

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

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16 **Abstract**

17

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

45

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

## 47 **1 Introduction**

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

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

71 A growing body of evidence is being collected on the impacts of AWD on both water use and rice  
72 yield, compared to either CF conditions or standard farmer practises (FP). For example, in a meta-  
73 analysis across a number of different field trials, when AWD was compared to FP, Lampayan et al.  
74 (2015) indicated that there was no overall significant decrease in yield, and in 16 out of 24 farmer  
75 participatory demonstration sites (across multiple countries) there was a significant increase in yield.  
76 This increase in yield ranged from 0.2-1.0 t ha<sup>-1</sup>. In the same analysis in the trials where water input  
77 was measured, all the AWD irrigated trials had lower water input compared to the FP trials. The  
78 percentage difference between the water management practices ranged from 17-38% less water  
79 used in the AWD trials (Lampayan et al., 2015). A number of other studies have also shown that  
80 AWD increases grain yield when compared to either CF or FP (Yang et al., 2009; Zhang, 2009; Wang

81 et al., 2014). However, in some studies, AWD either does not alter (Yao et al., 2012; Linqvist et al.,  
82 2015; Shaibu et al., 2015; Howell et al., 2015) or slightly lowers yield (Sudhir-Yadav et al., 2012;  
83 Linqvist et al., 2015; Shaibu et al., 2015). AWD has now been implemented and is recommended  
84 practise in a number of countries including Bangladesh, the Philippines, Myanmar, and Vietnam  
85 (Lampayan et al., 2015).

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

110 One of the impacts of soil drying is to make soils harder (Bengough et al., 2011). Hard soils impact on  
111 root growth (Bengough et al. 2011), and it has been established that soil hardening due to soil drying  
112 is likely to limit new root growth in droughted rice plants as much as reduced water availability  
113 (Cairns et al. 2004). It is important, therefore, to establish if AWD is likely to alter soil strength in a  
114 way that might impact new root growth. Drier, harder soil is also likely to alter vegetative growth

115 such as leaf elongation rate and tillering. Despite expectations that soil drying (Bouman & Tuong,  
116 2001) would decrease tiller initiation and cause more frequent tiller death under AWD (Yang &  
117 Zhang, 2010), tiller number was significantly higher under AWD than CF throughout development  
118 (Howell et al., 2015), and AWD plants had a greater number of productive tillers independent of  
119 whether tiller number during development was higher or lower (Chu et al., 2015). Increased tillering  
120 likely accelerated canopy development of AWD plants, unlike leaf elongation rate on the main tiller,  
121 which did not differ between AWD and CF plants (Howell et al., 2015). Vegetative growth processes  
122 such as leaf elongation and tillering have been correlated with differences in phytohormone  
123 concentrations (Liu et al. 2011; Yeh et al. 2015).

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

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

139

## 140 **2 Methods**

141

142 A field trial was conducted at the Bangladesh Agricultural University, Mymensingh over two years  
143 (2013 and 2014). Two different irrigation treatment were tested; for each treatment four replicate  
144 plots were used with cultivar randomly distributed in each plot. The water irrigation treatment used  
145 were continuously flooded (CF) and alternate wetting and drying (AWD), as described below. The  
146 AWD and CF areas containing the AWD and CF plots were next to each other within a field that for  
147 the last 40 years has been treated as one area. Importantly, this field has not been used for  
148 experiments for the last 15 years, during which time it has been used for general cultivation.  
149 Furthermore, when deciding on the chosen area importance was placed on the observation that no  
150 differences in plant performance had been perceived in that area. The selected field had a natural  
151 gentle slope going East to West of  $< 0.03\%$ . The AWD and CF plots were 14 m apart with the AWD  
152 plots placed on the Eastern side of the field while the CF plots were on the Western side, therefore if  
153 the CF plots leaked the water would naturally move down the field away from the AWD plots. To  
154 minimise seepage from the CF area in to the AWD area an additional precaution was taken.  
155 Drainage ditches were put around the AWD area. These drainage ditches were approximately 1 m  
156 away from the outer bund of the AWD area. Soil was collected from each of the plots prior to the  
157 start of the field experiment in 2013 and analysed for elemental composition.

158

### 159 **2.1 Field experiment 2013**

160

161 Rice seeds were sown in a nursery bed on 31<sup>st</sup> December 2012. The field site was ploughed on 8<sup>th</sup>  
162 February 2013, and then levelled. The day before transplanting (12<sup>th</sup> of February) the seedlings into  
163 the AWD and CF plots, the plots were fertilised with 40 kg ha<sup>-1</sup> nitrogen, 20 kg ha<sup>-1</sup> phosphorus, 70 kg  
164 ha<sup>-1</sup> potassium, 15 kg ha<sup>-1</sup> sulphur, and 3 kg ha<sup>-1</sup> zinc. A further 40 kg ha<sup>-1</sup> nitrogen was supplied  
165 during the tillering stage (26<sup>th</sup> March, 41 days after transplanting (DAT)), and another 40 kg ha<sup>-1</sup>  
166 nitrogen at the flowering stage (6<sup>th</sup> April, 52 DAT). The seedlings were transplanted into the eight  
167 plots on the 13<sup>th</sup> of February 2013. Each plot was 10 m x 24 m, and subdivided into 5 columns each  
168 2 m x 24 m. Plants were planted as two plants per hill in 2 m long rows with a distance of 20 cm  
169 between each plant in a row and a 20 cm distance between rows. Almost 300 rice accessions were  
170 planted in single rows within each plot, with the check cultivar BRRI dhan28 transplanted into every  
171 second row (a BRRI dhan28 row separated each of the 300 accessions). These 300 accessions make  
172 up a genome wide association mapping panel and will be described elsewhere. After the plants were  
173 transplanted the plots were flooded. For the four CF plots the surface water was kept at a depth of

174 between 2 cm and 5 cm above the soil surface from the time of transplanting to shortly before  
175 physiological maturity (13<sup>th</sup> April 2013, 59 DAT). For the four AWD plots plastic perforated tubes  
176 (pani pipe) were placed across the blocks to monitor the water depth. The aim was to allow the  
177 perched water table to drop to 15 cm below the soil surface. At that point the plots were irrigated to  
178 bring the water depth to between 2 cm and 5 cm above the soil surface. The AWD plots went  
179 through 4 cycles of soil drying (Figure 1A). Both the AWD and CF plots were kept under the same  
180 flooded conditions up until 18 DAT (3<sup>rd</sup> March) when water was withheld from the AWD plots (start  
181 of the first AWD cycle). The water depth in the AWD plots was allowed to drop to ~15 cm below the  
182 soil surface; for the first cycle the plots were re-flooded 29 DAT (14<sup>th</sup> of March). This cycling was  
183 conducted 3 more times with the AWD plots reflooded 40 DAT (25<sup>th</sup> March), 50 DAT (4<sup>th</sup> April), and  
184 57 DAT (11<sup>th</sup> April). At this point the rice plants had started flowering and the AWD plots were kept  
185 flooded and maintained the same as the CF plots until harvest.

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

206 Once the cultivars had flowered and the grain matured (as determined by 80% of the grains on the  
207 panicles developing a golden brown colouration), the grain and shoots from every 10<sup>th</sup> row of BRRI

208 dhan28 was hand harvested from the six central hills of each row. The grain was then hand threshed  
209 and weighed to determine the grain mass. Grain mass is determined as the combined grain mass of  
210 the 6 hills. The shoots were harvested approximately 5 cm above the soil, dried, and then weighed  
211 to determine the shoot weight. Shoot biomass is determined as the combined shoot biomass of the  
212 6 hills. Once dried the shoots were then cut into small pieces ~1-2 cm long. A sub sample of the  
213 grains and shoots was then sent to the University of Aberdeen, UK for chemical analysis.

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

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

225

## 226 **2.2 Field experiment 2014**

227

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

239 At periodic intervals, the youngest fully expanded leaves were collected for multi-analyte  
240 phytohormone analysis (Albacete et al., 2008). At midday, samples were taken from a single hill from  
241 six randomly selected hills in one plot each of AWD and CF. Samples were immediately frozen in



242 liquid nitrogen, freeze-dried, then ground to a fine powder before measurement. Cytokinins (*trans*-  
243 zeatin, *tZ*; zeatin riboside, *ZR*; and isopentenyl adenine, *iP*), indole-3-acetic acid (IAA), ABA, and the  
244 ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) were analysed according to  
245 Albacete et al. (2008) with some modifications. Briefly, 20 mg of homogenized dry plant material  
246 was dropped in 1 mL of cold (-20°C) extraction mixture of methanol / water (80/20, v/v). Solids were  
247 separated by centrifugation (20,000 *xg*, 15 min) and re-extracted for 30 min at 4°C in additional 0.5  
248 mL of the same extraction solution. Pooled supernatants were passed through Sep-Pak Plus  $\text{C}_{18}$   
249 cartridges (SepPak Plus, Waters, USA) to remove interfering lipids and plant pigments, and  
250 evaporated at 40°C under vacuum either to near dryness or until organic solvent was removed. The  
251 residue was dissolved in 1 mL methanol / water (20/80, v/v) solution using an ultrasonic bath. The  
252 dissolved samples were filtered through 13 mm diameter Millex filters with 0.22  $\mu\text{m}$  pore size nylon  
253 membrane (Millipore, Bedford, MA, USA).

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

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

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

269

## 270 **2.2 Pore water analysis**

271

272 Prior to elemental analysis of the pore water, the field-collected pore water was diluted 1:50 (in 1%  
273 nitric acid) for iron and manganese analysis, and 1:5 for all other elements. Elemental analysis was  
274 performed by inductively coupled plasma-mass spectrometry (ICP-MS, Agilent Technologies 7500)  
275 using hydrogen as the reaction gas at a rate of 1.4  $\text{mL min}^{-1}$ . Standards with the appropriate ranges

276 were made from 1000 mg L<sup>-1</sup> ICP-MS grade multi-element stock solution. For quality control, blank  
277 samples were included, as well as water certified reference material (CRM, BCR 610). An external  
278 line of 10 µg L<sup>-1</sup> rhodium was used as an internal control.

279

### 280 **2.3 Soil chemical analysis**

281

282 A transect was conducted across each of the eight plots (4 AWD and 4 CF), and five soil samples (~50  
283 g, from the top 15 cm) were collected along each transect prior to the start of the experiment in  
284 2013. The soil from each transect was then bulked to give a total of eight soil samples, one for each  
285 plot. The samples were air dried and sieved (2 mm); once sieved they were then oven dried at 105°C  
286 until they were at a consistent weight. A total of 0.1 g of soil was then used for digestion following  
287 the block digestion methodology of Adomako et al. (2009), using NCS ZC73007 as a quality control  
288 reference material. Once digested the samples were analysed for a range of elements using ICP-MS  
289 as described above. Soil pH was determined by shaking 1 g of dried soil with 10 mL of Milli-Q water  
290 and allowing the samples to stand for 30 minutes, then the pH measurement was made.

291

### 292 **2.4 Soil texture analysis**

293

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

297

### 298 **2.5 Rice shoot and grain analysis**

299

300 Rice grains were dehusked and oven dried (80°C). For digestion, 0.2 g of dehusked grains were  
301 accurately weighed out into 50 mL polyethylene centrifuge tubes. Shoot samples were oven dried  
302 (80°C) and then powderised using a ball mill. Shoot samples were accurately weighed (0.2 g) into 50  
303 mL polyethylene centrifuge tubes. Grain samples were digested with concentrated nitric acid and  
304 hydrogen peroxide as described in Norton et al., (2012). Shoot samples were digested by the  
305 following method: rice shoot samples (powdered) were transferred into Pyrex test tubes (16 x 100  
306 mm) and weighed (0.01g). Next, trace metal grade nitric acid spiked with indium internal standard  
307 was added to the tubes (1.16 mL per tube), and 1.2 mL hydrogen peroxide added. The samples were  
308 left overnight to pre-digest. They were then digested in dry block heaters at 115°C for 4 hours. The  
309 digested samples were diluted to 11.5 mL with 18.2 MΩcm Milli-Q Direct water. Total elemental

310 analysis (sodium, magnesium, phosphorus, potassium, calcium, manganese, iron, copper, zinc,  
311 arsenic, molybdenum, and cadmium) was performed by ICP-MS. Trace element grade reagents were  
312 used for all digests, and for quality control replicates of certified reference material (CRM) (Oriental  
313 basma tobacco leaves [INCT-OBTL-5], and rice flour [NIST 1568b]) were used; blanks were also  
314 included. All samples and standards contained 10  $\mu\text{g L}^{-1}$  indium as the internal standard.

315

## 316 **2.6 Statistical analysis**

317

318 To compare treatments, analysis of variance (ANOVA) has been considered justified as site history,  
319 close proximity of the treatment areas, and relevant measures of soil properties (see results)  
320 indicates equivalence between the two areas. A similar approach has been used by Devkota et al.  
321 (2013). For data analysis, ANOVA were performed using Minitab 17 Statistical Software. For the soil  
322 chemical analysis one-way ANOVA was conducted with the locations of the plots (AWD and CF) as  
323 the explanatory variable. For the soil particle size analysis one-way ANOVA was conducted with the  
324 locations of the plots (AWD and CF) as the explanatory variable for each of the three particle size  
325 categories. For the plant mass traits and the plant elemental concentration traits one-way ANOVA  
326 was conducted with AWD and CF as the explanatory variable. For the hormone analysis and pore  
327 water analysis two-way ANOVA were used with AWD and CF, and sampling point (occasion / date) as  
328 the explanatory variables. For the two-way ANOVA the presence of an interaction between the two  
329 explanatory variables was also determined.

330

331

332 **3 Results**

333

334 **3.1 Soil analysis**

335

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

343

344 **3.2 AWD cycling**

345

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

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

361

362 **3.3 Penetrometer results**

363

364 In the field trial in 2013 the penetration resistance of the AWD and CF plots was measured on two  
365 occasions. The first was 9 DAT (22<sup>nd</sup> of February); at this point both the AWD and CF plots were

366 under flooded conditions and before any AWD cycling had been conducted. At this time there was  
367 no significant difference between the CF and AWD plots (Figure 3A). The penetration resistance was  
368 between 40-100 kPa for the first 135 mm; this penetration resistance increased sharply to  
369 approximately 1500 kPa by a depth of 225 mm. When the plots were tested for penetration  
370 resistance at the end of the first AWD cycle, 28 DAT (13<sup>th</sup> March), there was a significant difference  
371 in the penetration resistance between the AWD and CF plots between 15-120 mm, with the AWD  
372 plots having increased penetration resistance (Figure 3B). The largest difference between the two  
373 different treatments was at a depth of 60 mm, where the soil under AWD had an average  
374 penetration resistance of 94 kPa compared to the CF soil which had an average of 61 kPa. After a  
375 depth of 135 mm there was no difference between the two treatments.

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

383

### 384 **3.4 Pore water**

385

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

397 In 2014 the concentrations of the same elements (manganese, iron, zinc, and arsenic) were  
398 determined in the pore water (Figure 5A-D). The sampling was performed from the second AWD  
399 cycle onwards. There was no significant difference between the AWD and CF plots for pore water

400 manganese and iron concentrations across all the time points (Figure 5A and 4B). There was a  
401 significant difference in the concentration of zinc in the pore water at 35, 40, 55, and 75 DAT, with  
402 the plots under AWD having a higher concentration of zinc (Figure 5C). There was a significant  
403 difference in the concentration of arsenic in the pore water at 37 and 75 DAT, with the plots under  
404 CF having a higher concentration of arsenic (Figure 5D). For both years the concentrations of  
405 cadmium were below the analytical limit of detection in the pore water samples ( $0.28 \mu\text{g L}^{-1}$  for year  
406 1 and  $0.38 \mu\text{g L}^{-1}$  for year 2), therefore these data are not presented. However, a new *in situ*  
407 sampling technique, DGT (diffusive gradients in thin-films), was used to measure the flux of cadmium  
408 from the soil solid phase to solution. The fluxes (not shown here) obtained in the plots of AWD were  
409 consistently higher than the results from CF plots and will be reported elsewhere.

410

### 411 **3.5 Physiological and phytohormonal measurements during vegetative growth**

412

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

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

426 Since there were minimal differences in leaf ABA concentrations in 2013, in the following year a  
427 larger range of phytohormones were measured. Again, measurements were taken at the end of a  
428 drying cycle (Measurement Occasions 2 and 4), and immediately after re-flooding the AWD plots  
429 (Measurement Occasions 3 and 5). Of the phytohormones measured, irrigation treatment had  
430 significant effect only on the cytokinins iso-pentenyladenine (iP) and trans-zeatin (tZ), with AWD  
431 increasing iP concentrations by 37% (averaged across Measurement Occasions 2-5) and decreasing  
432 tZ concentrations by 36% (averaged across Measurement Occasions 2-5). There was no consistent  
433 effect of re-flooding the soil on the concentrations of these, or other, phytohormones. Nevertheless,

434 the measurement occasion was highly significant ( $P < 0.001$ ) for the concentrations of all  
435 phytohormones measured, with significant increases in tZ, zeatin riboside (ZR), and ABA as the  
436 experiment progressed, and significant decreases in iP as the experiment progressed (Figure 6; Table  
437 3).

438

### 439 **3.6 Rice mass**

440

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

448

### 449 **3.7 Rice plant elemental concentration**

450

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

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

#### 467 **4 Discussion**

468 The reported effects that AWD has on rice grain yield varies between different studies. In this study,  
469 grain mass significantly increased in both years of this study (9.8% & 9.0%) for plants grown under  
470 AWD compared to CF. In this study the grain production was determined as the mass of grain  
471 produced by the 6 central plants of each row. Using this information an approximation of grain yield  
472 can be made, by scaling up the value based on the planting density, which must be used cautiously.  
473 This would result in a yield of 7.7 t ha<sup>-1</sup> for the plants grown under AWD and 7.0 t ha<sup>-1</sup> for plants  
474 grown under CF in 2013, and 9.1 t ha<sup>-1</sup> for the plants grown under AWD and 8.3 t ha<sup>-1</sup> for plants  
475 grown under CF in 2014. One of the factors that has been proposed to be responsible for an increase  
476 in grain yield is an increase in the proportion of productive tillers (Yang and Zhang, 2010). In this  
477 study, while an overall increase in plant biomass was observed in the plants grown under AWD,  
478 there was no significant difference in the total number of tillers between the plants grown under  
479 AWD and CF. This is in contrast to previous experiments where total tiller number decreased under  
480 AWD (Yang and Zhang, 2010; Chu et al., 2015). Although Howell et al. (2015) observed an increase  
481 (14%) in the number of productive tillers in one of the two rice varieties they tested under AWD  
482 compared to CF, there was also a decrease (11%) in the number of filled grains per panicle for that  
483 same variety. In the second year of the field trial, both the number of productive tillers (those that  
484 produced grain) and total tiller number were measured, and plants that were grown under AWD had  
485 significantly more productive tillers compared to the CF plants (Table 4). Again this is in contrast to  
486 the study by Yang and Zhang (2010), where they observed under moderate AWD there was no  
487 significant difference in the number of productive tillers when compared to CF. This increase in  
488 productive tiller number could be the main driver for the increase in the observed grain mass in the  
489 present study.

490 Considering the possible importance of tillering in regulating grain mass under different  
491 environmental stresses (including AWD), relatively few studies have attempted to understand its  
492 regulation. Phytohormones seem important since tillering mutants show altered hormone signalling  
493 (Lu et al., 2015), and applying chemical inhibitors of hormone action affects tillering (Seneweera et  
494 al., 2001). Although measuring phytohormone concentrations in tiller buds is technically difficult,  
495 previous studies show similar phytohormonal responses in rice roots, xylem sap, and leaves (Zhang  
496 et al., 2011). AWD decreased foliar cytokinin (both Z + ZR and iP + iPR) levels at the end of the drying  
497 cycle, but re-wetting increased cytokinin levels as long as soil drying was not too severe (Zhang et al.,  
498 2011). Severe soil drying also decreased foliar IAA levels and these changes were not responsive to  
499 drying and re-wetting cycles (Zhang et al., 2010). In our studies, tZ and iP showed opposite responses  
500 to AWD (Figure 6), which again were insensitive to drying and re-wetting cycles, as were the “stress



501 hormones” ABA and ACC. The relatively small impact of drying and re-wetting cycles on  
502 phytohormone concentrations is likely because only a small fraction of the root system (the upper 5-  
503 10 cm) is exposed to an appreciable soil drying given that at a depth of 15 cm the water content of  
504 the soil was never far below the water content of the deeper, still flooded soil (Figure 2). Indeed,  
505 split-root experiments with barley where half of the root zone was dried demonstrated that foliar  
506 ABA concentration significantly increased only if more than 30% of the root biomass was exposed to  
507 drying soil (Martin-Vertedor and Dodd, 2011). Thus, rice varieties which show a greater proportion  
508 of their root mass deeper in the soil profile might be expected to show more stable phytohormonal  
509 (and physiological) responses to AWD. BRR1 dhan28 is not one of these varieties as it has been  
510 developed for flooded conditions.

511 The main factor that appears to impact on grain yield production under AWD (in comparison to CF)  
512 is the severity of the soil drying phase of the AWD cycle. Studies which have imposed varying  
513 degrees of soil drying indicate that when more severe soil drying conditions are imposed during  
514 AWD, there is a reduction in grain yield (Yang et al., 2009; Sudhir-Yadav et al. 2011 Linquist et al.,  
515 2015). Although the severity of soil drying did not alter grain-filling rate and duration of grain filling  
516 of superior spikelets, both were decreased in inferior spikelets (Zhang et al., 2010). The severity of  
517 soil drying will also affect leaf growth and photosynthesis, but transient limitation of LER by AWD  
518 observed in this study (Figure 2) did not compromise final grain yield, suggesting a more important  
519 role of physiological processes occurring during grain filling. To date, the precise mechanism(s) by  
520 which AWD increases yield under moderate soil drying is unknown, but future studies should try to  
521 distinguish the relative importance of AWD effects on vegetative development and grain-filling,  
522 especially when AWD is only applied until anthesis, as occurred here.

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

531 The AWD treatment affected soil solution concentration of a number of elements, as well as the  
532 concentration of elements within the shoots and grain of the rice plants. Of particular note is the  
533 effect that AWD had on two toxic elements, arsenic and cadmium. Both these elements have been  
534 identified as accumulating in rice, making rice an important pathway of human ingestion (Zhao et al.,

535 2010; Meharg et al., 2013). In addition to the alterations in toxic elements, nutritionally important  
536 elements (such as iron) were affected by AWD.

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

547 When the plants were grown under AWD, there was a significant decrease in both shoot arsenic and  
548 grain arsenic in the rice plants compared to the plants grown under CF, although it was more marked  
549 in shoots in year 1 where both shoot and grain were measured. Rice plants have different uptake  
550 mechanisms for arsenite and arsenate. Ma et al. (2008) showed that arsenite is taken up through the  
551 Lsi1 silicon transporter while arsenate is accumulated via phosphate transporters (Meharg and  
552 Hartley-Whitaker, 2002). This is important in rice as it can accumulate up to 10% of its dry mass as  
553 silicon, reflecting the fact that the silicon uptake mechanism is very efficient (Ma et al., 2006).  
554 Growing rice plants in flooded conditions compared to non-flooded conditions results in a 10-fold  
555 greater arsenic accumulation in rice grains (Xu et al., 2008; Norton et al., 2012; Norton et al., 2013).  
556 When grown under AWD it was observed that the reduction in grain arsenic was only 9% and 25%.  
557 These reductions in grain arsenic are less than previously observed for AWD when compared to CF in  
558 a number of studies (Linguist et al., 2015; Somenahally et al., 2011; Chou et al., 2016), but a greater  
559 reduction in grain arsenic than the mildest of the three AWD treatment imposed by Linguist et al.  
560 (2015). The final concentration of inorganic arsenic in the grain is likely due to direct uptake from the  
561 soil rather than remobilisation of inorganic species from the rest of the plant, as inorganic arsenic in  
562 rice leaves is poorly remobilized (Carey et al., 2011). Therefore, key to reducing grain arsenic will be  
563 the degree of flooding at grain filling. If the soil is aerobic at grain filling, inorganic arsenic will be  
564 predominantly present as arsenate, which has a reduced mobility and uptake by rice plants, while if  
565 the soil is flooded arsenite will be dominant, which is more mobile and rapidly accumulated by rice  
566 plants. The method of AWD used in this study is referred to as safe AWD (Lampayan et al., 2015),  
567 where the AWD plots were re-flooded at the start of the reproductive stage (however, AWD was not  
568 implemented during grain filling which is an option for safe AWD). The study by Linguist et al., (2015)

569 directly addressed the issue of the effect of flooding during the reproductive stage by either  
570 extending the AWD cycling into the reproductive stage or by flooding at that stage. They observed  
571 that the AWD treatment with flooding at the reproductive phase had no effect on grain arsenic (or  
572 increased grain arsenic) in comparison to the CF treatment. However, when AWD was extended  
573 through the reproductive phase, a 64% reduction in grain arsenic compared to the plants grown  
574 under CF was observed (Linguist et al., 2015), but this more severe AWD treatment decreased grain  
575 yield by 12.6% (Linguist et al., 2015), clearly demonstrating a potential trade-off between large  
576 reductions in grain arsenic and yield.

577 The concentration of cadmium in the rice plants under AWD was not significantly different in the  
578 shoots compared to CF, however under AWD the concentration of cadmium was greater (up to  
579 67.3%) in the grain of the rice plants compared to CF. In contrast, Yang et al. (2009) observed a  
580 decrease in grain cadmium under a mild AWD treatment but increased grain cadmium under a  
581 severe AWD treatment. Cadmium can be present in soil naturally (0.1-1 mg kg<sup>-1</sup>) or soil can be  
582 contaminated with cadmium from anthropogenic sources (Smolders and Mertens, 2013). One  
583 source of anthropogenic cadmium to agricultural soils is P-fertilisers (Smolders and Mertens, 2013).  
584 Cadmium in the soil solution increased with increasing soil redox under oxidising conditions  
585 (Rinklebe et al., 2016). As soils become waterlogged, the increase in soil pH may contribute towards  
586 the immobilisation of cadmium in anaerobic soils (Smolders and Mertens, 2013). Under anaerobic  
587 conditions cadmium ions (Cd<sup>2+</sup>) may precipitate as cadmium sulphate, reducing the soil solution  
588 concentration of cadmium (Barrett and McBride, 2007). On the other hand, during AWD Fe<sup>2+</sup> is  
589 oxidised to Fe<sup>3+</sup>. Protons are released in the Fe<sup>2+</sup> oxidation process (eq 1), locally lowering the pH.



591 Cadmium is pH-sensitive and easily desorbed with decreasing pH. Therefore, it would be expected as  
592 the soil becomes more oxic during the AWD cycle, that the cadmium concentration increases in the  
593 soil solution (too low concentration to measure directly, but confirmed by DGT measurements) and  
594 this would lead to more cadmium being available to the plant to accumulate. However, it is  
595 interesting to note that the cadmium concentration in the shoots of the rice plants grown under  
596 AWD and CF are not different and it is only the grain cadmium concentration that is elevated. The  
597 concentrations of grain cadmium in this field experiment are low compared to other studies (Meharg  
598 et al., 2013). The highest average concentration of cadmium (year 2, plants grown under AWD) of  
599 0.019 mg kg<sup>-1</sup>, is below that of a survey of Bangladeshi rice grains where the average cadmium  
600 concentration was 0.099 mg kg<sup>-1</sup>. With a rice grain cadmium concentration of 0.099 mg kg<sup>-1</sup> it has  
601 been estimated that the weekly intake of cadmium from rice would lead to intakes deemed unsafe  
602 by international and national regulators (Meharg et al., 2013). Therefore, if AWD increased grain

603 cadmium further, this could have impacts on human health, suggesting either AWD might be best  
604 avoided in areas with high grain cadmium, and/or breeding for low cadmium should be pursued for  
605 AWD.

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

618

619 Conclusions

620

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

630

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634

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804 Zhao, F.-J., McGrath, S.P., Meharg, A.A. 2010. Arsenic as a food chain contaminant: Mechanisms of  
805 plant uptake and metabolism and mitigation strategies. *Ann Rev Plant Biol*, 61, 535-559.



806 Table 1. Soil elemental composition at the field site. Each value is the mean ( $\pm$  SD) across the 4 AWD  
 807 and 4 CF plots.

808  
 809

	P	Cr	Mn	Co	Ni	Cu	Zn	As	Mo	Cd
mg kg <sup>-1</sup>	574	76.4	665	17.5	51.2	40.7	99.7	4.63	0.57	0.19
	( $\pm$ 60)	( $\pm$ 12.6)	( $\pm$ 28)	( $\pm$ 0.6)	( $\pm$ 1.6)	( $\pm$ 1.9)	( $\pm$ 2.7)	( $\pm$ 0.30)	( $\pm$ 0.19)	( $\pm$ 0.15)

810  
 811

812 Table 2. Tiller number and plant height (measured at the end of the 4<sup>th</sup> drying cycle) exposed to  
 813 alternate wetting and drying (AWD) and continuous flooding (CF) in 2013. Data are means  $\pm$  SD of 9  
 814 plants, with P Values presented.

815

	Mean AWD ( $\pm$ SD)	Mean CF ( $\pm$ 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

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

817

818 Table 3. Two way ANOVA (F Values presented) to determine the effects of treatment (T),  
 819 measurement occasion (O) and their interactions on leaf hormone concentrations in 2014. Hormone  
 820 measured were cytokinins (*trans*-zeatin, tZ, zeatin riboside, ZR and isopentenyl adenine, iP), indole-  
 821 3-acetic acid (IAA), abscisic acid (ABA) and the ethylene precursor 1-aminocyclopropane-1-carboxylic  
 822 acid (ACC).

823

Hormone	F-values from 2-way ANOVA			Effect of treatment relative to CF
	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

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

825

826

827 Table 4. Mean total tiller number and shoot and grain mass for BRR1 dhan28 grown in the field  
 828

	Trait	Mean AWD ( $\pm$ SD)	Mean CF ( $\pm$ SD)	F value from ANOVA	Increase (+) or decrease (-) between AWD relative to CF
Year 1 <sup>a</sup>	Total tiller no.	13.6 (4.0)	12.8 (4.4)	NS	ND
	Shoot mass (g) <sup>#</sup>	119 (21.)	103 (15)	50.6***	+ 15.4%
	Grain mass (g) <sup>#</sup>	92.8 (20.2)	84.5 (15.3)	14.9***	+ 9.8%
Year 2 <sup>b</sup>	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) <sup>#</sup>	133 (24)	119 (22)	28.8***	+ 12.0%
	Grain mass (g) <sup>#</sup>	108 (24)	99(19)	12.9***	+ 9.0%

829 <sup>#</sup> shoot mass and grain mass for 6 hills

830 <sup>a</sup>(n=140 for AWD and CF); <sup>b</sup>(n=160 for AWD and CF).

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

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833

834 Table 5. Total shoot elemental concentrations for BRR1 dhan28 grown in 2013.

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

835

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

837

Table 6. Total grain elemental concentrations for BRR1 dhan28 grown in 2013 and 2014.

Trait	Year 1				Year 2						
	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 <sup>-1</sup> )	9.13 (3.99)	8.76 (4.59)	NS	ND	6.19 (3.04)	14.05 (4.59)	172***	- 56.0%			
Mg (mg kg <sup>-1</sup> )	1650 (150)	1660 (150)	NS	ND	1430 (120)	1500 (150)	23.0***	- 4.8%			
P (mg kg <sup>-1</sup> )	4200 (460)	4210 (420)	NS	ND	4500 (49)	455 (580)	NS	ND			
S (mg kg <sup>-1</sup> )	1240 (180)	1460 (150)	123***	- 15.4%	1330 (110)	1390 (120)	20.6***	- 4.2%			
K (mg kg <sup>-1</sup> )	2740 (320)	2830 (33)	5.02*	- 3.2%	3020 (250)	2960 (300)	NS	ND			
Ca (mg kg <sup>-1</sup> )	168 (17)	179 (24)	19.6***	- 6.3%	139 (9)	153 (12)	119***	- 8.7%			
Mn (mg kg <sup>-1</sup> )	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 <sup>-1</sup> )	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 <sup>-1</sup> )	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 <sup>-1</sup> )	26.1 (2.5)	25.7 (2.4)	NS	ND	24.6 (1.7)	24.9 (1.8)	NS	ND			
As (mg kg <sup>-1</sup> )	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 <sup>-1</sup> )	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 <sup>-1</sup> )	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 blocks during the rice growing season in 2013 (A) and 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 indicated 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  $\pm$  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), isopentenyladenine (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 1<sup>st</sup> drying cycle and halfway through (4) and at the end (5) of the 2<sup>nd</sup> drying cycle in 2014. Data are means  $\pm$  SE of 6 samples per treatment. Statistical analysis (two way ANOVA with treatment and measurement occasion as main factors) is presented in Table 2.