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Politécnico de Coimbra

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Microbiological Quality of Water from Lis Valley Rice Ecosystems

Orientadora: Professora Paula Amador

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Relatório de Estágio Profissionalizante apresentado à Escola Superior Agrária de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de mestre em Engenharia Agropecuária.

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ABSTRACT

Rice cultivation has an important economic and social value in Portugal, covering a total area of about 30 thousand ha. Rice agroecosystems are peculiar. They are located in low, flat and alluvial lands, close to the coast, with a natural difficulty in drainage and a salinity influence, with cultivation in level basins, irrigated by continuous submersion, in a very sensitive balance. Rice farming systems play a valuable environmental role, in harmony with the production of a human food of recognized quality, in a significant contribution to national food sovereignty. The main current constraints of rice irrigation are related to water scarcity, issues with a global dimension, with water saving being a much studied topic, with several alternative solutions already implemented in several countries.

This study aimed to analyse the microbiological quality of water and soil in rice cultivation in Vale do Lis with irrigation systems by continuous and intermittent submersion in level basins, and in drip irrigation, to evaluate the respective impacts on water resources, aiming to contribute to the establishment of good agricultural practices and public and environmental health regarding the use of water and soil in those agroecosystems. In the experimental methodology, the classic microbiological bioindicators of fecal contamination (total coliforms, fecal coliforms and fecal enterococci) were selected, as well as an analysis of antibiotic resistance (enumeration of ampicillin-resistant *Enterobacteriaceae*, phenotypic characterization of isolated resistant bacteria and molecular detection of their antibiotic resistance genes. The results of the classic bioindicators in flooding systems point to a positive environmental impact, as the drained water has better quality than the water used for irrigation. On the other hand, these rice paddies show signs of pollution by wild fauna , which reinforces the environmental service performed by these agroecosystems, as they provide habitat and food for several species of this type. Regarding the analysis of antibiotic resistance, the results indicate that the enumeration of ampicillin-resistant *Enterobacteriaceae* remains identical or increases in the water outlets, compared to the entrances, that the antibiotic resistance phenotypes tested are widely disseminated in these agroecosystems and are also highly prevalent in samples collected both at the entrances and at the exits, with a high percentage of multidrug-resistant isolates, and that the identification of *tet* and *sul* genes revealed the prevalence of *tet*A, *tet*L, *tet*M and *sul*2. In conclusion, in relation to fecal bioindicators, rice production systems by flooding have a significant purifying effect on water quality, contributing to improving the quality of drainage water from the collective network. However, antibiotic resistance analysis revealed that farmer health and rice ecosystems may be threatened as these environments act as a hotspot for the spread of genetic determinants of antibiotics. It is proposed that water management plans in hydro-agricultural development include monitoring the quality of irrigation water, with a sampling frequency according to the sources of pollution, to establish special hygiene and safety precautions.

Keywords List: rice crop, Lis Valley, total coliforms, fecal coliforms, fecal enterococci, ampicillin-resistant *Enterobacteriaceae*, sulfonamides, tetracycline.

RESUMO

A cultura do arroz tem um importante valor económico e social em Portugal, numa área total de cerca de 30 mil ha. Os agroecossistemas orizícolas são peculiares, desenvolvem-se em terrenos baixos, planos e aluvionares, na proximidade da costa, com uma natural dificuldade de drenagem e influência da salinidade, com o cultivo em canteiros regados por submersão contínua, num equilíbrio muito sensível. Os sistemas orizícolas desempenham um valioso papel ambiental, em harmonia com a produção de um alimento humano de reconhecida qualidade, num contributo significativo para a soberania alimentar nacional. Os principais constrangimentos atuais da rega do arroz prendem-se com a escassez de água, questões com uma dimensão mundial, sendo a poupança de água um tema muito estudado, com diversas soluções alternativas já implementadas na realidade de vários países.

Este estudo teve como objetivo analisar a qualidade microbiológica da água e do solo no cultivo de arroz no Vale do Lis em sistemas de rega por submersão contínua e intermitente em canteiros de nível, e na rega por gotejamento num talhão de ensaio, para avaliar os respetivos impactos nos recursos hídricos, visando contribuir para o estabelecimento de boas práticas agrícolas e de saúde pública e ambiental no uso da água e do solo naqueles agroecossistemas. Na metodologia experimental foram selecionados os bioindicadores microbiológicos clássicos de contaminação fecal (coliformes totais, coliformes fecais e enterecocos fecais), bem como uma análise de resistência a antibióticos (enumeração de *Enterobacteriaceae* resistentes à ampicilina, caracterização fenotípica de bactérias resistentes isoladas e deteção molecular de seus genes de resistência a antibióticos. Os resultados dos bioindicadores clássicos nos sistemas de alagamento apontam para um impacte ambiental positivo, pois a água drenada tem melhor qualidade do que a água utilizada na rega. Por outro lado, estes arrozais apresentam indícios de poluição por fauna selvagem, o que reforça o serviço ambiental desempenhado por esses agroecossistemas, pois fornecem habitat e alimento para diversas espécies deste tipo. Em relação à análise de resistência aos antibióticos, os resultados indicam que a enumeração de *Enterobacteriaceae* resistentes à ampicilina permanece idêntica ou aumenta nas saídas de água, em comparação com as entradas, que os fenótipos de resistência a antibióticos testados estão amplamente disseminados nesses agroecossistemas e também são altamente prevalentes em amostras colhidas tanto nas entradas quanto nas saídas, com alta percentagem de isolados multirresistentes, e que a identificação de genes *tet* e *sul* revelou a prevalência de *tet*A, *tet*L, *tet*M e *sul*2. Como conclusão, em relação aos bioindicadores fecais, os sistemas de produção de arroz por alagamento têm um efeito depurador significativo na qualidade da água, contribuindo para a melhoria da qualidade da água de drenagem da rede coletiva. No entanto, a análise de resistência a antibióticos revelou que a saúde do agricultor e os ecossistemas de arroz podem estar ameaçados, pois esses ambientes atuam como um hotspot para a disseminação de determinantes genéticos de antibióticos. Propõe-se que os planos de gestão da água no aproveitamento hidroagrícola incluam a monitorização da qualidade da água de rega, com uma frequência de amostragem de acordo com as fontes de poluição, para estabelecer cuidados especiais de higiene e segurança.

Lista Palavras-Chave: cultura do arroz, Vale do Lis, coliformes totais, coliformes fecais, enterecocos fecais, *Enterobacteriaceae* resistentes à ampicilina, sulfonamidas, tetraciclina.

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1. Introduction

1.1 General considerations about rice

Rice (*Oryza sativa* L.) is grown in at least 114 countries of the world, and more than 50 of them produce equal to or more than 100,000 t/year. World rice production is forecast to reach 518.2 million tons (milled basis), up 0.9 percent from the 2020 record harvest. Therefore, global rice utilization is seen growing by 1.6 percent in 2021/22 to a peak of 518.8 million tons, while world rice stocks at the close of 2021/22 marketing seasons hover around a historical high of 187.6 million tons (FAO, 2022).

Rice, maize (*Zea mays* L.), and wheat (*Triticum aestivum* L.) are the main staple food sources for human, but rice becomes the most important with respect to human nutrition and caloric intake as maize is used for purposes other than human consumption. Most of the countries consume their own rice; therefore, the economic importance of rice is different from other export commodities, and only 5–6 % export occurs worldwide (Datta et al., 2017).

This crop that belongs to the genus *Oryza* and the tribe *Oryzae* under the family *Poaceae* comprises more than 22 species distributed across the tropical, sub-tropical and temperate regions of Asia, Africa, Central and South America and Australia but only two species are cultivated: *O. glaberrima* and *O. sativa* (L.). The two main subspecies of *O. sativa*, *indica* (prevalent in tropical regions) and *japonica* (prevalent in the subtropical and temperate regions of East Asia), were domesticated 10,000 to 14,000 years ago.

While rice is the main food product, there are also other significant food products that are produced as part of paddy rice systems, it offers a number of ecosystem services, provides human livelihood and sustenance and enriched culture and provides home to a diverse assemblage of plant and animal life. Being semiaquatic, paddy fields are ideal for diverse biological organisms by offering shelter, food, breeding, and nesting habitat, it also serve as substitutes for wetlands for foraging cattle egrets during the breeding season when these feed on invertebrates, fish, and amphibians (Richardson & Taylor, 2003). The biological diversity is influenced by moisture and nutrient gradients within the rice fields and the growth stage of rice and the cropping season during which rice was grown (Chivenge et al., 2019).

The rice grain comes in many different colors, including brown, red, purple and even black. The colourful varieties of rice are considered valuable for their health benefits. The unpolished rice with its bran has high nutrient content than milled or polished white rice. However, rice consumers prefer to consume polished white rice, despite the fact that brown rice contains valuable nutrient content (Devi & Babu, 2015). A detailed analysis on the nutrient content of rice suggests that the nutrition value varies depending upon several factors such as the strain or variety (i.e. white, brown, red and black/purple), nutrient quality of the soil in which rice is cultivated, the degree of milling and the method of preparation before consumption.

In natura, the grain of this cereal consists of a protective outer layer (husk) and the caryopsis. The polished brown rice loses the layers of pericarp, aleurone,

subaleurone, the embryo, and some of the endosperm, leaving only the starchy endosperm, commonly known as white rice. The fractions that are lost with polishing form the bran, which comprises 6 to 10% of the weight of whole grain (Regina Storck, 2004).

The embryo represents 2 to 3% of the weight of the whole grain and is attached to the endosperm. The cells of the embryo contain stored starch, protein, and fat, which are used in germination as a source of energy and enzyme generation. Brown rice retains its bran layer (containing vitamins, minerals and fibre), as this has not been polished more to produce white rice. The coloured rice varieties are either semi-polished or unpolished. Red-coloured rice varieties are known to be rich in iron and zinc, while black rice varieties are especially high in protein, fat and crude fibre. Red and black rice get their colour from anthocyanin pigments, which are known to have free radical scavenging and antioxidant capacities, as well as other health benefits. Brown rice is highly nutritious. It has low calorie and has a high amount of fibre. Furthermore, it is a good source of magnesium, phosphorus, selenium, thiamine, niacin, vitamin B6 and an excellent source of manganese. The white rice raw and the long-grain white rice are a good source of carbohydrates, calcium, iron, thiamine, pantothenic acid, folate and vitamin E when compared with maize, wheat and potatoes. It does not contain vitamin C, vitamin A, beta carotene, lutein and zeaxanthin. It is also notably low in dietary fibre (Priya et al., 2019).

1.3 Rice Agroecosystems

Rice has a wide range of ecological amplitude and can be grown in a variety of habitats, figure 1, with different hydrological conditions, climate, and soil types (Bouman et al., 2007). The four main rice production environments based on its hydrology are summarized below:

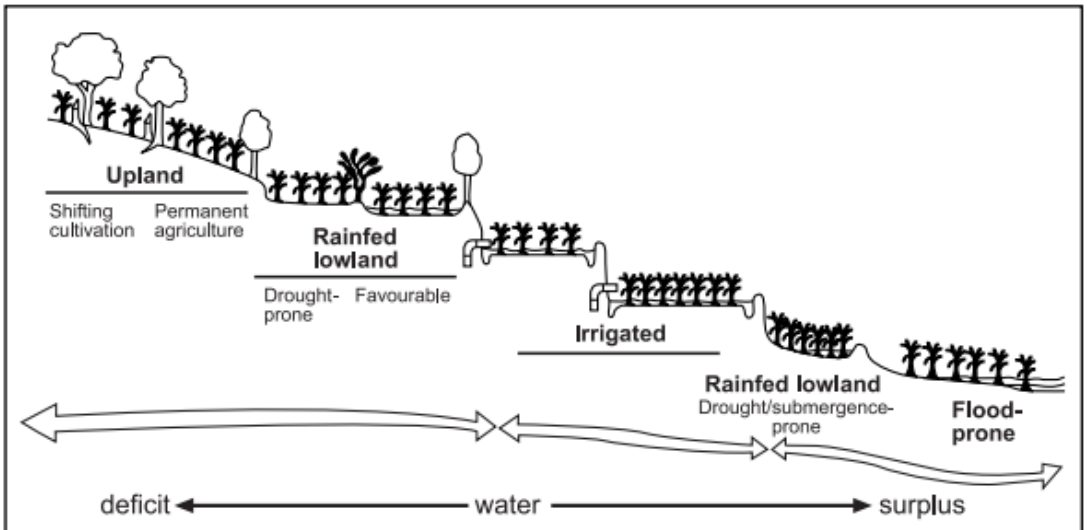
(i) Irrigated rice: Almost 75% of the world rice production comes from irrigated lowland rice with a cultivated area of about 85–90 m ha where the main source of water is irrigation for at least 80% of crop duration (Cirad, 2010). Global food security is largely dependent on this system. In India, farm size for this type of rice cultivation system is small (0.5–2 ha) where rice is cultivated once, twice, or thrice a year as monocrop or sometimes in rotation with other crops such as wheat.

(ii) Rainfed lowland rice: Rainfall is the only source of water for rainfed lowland rice production system. Fields for this system of cultivation are also bunded and are flooded with rainwater for at least part of the cropping season. The share of this system in global rice production is about 20 % with a cultivated area of about 40–45 mha. Some of the major yield-limiting factors for this system include drought, flood, weed infestation, iron toxicity, and disease (Seck et al., 2012).

(iii) Rainfed upland rice: Cultivation of rice like any other cereal is known as upland rice. Fields are characterized by well-drained, unsaturated, and aerobic soil without any ponded water for more than 80 % of the crop growth period. Broadcasting method is commonly used for this system where land is tilled prior to starting of rainy season. No water is held on the surface as there is no bunding. The

major constraints for this system include erratic rainfall, poor weed control, low fertilizer use, and high disease incidence (Seck et al, 2012).

(iv) Deep water or floating rice: Rice cultivated in areas where water depth reaches 1 m or more for a period of 10 days–5 months is called deep water or floating rice (Seck et al., 2012). Commonly available varieties of rice are not suitable for this system. Rice cultivated under this system has some special genetic characteristics. These are quick growth and stem/root elongation rate of as high as 10 cm day^{-1} with the rise in floodwater and formation of adventitious roots for direct absorption of nutrients from floodwater in addition to regular root system grounded in the soil (Balasubramanian et al., 2007).



Upland	Rainfed lowland	Irrigated	Flood-prone
Level to steeply sloping fields; rarely flooded, aerobic soil; rice direct seeded on plowed dry soil or dibbled in wet, non-puddled soil	Level to slightly sloping, bunded fields; non-continuous flooding of variable depth and duration; submergence not exceeding 50 cm for more than 10 consecutive days; rice transplanted in puddle soil or direct seeded on puddle or plowed dry soil; alternating aerobic to anaerobic soil of variable frequency and duration	Leveled, bunded fields with water control; rice transplanted or direct seeded in puddle soil; shallow flooded with anaerobic soil during crop growth	Level to slightly sloping or depressed fields; more than 10 consecutive days of medium to very deep flooding (50 to more than 300 cm) during crop growth; rice transplanted in puddle soil or direct seeded on plowed dry soil; aerobic or anaerobic soil; soil salinity or toxicity in tidal areas

Figure 1*- Rice land ecosystems (source: Halwart and Modadugu, 2014).*

Breeding rice varieties with tolerance to drought stress offers an economically viable and sustainable option to improve rice productivity (Pandey & Shukla, 2015).

Screening of thousands of germplasm has been conducted earlier for drought resistance in various corners of the world, however, only a few drought-tolerant varieties are yet recognized (Singh et al., 2015). The main reasons for the minimal success are non-availability of truly drought-tolerant genotypes and lack of suitable screening methods (Pandey & Shukla, 2015). During the last two decades, scientists from the International Rice Research Institute (IRRI), the Philippines, screened nearly 1000 Genebank accessions originated from 47 countries for drought tolerance (Rahman

& Zhang, 2016). They have identified 65 more drought-tolerant accessions, which are either *aus* or *indica* (Torres et al., 2013). The highest number of drought-tolerant *aus* accessions are originated from Bangladesh, followed by India, whereas the highest number of drought-tolerant *indica* accessions are originated from India followed by Bangladesh and Sri Lanka.

1.4 Crop characteristics and other issues

First of all, regarding rice agronomy, germination is understood as the appearance of the coleoptile tip emerging from the split husk of the grain. At this stage, the seed needs to absorb water in the proportion of 25 to 25% of its weight, which does not justify a water slide, hence it works essentially to regulate the environment and help in cleaning the waters and fighting pests. Sowing can be manual or mechanical. As in Portugal, most rice growers in California and Louisiana use airplanes and sow in 5-10cm deep water beds, using pre-drilled seeds (Pereira, 1989).

For the period from panicle initiation to flowering, the water requirements of the plants are highest. An abundant supply of water is recommended in order to prevent the damage that deficiency can cause. Water depths of 5 to 8 cm are desirable, but greater in areas subjected to low night temperatures in order to protect the developing panicle (Pereira, 1989).

The soils destined for rice cultivation are mainly lowland alluvial soils, where both zoning and gravity irrigation is relatively simple. In general, they have shallow water tables and are generally salinized, which makes them marginal for other crops. Rice is not demanding in terms of soil, and is grown in sandy as well as clay soils, as Silva (1969) states. However, in general, rice yields are higher relative to fine texture soils (clayey, loamy-clayey and sandy-clayey).

The influence of temperature on rice culture, when evaluated globally, presents results of some complexity, and sometimes contradictory. This is due in large part to the variation in the requirements of the plant with respect to growth and development, which depend on soil conditions and the variety grown. Each stage of development and each growth process responds differently to identical temperature conditions (Pereira, 1989).

Water deficiency in the post-beating period causes an increase in the number of imperfect grains, which affects maturation. However, the same author mentions that submergence is not necessary, as long as the soil is saturated or nearly saturated (80 to 95%). In this vein, under given conditions it is advisable to drain the beds 2-3 weeks after flowering. In the Portuguese case, the normal practice is to end irrigation 3-4 weeks after flowering, with the water retained in the bed functioning as a supplementary source for the water needs of the plant during this final period.

The concept of water management implies an important aspect related to water quality, both the water entering and leaving the bed. In fact, part of the water used in irrigating rice returns to natural or artificial waterways, and can be used for agricultural, industrial, domestic or recreational purposes. It is clear that the use of chemicals in rice cultivation is not an exception in modern agriculture. The problem is when application standards are not respected and non-biodegradable products are used, due to potential water mobility (Pereira, 1989).

Water needed for rice cultivation is more than any other arable crops. Nearly 75 and 80 % of the total existing water resources of the world and Asia, respectively,

are devoted to rice production (Bouman et al., 2007). Presently, the traditional ways of growing rice (flooding and puddling the soil followed by seedling transplanting) are increasingly becoming difficult due to acute water and labour shortage in major rice-producing areas of the world (Ishfaq et al., 2020). The benefits associated with growing rice under inundated conditions could be achieved even without this practice, for example, by plowing, fertilizing, and weeding through tractors and spraying of herbicides. High labour needs and emission of more greenhouse gases under wet conditions are the other problems associated with rice production under inundated conditions (Datta et al., 2017). Alternate wetting and drying (AWD) and aerobic rice provide a respective 38 and 40 % reduction in water input, (Lampayan et al., 2015), to rice over the conventional flooding; however, the farmers are reluctant to adopt these practices because of possible yield reduction and certain information gaps regarding these systems. The existing practice of rice cultivation where fields are kept continuously flooded is more popular among farmers because of weed suppression and higher yield. However, this practice causes excessive water loss due to higher seepage, percolation, and evapotranspiration (Grassi et al., 2009). Moreover, changing climate with a direct effect on agricultural water availability is a serious threat for the existing systems of rice cultivation (Yoo et al., 2012). Changing climate scenarios (warming in particular) is expected to cause a 13–23 % rise in irrigation water requirement for rice cultivation (Thomas, 2008).

1.5 Chemical environmental impacts of rice production

Irrigated rice systems contribute to the accumulation of reactive nitrogen (*N*) compounds in the environment. Reactive *N* is defined as all biologically, photochemically, and/or radiatively active forms of *N*. This diverse pool includes mineral *N* forms such as nitrate (NO_3^-) and ammonium (NH_4^-), gases that are chemically active in the troposphere (NO_x and ammonia, NH_3), and gases such as nitrous oxide (N_2O) that contribute to the greenhouse effect (Bouman et al., 2007).

Annually, irrigated rice consumes about 8–9 million t of fertilizer *N* or roughly 10% of global fertilizer *N* production. On average, only 30–40% of the applied *N* is recovered by the crop, leading to large losses of reactive and nonreactive *N* forms (Dobermann et al., 2003).

Ammonia (NH_3) volatilization from the application of urea fertilizer is the major pathway of *N* loss in flooded rice systems, often causing losses of 50% or more of the applied urea-*N* in tropical transplanted rice (Bouman et al., 2007).

The magnitude of NH_3 volatilization largely depends on climatic conditions and the method of *N* fertilizer application. Volatilized NH_4^+ can be deposited on the earth by rain, which can lead to soil acidification and unintended *N* inputs into natural ecosystems.

Techniques for upscaling of greenhouse gas emissions have improved with the use of simulation models coupled with Geographic Information System (GIS) databases on soil and land use (Matthews et al., 2000). However, the uncertainty about CH_4 emissions from rice fields is higher than about most other sources in the global CH_4 budget. Organic manure generally enhances CH_4 emissions. Flooding of the soil is a pre-requisite for sustained emissions of CH_4. Mid-season drainage, a common irrigation practice adopted in major rice-growing regions in China and Japan, greatly reduces

CH_4 emissions. Similarly, rice environments with an uneven supply of water, such as rainfed environments, have a lower emission potential than fully irrigated rice (Van Der Gon et al., 2000).

In irrigated rice systems with good water control, N_2O emissions are small except when excessively high fertilizer *N* rates are applied. In irrigated rice fields, the bulk of N_2O emissions occur during fallow periods and immediately after flooding of the soil at the end of the fallow period. However, in rainfed systems, nitrification during aerobic phases and denitrification during subsequent waterlogged phases might contribute to considerable emission of N_2O (Abao et al., 2000).

Changes in water quality associated with rice production may be positive or negative depending on the quality of the incoming water and management practices such as fertilizer and biocide use. The quality of the water leaving of rice fields may be improved as a result of the capacity of the wetland ecosystem to remove *N* and phosphorus (*P*) (Feng et al., 2004; Ikeda & Watanabe, 2002). Among the agrochemicals that pose the greatest threats to domestic use of groundwater are NO_3, biocides and their residues, and, more recently, arsenic (*As*). Salinization and acidification are other forms of water pollution that can be associated with rice cultivation.

Nitrate - NO_3 leaching from flooded rice fields is normally negligible because of rapid denitrification under anaerobic conditions. The relative contribution to this increase from rice, however, is not clear (Bouman et al., 2007).

Biocides - In traditional rice systems, relatively few herbicides are used as puddling, transplanting, and ponding water are effective weed control measures. The potential for water pollution by biocides is greatly affected by field water management. Different water regimes result in different pest and weed populations and densities, which farmers may combat with different amounts and types of biocides. Residual biocides interact differently with soil under different water regimes (Sethunathan, 1989).

Arsenic - *As* accumulates in the topsoil as a result of irrigation. Rice fields receive relatively high amounts of irrigation water, and therefore accumulate more *As* than non-rice fields. Under the flooded conditions in which rice is grown, redox potentials are low, making *As* potentially bioavailable. To date, however, it has not been possible to predict *As* uptake by plants from the soil (Meharg, 2004).

Salinization - In many areas, percolating water from lowland rice fields can raise the groundwater table and cause waterlogging. Where the groundwater is saline, this can salinize the root zone of nonrice crops (Bouman et al., 2007).

Acidic Pollution - Many rice-growing areas of the deltas of Southeast Asia include acid sulfate soils. Amelioration of these soils for rice cultivation entails the leaching of toxicities from the soil, which means transferring acidic pollutants from the soil to surrounding water (Tuong et al., 2003).

1.6 Microbiological Impacts in rice agroecosystems

1.6.1 General considerations

Rice plant yield is mostly dependent on the water type used for irrigation over the respective growth cycle. Throughout the growing season (aprox. 3 months), a volume of between 500 and 3000 mm of water is needed, and this figure differs with climatic conditions, soil type, and rice genotypes (Abedin et al., 2002). When dry conditions exist, water losses are relatively high and paddy soils become partially or completely dry.

In the case of irrigation for agricultural purposes, chemical constituents such as compounds of emerging concern can be absorbed by crops in the irrigation process, with this absorption being greater in foliage and roots than in fruits (Delli Compagni et al., 2020). The evaluation of ecosystem health requires the understanding and monitoring of bioindicators, which represent biological diversity (Bennett & Balvanera, 2007; Smukler et al. 2010, cited by Jang et al., 2013).

Functional groups of microorganisms consist of metabolically related organisms, e.g., oxygen-respiring bacteria, nitrate reducers, iron reducers, sulphate reducers, but also fermenting bacteria and CH_4 producing Archaea. The concerted action of all functional groups of microorganisms drives the carbon, nitrogen, sulphur, and iron cycle in rice paddies, but also in other soils and sediments (Liesack et al., 2000). Magurran, 2004, proposed that biodiversity is defined as the variety and abundance of species in a defined unit of study. Biological diversity is now increasingly recognized as a vital parameter to assess global and local environmental changes and the sustainability of developmental activities (Gabriel et al., 2006; Smukler et al., 2010).

Rice yield soil is studied in order to understand the processes leading to the production of methane, but also to gain general knowledge about both structure-function relationships between microbial groups and interactions of microorganisms with rice plants.

1.6.2 Antibiotic resistance

Antibiotics (AB) are widely used to protect the health of human and animals or to increase growth rate of animals as food additive. These AB have been intensively used over the last decades in human and animal therapy and livestock, resulting in serious environmental and public health problems. They are daily excreted into the environment through urine and feces, as a mixture of unchanged xenobiotic compounds and bioactive forms partially metabolized by humans and animals. This release, together with antibiotic-resistant bacteria (ARB), namely *Enterobacteriaceae*, can occur by several routes, the major of which are the network of municipal sewers and the soil farm fertilization with manure or sewage sludge. It is noteworthy that this reuse practice can lead to an increase of those pollutants in soil and in endophytic bacteria of crops grown in manured soil (Ji et al., 2012, Martínez-Carballo et al., 2007; Yang et al., 2016, cited by Amador et al., 2015).

Besides the chemical pollution by AB themselves, their long-term permanence in most water systems, pressures selection at sub-inhibitory concentrations upon microorganisms, resulting in the development of ARB and antibiotic resistance genes (ARG) (Kim & Aga, 2007; Korzeniewska et al., 2013, cited by Amador et al., 2019).

Concerning the presence of antibiotics in water resources, it can be noted that the wastewater and wastewater treatment plants (WWTP) can act as reservoirs of antibiotic resistance. They have also been proposed to be hotspots for horizontal gene transfer, enabling the spread of antibiotic resistance genes between different bacterial species (Kemper, 2008).

Previous studies have reported the higher occurrence of tetracycline and sulphonamide resistance genes in water, soil, and sediment (Peak et al., 2007; Chen et al., 2010; Luo et al., 2010; Li et al., 2012; Zhang & Zhang, 2011; Cheng et al., 2013, cited by Mu et al., 2015).

Regarding, sulphonamides class was the first drug to be used in veterinary medicine in therapeutic doses (Agyare et al., 2019, Lees et al., 2021) . This class are represented by synthetic AB, that competitively inhibits the enzyme dihydropteroate synthase (DHPS) which participates in folate synthesis, an essential mechanism for bacterial DNA and RNA synthesis (Maka et al., 2015; Xu et al., 2020). Their excessive usage imposed widespread selective pressures on bacteria, as seen by the high prevalence rates of sulphonamide resistance observed in mainly Gram-negative bacteria isolated from animals and humans all over the world in the past decade (Ben et al., 2017; Yuan et al., 2019). The accumulation of sulphonamides as environmental contaminants is potentiated by their resistance to degradation during conventional wastewater treatments (Felis et al., 2020). In addition to the direct environmental adverse impacts, high sulphonamide concentrations increase the risks of food chain contamination (Nunes et al., 2020).

Resistance to sulphonamides in Gram-negative bacteria is associated with the presence of four *sul* genes (*sul1*, *sul2*, *sul3* and *sul4*) (Maka et al., 2015; Xu et al., 2020). The *sul1* and *sul2* genes have previously been identified in *Enterobacteriaceae*, particularly *Escherichia* and *Salmonella*. In 2003, Perreten and Boerlin, reported the *sul*3 gene, detected in *Escherichia coli* isolated from pigs in Switzerland. In 2017, Razavi et al. described the *sul4* gene, which provided clinical resistance in *Enterobacteriaceae*. The *sul* genes can be transferred between bacteria via mobile genetic elements, such as, integrons, transposons or plasmids (Xu et al., 2020).

On the other hand, since the introduction of tetracyclines in 1950, their combination of broad-spectrum activity and low toxicity has led to their intensive use in human and animal infections therapy, and they have also been used for nearly as long to promote growth in food animal production systems (Adesoji et al., 2015). This extensive use favoured the emergence of tetracycline resistance in a diverse group of bacteria and caused restrictions on the clinical utility of these compounds (Marosevic et al., 2017; Sheykhsaran et al., 2019). Three principal tetracycline resistance mechanisms are efflux pumps, ribosomal protection, and enzymatic inactivation of tetracyclines drugs (Nguyen et al., 2014; Roberts & Schwarz, 2016). Several different *tet* genes have been described as conferring resistance to tetracyclines in bacteria. The most frequent types of *tet* genes belong to classes A, B, C, D and G (Xu et al., 2020), and these genes are responsible for encoding tetracycline efflux pumps (Grossman, 2016; Roberts & Schwarz, 2016).

There is a growing concern about the overall increase in bacterial resistance to antibiotics. Several studies have documented the transfer of antibiotic-resistant bacteria from animals to the human population, posing a serious threat to public health (Igbinosa, 2015; Zhu et al., 2017).

1.6.3 Detection and Characterization of Resistance Genes

For the past 70 years, research in antibiotic resistance has focused mainly on pathogens. Isolating pure cultures has been, and still is, the most important method in clinical microbiology. Antibiotic susceptibility testing of bacteria is relatively inexpensive and gives important data on resistance patterns that are needed for the clinical treatment of patients (Karkman et al., 2018).

Considering that ARGs are widespread in aquatic, soil and cultures environments mentioned above, there is a need for the development and application of molecular methods to investigate the occurrence, transport, and fate of the environmental ARGs. The methods more used for detection, typing, and characterization of ARGs are specific and multiplex polymerase chain reaction (PCR), real-time PCR, DNA sequencing, and hybridization-based techniques including microarray (Zhang et al., 2009).

PCR and quantitative PCR methods have been widely used in both pure cultures and mixed environmental samples for detection of specific ARGs. An important variant of PCR is the Multiplex-PCR. This method allows the simultaneous detection of multiple microorganisms potentially present in the samples, since a set of different primers pairs are added to the reaction, with different specificities which ultimately allows its reaction products to be distinguished based on the different molecular weight of the amplification products. Another method is the qPCR array, the simultaneous quantification of hundreds of ARGs and other genes of interest is possible as parallel assays in just one run. This creates an opportunity for quantification of many relevant ARGs, sequences related to mobile genetic elements, and genes specific to certain bacterial species (Zhang et al., 2009).

1.7 Objectives

The general objective of this study was the microbiological analysis of the water and soil of the rice cultivation system, to assess the respective impacts on water resources. The classic microbiological bioindicators of fecal contamination included the Total Coliforms, Fecal Coliforms and Fecal Enterococci, whereas the antibiotic resistance analysis resorted to the enumeration of ampicillin resistant *Enterobacteriaceae*, phenotypic characterization of resistant bacteria isolated and the molecular detection of their antibiotic resistance genes.

The irrigation systems studied were those of submersion in level basins by continuous and alternate wet and drying flooding and the drip irrigation system in the Lis Valley case study. This study aims to contribute to the establishment of good agricultural practices and public and environmental health in the use of water and soil in rice production systems.

2 Material and Methods

2.1 Study Sites and Experimental Treatments

The sites are located in the Lis Valley Irrigation District, which is a public irrigation district managed by a Water Users' Association (WUA), located in the Coastal Centre of Portugal (coordinates, 39°51'22.1"N 8°50'56.1"W), under Mediterranean Temperate climate, belonging to the counties of Leiria and Marinha Grande (Fig. 2a).

The total area is about 2000 ha, cultivated with forage corn, forage grass, vegetables, fruits and rice. The hydraulic infrastructures have the objectives of perimeter drainage defence through slope collectors and valley ditches, the irrigation water supply through a canal conveyance system and the field drainage based on a ditch network. Water is supplied by an open-channel conveyance network from weirs installed along the Lis river and tributaries, and by pumping from drainage ditches (Gonçalves et al., 2020). The soils are mainly alluvial with high agricultural quality, some are poorly drained, with waterlogging and salinization risks, particularly on the downstream areas. The structure of the on-farm parcel property is characterized mainly by small parcels (Ferreira et al., 2020). Rice is cultivated in traditional paddies, with ca. 10 cm of ponding depth, and an irrigation frequency varying from daily to a few days. Water in rice plays a main role in a temperature regulation and weed control. This crop is traditionally cultivated on lower soils with heavy texture, drainage problems, and a shallow groundwater table, totalizing an area of about 140 ha (Fig. 2b).

The water is supplied from irrigation canals, or in some fields, by farmer's pumping from ditches. The water shortage is an endemic problem during summer months in Lis Valley, being the major constraint to rice crop sustainability and expansion. Water-saving practices on rice irrigation are welcome to mitigate a problem with water scarcity.

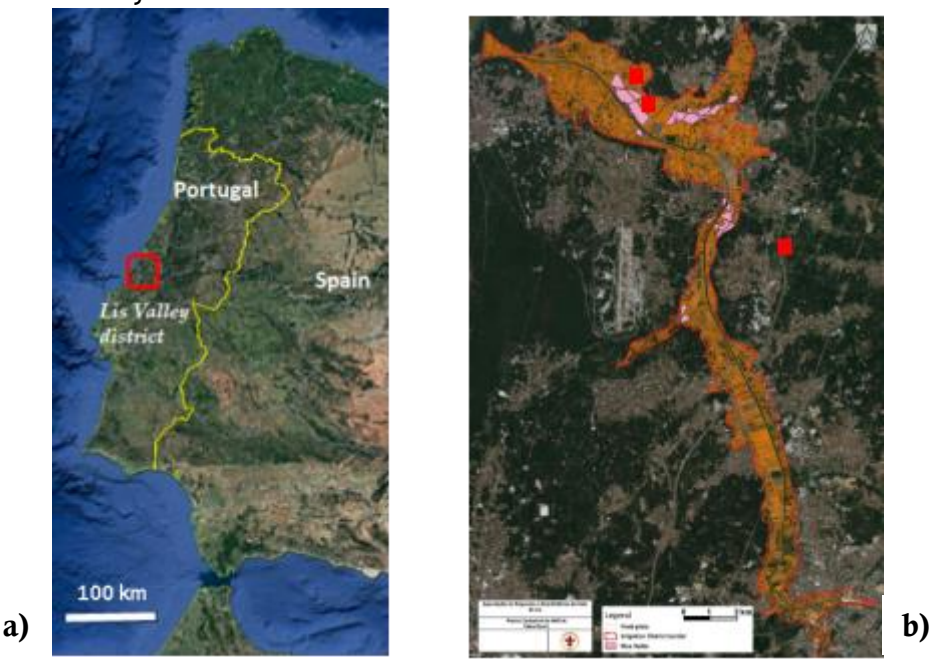


***Figure 2**- (a) Location of the Lis Valley Irrigation District in Portugal (red square) (source: Google Maps, https://maps.google.pt); (b) Location of rice fields (rose color) in Lis Valley Irrigation District, and the experimental fields (■) (source: Lis Valley Irrigation District Water Users).*

Three treatments, named continuous flooding (CF), alternate wet and drying (AWD) and surface drip irrigation (SDI), according to the irrigation systems used in each one were selected for field studies (Figure 3).

The CF (continuous flooding) essay was carried on a private farm, on a plot area of 2,8 ha, using traditional practices of flood irrigation full managed by the farmer, Lis Valley representative, used like the 'reference' irrigation method. The other plot, with

an area of 0,20 ha (20 m x 100 m), contiguous to the traditional one with identical agronomic practices, was irrigated by AWD. The soil has clay-loamy, neutral (pH=7.4), with a deep profile, and with a shallow groundwater table level, between 75 cm to 85 cm below the soil surface. The agronomic practices in both plots used were soil preparation, including the ploughing and harrowing, wet sowing of the rice cultivar "Ariete", carried out on 14th May, and the fertilizer doses of 90 kg N/ha, and herbicide treatments. The monitoring system installed followed the procedure described by Gonçalves et al., (2022). The evaluation of crop yield, considered the sampling of aerial part at harvest time, was done in several points of the plots, with a unit sampling area of 0.5 m^2.

The AWD (alternate wet and drying) essay, adapted the published by Bouman et al., (2007) with the adjustments to local agronomic practices: the initial flooding allowing the wet sowing, like the traditional practice (follows an initial drying event to favour the emergence), shallow ponding during the vegetative phase, considering the drying periods required for herbicide application, usually twice, with particular attention during the flowering period because it is very sensitive to water stress, the AWD technique during all stages after flowering, until last irrigation; the target was a flood water depth not higher than 5-7 cm; the irrigation schedule considered was an interval between 10 to 14 days of irrigation events; ensuring that the water level should not fall to 15 cm below the soil surface, measured in a water tube, and at least, the last irrigation should be about 20 days before the harvest.

The third treatment (SDI) was set in an area nearby the irrigation district, on a plot of a private farmer that usually produces horticultural crops. The soil has a light texture, very good internal drainage, acid (pH=5.3), 1.5% of organic matter, and with a groundwater level about 3-4 m depth below soil surface. The plot had an effective area of 240 m^2 (12 m width x 25 m length), was divided into 8 strips, with two drip lines per each strip, having each strip 6 rows of plants.

Rice was sown on 20th May in rows, to assess their performance to the drip irrigation condition. Spacing between rows was 20 cm, and the target plant spacing within row was 5-10 cm. Weeds were controlled with two applications of the herbicide Bentazona, complemented with two manual weeding.

The water was supplied using a submersible electric pump (Hidrobex, model Vetax-1000, 1 kW) and automatically controlled. The irrigation system comprised a sand-filter, complemented with two plastic mesh-filters, a fertigation injector, a water counter and two pressure gauges; a manifold of PE 50 mm, and PE drip lines of 16.2 mm diameter, brand NETAFIM, model Thyphon Plus 16150, non-regulated, dripper spacing of 0.30 m, with a flow of 1.00 L/h with pressure of 1.0 bar; the field installation had 16 drip lines with a length of 25 m, spaced of 0.60 m, working at a pressure of 1.0 bar, with a total discharge of 1.33 m^3/h. The monitoring system installed in this plot evaluated the following aspects: water use, soil moisture, crop development, yield quantity and quality. The evaluation of crop yield was considered the sampling of aerial part at harvest time, for each strip of 100 cm linear at row crop close to drip line, and identical sample on the row far from the drip line, to assess the medium yield.

A Portuguese cultivar of rice paddies, "Ariete", was used in three treatments.

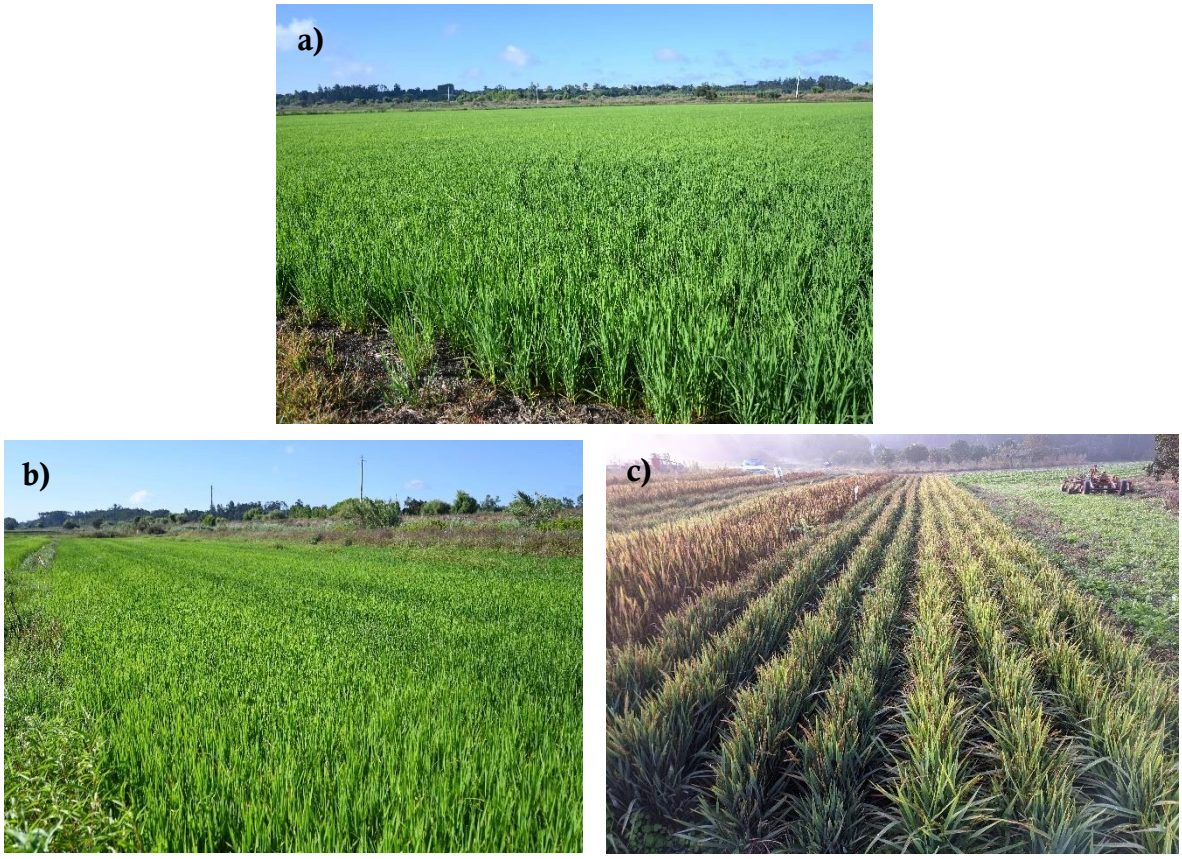


***Figure 3**- Overview of the three experimental sites; a) CF (continuous flooding); b) AWD (alternate wet and drying) ; c) SDI (subsurface drip irrigation) date of the photographs: a) 13/08/2020 ; b) 13/08/2020; c) 20/10/2021.*

2.2 Classical Microbiological Analysis

The culture media prepared and used throughout this study can be found in Annex 1.

2.2.1 Fecal Contamination Bioindicators

Microbiological analysis of water samples included the enumeration of Total Coliforms (TC), Fecal Coliforms (FC), and Fecal Enterococci (FE) by the dilution method with multiple fermentation tube technique.

2.2.1.1 Total Coliforms

The traditional multiple-tube technique was adopted for the analysis of coliforms (total or thermotolerant) and *E. coli*, (ISO 9308-2). This dilution method with fermentation in liquid substrates in at least three tubes at three dilution's, allows the quantification by "most probable number" (MPN) of microorganisms and is divided into two successive phases, a presumptive and a confirmatory phase. The latter is only performed if there is positive growth in the presumptive stage.

The presumptive phase procedure consists of homogenizing and transferring aliquots and/or dilutions of the sample to test tubes containing, at the bottom, an

inverted tube for gas collection (*Durhan* tube), and the appropriate culture medium, Brilliant Green Lactose Bile Broth (BGLB).

A test tube rack was prepared with 15 tubes, arranged 5 to 5, figure 4. The first 5 tubes with double concentration of BGLB culture medium and the next 10 with simple concentration of BGLB culture medium. In the first 5 tubes, 10mL of the water sample to be examined was inoculated using a sterile pipette. In the second 5 tubes, 1mL of the water sample was inoculated, and in the last 5 tubes, 0.1mL of the water sample was inoculated.

All tubes were incubated at 37°C ± 1ºC for 24 to 48 hours and subsequent identification of those that have growth (positive) of total coliforms, a result identified by the occurrence of acid reaction (yellowish color) or gas production (retained in the *Durhan* tube).

The results are expressed as the most probable number MNP/100mL of water, by reference to McCrady's Table, (Baird et al., 2017), and the positive tubes were subcultures on confirmation media.

In the confirmatory phase, the positive presumptive tubes were reinoculated (aliquots were transferred with a platinum loop) into tubes prepared in the same way as above. All the tubes were incubated at 37°C ± 1ºC for 24 to 48 hours and the subsequent identification of those that have growth (positive) of total coliforms, identified by the occurrence of gas production in the *Durhan* tubes.

***Figure 4**- Racks with freshly distributed BGLB culture medium into the test tube, with the inverted Durhan tubes.*

2.2.1.2 Fecal Coliforms

Using the tubes that tested positive in the presumptive test, a portion of the sample was inoculated in the Peptone Broth. Hold the test tube with the new culture medium, pass the hole through the flame, and dip the loop with the sample portion into it. The steps were repeated for each of the positive tubes. Tubes were incubated at 44.5°C ± 0.5ºC for 24/48h. At the end of the incubation period, 2 to 3 drops of *Kovacs* reagent were added to each test tubes.

When the result is revealed by Kovacs reagent in the tubes, it is considered positive for fecal coliforms if the presence of indole (one of the immediate degradation products of tryptophan deamination) is detected, showing a red ring (n-dimethylaminobenzaldehyde).

2.2.1.3 Fecal Enterococcus

The culture media were prepared in test tubes to inoculate the samples. The tubes with single and double concentration of Buffered Azide Glucose Glycerol Broth (BAGG) medium are arranged in the test tube racks, properly labelled.

A test tube rack is prepared with 15 tubes, arranged 5 to 5, the first 5 with double concentration of BAGG culture medium and the next 10 with single concentration of BAGG culture medium.

At the end, it is incubated at 37°C ± 1ºC for 48h. At the end of the incubation period, the tubes considered positive for fecal streptococci show a turbidity of the medium, or show a violet color change.

2.2.1.4 Fecal Index

The Fecal Index was determined according to Pepper and co-authors (2011) (Table 1).

Table 1- *Source of pollution according to the Fecal Index (FC/FE).*

FC/FE	Source of pollution
>4.0	Human pollution
2.0-4.0	Human waste in mixed pollution
0.7-2.0	Animal waste in mixed pollution
<0.7	Wild animal pollution

FC, Fecal Coliforms; FE, Fecal Enterococci; source: (Pepper et al., 2011).

2.3 Antibiotic resistance analysis

2.3.1 Ampicillin-resistant *Enterobacteriaceae*

The microbiological analysis of the soil and the water samples began with the determination of its microbial load. Soil samples were resuspended in a known volume of sterile saline solution (0.9% sodium chloride), stirred, and allowed to stand for 30 minutes before use. Water samples were vigorously homogenised. Decimal dilutions of the samples were prepared in sterile saline 0.9% NaCl, from which 100mL were filtered through cellulose membranes of 0.45µm (Millipore) and the filters placed on the surface of selective medium for *Enterobacteriaceae* VRBG (Violet Red Bile Glucose) agar (Oxoid, Hampshire, England) supplemented with 20mg/mL ampicillin (AppliChem, Darmstadt, Germany). After aerobic incubation of the plates for 24h at 37°C, all the *Enterobacteriaceae* isolates resistant to ampicillin (AMP^R) were counted. The colonies with different morphological types were selected, picked out three times and its purity.

2.3.2 Phenotype characterization

2.3.2.1 Antibiograms

A total of 57 cryopreserved isolates were used in the present investigation and were gathered in 2021 from different sampling sites. These isolates were provided by the research team of the GoLis project.

In summary, these isolates for phenotypic characterization 13 AB, had been cryopreserved after been selected, representing the main AB classes used in human medicine and livestock production in Portugal, namely: amoxicillin/clavulanic acid combination (AMC) 30µg/10µg, respectively; ceftazidime (CAZ) 30µg; cefotaxime (CTX) 30µg; cefpirome (CPO) 30µg; aztreonam (ATM) 30µg; cefoxitin (FOX) 30µg; imipenem (IPM) 10µg; meropenem (MEM) 10µg; chloramphenicol (CHL) 30µg; gentamicin (GEN) 10µg; ciprofloxacin (CIP) 5µg; trimethoprim/sulfamethoxazole (SXT) combination (1:19) and tetracycline (TET) 30µg. The Kirby-Bauer disk diffusion method had been performed in agreement with the guidelines for antimicrobial susceptibility tests defined by the Clinical Laboratory Standards Institute (CLSI, 2020). The isolates with a resistance phenotype against three or more structurally unrelated antimicrobial agents were defined as multidrug resistant (MDR) (Magiorakos et al., 2012).

2.3.2.3 Molecular characterization

2.3.2.3.1 DNA extraction

The screening for resistance genes was focused on a subset of isolates chosen according to their phenotypic profile of resistance. The presence of the most frequent *Enterobacteriaceae* resistance genes in these isolates was determined by different multiplex PCR. Therefore, total DNA of these isolates was extracted using two methods: part of isolates by GF-1 Bacterial DNA Extraction Kit (GF-1, Vivantis, Selangor, Malaysia) (Annex 2) and other part using a conventional extraction according Pitcher et al. (1989), where includes lysis by a enzymatic process, with lysozyme, and sarcosyl detergent and deproteinization with guanidine thiocyanate and phenol:chloroform:isoamilyc alcohol mixture (25:24:1). Ethanol precipitation and ethanol washing steps were made and the DNA samples were kept at -20ºC on ultra-pure water (Annex 3).

2.3.2.3.2 Multiplex PCR for genes detection

The DNA integrity was confirmed by agarose gel electrophoresis. A volume of 10µL of DNA was mixed with 2µL of loading buffer (Invitrogen; Life Technologies, California, USA) and resolved in a 1% (w/v) agarose gel (BioRad), at 25 V/cm for 1h. The 100 kb plus DNA *ladder* (Invitrogen; California, USA) was used as a molecular weight marker. DNA was visualized under a UV transillumination (Vilber Lourmat, France), after stainingwith ethidium bromide (125 µg/mL).

The search for 14 genes conferring three tetracycline resistance mechanisms, such as, efflux pump, ribosomal protection and tetracycline enzymatic alteration, was performed in two multiplex PCR: one for genes *tet*(A), *tet*(E), *tet*(G), *tet*(K), *tet*(L), *tet*(M), *tet*(O) and *tet*(S); and other for *tet*(B), *tet*(C), *tet*(D), *tet*(Q), *tetA*(P) and *tet*(X).

The detection of *sul1*, *sul2* and *sul3* genes conferring resistance to sulphonamide was performed by two multiplex PCR because of the need for a different annealing temperature for the detection of the *sul3* gene.

Two µL of each DNA sample was subjected to multiplex PCR in a 25µL reaction mixture containing 1x PCR buffer (200mM Tris-HCl, pH 8.4, 500mM KCl) and, according to the target gene, a variable concentration of primers (Table 2), MgCl2, dNTP and Taq DNA polymerase (0.5U, Invitrogen) (Table 3). The PCR conditions described by referenced authors in Table 2 were modified as specified in Table 3.

***Table 2** - Primers used for the identification of sulphonamides (sul) and tetracyclines (tet) resistance genes.*

Target gene	Primers sequences (5´- 3´) Fw/Rv	Amplicon size (bp)	Primers (µM)	Reference
***sul*1**	CGGCGTGGGCTACCTGAACG/GCCGATCGCGTGAAGTTCCG	433	0.4	Kerrn et al., 2002
***sul*2**	GCGCTCAAGGCAGATGGCATT/GCGTTTGATACCGGCTCCCGT	293	0.4	
***sul*3**	GAGCAAGATTTTGGAATCG/CATCTGCAGCTAACCTAGGGCTTTGGA	790	0.4	Perreten et al., 2003
***tet*(A)**	GCTACATCCTGCTTGCCTTC/CATAGATCGCCGTGAAGAGG	210	1.0	
***tet*(B)**	TTGGTTAGGGGCAAGTTTTG/GTAATGGGCCAATAACACCG	659	0.25	
***tet*(C)**	CTTGAGAGCCTTCAACCCAG/ATGGTCGTCATCTACCTGCC	418	0.25	
***tet*(D)**	AAACCATTACGGCATTCTGC/GACCGGATACACCATCCATC	787	2.0	
***tet*(E)**	AAACCACATCCTCCATACGC/AAATAGGCCACAACCGTCAG	278	1.0	
***tet*(G)**	CAGCTTTCGGATTCTTACGG/GATTGGTGAGGCTCGTTAGC	468	1.0	
***tet*(K)**	TCGATAGGAACAGCAGTA/CAGCAGATCCTACTCCTT	844	1.25	
***tet*(L)**	TCGTTAGCGTGCTGTCATTC/GTATCCCACCAATGTAGCCG	267	1.0	Ng et al., 2001
***tet*(M)**	GTGGACAAAGGTACAACGAG/CGGTAAAGTTCGTCACACAC	406	0.5	
***tet*(O)**	AACTTAGGCATTCTGGCTCAC/TCCCACTGTTCCATATCGTCA	515	1.25	
***tet*(S)**	CATAGACAAGCCGTTGACC/ATGTTTTTGGAACGCCAGAG	667	0.5	
***tet*A(P)**	CTTGGATTGCGGAAGAAGAG/ATATGCCCATTTAACCACGC	676	1.25	
***tet*(Q)**	TTATACTTCCTCCGGCATCG/ATCGGTTCGAGAATGTCCAC	904	1.25	
***tet*(X)**	CAATAATTGGTGGTGGACCC/TTCTTACCTTGGACATCCCG	468	1.25	

* Modified from Amador et al 2019.

***Table 3**- Multiplex PCR conditions for target genes.*

Target gene/group		Cycling conditions						Final concentrations		
		1st step[a]	Cycles	2nd step[b]	3rd step[c]	4th step[d]	5th step[e]	$MgCl_2$ (mM)	dNTP (µM)	*Taq* pol. (U)
sul	**1, 2**	94/5'	30x	94/15''	69/30''	72/60''	72/7'	2.0	200	0.5
sul	**3**				51/30''					
tet	**A, E, G, K, L, M, O, S**	94/5'	35x	94/60''	55/60''	72/90''	72/7'	3.0	300	2.5
tet	**B, C, D, A(P), Q, X**							4.0		

1st denaturation, b) 2nd denaturation, c) annealing, d) extension, e) final extension, all expressed with temperature in °C /time in minutes, ' or seconds, ''.

* Modified from Amador et al 2019.

Multiplex PCR were performed in a thermocycler (iCycler, Bio-Rad, Thermal Cycler, Hercules, CA, USA) and the amplification products obtained were separated by electrophoresis on a 1% agarose gel (BioRad) stained with ethidium bromide (125µg/mL) and visualized under a UV transillumination (Vilber Lourmat, France).

2.3.3 Identification of Bacteria

The species identification of isolates was carried out by VITEK 2 (BioMerieux) an automated microbiology system using colorimetric reagent cards. For this purpose, Gram-negative fermenting and non-fermenting bacilli cards (GN) were performed according to the manufacturer recommendations. In the first step, cell suspensions were prepared. With a sterile swab, enough colonies from a pure culture were transferred to a 12 x 75 mm clear polystyrene test tube containing 3.0 mL of sterile saline solution (0.45% to 0.50% aqueous NaCl, pH 4.5 to 7.0). Turbidity is adjusted according to the McFarland turbidity range, 0.50-0.63, and measured using a turbidity meter called the DensiChekTM. After this procedure, the cards are filled with these cell suspensions automatically by the apparatus and placed on a carousel, where the optical densities will be read at regular time intervals. Several metabolic activities are measured, such as acidification, alkalization, enzymatic hydrolysis, among others.

2.4. Crop monitoring

The irrigation volumes allocated to the pots were recorded, in parallel with the meteorological data provided by an automatic weather stations, installed near the experimental sites, with a set of sensors for air temperature and humidity, solar radiation, and wind speed, a Class A pan evaporimeter, and remote communication tool via GSM to the several data users.

After harvest, the samples with the aerial part of the plants were oven dried at 65°C, figure 5a), and the weight of straw, panicles and grain recorded separately. Number of panicles, weight of a thousand grains was also registered. A subsample of the rice grain (paddy) was mechanically husked in a rubber roller husker (bran), and a further subsample taken for bleaching in a polisher, i.e., for bran layer removal (white).

a)   b)

Figure 5- Photographs of the pos-harvest processing. a) the samples being oven dried at 65° C; b) measuring the length of the samples with aerial part.

3 Results and Discussion

3.1 Microbiological Parameters of Water Quality

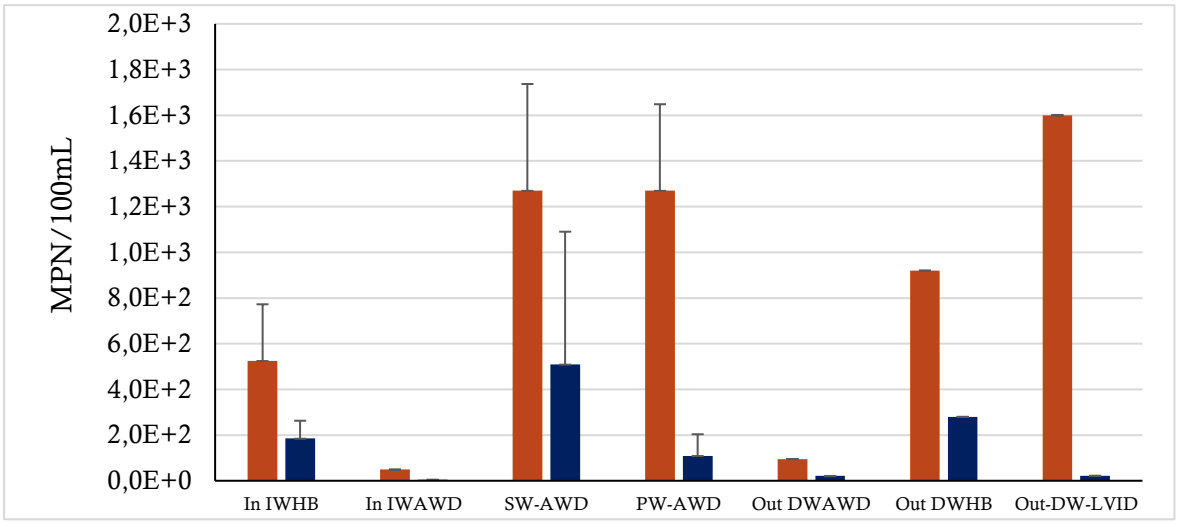
3.1.1 Fecal Contamination Bioindicators

The analysis of water microbiological quality, assessed through the enumeration of indicator groups at the main inlets and outlets of the paddies (Figures 6, 7 and 8), shows the quality of the water available for irrigation, as well as the effect of the rice ecosystems on the quality of drainage water.

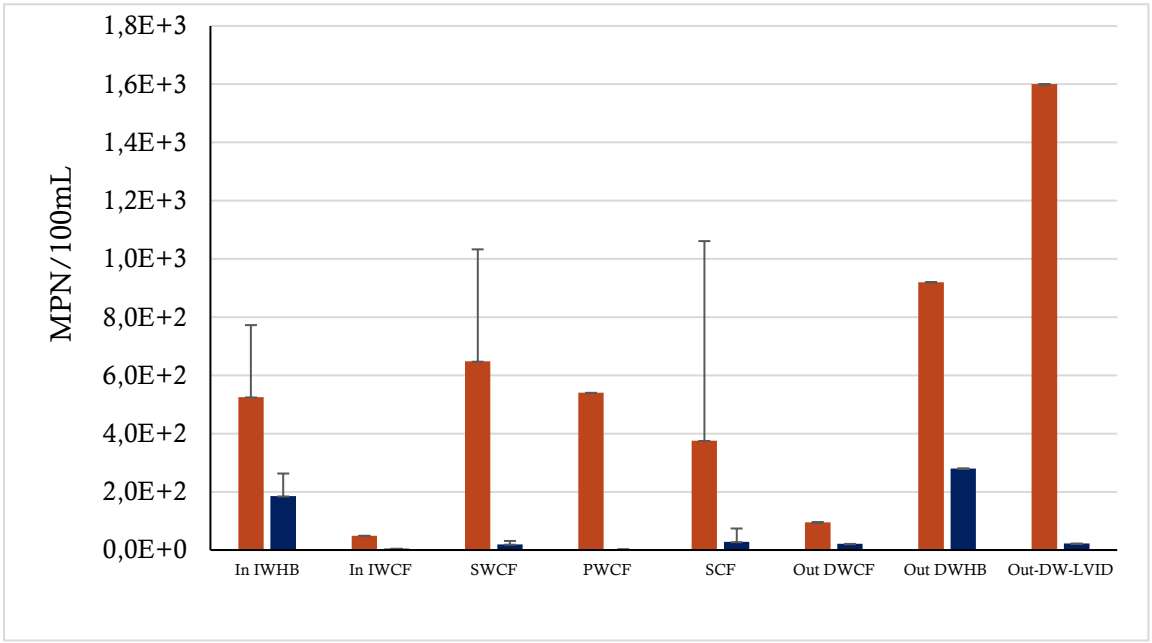
The Total Coliforms (**TC**) are widespread in the three paddies essayed with variable average counts among samples, being the counts in drainage samples 1.94 times higher than those in irrigation samples in the CF and AWD treatments (Figures 6A, 6B, 6C). Conversely, there was a drop in the TC counting's in SDI drainage water compared with the those at the paddies inlet, possibly due to the soil aerobic conditions that possibly unfavored the group of bacteria investigated. Nevertheless, those enumerations, both from irrigation and drainage water samples are below and in between the maximum recommended value (500 MPN/100 mL) and the maximum admissible value (10,000 MPN/100 mL) regarding aquaculture and bathing purposes (DL, 1989).

Fecal Coliforms (**FC**) counts in paddies' samples are higher in those from SDI treatment, then AWD, being below 28MPN/100mL in CF treatment (Figures 6A, 6B, 6C).

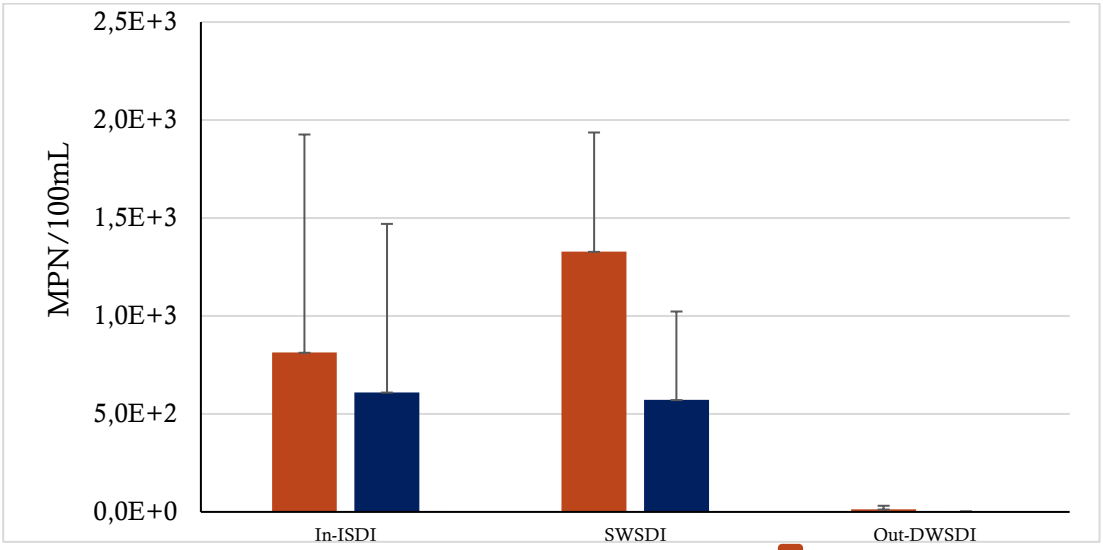
High average **FC** counts were registered in the inlet samples, namely in those collected at Ribeira da Aroeira (185MPN/100mL) and well water (610MPN/100mL). It is noteworthy that these **FC** counting's in the water available for irrigation are above 100MPN/100mL, set as the maximum recommended value, therefore not fulfilling the quality environmental objectives stablished for irrigation water (DL, 1989). These high values are explained by contamination from external sewage sources from irrigated area (Vieira et al., 2012). **FC** counts in the outlet water of SDI treatment dropped significantly compared with the inlet water, which might be explained by the soil effect on the reduction of *Enterobacteriaceae* numbers, (Tapias, 1997), and also due to rice soils are not being manure-amended. Considering aquaculture and bathing uses, the FC counts of effluent water are below the maximum recommended value (100MPN/100mL) (DL, 1989).


MPN/100mL
2,0E+3
1,8E+3
1,6E+3
1,4E+3
1,2E+3
1,0E+3
8,0E+2
6,0E+2
4,0E+2
2,0E+2
0,0E+0
In IWHB
In IWAWD
SW-AWD
PW-AWD
Out DWAWD
Out DWHB
Out-DW-LVID

A


MPN/100mL
1,8E+3
1,6E+3
1,4E+3
1,2E+3
1,0E+3
8,0E+2
6,0E+2
4,0E+2
2,0E+2
0,0E+0
In IWHB
In IWCF
SWCF
PWCF
SCF
Out DWCF
Out DWHB
Out-DW-LVID

B



C

Figure 6- *Average counts and standard deviation of Total Coliforms (orange bar and black line), Fecal Coliforms (blue bar and black line) per sampling site from treatments:* ***A****: AWD;* ***B****: CF;* ***C****: SDI.*

Legend of the sampling sites: ***A: In IWHB,*** Irrigation Water at the inlet of the Hydraulic Block; ***In IWAWD,*** Irrigation Water at the inlet of the paddies irrigated by Alternate Wet and Dryi ***SWAWD***, Soil Water of the paddies irrigated by Alternate Wet and Drying; ***PWAWD***, Piezometer Water of the paddies irrigated by Alternate Wet and Drying; ***Out DWAWD***, Drainage Water at the outlet of the paddies irrigated by Alternate Wet and Drying; ***Out DWHB***, Drainage Water at the outlet of the Hydraulic Block; ***Out DW LVID***, Drainage Water at the outlet of the Lis Valley Irrigation District. B: ***IWHB,*** Irrigation Water at the inlet of the Hydraulic Block; ***In IWCF,*** Irrigation Water at the inlet of the paddies irrigated by Continuous Flooding; ***SWCF***, Soil Water of the paddies irrigated by Continuous Flooding; ***PWCF***, Piezometer Water of the paddies irrigated by Continuous Flooding; ***SCF***, Soil of the paddies irrigated by Continuous Flooding; ***Out DWCF***, Drainage Water at the outlet of the paddies irrigated by Continuous Flooding; ***Out DWHB***, Drainage Water at the outlet of the Hydraulic Block. ***C: In IWSDI,*** Irrigation Water at the inlet of the paddies irrigated by Surface Drip Irrigation; ***SW SDI***, Soil Water of the paddies irrigated by Continuous Flooding; ***Out DWSDI***, Drainage Water at the outlet of the paddies irrigated by Surface Drip Irrigation. **Legend of the Irrigation treatments: AWD:** Alternate Wet and Drying; **CF:** Continuous Flooding; **SDI:** Surface Drip Irrigation.

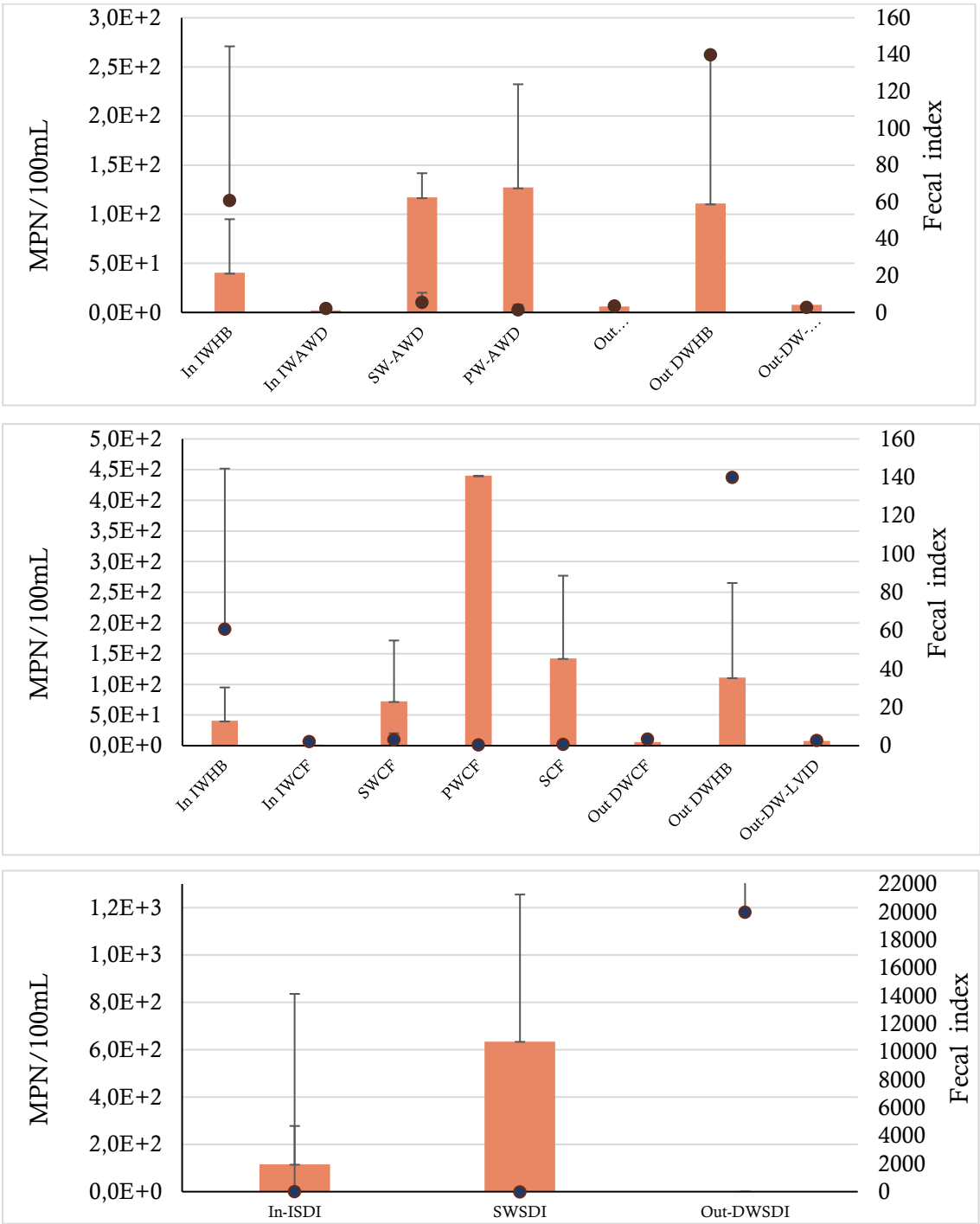


Figure 7- *Average counts and standard deviation of Fecal Enterococci , (salmon bar and black line); Fecal Index, (brown dot) per sampling site from treatments: A: AWD; B: CF; C: SDI.*

Legend: *cf*. Figure 6.

Regarding the Fecal Enterococci **(FE)** (Figures 7A, 7B, 7C), the higher average counts were recorded in the paddies regardless the treatment. The **FE** counts in the outlet water were lower than those at paddies, being below the maximum recommended value (100MPN/100mL), considering aquaculture and bathing uses of LVID effluent water (DL, 1998).

The analysis of the Fecal Index (**FI**), (Figures 7A, 7B, 7C), revel that the water available for irrigation in all treatments thus, entering the agroecosystem, has evidences fecal contamination of human origin. Differently, the samples from the paddies (AWD and SDI) have evidence fecal contamination of animal waste in mixed pollution, possibly related with the use of animal manure as soil fertilizer. The samples from the **CF** paddies have evidence with wild animal pollution (Pepper et al., 2011). In fact, this **CF** 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.

The mean counts of *Enterobacteriaceae* isolates resistant to ampicillin (**Amp^r**) showed high incidence (Figures 8A, 8B, 8C), with particularly high enumerations in paddies samples. The samples with higher mean counts were collected in the soil and soil water of the three essays, with average counts two or three order of magnitudes higher than the other samples.

The frequent resistance to antibiotics found in the aquatic systems, namely to the *β-lactams* (ex. ampicillin), is an additional critical point associated with fecal contaminants, placing the water quality and public health in a critical situation. The identification of situations 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 will also need to be assessed for the real influence of agricultural activity within the irrigation district, particularly at the drainage network level.

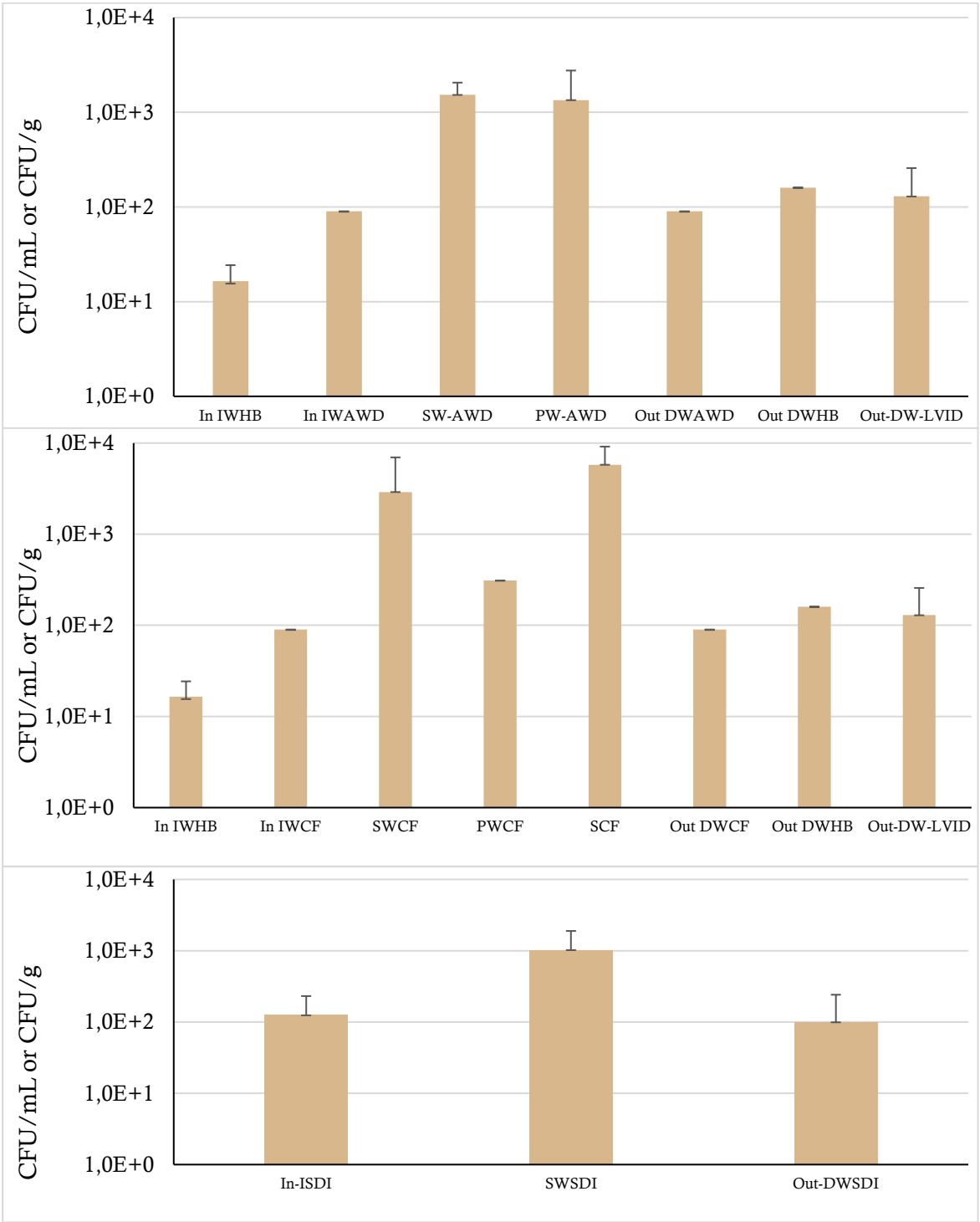


***Figure 8**- Average counts and standard deviation of Amp^r Enterobacteriaceae (light brown bar and black line) per sampling site from treatments: A: AWD; B: CF; C: SDI.*

Legend: *cf.* Figure 6.

3.1.2 Antibiotic Resistance

3.1.2.1 Phenotypic Characterization

Fifty seven isolates were selected among all samples of two treatments under study for this analysis, CF and SDI.

The 57 isolates obtained showed high resistance to most of the antibiotics tested (Figure 9). Among the ß-lactams, penicillin's (amoxicillin/clavulanic acid), 2nd generation cephalosporins (FOX), 3rd generation cephalosporins (CAZ and CTX) and 4th generation (CPO) were the AB for which more resistances was found.

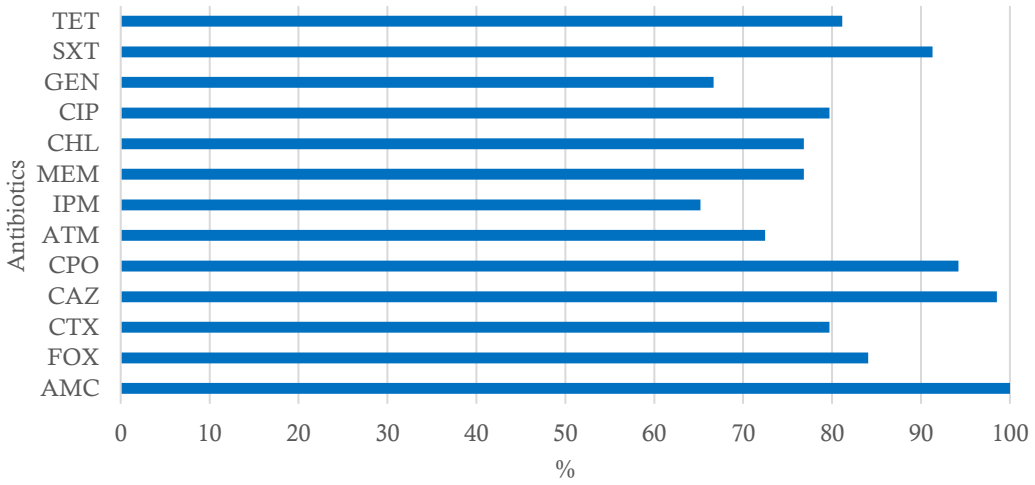


Figure 9- *Total percentage of the antibiotic resistant isolates for both treatments. Legend: (antibiotics): AMC, amoxicillin/clavulanic acid; FOX, cefoxitin; CTX, cefotaxime; CAZ, ceftazidime; CPO, cefpirome; ATM, aztreonam; IPM, imipenem; MEM, meropenem; CIP, ciprofloxacin; GEN, gentamicin; CHL, chloramphenicol; SXT, trimethoprim / sulfamethoxazole; TET, tetracycline.*

Regarding the non-ß-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.

The frequencies of AB resistance by sample origin (Table 4) reveals a higher prevalence of resistance for all AB in the isolates obtained from the Aroeira stream samples (In IWHB). This River provides the irrigation water to this hydraulic block, and the Rio Negro (Out DWHB) receives the drainage water from the entire hydraulic block, where the experimental plots for treatments CF were set.

***Table 4** - Isolates resistant percentage to the ß-lactam and non ß-lactam classes by sample origin from the continuous flooding treatment.*

Antibiotics		Sampling sites					
		In IWHB	In IWCF	SWCF	PWCF	Out DWCF	Out DWHB
ß-lactam	AMC	100	100	100	100	100	100
	FOX	100	60	80	100	90	100
	CTX	100	40	60	100	90	100
	CAZ	100	90	100	100	100	100
	CPO	100	90	70	100	100	100
	ATM	100	40	50	78	60	100
	IPM	100	80	40	11	70	100
	MEM	75	60	60	44	80	100
non ß-lactam	CHL	100	40	40	78	80	100
	CIP	100	60	70	67	60	100
	GEN	100	40	70	22	70	80
	SXT	100	60	90	100	100	100
	TET	100	60	40	89	80	100

Legend: (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; (sampling sites): In IWHB, In Irrigation Water Hydraulic Block; In IWCF, In Irrigation Continuous Flooding; SWCF, Soil Water Continuous Flooding; PWCF, Piezometer Water Continuous Flooding; Out DWCF, Out Drainage Water Continuous Flooding; Out DWHB, Drainage Water Hydraulic Block.

The isolates from the irrigation water samples of **CF** treatment (In IWCF), showed lower percentages of resistance, especially to non-ß-lactam AB, when compared to the isolates from the respective drainage water samples (Out DWCF). This fact 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, which drainages are all collected into the Rio Negro (Out IWHB). Therefore, the sample Out IWHB represent the resistance determinants that occur in the entire Hydraulic block, as Rio Negro works as a collector for all of them.

There is also a high resistance to ß-lactams for the isolates from the sample collected in the piezometer (PWCF), which represents a non-surface water. This high prevalence of isolates resistant to ß-lactams, especially to penicillin's and 2nd, 3rd and 4th generation cephalosporins is probably due to the use and abuse of this class of antibiotics for many years in livestock for the treatment of infections and as a growth promoter.

The isolated from the drip irrigation treatment (SDI) showed higher resistance percentages for both ß-lactams and non-ß-lactams than those from the CF treatment, exception for FOX, CTX and IPM. 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 (Table 5).

Table 5- *Isolates resistant percentage to the β-lactam and non β-lactam classes by sample origin from the drip irrigation treatment.*

Antibiotics		Sampling sites		
		In IWSDI	SWSDI	Out IWSDI
β-lactam	AMC	100,0	100,0	100,0
	FOX	70,0	90,0	70,0
	CTX	70,0	100,0	70,0
	CAZ	100,0	100,0	100,0
	CPO	100,0	100,0	100,0
	ATM	80,0	100,0	80,0
	IPM	100,0	50,0	100,0
	MEM	100,0	90,0	100,0
non β-lactam	CHL	100,0	100,0	100,0
	CIP	100,0	100,0	100,0
	GEN	100,0	80,0	100,0
	SXT	100,0	90,0	100,0
	TET	100,0	100,0	100,0

Legend: (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 / sulfamethoxazol; TET, tetracycline.

The samples that showed the greatest diversity in species were those collected in the Negro River, which, as previously stated, collects the drainage water from the entire hydraulic block, with rice paddies, and other crops, such as forage where manure is directly applied, and direct grazing is practiced.

Considering the 33 identified bacteria, isolated from the hydraulic block samples, the most prevalent species were not from the *Enterobacteriaceae* family, despite having been isolated in a selective medium for this family. The most frequent species were: *Aeromonas hydro/caviae* (21%), *Pseudomonas putida* (12%) and *Acinetobacter braakii* (12%) (Table 6). The higher prevalence of these Gram-negative bacteria is possibly due to their comfort in soil and water, as these are their natural habitat, and therefore have the optimal conditions to survive in these environmental conditions, unlike the *Enterobacteriaceae* that have the gastrointestinal tract as their natural habitat. Despite this, 27.3% of the species identified belong to the *Enterobacteriaceae* family, with the genus *Citrobacter* being the most frequent, followed, in decreasing order, by the genera *Serratia, Escherichia, Klebsiella and Enterobacter*.

Table 6- *List of AB resistance profile, AB resistance genes harbored per isolate species and sample origin from the continuous flooding treatment.*

Samples	Species	Resistance Phenotype														AB resistance genes	
		AMP	AMC	FOX	CTX	CAZ	CPO	ATM	IPM	MFM	CHL	CIP	GEN	SXT	TET	*sul*	*tet*
In IWHB	*Serratia marcescens*	X	X	X	X	X	X		X						X	___	____
	Serratia marcescens	X	X	X	X	X	X	X	X						X	___	G
	Acinetobacter haemolyticus	X	X	X	X	X	X	X	X	X	X	X	X	X	X	2	____
	Ralstonia insidiosa	X	X	X	X	X	X	X	X	X	X	X	X	X	X	2	A, M, L
	Escherichia coli	X	X	X	X	X	X	X	X		X	X	X	X	X	2	G
	Acinetobacter haemolyticus	X	X	X	X	X	X	X	X	X	X	X	X	X	X	2	A, M
	Aeromonas sobria	X	X		X	X	X	X	X	X	X	X	X	X	X	2	A, E, O
	Acinetobacter haemolyticus	X	X	X	X	X	X	X	X	X	X	X	X	X	X	2	L, M
In IWCF	*Aeromonas hydro/caviae*	X	X			X	X		X	X	X	X	X	X	X	2	A
	Aeromonas hydro/caviae	X	X	X	X	X	X	X	X	X		X	X	X	X	____	____
	Citrobacter freudii	X	X	X	X	X	X	X	X	X	X	X	X		X	____	____
	Aeromonas sobria	X	X			X	X		X	X	X	X		X	X	1,2	G, X
SWCF	*Pseudomonas putida*	X	X	X	X	X	X	X		X	X			X		1	D
	Pseudomonas putida	X	X	X	X	X	X	X		X	X	X	X	X	X	1	L
	Não identificada	X	X	X	X	X	X		X	X	X	X	X	X	X	____	A, A(P), L, O
	Pseudomonas putida	X	X	X	X	X	X	X		X	X	X	X	X	X	____	A, L
	Aeromonas hydro/caviae	X	X	X		X	X		X	X	X	X	X	X		____	A, L, O
PWCF	*Vibrio fluviae*	X	X	X	X	X	X	X			X			X	X	1, 2	A
	Aeromonas hydro/caviae	X	X	X	X	X	X		X	X	X	X	X	X	X	1, 2	A, M
	Klebsiella pneumoniae pneumoniae	X	X	X	X	X	X			X	X	X		X	X	____	____
Out DWCF	*Aeromonas hydro/caviae*	X	X	X	X	X	X		X	X	X		X	X	X	____	____
	Não identificada	X	X	X	X	X	X	X	X	X	X	X	X	X	X	____	A
	Citrobacter braakii	X	X	X	X	X	X	X	X	X	X	X	X	X	X	____	A, L
	Citrobacter braakii	X	X	X	X	X	X	X	X	X	X	X	X	X	X	2	A
	Aeromonas hydro/caviae	X	X	X	X	X	X		X	X	X	X	X	X	X	____	A
Out DWHB	*Acinetobacter haemolyticus*	X	X	X	X	X	X	X		X	X			X		2	G, Q, C
	Aeromonas hydro/caviae	X	X	X	X	X	X	X	X	X	X			X		2	____
	Klebsiella oxytoca	X	X	X	X	X	X	X	X	X	X	X	X	X	X	____	A
	Pseudomonas putida	X	X	X	X	X	X	X	X	X	X	X	X	X	X	2	A, B, C, M
	Citrobacter braakii	X	X	X	X	X	X	X	X	X	X	X	X	X	X	1,2	A
	Escherichia coli	X	X	X	X	X	X	X	X	X	X	X	X	X	X	2	A, X, O, S
	Enterobacter cloacae complex	X	X	X	X	X	X	X	X	X	X	X	X	X	X	2	____
	Sphingomonas paucimobilis	X	X	X	X	X	X	X	X	X	X	X	X	X	X	2	____

Legend: (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 / sulfamethoxazol; TET, tetracycline; (sampling sites): In IWHB, In Irrigation Water Hydraulic Block; In IWCF, In Irrigation Water Continuous Flooding; SWCF, Soil Water Continuous Flooding; PWCF, Piezometer Water Continuous Flooding; Out DWCF, Out Drainage Water Continuous Flooding; Out DWHB, Drainage Water Hydraulic Block.

Regarding the isolates collected from the SDI treatment samples (Table 7), the genus *Aeromonas* was the most prevalent, followed by the *Enterobacter cloacae* complex and *Pantoea* spp. The samples *In IWSDI* and *Out IWSDI* contained less diversity of species. An explanation for this result is possibly related to the hydraulic conditions of this experimental plot. Here water is pumped from a nearby well for rice irrigation (SDI), percolating the excess, ending up back into the well, thus being reused in a closed circuit.

Table 7- *List of AB resistance profile, AB resistance genes harbored per isolate species and sample origin from the surface drip irrigation treatment.*

Samples	Species	Resistance Phenotype														AB resistance genes	
		AMP	AMC	FOX	CTX	CAZ	CPO	ATM	IPM	MEM	CHL	CIP	GEN	SXT	TET	*sul*	*tet*
In IWSDI	*Aeromonas hydro/caviae*	X	X	X	X	X	X	X	X	X	X	X	X	X	X	1	____
	Aeromonas sobria	X	X			X	X		X	X	X	X	X	X	X	____	____
	Aeromonas hydro/caviae	X	X	X	X	X	X	X	X	X	X	X	X	X	X	____	A
	Aeromonas hydro/caviae	X	X	X	X	X	X	X	X	X	X	X	X	X	X	____	A
	Aeromonas hydro/caviae	X	X	X	X	X	X	X	X	X	X	X	X	X	X	____	A
SWSDI	*Pseudomonas putida*	X	X	X	X	X	X	X		X	X	X	X	X	X	____	A, L
	Pantoea spp.	X	X		X	X	X	X	X	X	X	X	X	X	X	____	____
	Enterobacter cloacae complex	X	X	X	X	X	X	X	X	X	X	X	X	X	X	____	A
	Enterobacter cloacae complex	X	X	X	X	X	X	X	X	X	X	X	X	X	X	2	____
	Pantoea spp.	X	X	X	X	X	X	X		X	X	X	X	X	X	____	____
Out IWSDI	*Aeromonas hydro/caviae*	X	X	X	X	X	X	X	X	X	X	X	X	X	X	1	____
	Aeromonas sobria	X	X			X	X		X	X	X	X	X	X	X	____	____
	Aeromonas hydro/caviae	X	X	X	X	X	X	X	X	X	X	X	X	X	X	____	A
	Aeromonas hydro/caviae	X	X	X	X	X	X	X	X	X	X	X	X	X	X	____	A
	Aeromonas hydro/caviae	X	X	X	X	X	X	X	X	X	X	X	X	X	X	____	A

Legend: In IWSDI: , In Irrigation Water Subsurface Drip Irrigation, SWSDI: Soil Water Subsurface Drip Irrigation, Out IWSDI: Out Irrigation Water Subsurface Drip Irrigation.

The resistance phenotypes of all the 57 isolates characterized revealed a prevalence of 84.1% of multidrug-resistant bacteria (MDR). An isolate is considered multidrug resistant when it presents the resistance phenotype against three or more structurally unrelated antimicrobial agents (Magiorakos et al., 2012).

It is worth noting that all the isolates obtained from the SDI treatment were MDR. Likewise, all isolates from the Aroeira stream, as well as those from the Rio Negro were MDR. Probably the justification for these results has to do with the characteristics of these two sampling sites, both collect drainage water from various areas of the Lis valley, where different cultural activities are practiced, which allows all resistance to be accumulated. The *In IWCF* site fewer MDR isolates were recorded (60%), followed by the *SWCF* site (70%).

3.1.2.2 Genotypic Characterization

The screening of the 14 most frequent *tet* genes conferring resistance to TET revealed that genotypes are consistent with the phenotypes observed, except for eight isolates (Table 6). The TET resistance in the isolates without *tet* genes, might be due to other resistance genes, not targeted in this study. There are at least 47 distinct genes identified, responsible for four main mechanisms by which the bacteria acquire resistance to tetracyclines (Nguyen et al., 2014). On the other hand, there are three isolates not showing phenotypic resistance, but harbouring resistance genes. In this case, the antibiogram should be repeated to confirm these results.

The most incident genes were *tet*(A), tet(L) and *tet*(M) (Table 6). The prevalence of *tet*(A) gene in this study agrees with the dominant *tet* gene type in different animal and environmental samples reported worldwide in a myriad of studies (Karczmarczyk et al, 2011; McNeece, et al 2014; Zhang, et al 2015).

Once again, the samples at the inlet and outlet of the Hydraulic Block present a greater diversity of tetracycline resistance genes in the same isolate. These accumulation of genes in one isolate is possibly favoured by the great diversity of bacteria at these sites.

The most prevalent resistance genes to the sulphonamide class were *sul*2, followed by *sul*1 (Table 7). Additionally, no isolates harboured *sul*3. This ranking varies according to the sample source. These genes prevalence can vary greatly with the matrixes, as well as the species. These results are in accordance with those of a previous study carried out in the Centre of Portugal, where the genes *sul*1 and *sul*3 were more frequent than *sul*2 among poultry farm isolates. Curiously, the isolates from dairy farms presented a higher incidence of *sul*1, whereas *sul*3 prevailed in slaughterhouses isolates and *sul*2 in those from pig farms (Amador et al., 2019). In this way, the results obtained in the present study, with prevalence of *sul*2 might be explained by the presence of intensive pig farms in the surrounding areas of the Lis Valley.

3.2 Crop and water productivity

The average values of water production and productivity are presented in Figure 10.

Rice production in 2021 was lower than the potential production of Ariete variety, which is in between 6000 and 7500g/ha (Almeida et al., 2021; Figueiredo et al., 2013). The production of rice fields irrigated by SDI was significantly lower than that of CF and AWD treatments. This pattern is accompanied by the weight of a thousand grains (Figure 10A).

The opposite situation is found in straw production, where significantly higher values were produced in rice fields irrigated by SDI (Figure 10B). Regarding water productivity, it was higher in rice fields irrigated by SDI, followed by AWD and CF, as expected (Figure 10C).

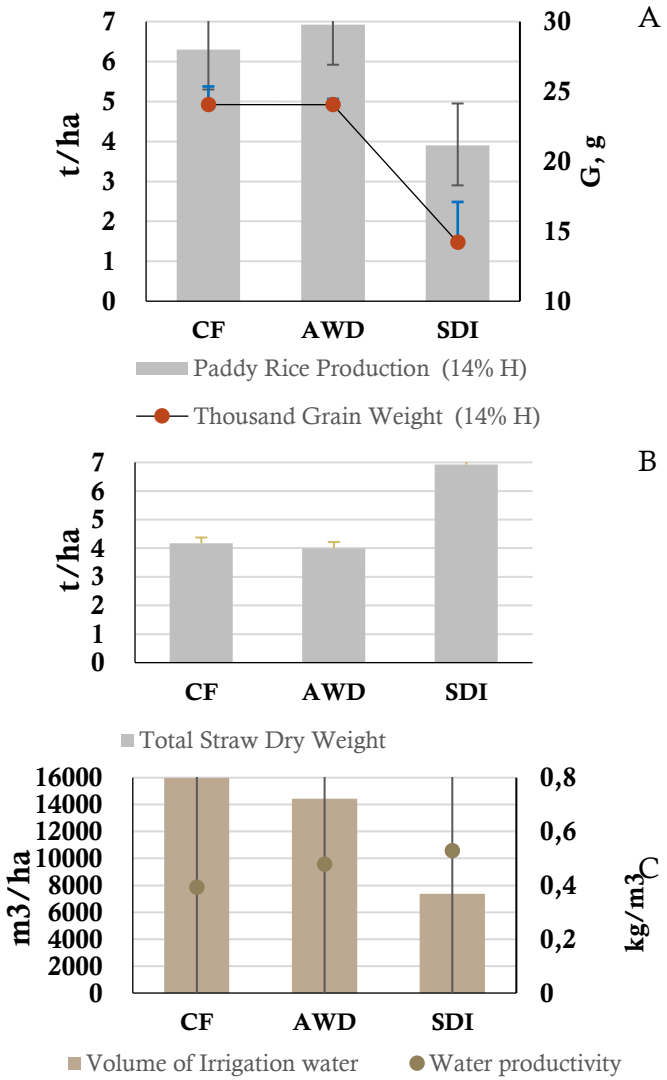


***Figure 10**- Average values and standard deviation of: **A**, yield, t/ha at 14% humidity of paddy rice (light grey bar and black line), and thousand grain weight, g (●); **B**, straw dry weight, t/ha (grey bar and red line); **C**, irrigation water and water productivity of rice produced under the Irrigation systems: **CF**, continuous flooding; **AWD**, alternate wet and drying; **SDI**, superficial drip irrigation.*

4. Conclusions

The results of this experimental study allowed assessing the microbiological quality of water in rice farming systems, both in terms of classical bioindicators and of antibiotic resistance analysis.

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, both in CF and AWD, 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 relatively to the irrigation entrance, proved a positive impact of these rice irrigation systems on this bioindicator (FC). As for fecal enterococci, their counts increased significantly inside the soil water of rice fields, however at the outlets, a decrease in these counts was recorded. 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, which does not fulfil the maximum recommended value of 100MPN/100mL of FC, the quality bioindicator parameter provided by the law. Besides, the irrigation water has evidence of fecal contamination of human origin. In addition, rice fields show evidence of pollution from wild animals, which is also an environmental service, as the flooding irrigation system, and eventually the AWD, in varying stages of flooding and drainage, provide habitat and food for several species of birds. As a conclusion regarding these indicators, it is shown that in the analysed systems, rice production systems in flooded level basins have a significant purifying effect on water quality, contributing to the improvement of collective drainage water.

The analysis of the classical indicators in the drip irrigation treatment led to similar conclusions as for the previous treatments, except for the parameter total coliforms, which enumerations decrease between the irrigation water and the internal soil drainage water, possibly due to the effect of soil aerobic activity, prevalent in this cropping system.

Regarding the antibiotic resistance analysis focused on flooding irrigation in level basins systems, as well as the drip irrigation trial, 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 antibiotic resistance phenotypes to all 13 antibiotics assayed are widely disseminated in these agroecosystems and are also highly prevalent in samples taken from both inlets and outlets, with a high percentage of the multidrug resistant isolates. The search for *tet* and *sul* genes revealed the prevalence of *tet*(A), *tet*(L), *tet*(M) and *sul*2. Therefore, it can be considered that the quality of irrigation, regarding these parameters, not included in the legislation, might endanger the farmer health and that rice ecosystems act as a sink for dissemination of AB genetic determinants.

In summary, 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, where and when microbiological contamination of water is at greatest risk. Based on the knowledge obtained, it is proposed that the sampling for analysis incorporates the irrigation water management plans, with a sampling frequency according to the sources of pollution.

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ANNEX 1

Bacterial growth media for preparatory cultures:

Ringer's Solution (2.25g/L of sodium chloride; 0.105g/L of potassium chloride; 0.12g/L of hydrated calcium chloride; 0.05g/L of sodium hydrogen carbonate; 1000mL of distilled water); provider: OXOID.

Brilliant Green Lactose Bile Broth (BGLB), (10.0g/L of peptone; 20.0g/L of Oxgall; 10g/L of lactose; 13,3mg/L of brilliant green); provider: HIMEDIA.

Peptone Broth (PB) (5.0g/L of meat extract, provider: HIMEDIA; 15.0g/L of peptone, provider: HIMEDIA; 5.0g/L of sodium chloride); provider: SIGMA-ALDRICH.

BAGG Broth (20.0g/L of tryptose; 5.0g/L of dextrose; 4.0g/L of dibasic potassium phosphate; 1.5g/L of monobasic potassium phosphate; 5.0g/L of sodium chloride; 0.5g/L of sodium azide; 0.015g/L of bromocresol purple); provider: HIMEDIA.

Plate Count Agar (PCA) (5.0g/L of digested casein; 2.5g/L of yeast extract; 1.0g/L of dextrose; 15.0g/L of agar; pH=7,0±0,2 a 25ºC); provider: HIMEDIA.

Violet Red Bile Glucose (VRBG) Agar (7.0g/L of enzymatic digest of animal tissues; 3.0g/L of yeast extract; 1.5g/L of bile salts; 5.0g/L of sodium chloride; 0.03g/L of neutral red; 0.002g/L of crystal violet; 10.0g/L of glucose; 12.0g/L of agar); provider: HIMEDIA.

Mueller-Hinton Agar (2.0g/L of beef extract; 17.5g/L of acid hydrolysate of casein; 1.5g/L of starch; 17.0g/L of agar); provider: HIMEDIA.

- **Luria-Bertani Medium Broth** (LB; 10.0g/L tryptone from casein, 5.0g/L yeast extract, 5.0g/L sodium chloride); provider: HIMEDIA.

- **2xYT Medium Broth** (2Xyt; 16.0g/L tryptone from casein, 10.0g/L yeast Extract, 5.0g/L sodium Chloride); provider: SIGMA.

- **Tryptic Soy Agar** (TSA; 15.0g/L of enzymatic digest of casein; 5g/L of enzymatic digest of soybean; 5.0g/L of sodium chloride; 15.0g/L of agar); provider: SIGMA.

- **TBE** (50mmol/L of Tris-Borate-EDTA buffer; 45mmol/L of boric acid; 0.5mmol/L of EDTA); provider: SIGMA.

Stock solutions:

- GES solution (5M of guanidine thiocyanate; 100mM of EDTA; 0.5% of sarcosyl); provider: HIMEDIA.

- TAE buffer, 50X stock solution in 1L of distilled H2O (100mL of 0.5M of EDTA, pH 8.0; 242g of Tris-acetate base; 57.1 mL of acetic acid glacial).

- RNase (20mg/mL) and lysozyme (50mg/mL).

- NH_4Ac (10 M), NaAc (3 M).

ANNEX 2

GF-1 Bacterial DNA Extraction Kit

Procedures

Reminder

- All steps are to be carried out at room temperature unless stated otherwise.
- Wash Buffer (concentrate) has to be diluted with absolute ethanol before use. Please refer to Reconstitution of Solutions.
- If precipitation forms in Buffer BG, incubate at 55°C - 65°C with occasional mixing until completely dissolved.

Pre-set waterbath to 37°C and the second waterbath to 65°C.

Pre-heat Elution Buffer to 65°C (optional).

1. **Centrifugation Pellet:** 1 - 3ml of bacteria culture grown overnight or culture grown to log phase by centrifugation at 6,000 x g for 2 min at room temperature. Decant the supernatant completely. Thorough removal of supernatant is essential as residual culture media may affect both yield and purity.

2. **Resuspension of pellet:** Add 100µl Buffer R1 to the pellet and resuspend the cells completely by pipetting up and down. Ensure complete cell resuspension. Lysis will not occur if clumps of bacteria remain following an inefficient resuspension procedure.

3. **Lysozyme treatment:** For Gram-negative bacteria strains, add 10µl lysozyme (50mg/ml) into the cell suspension. For Gram-positive bacteria strains, add 20µl lysozyme (50mg/ml) into the cell suspension. Mix thoroughly and incubate at 37°C for 20 min. Some bacterial strains may require longer incubation time in lysozyme.

4. **Centrifugation Pellet:** Digested cells by centrifugation at 10,000 x g for 3 min. Decant the supernatant completely.

5. **Protein denaturation:** Resuspend pellet in 180 µl of Buffer R2 and add 20 µl of Proteinase K. Mix thoroughly. Incubate at 65°C for 20 min in a shaking waterbath or with occasional mixing every 5 min. Lysate should be clear at the end of incubation or else extend the incubation time to 30 min.

Optional: Removal of RNA If RNA-free DNA is required, add 20 µl of RNase A (DNase-free, 20mg/ml). Mix and incubate at 37°C for 5 min. Residual RNA fragments will be removed during column washing.

6. **Homogenization:** Add 2 volumes (~400 µl without RNase A treatment, ~440 µl with RNase A treatment) of Buffer BG and mix thoroughly by inverting tube several times until a homogeneous solution is obtained. Incubate for 10 min at 65°C.

7. **Addition of Ethanol:** Add 200 µl of absolute ethanol. Mix immediately and thoroughly. Mix immediately to prevent uneven precipitation of nucleic acid due to high local ethanol concentrations.

8. **Loading to column:** Transfer the sample (max.650μl) into a column assembled in a clean collection tube (provided). Centrifuge at 10,000 x g for 1 min. Discard flow through. If column clogs, add 200μl Buffer BG into column and centrifuged as above.

9. **Column washing:** Wash the column with 650 μl of Wash Buffer and centrifuge at 10,000 x g for 1 min. Discard flow through. Ensure that ethanol has been added into the Wash Buffer before use (refer to Reconstitution of Solutions).

10. **Column drying:** Centrifuge the column at 10,000 x g for 1 min to remove residual ethanol. This step has to be carried out to remove all traces of ethanol as residual ethanol can affect the quality of DNA and may subsequently inhibit enzymatic reactions.

11. **DNA elution:** Place the column into a clean microcentrifuge tube. Add 50 - 100 μl of preheated Elution Buffer, TE buffer or sterile water directly onto column membrane and stand for 2 min. Centrifuge at 10,000x g for 1 min to elute DNA. Store DNA at 4°C or -20°C. Ensure that the Elution Buffer is dispensed directly onto the center of the membrane for complete elution. TE Buffer can also elute DNA although EDTA may inhibit subsequent enzymatic reactions. If water is used for eluting DNA, maximum elution efficiency is achieved between pH 7.0 and 8.5. Store DNA at -20°C as DNA may degrade in the absence of a buffering agent.

ANNEX 3

Extraction of genomic DNA – cited by Pitcher et al., 1989

Procedure

In order to protect the nucleic acids from the endonucleases existing on our hands, it is important to use gloves when lysing the cells.

0. Grow the cells overnight in LB medium at 37°C with agitation.

1. Harvest the bacterial cells (2 mL) by centrifugation at 8000 rpm for 10 minutes, discard the supernatant, and solubilize the pellet with 1 mL TE (epp 2 mL)

2. Centrifuge again at 8000 rpm for 10 minutes, solubilize the pellet with 1 mL TE. Ensure that the pellet is completely resuspended but do not use the vortex.

3. Repeat the centrifugation and discard the supernatant.

4. Resuspend in 250 µL of TE with lysozyme (20mg/mL). Incubate in a 37°C waterbath for 1 to 2 h.

5. Add 500 µL of GES reagent, shake by inversion and place on ice for about 5 to 10 min (confirm lysis by checking that the solution becomes transparent).

6. Add 250 µL of cold 10 M NH_4Ac and place on ice 10 min; add 1 mL of the 25:24:1 mixture of phenol/chloroform/isoamyl alcohol and mix by inversion.

7. Centrifuge at maximum speed for 10 min and recover the supernatant to a new tube (epp 2mL). Be careful not to entraining interphase.

8. Add an equal volume of cold isopropanol by inversion (a balloon should be observed).

9. Roll up the nucleic acid pellets with a loop or, if no pellets are observed, centrifuge (v. Max, 10 min) and wash the pellet with 1 mL of 70% ethanol.

10. After 5-10 min of drying, solubilize the nucleic acids in 500 µL of TE with RNase (50 µg /mL). Incubate in a 37°C bath for 30 min).

11. Add 500 µL of phenol/chloroform/isoamyl alcohol, mix by inversion and centrifuge at maximum speed for 10 min).

12. Remove the supernatant to a new tube (epp 2 mL) and add 1/10 volume of cold 3 M NaAc pH 5.2. Mix by inversion.

13. Add 2.5 volumes of cold (-20°C) absolute ethanol and mix by inversion.

14. Centrifuge (v. max, 10 min) or roll the pellet with the loop. Wash the novel or pellet with 1 mL of 70% ethanol.

15. After 5 to 10 min of drying solubilize in 100 µL of TE.

16. Observe a 5 µL aliquot on an agarose gel to assess the quality of the DNA in terms of average fragment size obtained.