobic (reduced) conditions iron is largely present as Fe^{2+}, however under oxidised conditions it is present as Fe^{3+}, with Fe^{2+} being more soluble than Fe^{3+}. When Fe^{2+} encounters dissolved oxygen it is oxidised to Fe^{3+}, which primarily precipitates as amorphous ferric hydroxide.

Conclusions

This study confirms previous findings that AWD water management can increase grain production when compared to CF. We present evidence that AWD has quite subtle effects on plant physiology, specifically leaf elongation, the concentration of ABA and two cytokinins, and increases the number of productive tillers. The combination of all these subtle effects could be the reason that there are detectable differences in grain production between plants grown in AWD and CF. Impacts of AWD on many elements in the grain were detected: crucially, arsenic decreased in AWD-grown grain, which is positive for human health, but cadmium increased and iron decreased, which are not desired outcomes. These impacts on grain quality needs to be carefully considered when AWD is implemented.

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Table 1. Soil elemental composition at the field site. Each value is the mean (± SD) across the 4 AWD and 4 CF plots.

	P	Cr	Mn	Co	Ni	Cu	Zn	As	Mo	Cd
mg kg^{-1}	574 (±60)	76.4 (±12.6)	665 (±28)	17.5 (±0.6)	51.2 (±1.6)	40.7 (±1.9)	99.7 (±2.7)	4.63 (±0.30)	0.57 (±0.19)	0.19 (±0.15)

Table 2. Tiller number and plant height (measured at the end of the 4th drying cycle) exposed to alternate wetting and drying (AWD) and continuous flooding (CF) in 2013. Data are means ± SD of 9 plants, with P Values presented.

	Mean AWD (± SD)	Mean CF (± SD)	Significance test
Plant Height (cm)	76.0 (6.0)	84.0 (6.0)	*
Tiller Number	21.7 (3.9)	19.8 (3.9)	NS

*P<0.05; **P<0.01; ***P<0.001; NS = not significant

Table 3. Two way ANOVA (F Values presented) to determine the effects of treatment (T), measurement occasion (O) and their interactions on leaf hormone concentrations in 2014. Hormone measured were cytokinins (*trans*-zeatin, *t*Z, zeatin riboside, ZR and isopentenyl adenine, iP), indole-3-acetic acid (IAA), abscisic acid (ABA) and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC).

	F-values from 2-way ANOVA			Effect of treatment relative to CF
Hormone	Treatment (T)	Occasion (O)	T x O	
tZ	5.36*	5.60***	NS	AWD decreased by 36%
iP	9.00**	5.68***	NS	AWD increased by 37%
ZR	NS	17.53***	NS	ND
ABA	NS	14.92***	NS	ND
ACC	NS	6.21***	NS	ND
IAA	NS	4.85***	NS	ND

*P<0.05; **P<0.01; ***P<0.001; NS = not significant; ND = no difference

Table 4.Mean total tiller number and shoot and grain mass for BRRI dhan28 grown in the field

	Trait	Mean AWD (± SD)	Mean CF (± SD)	F value from ANOVA	Increase (+) or decrease (-) between AWD relative to CF
Year 1[a]	Total tiller no.	13.6 (4.0)	12.8 (4.4)	NS	ND
	Shoot mass (g)[#]	119 (21.)	103 (15)	50.6***	+ 15.4%
	Grain mass (g)[#]	92.8 (20.2)	84.5 (15.3)	14.9***	+ 9.8%
Year 2[b]	Total tiller no.	17.3 (4.6)	16.6 (3.1)	NS	ND
	Productive tiller no.	15.1 (4.2)	14.2 (2.8)	6.3*	+ 6.3%
	Shoot mass (g)[#]	133 (24)	119 (22)	28.8***	+ 12.0%
	Grain mass (g)[#]	108 (24)	99(19)	12.9***	+ 9.0%

[#] shoot mass and grain mass for 6 hills

[a](n=140 for AWD and CF); [b](n=160 for AWD and CF).

*P<0.05; **P<0.01; ***P<0.001; NS = not significant; ND = no difference

Table 5. Total shoot elemental concentrations for BRRI dhan28 grown in 2013.

Trait	Mean AWD (± SD)	Mean CF (± SD)	F value from ANOVA	Increase (+) or decrease (-) between AWD relative to CF
Na ($mg\ kg^{-1}$)	1110 (560)	1360 (800)	9.04**	- 18.6%
Mg ($mg\ kg^{-1}$)	2220 (320)	2660 (440)	87.6***	- 16.6%
P ($mg\ kg^{-1}$)	934 (337)	880 (293)	NS	ND
K ($mg\ kg^{-1}$)	21100 (3700)	20400 (2800)	NS	ND
Ca ($mg\ kg^{-1}$)	3250 (650)	3510 (730)	9.79**	- 7.5%
Mn ($mg\ kg^{-1}$)	511 (113)	384 (79)	114***	+ 33.1%
Fe ($mg\ kg^{-1}$)	378 (188)	444 (301)	4.65*	- 14.9%
Cu ($mg\ kg^{-1}$)	3.63 (1.14)	2.63 (1.00)	58.8***	+ 38.0%
Zn ($mg\ kg^{-1}$)	34.9 (10.7)	28.1 (7.0)	38.9***	+ 24.4%
As ($mg\ kg^{-1}$)	1.38 (0.28)	1.81 (0.44)	94.2***	- 24.1%
Mo ($mg\ kg^{-1}$)	0.77 (0.34)	1.08 (0.59)	25.0***	-28.4%
Cd ($mg\ kg^{-1}$)	2.65 (0.59)	2.73 (0.64)	NS	ND

*P<0.05; **P<0.01; ***P<0.001; NS = not significant; ND = no difference

Table 6. Total grain elemental concentrations for BRRI dhan28 grown in 2013 and 2014.

	Year 1				Year 2			
Trait	Mean AWD (± SD)	Mean CF (± SD)	F value from ANOVA	Increase (+) or decrease (-) between AWD and CF	Mean AWD (± SD)	Mean CF (± SD)	F value from ANOVA	Increase (+) or decrease (-) between AWD and CF
Na (mg kg^{-1})	9.13 (3.99)	8.76 (4.59)	NS	ND	6.19 (3.04)	14.05 (4.59)	172***	- 56.0%
Mg (mg kg^{-1})	1650 (150)	1660 (150)	NS	ND	1430 (120)	1500 (150)	23.0***	- 4.8%
P (mg kg^{-1})	4200 (460)	4210 (420)	NS	ND	4500 (49)	455 (580)	NS	ND
S (mg kg^{-1})	1240 (180)	1460 (150)	123***	- 15.4%	1330 (110)	1390 (120)	20.6***	- 4.2%
K (mg kg^{-1})	2740 (320)	2830 (33)	5.02*	- 3.2%	3020 (250)	2960 (300)	NS	ND
Ca (mg kg^{-1})	168 (17)	179 (24)	19.6***	- 6.3%	139 (9)	153 (12)	119***	- 8.7%
Mn (mg kg^{-1})	35.3 (4.5)	29.8 (3.7)	119***	+ 18.5%	31.5 (3.1)	24.7 (2.6)	446***	+ 27.5%
Fe (mg kg^{-1})	11.6 (1.9)	13.7 (2.0)	80.6***	- 15.5%	10.2 (1.5)	11.5 (1.4)	57.5***	- 10.7%
Cu (mg kg^{-1})	4.11 (0.95)	2.27 (0.80)	293***	+ 80.8%	3.97 (1.04)	2.90 (1.03)	85.0***	+ 36.7%
Zn (mg kg^{-1})	26.1 (2.5)	25.7 (2.4)	NS	ND	24.6 (1.7)	24.9 (1.8)	NS	ND
As (mg kg^{-1})	0.245 (0.026)	0.284 (0.028)	147***	- 13.7%	0.226 (0.026)	0.304 (0.035)	512***	- 25.7%
Mo (mg kg^{-1})	0.59 (0.13)	0.74 (0.11)	93.0***	- 19.5%	2.01 (0.29)	1.94 (0.25)	5.42*	+ 3.7%
Cd (mg kg^{-1})	0.017 (0.003)	0.013 (0.003)	88.1***	+ 27.8%	0.019 (0.008)	0.011 (0.006)	99.0***	+ 67.3%

*P<0.05; **P<0.01; ***P<0.001; NS = not significant; ND = no difference

Figure 1. Water depth in the AWD blockss during the rice growing season in 2013 (A) ad 2014 (B). Each point is the mean of the water depth at the four field tubes in each year. The length (time) of each of the AWD cycles is indicate by a grey bar. The water depth in the CF plots was maintained at 2-5 cm above the soil surface. Error bars are SE.

Figure 2. Height of the water table (a) and volumetric soil moisture content at 4 depths below the soil surface (b) in the alternate wetting and drying (AWD) treatment and mean leaf elongation rate (c) and ABA concentration (d) of plants exposed to AWD (filled symbols) and continuous flooding (hollow symbols) in 2013. Data are means ± SE of 5 water tubes (a), 4 measurements at each soil depth recorded hourly with error bars omitted for clarity (b), 9 plants (c) and 6 samples per treatment taken at two hourly intervals between 1030 and 1630h on each day (there was no significant diurnal variation in ABA concentration in either treatment) comprising 24 ABA determinations in total. Vertical dotted lines indicate when the AWD treatment was re-flooded. Asterisks in c and d denote statistical significance at $p<0.05$ (*), <0.01 (**) and 0.001 (***).

Figure 3. Penetration resistance of the soils at depth across the AWD (filled symbols) and CF (open symbols) across each of the plots. Penetration resistance was measured prior to the first AWD cycle in year 1 (A), after the first AWD cycle in year 1 (B), and after the final AWD cycle, when both treatments had been under flooded conditions for 11 days, in year 2 (C). The individual data points are the mean penetration resistance for each depth across the four replicated blocks for each treatment. Error bars are SE. Asterisks denote statistical significance at $p<0.05$ (*).

Figure 4. Pore water concentrations of manganese (A), iron (B), zinc (C) and arsenic (D) in the pore water sampled from the AWD (filled symbols) and the CF (open symbols) across the AWD cycling period in 2013. The grey shading marks the AWD cycle treatments. Error bars are SE.

Figure 5. Pore water concentrations of manganese (A), iron (B), zinc (C) and arsenic (D) in the pore water sampled from the AWD (filled symbols) and the CF (open symbols) across the AWD cycling period in 2014. The grey shading marks the AWD cycle treatments. Error bars are SE.

Figure 6. Leaf *trans*-zeatin (tZ) (a), isopentenyadenine (iP) (b), zeatin riboside (ZR) (c), abscisic acid (ABA) (d), 1-aminocyclopropanecarboxylic acid (ACC) (e) and indole-acetic acid (IAA) (f) concentrations in plants exposed to AWD (filled symbols) and continuous flooding (open symbols) on 5 measurement occasions comprising prior to imposing AWD (1), halfway through (2) and at the end (3) of the 1st drying cycle and halfway through (4) and at the end (5) of the 2nd drying cycle in 2014. Data are means ± SE of 6 samples per treatment. Statistical analysis (two way ANOVA with treatment and measurement occasion as main factors) is presented in Table 2.