applied sciences

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Evaluation of the Microbiological Quality of Water in the Rice-Growing System of the Lis Valley, Portugal

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Abstract: Society's demand for the preservation of water resources has stimulated technological advances and improved agricultural management, to limit its negative impacts, favor socio-economic development and promote the conservation of natural resources. This study assessed the impact of a rice farming system in the Mediterranean area on the microbiological quality of water, representing the irrigation and cultivation conditions of the Lis Valley Irrigation District (LVID). Indicators used include the counting of total coliforms (TC), fecal coliforms (FC), fecal enterococci (FE) and ampicillin-resistant *Enterobacteriaceae* (Amp^r), and a phenotypic characterization of antibiotic (AB) resistances. The results show that the water at the LVID inlets contained high microbiological contamination due to sources outside the perimeter. The rice paddy agroecosystem had a beneficial impact on the load of TC, FC and FE of the respective drainage water, with a decrease of two orders of magnitude between the values of these counts at the inlets and at the outlets of the rice fields. However, the relative frequency of *Enterobacteriaceae* Amp^r increased in the rice paddy soil, possibly as a result of the favorable conditions provided by this wetland ecosystem for the transfer of AB resistance genes. The analysis of AB resistance patterns revealed high relative resistance percentages for both β-lactams and non-β-lactams in all samples. This study made it possible to assess the risks of microbiological contamination of irrigation water and the beneficial effect of the rice-growing system in controlling the applied indicators.

Keywords: irrigation water quality; microbiological indicators; coliforms; enterococci; *Enterobacteriaceae*; MEDWATERICE

Citation: Duarte, I.M.; Marques, H.; Amador, P.; Vasconcelos, T.; Gonçalves, J.M. Evaluation of the Microbiological Quality of Water in the Rice-Growing System of the Lis Valley, Portugal. *Appl. Sci.* **2022**, *12*, 9856. https://doi.org/10.3390/app12199856

Academic Editors: Jesús Montero Martínez, Adriana Correa and María Dolores Gómez-López

Received: 30 August 2022
Accepted: 27 September 2022
Published: 30 September 2022

1. Introduction

The development, sustainability and business competitiveness of Portuguese agriculture are highly dependent on irrigation [1]. In turn, global changes lead to a decline in available water resources [2], implying that water saving is strategic for the development of agriculture. The management of hydro-agricultural perimeters with river gravity-based supply has specific characteristics, especially if the drainage water is reused to mitigate water scarcity and improve the water distribution, as is the case of the Lis Valley Irrigation District (LVID). This practice raises concerns regarding farmer and consumer safety. It is therefore important to monitor the microbiological quality of water to obtain information for its rational management, reduce environmental and public health risks, and support farmers in the adoption of more appropriate practices; in short, to contribute to the sustainability of irrigated agriculture [3].

Fecal coliforms, e.g., *Escherichia coli*, mainly found in the intestine of human beings and warm-blooded animals, are used as indicators of the water microbiological quality. These bacteria, indicating fecal contamination, and thus the putative presence of pathogenic microorganisms, are highly relevant in medical microbiology, due to the water-carried diseases (sanitation conditions and drinking water quality), as well as in the assessment of environmental impacts caused by anthropological action. As surface and subterranean water resources can be affected by various activities developed in the hydrographic basins, it is important to study the quality of the water entering a system and returned to the aquifer after use. There is also an emergent global health concern regarding antibiotic (AB) resistances, involving the transfer of bacteria and genes between humans, animals and the environment. The transmission events of widespread AB resistant strains are common [4]; therefore, quantifying the pathways and identifying the sinks and bottlenecks for environmental transmission are fundamental to manage the global AB resistance crisis [5].

Rice cultivation has a very important economic and social value in Portugal; rice is a strategic crop for food security in some countries, and consumption throughout the Mediterranean is increasing steadily. Traditionally, rice is grown in level basins and irrigated by continuous flooding, requiring a higher irrigation allocation than most crops, thus implying greater pressure on water resources [6]. On the other hand, paddies allow high productivity in soils with very poor drainage and high risk of salinity. These facts explain the need to save water in rice production and to safeguard the environmental sustainability of rice-growing agroecosystems, to ensure the viability of this crop; this topic concerns farmers and political authorities, and therefore is the object of study and experimental development [7].

Considering the growing concerns about pollution of surface and ground waters in general, the knowledge of the environmental impacts of rice production on the ecosystem is relevant for its sustainability. However, there are scarce studies on the microbiological impacts of rice fields in Portugal, especially considering that information is required at the level of each rice-growing ecosystem. Contributing to a greater knowledge on the subject, the objective of this study was to determine the impact of rice farming activity, at the small watershed scale in a Mediterranean area, on the microbiological quality of superficial water. This information, available for water management organizations, will support measures to improve and minimize the risks regarding the conservation of surface and groundwater bodies. This work took place under the scope of the Lis Valley Water Management Operational Group [8] and the MEDWATERICE Project [9].

2. Materials and Methods

The study was carried out in the LVID, whose soils are mostly modern alluvial soils of high agricultural quality, some of which have poor drainage conditions. The main crops are maize and forage pastures, vegetables, orchards and rice. The climate results from the Mediterranean influence, mainly in the summer, because of high temperatures and sunshine and very little precipitation, and from the Atlantic influence, by the frontal surfaces, predominant in winter, responsible for most of the precipitation [10]. LVID is a public irrigation district, managed by the Water Users Association (WUA), located in the municipalities of Leiria and Marinha Grande, in the Coastal Center of Portugal.

Irrigation water is supplied through small temporary weirs of the Lis River and its tributaries, with gravity derivations. The distribution system by channel, for each sector, has reinforcements by pumping from the drainage network in the peak irrigation period [10,11]. There is a significant increase in the salinity of the water of the Lis River in the downstream section, explained by the hydrogeological characteristics of the saline springs in the downstream area, by the drainage effluent, and by the influence of the tides. The water from the river downstream of the Salgadas weir is no longer suitable for irrigation [10].

The study was conducted in paddy fields of the Hydraulic Block II (hereinafter referred to as Block II), in the downstream part of the LVID, where rice fields from local producers were selected for the present study (Figure 1). This Block is irrigated by water from the Aroeira stream and drained by the Rio Negro. Its main crops, in addition to rice, are maize and pastures, intended, in part, for direct grazing of cattle and horses.

Figure 1. Location of the sampling sites (white figures, 1–8), the rice plots (green) in Block II (green dashed line) of Lis Valley Irrigation District (Source: Google Maps, https://www.google.pt/maps/, accessed on 6 July 2022). Legend: 1, LVID at upstream inlets (Lis River, at Arrabalde section, Lena River at the mouth and Milagres riverside mouth); 2, LVID inlet, at Aroeira riverside upstream section; 3, Block II inlet; 4, Paddies' inlet; 5, Paddies (surface water, soil, and groundwater); 6, Paddies' outlet; 7, Block II outlet; 8, LVID outlet (Lis River, at Bajanca bridge section).

Rice is produced in private farms, with an average area of 70 ha, in plots of about 3–4 ha. The soils have a clay loam texture, with a typical proportion of 56% clay and 37% silt, the pH is about 7.0–7.4, organic carbon content is 2.6–2.8% and bulk density is about 1.25 g/cm^3. The ground table level is 70–90 cm below the soil surface, with a saturation soil water content of 0.50–0.52 cm^3/cm^3 [12].

In the autumn—winter period with low temperatures, precipitation and topmost rivers flow, crops are not irrigated, with the runoff drained to the ditches, river and sea. Soils are amended with crop stubble, but not with manure and slurry, in compliance with the Portuguese law [13]. There is some free grazing by extensive livestock systems (characterized by a low antibiotic administration and, consequently, a lower incidence of bioindicator bacteria groups, including those with antibiotic resistances (unpublished results of our research) in the organic residues left in the soil). Conversely, in the spring–summer period, the hot dry season, irrigation water from the river is reinforced with the runoff from upstream regions. The runoff is exclusively reused during water shortages in the summer. The organic manure fertilization is generally used in other crops, except in rice. Due to the described characteristics of this ecosystem, the water scarcity peak was selected as the period for sampling, as it is considered the time with the highest expected microbial loads at sampling sites, thus representing the worst scenario, with greater risks of contamination for the environment and humans. For this reason, sampling to assess the microbiological quality was carried out from 2019 to 2021, during the dry season, in the summer, when the lowest flows occur in the water lines, during the peak irrigation period.

The sampling sites for the microbiological study focused on the inlets and outlets of the LVID, Block II and the respective rice fields, in accordance with the objective of evaluating the impact of rice farming activities on the quality of water drained into the environment. The adopted methodology for monitoring the water quality of irrigation and drainage networks followed previously described guidelines [14]. The locations of the main inlets to the LVID include the Lis River, in Leiria (Arrabalde) and the mouth of its tributaries: Milagres Stream, Aroeira Stream and Lena River; and the perimeter outlet: the Lis River section, at the Bajanca bridge (Figure 1).

Sampling downstream of the Lis River was always carried out on the seashore, to avoid the influence of brackish sea water. Water samples were collected in sterile plastic bottles and kept at 4 °C until microbiological processing, which was carried out up to four hours after collection.

The physicochemical quality of these water samples was evaluated with a precalibrated in situ portable multiparametric probe (SmarTROLL RDO Handheld, Fort Collins, CO, USA), for the following parameters: pH, Electrical Conductivity (EC, µS/cm), Saturation of Dissolved Oxygen (SDO, %) and Total Dissolved Solids (TDS, ppm). Chloride (CL, mg/L) was evaluated with suppressed ion chromatography [15]. The SAR indicator was calculated from the measurement of the elements calcium, magnesium and sodium, evaluated by inductively coupled plasma optical emission spectrometry [16]. The results obtained were compared to the maximum recommended values (MRVs) of the Portuguese Irrigation Water Quality Standards [17].

The microbiological analysis of the water samples included the enumeration of Total Coliforms (TC), Fecal Coliforms (FC) and Fecal Enterococci (FE) by the dilution method with the multiple tube fermentation technique, with the results expressed by the Most Probable Number (MPN)/100 mL, according to the reference analytical methods [17,18].

The count of ampicillin-resistant *Enterobacteriaceae* in the water samples was performed by the membrane filtration technique. Decimal dilutions of each sample were prepared in sterile 0.9% saline, from which a volume of 100 mL was filtered through a cellulose membrane of 0.45 µm pore size (Millipore, Bedford, MA, USA) under vacuum, with the filters placed aseptically on the surface of the selective medium for *Enterobacteriaceae*, Violet Red Bile Glucose (VRBG) agar (Oxoid, Hampshire, England) supplemented with 20 µg/mL sodium ampicillin (AppliChem, Darmstadt, Germany). After aerobic incubation of the plates at 37 °C for 24 to 48 h, isolated colonies of ampicillin-resistant *Enterobacteriaceae* (Amp^r) were counted [19].

Antibiograms were performed directly on the isolates in mixed culture, using the direct antimicrobial susceptibility tests, according to Zebouh et al. [20], to determine the most resistant phenotypes present in a mixed population.

In summary, the samples with mixed isolates were phenotypically characterized for 13 AB, representing the main AB classes used in human medicine and livestock production in Portugal, namely: amoxicillin/clavulanic acid combination (AMC) 30 µg/10 µg, respectively; ceftazidime (CAZ) 30 µg; cefotaxime (CTX) 30 µg; cefpirome (CPO) 30 µg; aztreonam (ATM) 30 µg; cefoxitin (FOX) 30 µg; imipenem (IPM) 10 µg; meropenem (MEM) 10 µg; chloramphenicol (CHL) 30 µg; gentamicin (GEN) 10 µg; ciprofloxacin (CIP) 5 µg; trimethoprim/sulfamethoxazole (SXT) combination (1:19) and tetracycline (TET) 30 µg. The Kirby–Bauer disk diffusion method was performed according to the guidelines for antimicrobial susceptibility tests defined by the Clinical Laboratory Standards Institute [21]. Resistance was recorded as any bacterial morphology growing to the edge of the AB disc, while sensitivity was defined as a complete and clear zone of inhibition for all bacterial taxa represented on the susceptibility agar.

3. Results and Discussion

3.1. Physicochemical Indicators of Water Quality

The average pH values of the irrigation and drainage water samples are within an acceptable range [17], in general slightly above 7.0, except for the Aroeira riverside, at the section of the Block II inlet (Table 1).

Table 1. Physicochemical parameters of water samples, per sampling site (average ± standard deviation).

Sampling Site	pH (6.5–8.4) *	Cl, mg/L (70) *	TDS, ppm (640) *	SDO, %	EC, µS/cm (1000) *	SAR (8) *
LVID—Inlets upstream	7.8 ± 0.5	69.4 ± 15.7	444 ± 47	92.7 ± 15.0	666 ± 117	1.4 ± 0.2
Block Inlet	6.9 ± 1.1	39.1 ± 14.1	179 ± 7	97.1 ± 4.8	285 ± 32	1.8 ± 0.1
Paddies' Inlet	7.3 ± 0.2	41.5 ± 15.0	428 ± 137	91.1 ± 14.3	651 ± 211	1.8 ± 0.2
Paddies—Soil	7.2 ± 0.1	71.1 ± 10.1	550 ± 115	48.5 ± 26.3	827 ± 26	2.2 ± 0.3
Paddies—Groundwater	7.0 ± 0.3	143.0 ± 11.3	870 ± 217	53.9 ± 29.1	1336 ± 336	1.9 ± 0.1
Paddies—Soil water	7.2 ± 0.3	62.0 ± 16.9	827 ± 299	56.2 ± 19.1	1327 ± 518	1.9 ± 0.1
Paddies' Outlet	7.4 ± 0.1	45.2 ± 15.1	377 ± 100	71.7 ± 6.0	582 ± 153	1.8 ± 0.2
Block II Outlet	7.3 ± 0.3	259.3 ± 112.4	670 ± 267	76.3 ± 13.6	1087 ± 387	6.3 ± 2.2
LVID Outlet	7.4 ± 0.3	115.2 ± 23.8	757 ± 474	84.3 ± 8.9	1224 ± 769	1.9 ± 0.3
LVID—Inlets upstream	7.8 ± 0.5	69.4 ± 15.7	444 ± 47	95.1 ± 15.0	666 ± 117	1.4 ± 0.2

* Maximum recommended values, according to the Portuguese Irrigation Water Quality Legislation [12]. Legend: Cl—Chloride; TDS—Total Dissolved Solids; SDO—Saturation of Dissolved Oxygen; EC—Electrical Conductivity; SAR—Sodium Adsorption Ratio.

The SDO values of inlet and outlet water samples are above 76%, except for those from paddies and paddies' outlets (Table 1), as expected for still water with less contact with the air. Although there are no references to irrigation water [17], a value higher than 50% is recommended for drinking water; therefore, we can conclude that dissolved oxygen in these water bodies is good or acceptable.

The average EC values in the water paddies and the paddies' affluent are above the MRV for irrigation, as expected; however, the EC of irrigation water is quite low and acceptable for irrigation of rice and other crops. The SAR values are relatively low, revealing no risks of soil conservation or crop damage due to the high content of sodium. The highest SAR values of the samples collected in the Rio Negro ditch (Block II Outlet) are explained by the drainage function of this ditch, where higher sodium concentration occurs.

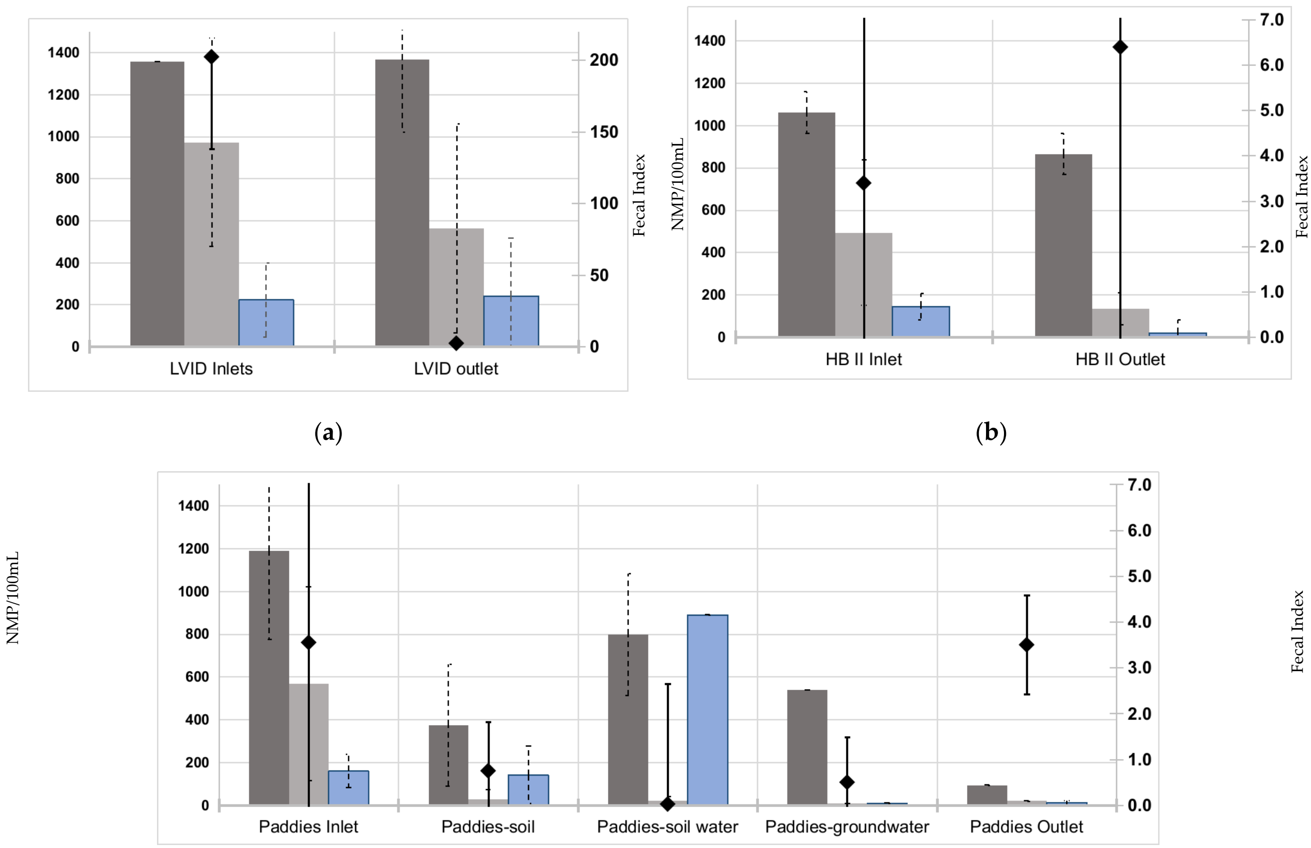
3.2. Microbiological Indicators of Water Quality

The analysis of the microbiological quality of the water, evaluated through the enumeration of groups of indicators in the main inlets and outlets of the LVID (Figure 2 and Table 2), shows the effect of the different agricultural activities conducted here on the quality of the water at its outlet.

Table 2. Ratio of the mean values of Total Coliforms, Fecal Coliforms, Fecal Enterococci and *Enterobacteriaceae* Amp^r counts at the outlet and inlet of the: LVID; Block II; rice fields of Block II.

Microorganism Group	Outlet/Inlet Ratio		
	LVID [1]	Block II [2]	Rice Fields
Total Coliforms (MPN/100 mL)	1.01	0.82	0.08
Fecal Coliforms (MPN/100 mL)	0.58	0.28	0.04
Fecal Enterococci (MPN/100 mL)	1.09	0.15	0.04
Enterobacteriaceae Amp^r (CFU/mL)	0.06	102,094	3

[1] LVID, Lis Valley Irrigation District; [2] Block II, Hydraulic Block II.



(**a**) (**b**) (**c**)

Figure 2. Mean values of counts (and standard deviation) of Total Coliforms (■), Fecal Coliforms (■), Fecal Enterococci (■), expressed in MPN/100mL, and Fecal Index (◆) at the inlet and outlet of: (**a**) LVID; (**b**) Block II (HB II); (**c**) rice paddies of Block II, including at the soil, soil water and groundwater.

TC values are very widespread, reaching averages above 700 MPN/100 mL in all sampled locations, aside from paddy drainage, with no significant differences between irrigation and drainage water counts. Between the inlet and outlet of Block II there was an 18% decrease in TC counts, and a 92% decrease in rice fields. These values are between the maximum recommended in the legislation (500 MPN/100 mL) and the maximum admissible (10,000 MPN/100 mL) for bathing purposes [17]. There is no specific regulation for agricultural drainage water regarding this parameter, but the proximity of Vieira de Leiria Beach about 3 km downstream from the LVID (Figure 1, site 8) justifies this concern.

The FC counts (Figure 2 and Table 2) in all samples, except for rice paddies' samples, are above 100 MPN/100 mL. Therefore, irrigation water does not fulfill the quality environmental objectives established [17]. The highest mean values were recorded in the samples at the inlets of the LVID (Figure 1, sampling sites 1 and 2). These high concentrations of FC in the water available for rice irrigation (569 MPN/100 mL) might be explained by sewage contamination, external to the LVID [22]. However, the average FC counts in the water at the outlet of the perimeter (564 MPN/100 mL) are 42% lower than those at the inlet. Sharper drops in FC counts were recorded at the Block II (72%). Considering bathing use, the FC counts of LVID effluent water are above the maximum recommended value (100 MPN/100 mL), but still below the maximum allowable value (2000 MPN/100 mL) [17].

The counts of FC in irrigation are significantly higher than those in samples of soil-water, the root zone, the groundwater (depth of 1 to 2 m) and at the outlet of the level basins (21 MPN/100 mL) (96%). This fact might be explained by the soil effect on the reduction in this group's values [23]. Our data agree with a study carried out in Brazil demonstrating that paddy agro-ecosystems have the potential ability to circulate the nutrients, thus leading to lower levels of those contaminating microbial agents in the drainage water than in the irrigation water [24]. These results may additionally be explained by the kind of crops grown at the outflow of the LVID (rice and pastures), where the soils are not manured, contrary to the upstream areas of the valley, where other crops are grown.

The quality standard established for irrigation water refers to the quality requirements for the reuse of treated urban wastewater in the irrigation of agricultural, forestry and ornamental areas, nurseries, lawns and other green spaces [25]. Regarding microbiological quality requirements, this standard [26] considered the utilization of irrigated crops (e.g., for industry or human or animal consumption) and the adopted irrigation method, which is less restrictive and more flexible than that established in Portuguese legislation [12]. According to Monte and Albuquerque [27], Portuguese legislation [17] is very demanding since the water in most rivers does not have fecal coliforms below 100 MPN/100 mL.

The highest average concentrations of FE (Figure 2 and Table 2) were recorded at the inlet of the LVID, with an explanation like that presented for the FC values [22]. The average counts of FE in the water at the outlet and at the inlets of the LVID do not differ significantly and are above the maximum recommended value (100 MPN/100 mL), considering bathing uses of the effluent water from the LVID. A different situation was found between the inlet and outlet of Block II, in which there was an 85% decrease in the FE counts. Significantly higher counts were recorded in the rice field samples (569 MPN/100 mL), either at the inlet to the paddies or in the soil, despite the FE values being significantly reduced in groundwater and drainage (5 MPN/100 mL).

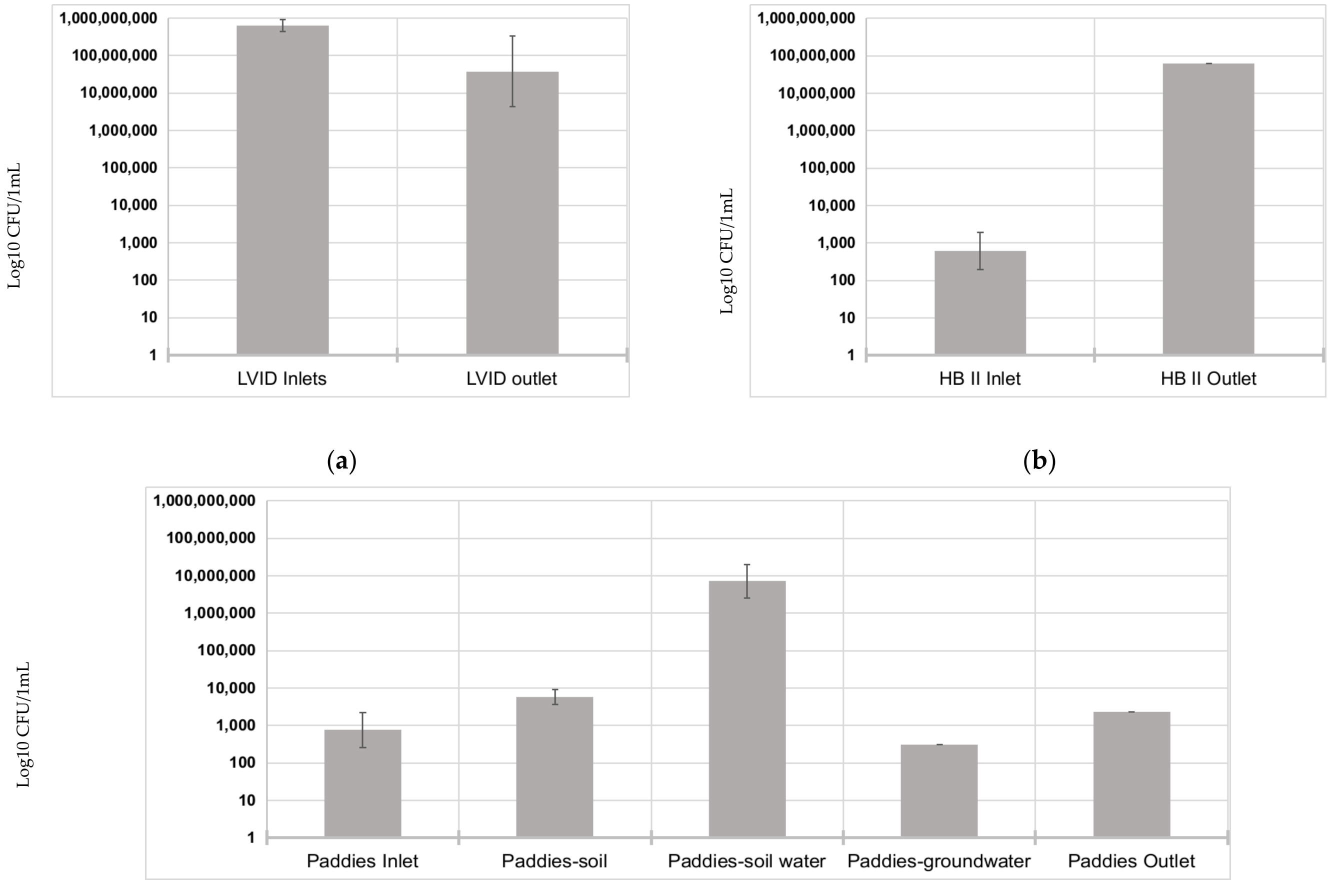
The analysis of the Fecal Index, proposed by Gerba and co-authors [28], reveals that at the LVID inlets there are signs of human fecal contamination. In contrast, the rice paddy samples indicate a strong influence of the wildlife effect. The final section of the Lis River, which has regularized banks with arboreal and shrubby vegetation and a predominance of rushes, ash and willows, which shelter a diversity of birds. In addition, the wetland attracts wading birds, which might explain these results. In fact, these rice landscapes can constitute habitats for wildlife, as this is a low-intensity production system contiguous with patches of native vegetation, harboring diverse wetland dependent bird species, commonly observed in the paddies.

In turn, the water sampled in the downstream stretch of the Lis River shows evidence of fecal contamination of animal origin in mixed pollution, possibly associated with the utilization of animal manure as a soil fertilizer, collected within the ditch.

Although no studies have been carried out on the impact of water physicochemical characteristics on microbial growth, it appears that higher salinity in water does not affect microbial activity. The sampling sites with higher salinity (water in the rice field soil and in the drainage of Rio Negro ditch) present EC values slightly above the VMR for irrigation water (Table 1). In addition, there have been no problems arising from salinity of the soil or the crops, which is due to the efficiency of the drainage system.

3.3. Ampicillin-Resistant Enterobacteriaceae

The average counts of ampicillin-resistant *Enterobacteriaceae* isolates revealed a high variability throughout the irrigation campaigns, namely at some inlets and at the LVID outlet (Lis River at the Bajanca section). The samples with the highest average counts were collected at the LVID inlets (seven orders of magnitude difference among the river section). Despite this high variability, there was an average reduction of two orders of magnitude between the Amp^r counts in the inlets and the outlet of the LVID (Figure 3a). Conversely, in Block II, there was an increase of five orders of magnitude (85%) between the inlet and outlet (Figure 3b), which can be explained by the effect of drainage water discharges from other sources, which occurs downstream of the rice fields, with high concentrations of *Enterobacteriaceae* Amp^r.



(c)

Figure 3. Mean values of *Enterobacteriaceae* Amp^r counts, expressed in CFU/mL (and standard deviation) at the inlet and outlet of: (**a**) LVID; (**b**) Block II (HB II); (**c**) rice paddies of Block II, including at the soil, soil water and groundwater.

In fact, the other cultures practiced in Block II (maize, pasture) are manured. Thus, the microbiological load from livestock, delivered directly into the soil of some areas of the LVID, makes the microbiological risk emergent, possibly due to the presence of AB resistance genes [29]. These results demonstrate that Good Agricultural Practices must be observed in terms of manure and slurry management [26]. Intensive livestock activity should fully compost these residues before being incorporated into the soil, mainly for corn and pasture production.

Significantly higher Amp^r counts were recorded in rice paddy soils than in the irrigation water at the inlet of the paddies (five orders of magnitude difference), but in groundwater and in drainage from the paddies these counts decrease significantly (Figure 3c), which may be explained by the effect of the soil in reducing the number of *Enterobacteriaceae* [23].

An additional critical aspect associated with fecal contaminants is the frequent resistance to ABs found in the aquatic systems, namely to the β-lactams (e.g., ampicillin), placing the water quality and public health in a critical situation. Once contaminated, the soils from agricultural systems can become a source of environmental contamination through the runoff into water bodies, such as aquifers and rivers, and the leaching of these AB-resistance determinants deposited on the soil surface to groundwater [19]. The identification in some cases of microbiological contamination risk in irrigation water of LVID, whose main responsibility is external to the Lis valley, requires special precautionary measures, in particular regarding the safety of farmers and consumers. These risks need to be evaluated for the real knowledge of the influence of agricultural activity within the irrigation district, particularly at the drainage network level.

3.4. Phenotypic Characterization of Antibiotic Resistance

The *Enterobacteriaceae* isolates obtained from the samples collected for three consecutive years showed high resistance to most of the ABs tested (Figure 4). Among the β-lactams, penicillin (amoxicillin/clavulanic acid), 2nd generation cephalosporins (FOX), 3rd generation cephalosporins (CAZ and CTX) and 4th generation (CPO) were the ABs for which more resistances were found. Regarding the non-β-lactam AB classes, a higher percentage of isolates resistant to tetracycline and sulphonamides AB (TET and SXT, respectively) was observed. This result can be explained by the intensive use of this class of AB for many years in veterinary medicine and intensive animal production in the region [4].

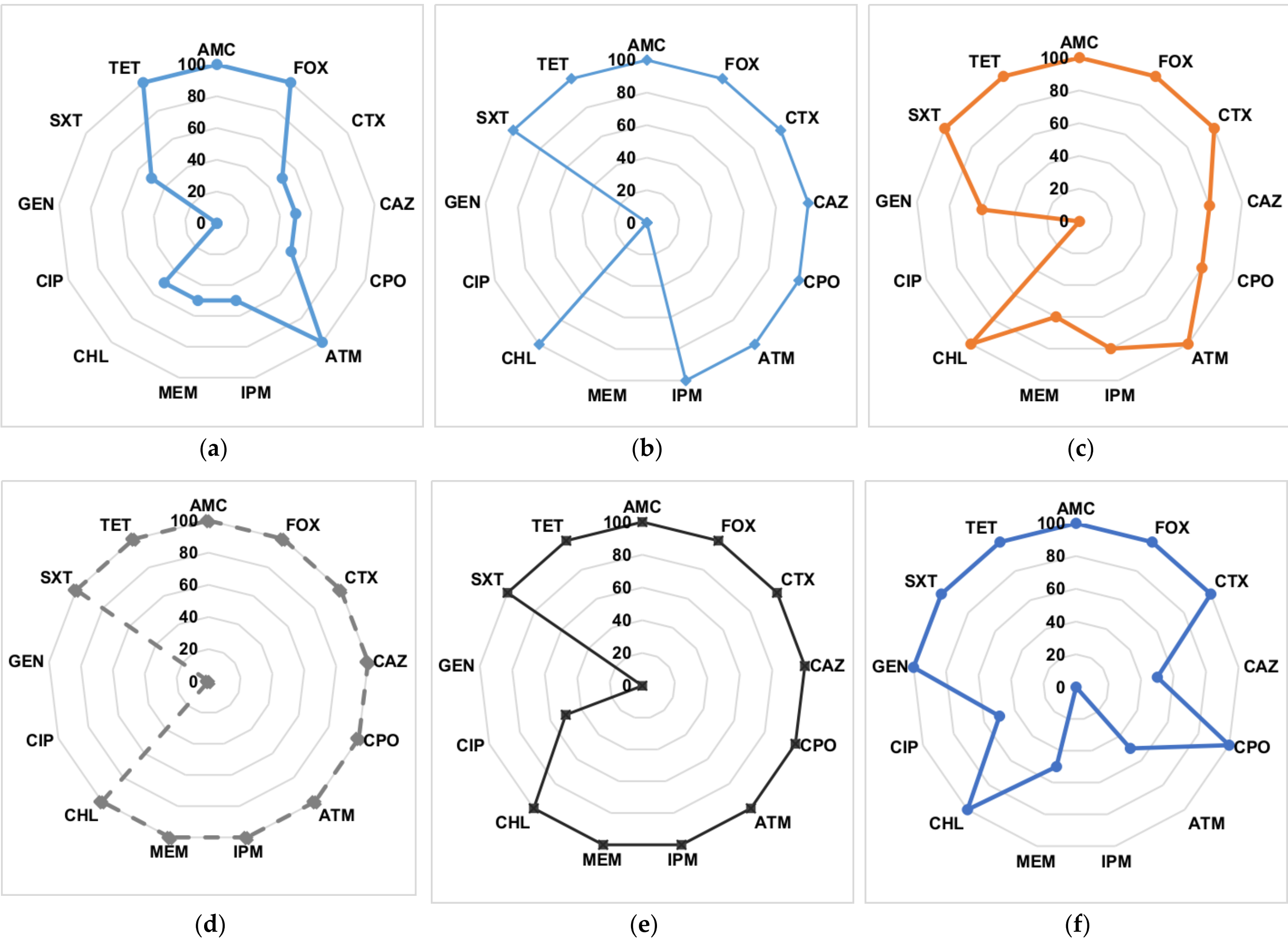


Figure 4. Relative frequency of antibiotic resistance patterns of *Enterobacteriaceae* isolates obtained from samples collected in 2019, 2020 and 2021 from: (**a**) Block II Inlet; (**b**) Paddies' Inlet; (**c**) Rice paddies—soil water; (**d**) Paddies' Outlet; (**e**) Block II Outlet; (**f**) LVID Outlet. AB: Nine β-lactams: ampicillin (AMP), amoxicillin/clavulanic acid (AMC), ceftazidime (CAZ), cefotaxime (CTX), cefpirome (CPO), aztreonam (ATM), cefoxitin (FOX), imipenem (IPM), meropenem (MEM) and five **b**: chloramphenicol (CHL), tetracycline (TET), gentamicin (GEN), trimethoprim/ sulfamethoxazole (SXT) and ciprofloxacin (CIP).

The AB resistance patterns of samples from the inlet of Block II showed 100% resistance to AMC, FOX, ATM and TET, but lower resistance to the other ABs tested (Figure 4a). Soil water samples from the paddies, in addition to those resistances, showed 100% resistance to CTX, CHL and SXT, which had already been detected in the irrigation water, although less prevalently (Figure 4b,c). The highest resistances were detected at the outlet of Block II (Figure 4e).

The higher percentages of resistances to ABs detected in drainage water samples (Figure 4d,e), compared with irrigation water and paddies' samples, might be explained by the leaching of native microorganisms from the soil and water that have been accumulating resistances due to the cultural practices used in this hydraulic block, namely direct grazing in forage crops, and the soil incorporation of manure to some other non-rice-crops, for which drainages are all collected in the Rio Negro ditch (Block II Outlet). Therefore, this sampling site represents the resistance determinants that occur in the entire Block II, because the Rio Negro ditch works as a collector for all of them.

The isolates from the paddies showed higher relative resistance percentages for both β-lactams and non-β-lactams than those from the irrigation water at the Block inlet, except for CIP, at which no resistances were detected. At the outlet of the Block, these values are even higher. An explanatory hypothesis for these results lies in the fact that, as drainage water is reused for irrigation, it enters a cycle that allows the accumulation of resistant microorganisms, thus favoring the transfer of AB resistance genes by bacterial recombination. The increase in the relative variation frequencies of AB resistances in soil water of the paddies (Table 3) is also possibly due to the conditions of the rice production ecosystem, facilitating AB resistance gene transference.

Table 3. Relative variation of the resistant percentage to the β-lactam and non β-lactam classes of samples collected on several sites of the water paths of paddies.

Antibiotics [1]		Paddies' Inlet vs. Block II Inlet	Paddies vs. Paddies' Inlet	Paddies' Outlet vs. Paddies	Block II Outlet vs. Paddies' Outlet	Paddies' Outlet vs. Paddies' Inlet	Block II Outlet vs. Block II Inlet
β-lactam	AMC	0	0	0	0	0	0
	FOX	0	0	0	0	0	0
	CTX	1	0	0	0	0	1
	CAZ	1	0	0	0	0	1
	CPO	1	0	0	0	0	1
	ATM	0	0	0	0	0	0
	IPM	1	0	0	0	0	1
	MEM	−2	2	1	0	2	1
non β-lactam	CHL	1	0	0	0	0	1
	CIP	*	*	*	2	*	2
	GEN	*	2	−2	*	*	*
	SXT	1	0	0	0	0	1
	TET	0	0	0	0	0	0

Relative variation (RV) calculated as: RV = (f2 − f1)/((f1 + f1)/2), where f1, is the mean value of the antibiotic resistant percentage at an upstream site; and f2, at a downstream site, applied to the antibiotics listed below. [1] Antibiotics: AMC, amoxicillin/clavulanic acid; FOX, cefoxitin; CTX, cefotaxime; CAZ, ceftazidime; CPO, cefpirome; ATM, aztreonam; IPM, imipenem; MEM, meropenem; CIP, ciprofloxacin; GEN, gentamicin; CHL, chloramphenicol; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline. * not defined, due to both values being null; therefore, with a null absolute variance.

There is also a high resistance to β-lactams for the isolates from the samples collected at the paddy inlet and outlet and at the Block II outlet (Figure 4b,d,e), which represents surface water. This high prevalence of isolates resistant to β-lactams, especially to penicillin and 2nd, 3rd and 4th generation cephalosporins is probably due to the use and abuse of this class of ABs for many years in livestock for the treatment of infections and as a growth promoter. The livestock production in the area surrounding the LVID possibly explains these high AB resistance frequencies. Farm animals are frequently a major source of pathogenic microorganisms in the soil and watershed of aquatic systems [30]; in addition, the intensive livestock in the region resorts to AB administration for veterinary purposes [31]. Moreover, at the outlet of LVID (Bajanca) (Figure 4f), the relative reduction is probably due to the flow dilution effect of the Rio Negro ditch stream in the Lis River.

The dissemination of resistances throughout the environment represents a risk to human health [32–34]. Antibiotic resistance has been identified as a global health concern, involving the transfer of resistant bacteria and their genes between humans, plants, animals and their shared environment. As bacteria and genes often cross environments and species boundaries, it is critical to understand the connections between the microbiota of humans, animals, plants and the environment to manage this global health challenge with an interdisciplinary and unified approach, based on One Health principles. Human–animal–environment interfaces create chances for one population or another to be a reservoir of resistant bacteria that can spread in any direction [35]. Multiple environmental reservoirs including soil, agricultural zones, wastewater and other aquifer resources such as rivers, estuary sediments and urban ponds are primarily responsible for this spread.

The investigated paddies and the surrounding fields in Block II, with increased abundance of AB resistant bacteria at the outlet in comparison with the inlet, showed that it has the potential to disseminate determinants of antibiotic resistance, which could pose a major threat to public health.

One of the main routes for the dissemination of antibiotic resistant bacteria (ARB) and resistance genes (ARGs) is the aquatic environment [36,37]. Unfortunately, conventional water treatment processes cannot entirely eliminate the determinants of resistance to AB from raw water, and for this reason the water bodies represent an important source of ARG dissemination [38]. Consequently, wastewater reused for irrigation not only in the rice fields, but also in the surrounding agricultural land, can have a return in the cycle and reach animals and humans through the food chain [39]. In fact, another possible exposure route, usually dominating the transmission of certain resistant bacteria, is via food, including raw vegetables [40,41]. This via is blocked for rice, as it is eaten cooked, but the maize for animal feed is a route via food for livestock. Additionally, it can still colonize other animals that surround this area of rice fields, such as the various species of typical birds dependent on wetlands in rice fields and wildlife from the surrounding forest. Several studies have reported the role of wild birds in the global transmission of resistance genes, due to their frequent movement, as in the case of migratory birds, contacting landfills and wastewater and the ease with which they collect food from various locations. This may contribute to fecal contamination of natural water reservoirs or food with which they come in contact [42–44]. There are also studies pointing to insects as vectors for the dissemination of AB resistances from rural to urban areas. Since there are several livestock farms in the vicinity of the sampling sites, flies contaminated with AB-resistant bacteria in the livestock environment may disperse them in urban areas, since the flight distance of houseflies (*Musca domestica*) varies between 5 and 7 km [45]. Humans can also be directly exposed to contaminated surface water during occupational or leisure activities [46]. In addition, microorganisms present in the soil and wetland surfaces can be carried in dust, depending on particle size and wind conditions, and redistributed by wind erosion processes at regional or field scales [47].

3.5. Discussion about Sampling Procedures and Mitigation of Microbiological Risk

Here, a proposal is presented, based on the knowledge gathered in this study, aiming to optimize the water sampling methodology in the LVID, in terms of time and frequency, to reliably and timely monitor the effects of irrigation water quality and cultivation practices, to allow eventual adaptations throughout the cultivation cycles, and without neglecting the need for further studies. As the sources of pollution outside the LVID are not uniform in time, it is proposed to increase the irrigation water sampling to three periods to assess the water quality in this agroecosystem: (i) One at the beginning of the irrigation season, usually in May, to assess the baseline situation. Information provided allows inferences about the means of water conduction to be adopted during the irrigation season that is about to begin. (ii) A second one at the peak of the irrigation period, in July or the middle of August, which will provide information during the critical period of irrigation, thus allowing adjustment in the conduct of irrigation. From a microbiological point of view, this is the period with higher risk, so these data are required to manage the risk control measures for crops, farmers and consumers. (iii) A third one at the end of the irrigation campaign, after the first autumn rains, usually in September, which allows assessing the impact of the production system, specifically regarding the organic fertilization and the irrigation system on the drainage water into the aquifer.

For risk mitigation practices undertaken to reduce microbiological contamination in the LVID via the direct contact of the irrigation and drainage water, and the organic fertilizer with the farmer and the consumer (through the edible parts of the plant), adaptions regarding the following may be considered:

The irrigation method, selected according to the type of crop, for example: (i) preference for drip or furrow irrigation, operated with due care, particularly in the case of vegetables for raw human consumption, to minimize the risk of contamination; (ii) limit the use of sprinkler irrigation to crops undergoing further processing, and consider the nearby crops for consumption in natura, which can be reached by drift, in addition to the farm workers.

The drainage practices should control the runoff from manured fields, mainly during the rainy seasons, through the soil water retention, promoting its infiltration, and thus reducing the microbial load reaching the surface drainage network. The free access of people and animals to the drainage network should be limited.

Planning of organic fertilization should be conducted, resorting to a fully composted manure, to minimize the incorporation of pathogenic microorganisms, including those harboring antibiotic resistance genes. Slurry and manure, if spread on the soil surface, should be immediately incorporated.

Based on the results obtained in this study, it is up to WUA and farmers to adapt the mode of operation and the technologies to minimize the negative impacts of water usage on the conservation of water resource quality. Monitoring water quality within the irrigation district is crucial to know the local reality over time and allow adjustments to be as effective as possible [14,31,48,49], and to inform farmers about special precautions and safety areas where microbiological contamination is at greatest risk [50].

4. Conclusions

Considering that studies are needed at the level of the particularity of each rice-growing ecosystem to provide baseline knowledge, this is an innovative work in terms of rice fields in Portugal.

Regarding the classic indicators related to flooding irrigation systems in level basins, it was concluded that the enumerations of the total coliforms in the surface drainage water increased in comparison with those of the irrigation water from the distribution channels. In turn, the reducing counts of fecal coliforms at the drainage outlet relative to the irrigation entrance proved a positive impact of these rice irrigation systems on this bioindicator. Regarding fecal enterococci, their counts increased significantly inside the soil water of rice fields; however, at the outlets, a decrease in these counts was recorded.

This analysis of the microbiological quality of the water samples reveals that a significant source of problems is external to the LVID, as several measurements demonstrate. Therefore, it can be considered that these rice ecosystems provide an environmental service, since the water that is returned through the drainage back into the collective ditch network has much better quality than that available for irrigation and does not reach the maximum recommended value of 100 MPN/100mL of FC, the quality bioindicator parameter provided by the law. In addition, rice fields show evidence of pollution from wild animals, which is also an environmental service provided by rice growers, as the flooding irrigation system provides a habitat and food for several species of birds. As a conclusion regarding these indicators, it is shown that, in the analyzed systems, rice production systems in flooded level basins have a significant purifying effect on water quality, contributing to the improvement in collective drainage water.

However, regarding the AB resistance analysis, the opposite situation was revealed in relation to the classic analysis. The enumeration of ampicillin-resistant *Enterobacteriaceae* remains identical or increases at the outlets in comparison with the inlets. The AB resistance phenotypes to all 13 ABs assayed are widely disseminated in these agroecosystems and are also highly prevalent in samples taken from both inlets and outlets. The frequent resistance to ABs found in these aquatic systems, namely to β-lactams (e.g., ampicillin), is a critical point, which can put water quality and public health at risk.

The analysis of the microbiological quality of the water samples reveals that a significant source of the problems is external to the rice crop systems, as the various measurements demonstrate. Therefore, to minimize the negative impacts of these problems, monitoring the quality of irrigation water is crucial to know the local reality over time and allow farmers to be informed about special hygiene and safety precautions, and where and when there is greatest risk of microbiological contamination of water.

Author Contributions: Conceptualization, J.M.G., I.M.D. and P.A.; Methodology, J.M.G., H.M., I.M.D. and P.A.; validation, all; investigation, all; newsroom, J.M.G., I.M.D., T.V. and P.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by: Project "Lis Valley Water Management Operational Group" ("PDR2020-1.0.1-FEADER-030911—Operational Group for water management in the Lis Valley"), funded by IFAP/PDR2020, Measure Innovation, Groups Intervention Operations. Project "MEDWATERICE, Towards a sustainable use of water in Mediterranean rice-based agroecosystems". PRIMA Program—PRIMA-Section-2018; Topic: 1.1.3: Irrigation technologies and practices: MEDWATERICE-PRIMA-0005-2018 (www.medwaterice.org, accessed on 30 June 2022).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: Rice growers, Nuno Guilherme, for providing the paddies for the field trails.

Conflicts of Interest: The authors declare no conflict of interest. Funders played no role in the study design; in the collection, analysis or interpretation of data; in writing the manuscript, or in the decision to publish the results.

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