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RESEARCH PAPER

Can the irrigation of soils with Amoxicillin-enriched water cause the proliferation of Bacteria resistant to antibiotics among culturable heterotrophic aerobic soil bacteria?

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هل يمكن أن يؤدي ري التربة بالمياه الغنية بالمضاد الحيوي الأموكسيسيلين (Amoxicillin) إلى انتشار البكتيريا المقاومة للأموكسيسيلين بين بكتيريا التربة الهوائية غير ذاتية التغذية (البكتيريا العضوية)؟

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ABSTRACT. This study investigated the short-term impact of irrigation with Amoxicillin solutions on the presence of the amoxicillin-resistance trait among culturable soil heterotrophic aerobic bacteria. The microcosm experimental design consisted of 15 days of incubation of 10 g soil samples irrigated daily with distilled water containing increasing doses of amoxicillin (0, 0.1, 1, 10, 100, 1000 µg g^{-1} of soil day^{-1}). The hypothesis was that continuous daily addition of antibiotics would increase the proportion of antibiotic-resistant bacteria in soils. After the incubation period, the total and antibiotic resistance heterotrophic aerobic bacteria communities were assessed through serial dilution of soil suspensions, followed by agar plate culture enumeration, isolation, identification and microscopy observation. The presence of antibiotic-resistant bacteria was also evaluated directly on treated wastewater used for field irrigation before this microcosm study to assess the amoxicillin-resistant bacteria bioaugmentation hypothesis. Results indicated that the Amoxicillin resistance was widespread among bacteria present in both treated wastewater used for irrigation and in the receiving soil. A microcosm experiment was attempted as a 'proof of concept' to demonstrate that irrigation with treated wastewater containing antibiotics would exert selective pressure and promote the proliferation of antibiotic resistance bacteria. Unexpectedly, the results from the microcosm incubations indicated the daily addition of amoxicillin did not increase bacterial antibiotic resistance trait abundance in soils, which even significantly decreased for all tested doses. The antibiotic-resistant species identified among the isolates *were Pseudomonas mosselii, P. otitidis, P. mendocina, P. flavescens, Stenotrophomnas maltophilia, Bacillus thuringiensis, Aeromonas veronii, Candida parapsilosis, Streptomyces violaceoruber and Microbacterium barkeri.*

KEYWORDS: Treated wastewater, Amoxicillin Antibiotic, Antibiotic Resistance Bacteria.

الملخص: بحثت هذه الدراسة في التأثير قصير المدى لري التربة بالمياه المعالجة الغنية بالمضاد الحيوي أموكسيسيلين (Amoxicillin) على وجود سمة مقاومة للمضاد الحيوي أموكسيسيلين بين البكتيريا الهوائية غير ذاتية التغذية (Amoxicillin resistance heterotrophic bacteria) في التربة القابلة للزراعة. تم تصميم الدراسة التجريبية باستخدام ١٠ جرام من التربة المروية يوميا بالماء المقطر التي تحتوي على جرعات متزايدة من المضاد الحيوي أموكسيسيلين (٠، ٠,١، ١، ١٠، ١٠٠، ١٠٠٠ ميكروغرام /اليوم/ التربة) لمدة حضانة ١٥ يوما. كانت الفرضية المبدئية هي أن الإضافة اليومية المستمرة للمضادات الحيوية من المحتمل أن تزيد نسبة البكتيريا المقاومة للمضادات الحيوية في التربة. بعد انتهاء فترة الحضانة، تم تقييم مجمل مجموع المجتمعات البكتيريا الهوائية غير ذاتية التغذية المقاومة للمضادات الحيوية من خلال التخفيف المتسلسل لعينات التربة، تليها حساب صفيحة قطعة هلام الآجار، والعزل البكتيري، والتعرف والكشف عن الأنواع البكتيرية، والملاحظة المجهرية. تم أيضًا تقييم وجود البكتيريا المقاومة للمضادات الحيوية مباشرة على مياه الصرف الصحي المعالجة المستخدمة للري الميداني قبل هذه الدراسة المصغرة لتقييم فرضية الزيادة الحيوية للبكتيريا المقاومة للأموكسيسيلين (Amoxicillin resistance heterotrophic bacteria). أشارت النتائج إلى انتشار البكتيريا المقاومة للأموكسيسيلين في مياه الصرف الصحي المعالجة المستخدمة في الري وفي التربة المستقبلة للمياه. تم تطبيق الدراسة المصغرة «كدليل على المفهوم» لإثبات أن الري بمياه الصرف الصحي المعالجة المحتوية على مضادات حيوية من شأنها أن تمارس ضغطًا انتقائيًا وتعزز من تكاثر ونمو البكتيريا المقاومة للمضادات الحيوية. وبشكل غير متوقع، أشارت النتائج أيضا إلى أن الإضافة اليومية لمضادات الأموكسيسيلين لا تزيد من مقاومة البكتيريا للمضادات الحيوية في التربة، والتي انخفضت بشكل ملحوظ لجميع الجرعات المختبرة. العزلات البكتيرية المقاومة التي تم تعريفها فصائلها هي: *Pseudomonas mosselii, P. otitidis, P. mendocina, P. flavescens, Stenotrophomnas maltophilia ,Bacillus thuringiensis, Aeromonas veronii, Candida parapsilosis, Streptomyces violaceoruber Microbacterium barkeri.*

الكلمات المفتاحية: مياه الصرف الصحي المعالجة، المضاد الحيوي أموكسيسيلين (Amoxicillin) ، البكتيريا المقاومة للمضادات الحيوية.

Introduction

The reuse of treated wastewater is one of the alternative methods for increasing water conservation and sustainability, especially the use of treated wastewater for irrigation can be highlighted. The use of treated wastewater for irrigation may positively affect soil fertility and increase crop yields (Asano, 1988). There are nonetheless safety issues associated with soil irrigation with treated wastewater, namely the presence of microbial pathogens, heavy metals, organic pollutants (Asano, 1988; EPA, 2012), and pharmaceuticals such as antibiotics (Wu et al., 2014). Antibiotics are bioactive compounds secreted by certain microbial species, which directly inhibit the growth of other competing bacteria (Negreanu et al., 2012). Amoxicillin belongs to the *β-lac-*

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tam group of antibiotics, and it is one of the most widely used antibiotics in human and veterinary medicine (Diz Dios et al., 2006). Amoxicillin is an antibiotic of the amino-penicillin group used against a broad spectrum of Gram-positive and Gram-negative bacteria (Croydon and Sutherland 1970). Clavulanic acid is a natural substance produced by *Streptomyces clavuligerus* and has slight antibacterial activity but a very high inhibition of many *β-lactamases*. It is usually added to amoxicillin to increase its half-life. Low concentrations of clavulanic acid (0.05-2 μg mL^{-1}) cause irreversible time-dependent inactivation of many of the *β-lactamases*, including penicillinases produced by *Staphylococcus aureus* (Reading and Cole, 1977). There is increasing societal concern about the evolving antibiotic resistance of natural bacteria, with special concerns expected to occur when antibiotics are continuously released into soil and water environments (Bisht et al., 2009). Therefore, leading to the continuous search for new generations of antibiotic compounds (Czekalski et al., 2012). In this short-term study, amoxicillin was used because it has been reported to have a short half-life compared to other antibiotics (Diz Dios et al., 2006).

Many antibiotics are poorly absorbed in the human digestive tract, and it is estimated that 25% to 75% of the ingested antibiotics are excreted in faeces (Ash et al., 2002). In both animals and humans, up to 95% of antibiotics can be excreted in an unaltered state (Elmund et al. 1971). Most of the antibiotics used for animals and humans are not entirely removed in wastewater treatment plants (WWTPs). As a result, treated wastewater are a natural reservoir of residual concentrations of antibiotic and antibiotic-resistant bacteria (AR). Wastewater treatment plants are not designed to remove or monitor pharmaceuticals (Pruden et al., 2013). Due to this low removal efficiency, multiple antibiotics in municipal wastewater and treatment plants were frequently detected in previous studies, e.g. Zhang and Li (2011). The combination of antibiotics, nutrients, and bacteria in treated domestic sewage wastewater could potentially result in the selection of antibiotic resistance among bacterial populations present in the environment (Negreanu et al., 2012).

Soil organic matter represents an important source of energy and nutrients for microorganisms and plants (Fahrenfeld et al., 2013; Menezes-Blackburn et al., 2013). Soil microbial biomass, activity, taxonomic and functional diversity have strong correlation with soil health and have been considered as good biological indexes (Menezes-Blackburn et al., 2020). The application of irrigation water containing antibiotic resistant bacteria to soil represents a possible contamination source for crops, groundwater and receiving water bodies. Many environmental factors such as temperature, pH, moisture, organic matter content, and others, may enhance the survival of pathogenic microbes on agricultural lands (Trevisan et al., 2002). Antibiotic-resistant pathogenic bacteria may spread across the environment by several different routes, including treated and untreated sewage effluents, sludge and agricultural runoff (Al-Bahry et al., 2007). However, antibiotic resistances are often formed when antibiotics are misused or overused; new bacteria strains arise that are no longer sensitive to these drugs. The presence of selective pressure of antimicrobial drug usage is responsible for the natural development of bacterial antibiotic resistance (Al-Bahry et al. 2015). Under optimal conditions, bacteria multiply very rapidly, with generation times ranging from minutes to hours. The high number and short generation times allow for a high likelihood of spontaneous mutations to occur, allowing any given population of bacteria a significantly high genetic diversity to naturally occur (Al-Bahry et al., 2015).

Treated and untreated sewage effluents may pose risks for groundwater pollution. In Oman, *E. coli* isolated from contaminated wells were found to be resistant to multiple antibiotics, possibly due to the contamination with sewage effluents (Al-Bahry et al., 2014). Introducing antimicrobial agents into the environment might influence the change in the physiological traits of bacteria through the selection pressure over naturally occurring bacterial communities (Martinez, 2009). The bactericidal or bacteriostatic agents (AB) inhibit microbial growth either by: a) targeting membranes or cell walls; b) targeting protein synthesis through the ribosomal subunits as tetracycline, and fluoroquinolones; or c) interfering with the nucleic acid synthesis (Sengupta et al., 2013). Nowadays, the overuse of antibiotics is considered the main cause of the emergence of antibiotic-resistant pathogens, resulting in direct risk for human health (McArdell et al., 2003).

This short-term study investigated the scenario in which irrigating soil with antibiotic containing treated wastewater could propagate antibiotic resistance in soils. The specific objectives were to: a) Evaluate the response soil bacteria enumeration for increasing doses of amoxicillin and the relative proportion of amoxicillin antibiotic resistant strains among the culturable heterotrophic aerobic bacterial community; and b) Evaluate the abundance and proportion of amoxicillin antibiotic resistance within the heterotrophic aerobic bacterial community present on the treated wastewater currently used for irrigation, to verify if this is a possible source of antibiotic-resistant bacterial inoculum to soils.

Methodology

Site of Soil Sample and Treated Wastewater Collection

The soil sample used from the microcosm incubation study was collected from Sultan Qaboos University agricultural experiment station from a site irrigated with treated wastewater for five months (23°35’52.7”N 58°09’50.4”E) during spring 2019. The sampling location is 49 mamsl and 9.27 km from the coastline, free from the influence of seawater intrusion. Approximately one kg of soil was collected from the top 5 cm of and sieved to below 2 mm particle size. The soil background physicochemical properties were 44.24% sand, 54.84 % silt, 0.92 % clay, electrical conductivity on the saturated paste extract (ECe) of 8.3 dS m^{-1}, pH 8 and 0.33 % soil organic matter by loss on ignition.

The water holding capacity was measured as the soil moisture content (g g^{-1}) remaining after one day of free drainage of a saturated 20 g soil sample covered with a plastic film to prevent evaporation. Seventy per cent of this moisture content was used during initial microcosm incubation conditions. The treated wastewater was collected from the irrigation system at the same site and moved to the laboratory for immediate microbial analyses.

Soil microcosm Incubation with Amoxicillin

Amoxicillin-clavulanic acid (AM-CL) was chosen for being the most commonly used antibiotic, due to its common occurrence in treated wastewater effluents, and as a result of its short half-life and high soil degradation (up to 80%) (Braschi et al., 2013). The human adult's standard dosage of amoxicillin (0.5 mg/kg/day) was taken as a reference maximum dosage treatment. To simulate a constant addition of Amoxicillin through daily irrigation with treated wastewater, a logarithmic increase dosage was designed as 0, 0.001, 0.01, 0.1, 1 and 10 mg kg^{-1} day^{-1}. These antibiotic solutions (0.1 mL 10 g^{-1} soil) were added daily for 15 days in triplicate incubations. Each of the 10 g incubation replicates was kept at open-air room temperature (25±1.34 °C) to avoid moisture buildup and anoxic conditions and were daily mixed after Amoxicillin application.

Enumeration of Amoxicillin-resistant Heterotrophic Aerobic Bacteria

Soil bacterial colonies were examined from soil matrices by suspending 10 g of freshly collected soil samples (kept at 4 °C) in 95 mL of saline solution (0.85% $NaCl_2$). Samples were shaken at 100 rpm for 10 min, and soil suspensions were then subjected to four sequential 10× dilutions. For each sample, four replicates were used to increase accuracy. Luria Bertani (LB) agar was used a basal general-bacteria-growth-media in three conditions, either: a) unamended (control); (b) amended with 0.5 mg mL^{-1}, or (c) 20 mg mL^{-1} of (AM-CL) , and were used to cultivate heterotrophic aerobic bacteria without and with antibiotic resistance respectively. The LB agar media plates were inoculated with 0.1 mL of the freshly prepared soils suspensions and incubated at room temperature for 72 hours, to ensure enough time for bacterial growth. The bacteria colonies were enumerated (abundance) and calculated as Colony-Forming Units per gram of soil (CFU g^{-1}). The relative proportion of antibiotic-resistant culturable bacteria was calculated by comparing the number of CFUs growing on plates with antibiotics compared to the number of CFUs growing on control plates without antibiotics. The same agar medium and growth conditions were used for soil suspensions and the fresh treated wastewater sample.

Bacteria Strain Isolation, Identification and Microscopy

Antibiotic-resistant bacterial colonies able to grow on the amoxicillin-supplemented LB-Agar medium were isolated in pure culture by standard streak-clean procedure on fresh LB-Agar medium supplemented with 20 mg ml^{-1} (AM-CL). The bacterial colonies' morphology was examined both through direct naked eye observation of

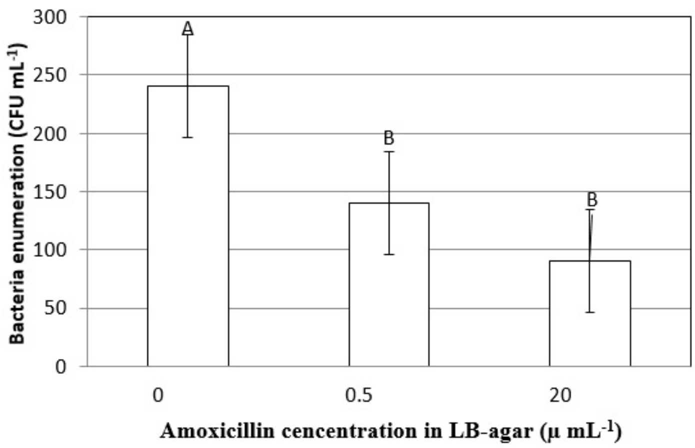

Figure 1. Average culturable bacteria enumeration in Luria Bertani Agar (CFU mL^{-1}) medium inoculated of Treated Wastewater used for soil irrigation at the agricultural experiment station at Sultan Qaboos University. Columns labelled with different letters are statistically different (Tukey p≤0.05).

colony colour, shape and texture, and through brightfield microscopy with gram-staining for distinguishing between the different isolates. Bacterial species identification was performed by using a MALDI Bio-typer (Bruker Co., UK) based on proteomic fingerprinting analysed by high-throughput MALDI-TOF mass spectrometry.

Statistical Analysis

The data obtained were subjected to analysis of variance (one way ANOVA) and subsequently, Tukey's HSD test was used to separate the means of the selected soil properties. The significance level was defined as $p \leq 0.05$.

Results

Amoxicillin-resistant Heterotrophic Aerobic Bacteria in Treated Waste Water

A considerable fraction of wastewater bacteria were able to grow in LB medium supplemented Amoxicillin-clavulanic acid (0.5 or 20 µg mL^{-1}) as shown in Figure S1. The average bacterial cells present in the control LB medium (without Amoxicillin-clavulanic acid) was 240 CFU mL^{-1} (Figure 1). In the plates containing 0.5 and 20 µg mL^{-1} of Amoxicillin, the abundance of bacteria colonies was 140 and 90 CFU mL^{-1}, respectively. This corresponds to a very high proportion of the total CFU in control plates, approximately 58.3% and 37.5% for plates containing 0.5 µg mL^{-1} and 20 µg mL^{-1} of Amoxicillin, respectively. Considering a hypothetical irrigation with 5 L m^{-2} day^{-1}, a total of 1.20×10^6 CFU m^{-2} would be the daily inoculation of these soils with highly Amoxicillin-clavulanic acid-resistant bacteria.

Average culturable bacteria enumeration in Luria Bertani Agar (CFU mL^{-1}) medium inoculated of Treated Wastewater used for soil irrigation at the agricultural experiment station at Sultan Qaboos University. Columns labelled with different letters are statistically different (Tukey $p \leq 0.05$).

In soils, the average soil bacterial CFU g^{-1} enumeration in LB medium supplemented with 0.5 µg mL^{-1} was not significantly reduced with regards to the control (Figure 2). Whereas in LB medium supplemented with 20 µg mL^{-1} Amoxicillin the reduction in CFU g^{-1} was significantly decreased by two orders of magnitude. Therefore, the LB medium supplemented with 20 µg mL^{-1} was used for further soil studies. Curiously, in the soils irrigated with treated wastewater for five months before the experiment, the proportion of bacterial cells culturable in LB medium supplemented with 20 µg mL^{-1} was only one per cent of the control LB-agar medium; much lower than the 37.5% observed directly on the treated wastewater. It is worth noticing that Amoxicillin-resistant bacteria were found to be present in the soil even when no antibiotic or wastewater was applied. The visual aspect LB agar plates of heterotrophic aerobic showing total bacterial cell growth with much lower abundance at 20 µg mL^{-1} of Amoxicillin can be seen in figure S2. Average culturable bacteria enumeration in Luria Bertani Agar (CFU mL^{-1}) medium inoculated suspensions of soils irrigated with treated wastewater at the agricultural station at Sultan Qaboos University. Columns labelled with different letters are statistically different (Tukey $p \leq 0.05$).

Amoxicillin-resistant Heterotrophic Aerobic Bacteria in Soil Irrigated with Amoxicillin solutions - Microcosm assays

In this microcosm experiment, the abundance of antibiotic resistance bacteria was evaluated after 15 days of soil incubation with Amoxicillin-clavulanic acid added daily with the irrigation water (0, 0.001, 0.01, 0.1, 1 and 10 mg Amoxicillin kg^{-1} soil day^{-1}). The same LB medium

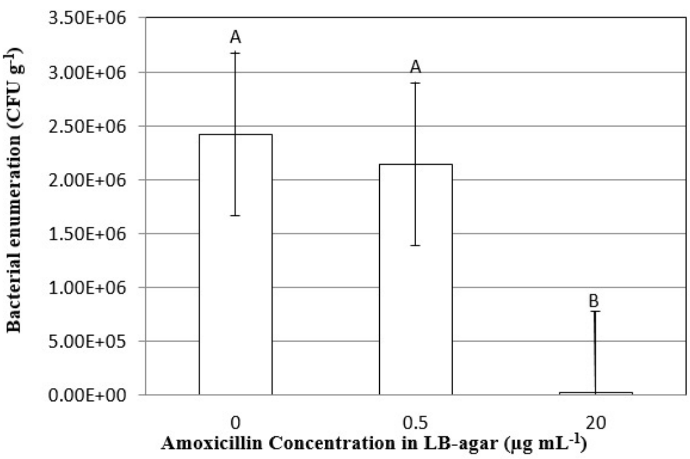

Figure 2. Average culturable bacteria enumeration in Luria Bertani Agar (CFU mL^{-1}) medium inoculated suspensions of soils irrigated with treated wastewater at the agricultural station at Sultan Qaboos University. Columns labelled with different letters are statistically different (Tukey $p \leq 0.05$).

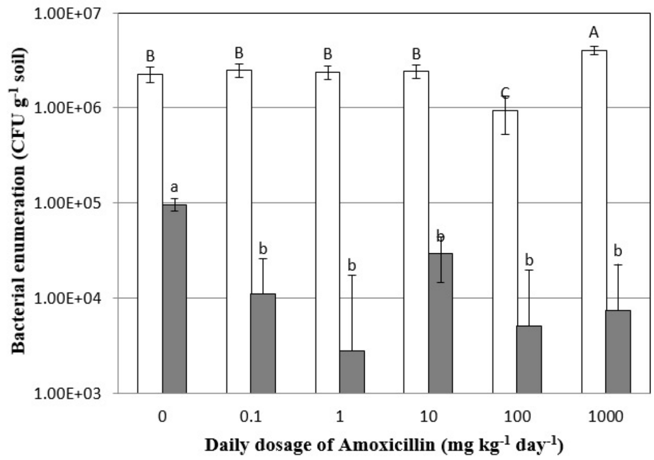

Figure 3. Enumeration of bacteria colony forming units (CFU g^{-1} soil) in Luria Bertani Agar medium LB medium with or without Amoxicillin supplementation (0 and 20 µg amoxicillin mL^{-1}, unshaded and unshaded bars respectively). Soil samples treated with 0, 0.1, 1, 10, 100, 1000 mg kg^{-1} of soil sample day^{-1} for 15 days. Columns labelled with different letters are statistically different (Tukey p≤0.05).

cultivation conditions described herein was followed for the microcosm soil samples (0.5 and 20 µg Amoxicillin mL^{-1}). Amoxicillin only negatively affected the total culturable bacteria CFU g^{-1} soil at the 1 mg kg^{-1} soil day^{-1} dose, and at 10 mg kg^{-1} soil day^{-1}, the CFU g^{-1} soil even significantly increased. There was an intensive and significant decrease in the total and relative abundance of antibiotic-resistant bacteria in soils treated with higher doses of amoxicillin (Figure 3). This effect was contrary to our central hypothesis that the addition of AM-CL would increase the relative abundance of this trait. The total bacterial CFU g^{-1} soil in all samples where Amoxicillin-clavulanic acid was added was below 10^5 CFU g^{-1}. On the other hand, the average bacterial CFU g^{-1} soil was $2.42x10^6$ CFU g^{-1} at incubation conditions without Amoxicillin addition.

Enumeration of bacteria colony forming units (CFU g^{-1} soil) in Luria Bertani Agar medium LB medium with or without Amoxicillin supplementation (0 and 20 µg amoxicillin mL^{-1}). Soil samples treated with 0, 0.1, 1, 10, 100, 1000 mg kg^{-1} of soil sample day^{-1} for 15 days. Columns labelled with different letters are statistically different (Tukey p≤0.05).

The relative proportion (%) of antibiotic resistance bacteria was dramatically and significantly decreased nearly ten folds for soils receiving amoxicillin (Figure 4). . The daily application of AM-CL reduced the % of amoxicillin-tolerant heterotrophic aerobic bacterial

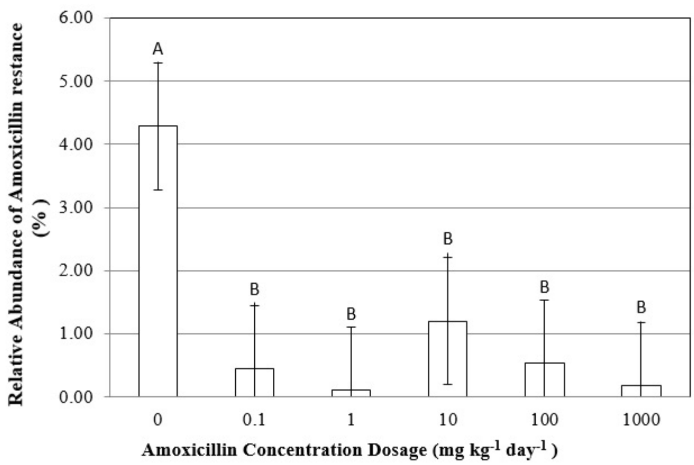

Figure 4. Relative abundance (%) of bacteria colony forming units (CFU g^{-1} soil) able to grow in Luria Bertani Agar medium LB medium containing 20 µg Amoxicillin mL^{-1} medium with regards to 0 µg Amoxicillin mL^{-1} controls. Soil samples were treated with 0, 0.1, 1, 10, 100, 1000 mg kg^{-1} of soil sample day^{-1} for 15 days. Columns labelled with different letters are statistically different (Tukey p≤0.05).

abundance in all treatments. The percentage differences in antibiotic resistant bacteria were not statistically significant among the AM-CL treated soil samples. Moreover, the relative bacterial abundance in LB-agar supplemented with 20 µg AM-CL mL^{-1} was on average 0.5% for Amoxicillin treated soils with respect to control LB plates. Whereas it was over 4% for the control soils without Amoxicillin.

Relative abundance (%) of bacteria colony forming units (CFU g^{-1} soil) in Luria Bertani Agar medium LB medium containing 20 µg Amoxicillin mL^{-1} medium compared to 0 µg Amoxicillin mL^{-1} controls. Columns labelled with different letters are statistically different (Tukey p≤0.05).

Antibiotic-resistant Bacteria Isolation, Identification and Microscopy

The isolated AM-CL resistant microorganisms were identified at the species level using MALDI Bio-typer (Brooker-UK) and observed by bright light microscopy. Based on these tests, the majority of the AM-CL resistant bacteria isolated from treated wastewater were gram-negative, and in soil, most were gram-positive. The bacterial shape, arrangement, and structure of pure isolated colonies *were also recorded. Among isolates, the positively identified strains were Pseudomonas mosselii, Pseudomonas otitidis, Pseudomonas mendocina, Stenotrophomnas maltophilia (Pseudomonas beteli), Pseudomonas flavescens, Bacillus thuringiensis Aeromonas veronii, Candida parapsilosis, Streptomyces violaceoruber, Microbacterium barkeri.*

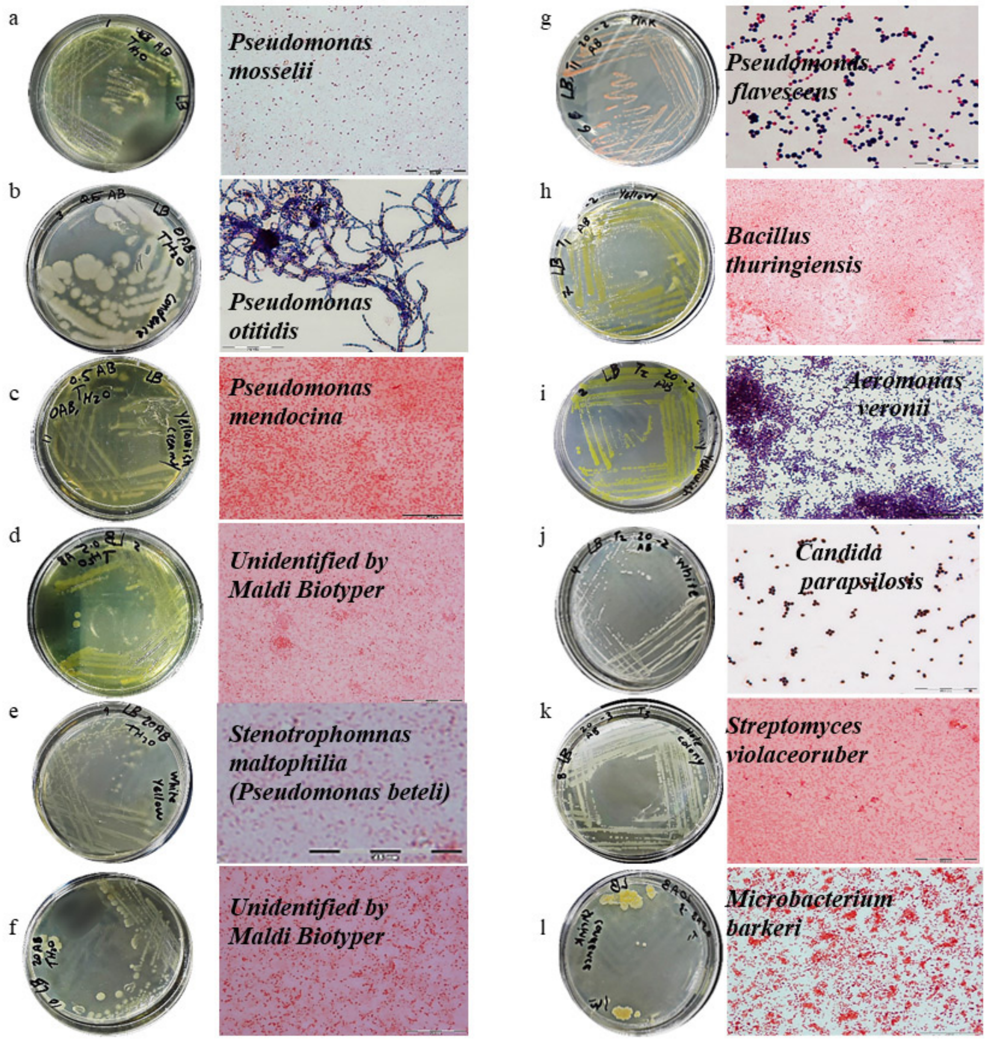

Figure 5. Petri dish LB-agar-streaked colonies image and gram-stained bright light microscopy (1000x amplification) image of the microbial strains isolated in this study. The species identification was performed using Maldi-Biotyper.

Amoxicillin-clavulanic acid-resistant strains isolated from treated wastewater (a to f) and from soils (g to l) irrigated with the same treated wastewater. Isolation, purification and continuous propagation of these strains were performed using Luria Bertani-Agar medium supplemented with 20 μg Amoxicillin mL^{-1}. Species identification was performed with MALDI Bio-typer (ID>97%).

Discussion

Hundreds of different bacteria species can be isolated even from tertiary treated wastewater (Wu et al., 2014). Based on the work from Karthikeyan et al. (2006), during the sewage treatment process, only partial antibiotic removal is achieved, and treated wastewater contains a trace amount of a variety of antibiotics. Bacteria exposed to antibiotic compounds may be selected for their antibiotic resistance traits. Nevertheless, minimal inhibitory concentrations may not be exceeded in treated wastewaters (Martinez, 2009). Bacterial communities exposed to inhibitory antibiotic concentrations can be enriched for antibiotic resistance, and this is related to an overall higher antibiotic concentration in natural environments (Gullberg et al., 2011). Contrary to our hypothesis and the current literature, in our study, less antibiotic resistance was obtained with the addition of AM-CL through irrigation. Hereby, we cannot suggest Amoxicillin-clavulanic acid in treated wastewater would increase the abundance of antibiotic resistance bacterial traits in the soils. Amoxicillin-clavulanic acid stored at room temperature is known to be labile to bacterial degradation, and more than 30% of Amoxicillin-clavulanic acid degraded can be degraded after seven days (Gullberg et al. 2011). The dissolved antibiotic in water is highly affected by environmental conditions, such as temperature ad pH (Gullberg et al., 2011). Amoxicillin clavulanic may be degraded and become a nutrient source to bacterial cells. We speculate here that this may have caused an overgrowth of low generation time bacteria sensitive to Amoxicillin that benefited from the rapid degradation of this antibiotic and were stimulated by the freshly added carbon source.

Moreover, several studies demonstrated that other compounds other than antibiotics could cause the proliferation of antibiotic-resistant bacteria in a process known as cross-resistance. These are usually due to the presence of a high concentration of quaternary ammonium compounds (QACs) (Sidhu et al. 2001), triclosan and triclocarban (used in soaps and other household compounds) (Aiello et al., 2005), or even heavy metals (Baker-Austin et al., 2006).

The average bacteria biomass is about four times higher in the treated wastewater samples than in freshwater samples, and their diversity can be ten folds higher (Aiello et al., 2005; Diz Dios et al., 2006). Chlorination, ozonation, and UV radiation post-treatment disinfection processes were successful treatments against microbial agents, and pharmaceutical ingredients can significantly reduce the risks associated with soils irrigated with treated wastewater (EPA, 2012).

Using classical methods to quantify antibiotic-resistant bacteria can only evidence a small fraction (<1%) of the total community, and due to this fact, serial dilution and agar culturing methods are often inaccurate to represent the whole soil system (Amann et al., 1995). The data presented reflect changes in the culturable antibiotic-resistant aerobic heterotrophic bacteria, and may not describe trends in the rest of the soil community.

Among the isolated Amoxicillin-resistant strains, two were unidentifiable by Maldi Biotyper. Four were Pseudomonas strains, gram-negative, rod-shaped, and motile by a single polar flagellum. *Pseudomonas mosselii, Pseudomonas otitidis, Pseudomonas mendocina, Pseudomonas mendocina, Pseudomonas flavescens*. Some of them are known opportunistic pathogens, but mostly these are commonly found in soil, water, plants, sewage and animals. Little is known about the antimicrobial mechanisms (Rapsinski et al. 2016). Other gram-negative antibiotic-resistant bacteria found were *Stenotrophomnas maltophilia (Pseudomonas beteli), Aeromonas veronii and Streptomyces violaceoruber*. Two Amoxicillin-resistant Gram-positive bacteria were isolated: *Microbacterium barkeri*, common in sewage samples, and *Bacillus thuringiensis* used to control insects for agriculture and public health purposes (Mizuki et al., 1999).

Surprisingly not all isolated microbes were bacteria. One isolate from a soil sample treated with 0.1 μg Amoxicillin g^{-1} soil day^{-1} and cultivated/propagated in LB plates containing 20 μg mL^{-1} was identified as *Rhodotorula mucilaginosa*. A common environmental yeast found in air, soil, lakes, and ocean water. It produces pink to red colonies that are unicellular lacking pseudohyphae and hyphae. It is the most common microorganism isolated from the hands of hospital employees and patients (Strausbaugh et al., 1996).

Conclusion

The effluent of treated wastewater used for irrigation carry antibiotic-resistant bacteria that may be an inoculum source to soil environments. A significant fraction of bacterial colonies isolated from soils (4%) resists Amoxicillin and clavulanic acid antibiotics. The total antibiotic-resistant bacteria abundance was significantly decreased when the soil was incubated with increasing dose of antibiotics. This study sets an important precedent that irrigation with wastewater containing Amoxicillin-clavulanic acid does not propagate this trait in soils by directed evolution. This is likely due to the quick degradation of this antibiotic in soil environments. In addition, more studies are required for clarifying what the antibiotic resistance traits present are, and if there is an effective transfer and survival of wastewater antibiotic-resistant communities in soils.

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