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INFLUENCE OF SUBSTRATE TYPE ON THE PHYSIOLOGICAL PROFILE OF THE HETEROTROPHIC BACTERIAL COMMUNITY IN RECIRCULATING AQUAPONIC SYSTEMS

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Abstract

Aquaponics incorporate fish culture and hydroponically grown plants into one intensive production system. In this productive system, the microbial community develops everywhere being responsible of carrying out the nutrient between different compartments. Currently, the state of knowledge regarding the dynamics of microbial communities in recirculating aquaponic systems is stilllimited, due to poor understanding of interactions between bacterial population established on different substrates; therefore, in order to clear a number of uncertainties, especially at the level of heterotrophic communities, further studies are required. The present paper assessed the dynamics of heterotrophic microbial communities in recirculating aquaponicsystems. The studyis based on the assessment of activity and, especially, of the metabolic diversity of bacterial community attached to the root area of hydroponically cultivated species. Examination of the data gave the first indications to functional groups of organisms in the different compartments of an experimental aquaponic system.

Key words: *aquaponic systems, heterotrophic bacteria, substrate type, wastewater treatment.*

INTRODUCTION

Aquaponic systems are highly engineered aquaculture-agriculture systems that use fish effluent (which comprises both particulate waste solids and dissolved nutrients) from intensive closed aquaculture system as nutrient medium to grow edibleplants in attached hydroponic subsystems (Bartelme et al., 2018). The treatment of waste water from intensive aquaculture, using phytoremediation strategy, is of increasing interest for the international scientific community, as it has been materialized in a series of studies published in prestigious journals in recent years (Graber and Junge, 2009; Sikawa and Yakupitiyage, 2010; Endut et al., 2010; van Kessel et al., 2010; Hu et al., 2015; Suhl et al., 2016; Filep et al., 2016; Forchino et al., 2017).

In addition to the clear advantage of utilizing residual nutrients and purifying technological water (Turciosa and Papenbrock, 2014; Simionov et al., 2017), aquaponic integrated systems provides a significant support surface, represented by plant's root system, to form biofilm. Moreover, this renewable surface ensures a permanent oxygenation of the culture medium while generating a series of valuable organic compounds such as enzymes and vitamins (Bertin et al., 2003). Microbial community (MC) from aquaponic system is the invisible link betwwen fish excrements, highly concentrated in ammonium, and plant fertilizer, which should be a combination of low ammonium and high nitrate (Somerville et al., 2014).

Despite the importance of MC in aquaponic systems onlyfew investigations have been undertaken to determine the microorganisms whitin the systems or microorganisms that colonize the plant rhizosphere and to evaluate their activity (Kuhad et al., 2004; Munguia-Fragozo et al., 2015; Schmautz et al., 2017). In recent years, with the development of modern molecular techniques, it has been possible to elucidate some aspects of biofilm structure attached to different filter media (inert) and characterize bacterial population dynamics in biological filters with different configurations integrated in classical recirculating systems (Brazil, 2006; Malone and Pfeiffer, 2006; Eding et al., 2006). However, these studies involved mainly nitrifying bacteria (ammonium-oxidizing bacteria and nitrite-oxidizing bacteria) or organisms that perform

denitrification, ANAMMOX (Rurangwa and Verdegem, 2015).

Aquaponic systems incorporate different compartments operating under different technological conditions and, therefore, they develop different microbial communities worth studying. Understanding rhizosphere microorganism associations and MC activity can be an important step in optimizing processes within aquaponic systems.

Community-level physiological profiling (CLPP), assessed using BiologEcoPlates, is a technique detecting multiple microbial metabolic activitiesgiving valuable information about functional adaptations over space and time of these communities that can be compared and classified based on sole carbon source utilization patterns. The population of microorganisms gives a characteristic response pattern called a metabolic fingerprint (Gryta et al., 2014).

The present paper assessed the dynamics of heterotrophic microbial communities in different compartments of recirculating aquaponic systems. The main goal was to assess the metabolic activity and diversity of bacterial community attached to the root area of hydroponically cultivated plants.

MATERIALS AND METHODS

Experimental system

The experiment was carried out in an experimental aquaponic system (AS) with a total volume of 1.8 m^3. The aquaponic recirculating system incorporates four fish rearing tanks (268 l) connected to three hydroponic units, each of which consisted of three deep water culture basins. For total solids (TS) control, the recirculating system has been provided with a backwash sand filter where wastewater from the sump tank was continuously pumped with a constant flow of 48 l/min. Water, free of solids, was pumped to the top of the biofilter via a 'spray bar', then trickled across the biological filter medium (0.4 m^3, Bactoballs, 200 m^2/m^3). Biologically treated water was pumped to hydroponic modules that consist of three units (60x90x30 cm), with independent inlet provided with valves which permitted flow control and adjustments. The flow rate through hydroponic units was 8 l/m. Before returning to the fish tanks, the water was passed through a degassing column for CO_2 striping. The sterilization and disinfection process was realized with an UV filter. For the oxygen supply, the recirculation system was provided also with an oxygenation unit represented by one compressor Resun Air-Pump, Model: ACO-018 A with a flow of 260 l/min.

The experiment was designed to characterize microbial communities developed at different compartments of AS (biological filter, mechanical filter, basins, rhizosphere of two types of plants: *Lactuca sativa* and *Ocimum basilicum*). Before starting the experiment, biofilter was activated in order to develop a suitable population of nitrifying bacteria. After three weeks, the system was populated with carp, which was maintained in the system for 2 weeks, sufficient for nutrient accumulation to provide the nutritional needs of the plants used (*Lactuca sativa*, Clarion and Lollo Rosa varieties and *Ocimum basilicum*).

The experiment started by stocking 146 fish of approximately 96 g/ex in the four rearing units (Table 1). The fish were fed with commercial pellets of 41% protein content, in a ratio of 2% of body weight/day. The total amount of food calculated for one day was administered in three meals that were manually distributed over a period of 4-5 hours, starting at 9.00 in the morning.

Table 1. Biometry of carp yearlings used in the experiment

Tank	Parameter	Med.±Stde.
B1	Weight (g/ex.)	95.22±48.45
	Lenght (cm)	18.00±3.13
B2	Weight (g/ex.)	92.10±48.38
	Lenght (cm)	17.75±3.33
B3	Weight (g/ex.)	98.45±55.16
	Lenght (cm)	18.08±3.687
B4	Weight (g/ex.)	96.78±55.84
	Lenght (cm)	18.14±3.31

Salad and basil seedlings (4-6 leaves) were planted at a density of 44 plants/m^2 (24 plants per unit), each specie/variety being distributed in one of the three hydroponic units connected to the recirculating system. The lighting regime for plant growth was 10 h/day. The water level in each hydroponic unit was maintained at 15

cm. Before being introduced into the system, the plants were weighed and the roots were washed with an antibiotic solution. Dehydrated and sterilized coconut fiber was used as the physical support. All plants were then placed on the floating rafts, suspended at the surface of the water so as to allow 3-4 cm immersion of the container into solution.Nutrient supplements (iron DTPA, microelements) were added to meet the nutrient requierments for plants.

Sampling

Samples were taken every week during five weeks.The sample representing the biofilm in the biofilter (BACTO) was scraped off directly from the 10 bioballs (black polypropylene) with a pincer. The sample of plants rootswas assembled by cutting one root hair from 3 random plants in each of the three basins (SR- *Lactuca sativa* variety Lollo Rosa, SV- *Lactuca sativa* variety Clarion, B – *Ocimum basilicum*). All samples were placed immediately into a 50 ml Falcon tube and transferred to the laboratory. For the analysis of the microbial communities from the water, the samples were taken from the outlet of biological filter (FB), from the outlet of mechanical filter (FM) and directly from the fish basins (APA).

The dissolved oxygen (DO), conductivity and pH levels were determined daily with hand-held devices (WTW Oxi 315 i, Conductimeter WTW and WTW pH meter 340 respectively) Determination of $NH4^-$, NO_2^-, NO_3^- and PO_4^{3-} was carried out weekly using the spectrophotometric method (Specol UV-VIS). All water parameters were kept within optimal range for plants and carp rearing.

Microbial community profiling

To evaluate the metabolic profile and diversity of microbial communities (MC) in different compartments of the recirculating aquaponicsystem (including rhizosphere), Biologecoplates were used. These plates were designed for microbial communities and for microbial ecology studies. The Biolog plates consist of 96 wells, 31 carbon substrates and a three-loop control. The rate of utilization of the carbon sources was pointed by the reduction of tetrazolium violet redox dye, which changed from colorless to purple if added microorganisms utilize the substrate (Pohland and Owen, 2009).

To be able to capture environmental-induced variability 3 replicas/plate were inoculated with the same sample. Water samples were inoculated directly into the wells (100 µl). To assess the microbial communities developed at the rhizosphere, the samples (10 cm^3 of root) were transferred to 90 ml of sterilized distilled water enriched with 0.2% nutrient agar. Subsequently, they were shaken at 125 rpm for 15 minutes and held for one hour at room temperature prior to inoculation.The slurry was then poured into sterilize, and then passed through four layers of sterilized gauze and then inoculated into microplates. All instruments, equipment and glassware used were sterilized before use.A Tecan Sunrise reader was used to read the microplates at 24, 48 and 72 hours during incubation (25^0C) at an optical density (OD) of 590 nm.

Optical density values obtained at 48 h of incubation represented the optima range of optical density readings, so 48 h of incubation results was used for the assessment of microbial functional diversity and statistical analyses. In Biolog pates, substrates are subdivided into five group substrates represented by carbohydrates, carboxylic and ketonic acids, amines and amides, amino acids, and polymers, according to Weber and Legge (2009). Microbial activity in each microplate was expressed as average well color development (AWCD):

$$AWCDjt = \frac{1}{31}\sum_{i=1}^{31} ODijt$$

where: ODijt is optical density value from each i well and j replicate at time t corrected subtracting the blank well (inoculated, but without a carbon source) values from each plate well.

OD values were standardized according to Grove et al. (2004) with the following formula:

$$\overleftarrow{ODijt} = \frac{ODijt}{AWCDjt}$$

The final values used to indicate activity in each well were obtained after extracting the

OD value of the control.In order to quantify microbial biodiversity Shannon's diversity index (H) was calculated with formula H=−Σpi(lnpi), where pi = proportional color development of the well over total color development of all wells of a plate.

Statistical analisys

The statistical analysis was performed using the following programs: SPSS 15.0 for Windows, and Biodiversity Pro 2. The distribution normality was verified using the Kolmogorov-Smirnov Z test. The statistical differences between the variables were tested using ANOVA test (α=0.05). The homogeneity of the variance was tested using the Levene test. The physiological profile of microbial communities has been assessed through the analysis of principal components (PCAs) that allowed microbial samples to be compared based on differences in patterns of use of different carbon sources.

RESULTS AND DISCUSSIONS

Biological treatment is one of the most important parts of wastewater treatment in a recirculanting aquaponic system. Therefore, knowing the activity of microorganisms developed in different compartiments of an AS may be important for the operational management and for keeping the balance of nutrients within the system. The Biolog plate was used for studying metabolicresponse of microbial communities from an experimental AS.

The analysis of the data recorded during different stages/weeks revealed that, in the case of the studied plants, the mean values of the optical densities differ significantly ($p<0.05$; Anova repeated measures) from one stage to the other, the icreasing rate of the bacterial density being also different among plant speciesor system compartiments (Figure 1). Unlike microbial community developed on plant roots, biofilm developed on the inert support media was characterized by a relatively constant bacterial density over the experimental stages, with mean AWCD values ranging from 1.03 to 1.07. In the last two experimental stages the biological community developed on the roots of the plants (SV and B) is characterized by a higher bacterial density compared to the biological film developed on bactoballs (inert support). The mean AWCD for the last experimental stagerecordedsignificant (post-hoc Anova, $p<0.05$) higher value for B (1.35 ± 0.23) comparing with SV (1.09 ± 0.34), BACTO (1.03 ± 0.37) and SR (0.93 ± 0.42).

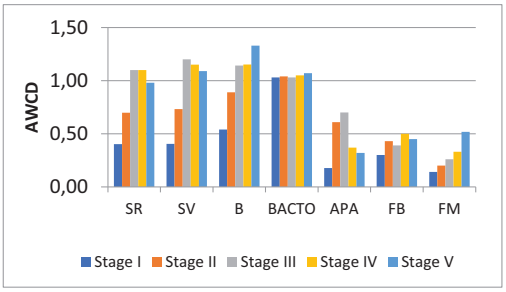

Figure 1. The dynamics of mean AWCD values for all sampling points and stages during experiment

Unlike the water from biological filter, where practically no significant variation in microbial density was registered, in the last week there was a tendency of microbial abundance in the mechanical filter most likely due to increase of nutritive substrate in this compartment.

Concerning the diversity of bacterial communities, this was analyzed using the Shannon (H) diversity index. As can be seen from Table 2, lowest H values were found for the samples taken from the mechanical filter (FM) in the first three experimental stages, for the water samples (APA) and, surprisingly, for the biological filter biofilm (BACTO) samples taken in the third stage.In the fourth stage at the level of the biological filter, both abundance and bacterial diversity are restored. A possible explanation for this phenomenon is that periodically, some of the bacteria attached to the biological film detach themselves and form the so-called planktonic bacterial communities (Michaud et al., 2006). This could be also the explanation for higher bacterial diversity from water samples from biological filter collected in the third stage of the experiment. The greatest microbial diversity is observed, regardless the stage, at the biofilm bound to the plantrizoshere. This implies that the microbial communities from the roots of plants had aheterogenous bacterial comunity.

Over all stages, analysis of the diversity of microbial communities using the Shannon Diversity Index highlights the fact that fixed

microbial community (roots and inert media) are significantly more diverse ($p<0.05$) than MC from water samples (Table 2).
Also, it can be noticed that, over time, microbial diversity is amplified, a sign that they are also composed of fast growing species and slow growing species.

The statistical comparison of the Shannon -H biodiversity index for all sampling points sugest significant differences ($p<0.05$) with BACTO, SV, SR and B framing in the same subset of values, while FM, APA and FB were classified into distinct subsets.

Table 2. Mean values of Shannon index (H) based on 48-h incubation (means±standard errors)

Shannon H index	SR	SV	B	APA	FB	FM	BACTO
Stage I	0.9±0.01	0.95±0.02	0.98±0.03	0.78±0.01	0.85±0.01	0.57±0.01	1.04±0.11
Stage II	1.13±0.04	1.31±0.01	1.24±0.06	0.98±0.04	0.96±0.06	0.58±0.03	1.32±0.08
Stage III	1.28±0.02	1.34±0.04	1.45±0.07	0.78±0.03	1.06±0.05	0.69±0.04	0.73±0.09
Stage IV	1.45±0.03	1.46±0.07	1.45±0.09	0.99±0.05	0.95±0.01	0.90±0.05	1.12±0.10
Stage V	1.43±0.02	1.47±0.02	1.47±0.02	1.09±0.04	1.15±0.03	1.24±0.01	1.15±0.12

Regarding the metabolic profile of the microbial communities in the recirculating system, in general, and from the biological film, in particular, it was characterized after the heterotrophic bacteria preference analysis for a particular carbon compound or for one or more groups of compounds, which we will further define as chemical guilds.At the biolog plate, the 31 carbon sources can be grouped into six categories: amines, amino acids, carboxylic acids, carbohydrates, phenols, and polymers.
The patterns of using different carbon sources by the bacterial communities developed at the different compartments of the AS and on the rhizosphere of horticultural plants, cultivated in the hydroponic modules having as nutritive support the waste water from the intensive growth of *Cyprinus carpio*, was analyzed by PCA. This method allows highlighting the pattern of associations (correlations) between variables, but also to determine possible latent variables that would explain a part of the measured variance.
PCA analysis for the entire experimental period shows a clear separation of the seven sampling points along the first two components. In this case, PC1 explains 50.09% of the variance, while PC2 explains 22.74%.
Figure 2 shows that the metabolic profile of bacterial communities in recirculated water (APA), biological filter (FB) and bactoballs (BACTO) is relativelly similar, differing from the metabolic profile of the plant rhizosphere. However, if we consider also nitrifying bacteria, the composition of the microbial community attached to the biofilter differed from the composition of the suspended bacteria (Blancheton et al., 2013).
In the case of a complete analysis, which also takes into account the average AWCD values for different chemical guilds, for each experimental stage, it was observed that there is a similarity of the metabolic profile of all components at the beginning of the experiment, the diversity increasing with the maturation of the biological films.

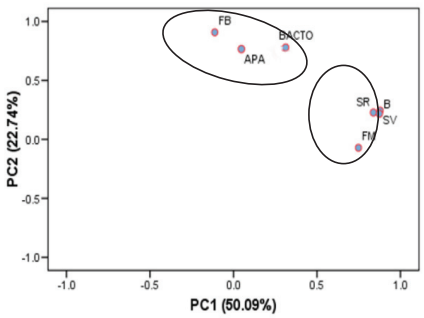

Figure 2. Principal component analysis for AWCD for all samples during experiment

Analysis of AWCD mean values reveals significant differences (Anova, $p<0.05$) when comparing different trophic types preferred by microbial communities in different RAS compartments.
It is also noted that carbohydrates represent, with the exception of FM, the preferred carbon source of MCfromwater or attached as biofilms in the aquaponic recirculating system.

The statistical analysis revealed insignificant (t test, $p>0.05$) differences between the chemical guilds at the root level of the salad, both varieties having relatively the same organizational structure regarding the metabolic profile of the present microorganisms.

Unlike salad, in the rhizosphere of *Ocimum basilicum* species, AWCD average values for phenols and polymers were significantly lower than those for the rest of the substrates. At the level of the biological filter, communities developed as biofilm preferred as nutritive substrat polymers and carbohydrates, followed by amino acids and amines. Also, it was observed that MCs having the preferred substrate phenols have diminished in the last two experimental stages.

The same tendency of decreasingMC that prefers the substrate of phenols is also observed in red salad plants. During experiment, at the level of the rhizosphere, there was a decrease in the response of the wells with source of carbon polymers.

Table 3. Pattern of substrate utilization for anlysed samples

Chemical guilds	**Carbon Sources**				*SR*	*SV*	*B*	*APA*	*FB*	*FM*	*BACTO*
Amines (A)	Putrescine										
	Phenylethylamine										
Aminoacids (AA)	L-Arginine										
	L-Asparagine										
	L-Phenylalanine										
	L-Serine										
	Glycyl-L-glutamic acid										
	L-Threonine										
Carbohydrates (CH)	α-D-Lactose										
	β-Metil-D-Glicoside										
	D- Cellobiose										
	D-Mannitol										
	i- Erythritol										
	Glucose-1-phosphate										
	D- Galactonic Acid γ-Lactone										
	N-Acetyl-D-Glucosamine										
	D,L-α-Glycerol phosphate										
	D-Xylose										
Carboxylic acids (CA)	Acid α-Ketobutiric										
	D-Glucosaminic acid										
	D-Malic acid										
	γ-Hydroxybutyric acid										
	Pyruvic acid methyl ester										
	D-Galacturonic acid										
	Itaconic acid										
Polymers (P)	α-Ciclodextrin										
	Tween 40										
	Tween 80										
	Glycogen										
Fenols (F)	2- Hydroxybenzoic acid										
	4- Hydroxybenzoic acid										

Legend

In this paper, both the structure and the dynamics of the various bacterial communities, both attached and suspended, developed at the different compartments of an aquaponic recirculating system were assesed. The variation in the pattern of metabolic response,

indicated by CLPP, suggested a complex interaction between bacterial communities and the type of support medium, treatment process, biofilm age, and perhaps other environmental variables. Metabolic imprinting using redox technology embedded in Biolog plates has already been demonstrated as an effective method in differentiating heterotrophic microbial communities from different wastewater treatment systems (Hench et al., 2004).

Five weeks after the start of the experiment, significant differences ($p<0.05$) among metabolic profiles of biofilms formed at different compartments of the aquaponic recirculating system were detected. The highest intensity response came from the salad and basil rizosfere microbial community. Analysis of the different ages of biofilms reveals pronounced differences in both, the metabolic profile and the diversity of microbial communities.

These results suggest that the support medium plays an important role in determining the structure of the microbial community in aquaponic systems, and that the incorporation of plants (roots) in the recirculation system configuration could provide unique connectivity sites for certain microbial populations.

The fact that the structure of the bacterial communities differs depending on the media environment, the specific conditions and the operating period of a reactor has already been demonstrated. Also, some studies conducted with the purpose to include different plant species in wetlands and assess their waste water treatment potential have highlighted the fact that macrophytes can stimulate the development of specific microbial communities (Vacca et al., 2005). Vymazal (2010) also showed differences in microbial activity at the root of the different plant species within the same wetlands.

In this study, differences in the metabolic profile of biofilms extracted from different support media were detected although there were no significant differences regarding water parameters from different compartiments. Thus, the main carbon sources preferred by the bacterial communities at the level of the rhizosphere were:

- SR: i-Eritriol (CH), Piruvic Acid Methyl Ester (CA), Tween 40 (P).
- SV: L-Argininеand L-Asparagine (AA), Glucose-1-Phosphate (CH).
- B: L-Asparagine and L-Phenylalanine (AA), D- Cellobiose and Glucose-1-Phosphate (CH).

The metabolic profile of communities in the inert environment is suggested by the following substrates: L-Phenylalanine (AA), Glucose-1-Phosphate (CH), Phenylethylamine (A), α-Cyclodextrine(P) and i-Eritritol (CH).

As for microbial diversity, there were statistical differences ($p<0.05$) between the bacterial films attached to the inert environment comparing with those from the plant rhizosphere. However, among plant species/varieties there were no significant differences ($p>0.05$) regarding diversity of microbial community prolipherating in the rizosfere although the metabolic profile was sligly diferent.

CONCLUSIONS

The results show the importance of the substratetype in determining the structure of heterotrophic microbial communities in recirculating aquaponic systems. The plant roots (as a support medium) contribute to the microbial community development and thus, indirectly, influences waste water treatment processesin aquaponic systems since microbioms are governing nutrient cycling. The present study suggests microbial niche differentiation within the aquaponics components.

In conclusion, the obtained results highlighted the importance of understanding the metabolic pathways and structure of microbial communities within aquaponic systems. At rizosphere microbiom level may also proliferate plant growth promoting microorganisms which, especially in aquaponic systems, may reduce nutrient supplementation if those communities could be properly manipulated and integrated as processes into AS design. However, the contribution of plant roots (as a support medium) to the efficiency of the process of treatment of aquaculture effluent remains to be elucidated. The way in which heterotrophic bacteria interfere in improving growth conditions is unclear and requires further investigation. Applying modern methods of study to microbial communities will make possible the correlation of microbial systema-

tics and dynamics with management of aquaculture activities.

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