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BIOFUNGUS: FUNGUS MBBR PILOT PLANT ON MURCIA ESTE WWTP

E. Mena[1], A. Gadea[2], A. Monreal-Bernal[3], S. López-García[4], V. Garre[5] and A. J. Lara-Guillén[6]

[1,2]Emuasa, 30008, Murcia, (Spain)
[3]Aquatec, Proyectos para el Sector del Agua S.A.U., 30100, Murcia, (Spain)
[4,5]Dpto. de Genética y Microbiología, Facultad de Biología, Universidad de Murcia, 30100, Murcia, (Spain)
[6]Technology Centre for Energy and the Environment (CETENMA), 30353 Murcia, (Spain)

[1]*eva.mena@emuasa.es*

Abstract

Concerns about energy efficiency and contaminants of emerging concern (CECs) in wastewater treatment plants (WWTP) lead development of new and alternative processes. Conventional activated sludge systems have a high energy consumption and footprint. Alternative processes are nowadays implemented to reduce them.

In this study, we present the results of Biofungus project. Influent wastewater is treated under real conditions and continuous operation in a two-step Moving Bed Biofilm Reactor (MBBR) pilot plant based on Mucor fungus. Several strains and spontaneous mutants of the Mucor fungus were investigated. Those showing improved growth performance, wastewater resistance and nitrate consumption rate were isolated in laboratory and used at the pilot plant. Moreover, the in-situ growth at the pilot plant of the fungus from spores is implemented at the plant as a parallel process. The obtained effluent water meets regulations requirements, showing a high COD and suspended solids (SS) removal (87% and 94% on average respectively) and total nitrogen removal of 35% on average.

The Biofungus pilot plant treatment works analogous to a conventional activated sludge process. One of the difficulties observed is the retention of the fungi during the process. The volume of carriers used at the MBBR is between 25-30% of the aeration tank. They allow the fungi to grow attached to them avoiding the dilution of its concentration by flotation and loss from this tank. This stage is followed by a settling tank, where the biomass is either recirculated to the aeration tank or purged to a sludge thickener tank. The process is a two-stage process with aerobic and anoxic reactors. The Mucor fungus specialized in the nitrate consumption is dosed into the anoxic reactor after a different Mucor string has consumed the DQO at the MBBR. Both stages are connected by a primary settling tank and a secondary decanter clarifies water after anoxic stage. After this secondary decanter the treated water is obtained. The retention time on the plant is between 6 and 10 hours and the treated volume is 3,6m3/day.

The *Mucor* fungus has proved able to eliminate high ammonia and nitrate concentrations in sort periods of time, resulting on consumption rates of 1,6 mgN-NO^3/h. Finally, its resistance to CECs has been evaluated under a wide range of contaminants including drugs, pesticides, herbicides, fungicides and hormones. Almost no toxicity to contaminant concentrations as high as 20 mg/l was observed. The water treatment performance has been also tested with a combination of CECs at 200µg/l and no influence has been observed after a week over the effluent water quality.

Keywords
Innovation, wastewater, Moving Bed Biofilm Reactor, fungus, denitrify, carriers.

1 INTRODUCTION

Activated sludge process has been the most applied biological treatment for urban wastewater since its discovery at the beginning of 20 century. Different bacterial communities are able to transform organic matter and nitrogen compounds into gas nitrogen and carbon dioxide. However, with the aim of reducing the footprint of the wastewater treatment plants (WWTP), the energy consumptions and improving the performance of conventional processes, the last two decades a lot of effort has been carried out investigating other microorganism and alternative technologies. Fungi and algae are some of the organisms' candidates as bacteria substitutes for wastewater treatment processes.

The use of filamentous fungus in wastewater treatment is still a novel approximation. Various studies have described the advantages related with the use of these organisms. Some of the benefits are: a) an improvement in nitrogen compounds elimination by the fungus or by synergistic processes with the bacteria; b) the improved flocculation and suspended solids separation; c) the reduction of produced sludge during the wastewater treatment process; d) improved performance during the dehydration stage of the sludge; e) increase the biogas production by digestion of fungal sludge; f) great resistance to contaminants of emerging concern and it assimilation and degradation.

Several fungi species have been researched in the past. Some promising results for wastewater treatment application have been obtained with *Mucor* and *Fusarium* species demonstrating a high performance for suspended solids, turbidity and chemical oxygen demand (COD) removal [1]. Although these are the most studied and known species other fungus, such as the *Aspergillus niger,* have been studied, reporting COD removal of 72% [2]. Denitrification process for wastewater treatment have been also investigated resulting in an application that worth more in-depth investigation [3].

Besides the degradation of organic matter and nitrogen compounds (ammonia and nitrate), it has been demonstrated that fungal sludge could be and excellent source of valuable subproducts such as amylase, chitin, chitosan, glucosamine, lactic acid and several antimicrobial compounds [4].

The fungus, due to its growth structure by hyphae and mycelium development (being the mycelium the aggregate of hyphae), allows the suspended solid retention at its external layer and the nutrient harvesting. Moreover, due to its higher number of gens and superior complexity as organism it has developed an improved reproductive selectivity, that translates into an enhanced flexibility and adaptation to the changing environment [5]. Another interesting aspect from fungus structure is that it produces extracellular enzymes responsible of facilitating the degradation of different refractory compounds such as phenolics, polyhydroxyalkanoates (PHAx) or pharmaceutical active compounds (PhACs), frequently present in wastewaters. This fungus peculiarity has led to several studies of complex wastewater treatment effluents or the elimination performance of contaminants of emerging concerns [6], [7], [8].

But, all these mentioned advantages sometimes do not materialized when real conditions and large scale pilots are tested. The aim of BIOFUNGUS project is demonstrating the benefits of a fungal wastewater treatment at semi-large scale in real-life operating conditions. A Moving Bed Biofilm Reactor (MBBR) pilot plant has been designed and built for testing the two-step process based on *Mucor circinelloides* fungi.

The project, ended in June 2022, demonstrates the viability of pilot plant in-situ fungus culture at large scale, enabling the continuous dosing by demand of the process. This result allows to keep the treatment performance in range of the conventional bacterial activated sludges process. Besides that, the fungus has shown good tolerance to high contaminants concentrations, such us drugs, herbicides or pesticides, with almost no inhibition of the wastewater treatment process.

2 MATERIALS AND METHODS

Several strains and spontaneous mutants of the *Mucor* where investigated. Those showing the best growth performance where used. The optimal culture conditions for the *Mucor* fungus have been identified at laboratory and later applied at the pilot plant. These strains and mutants were tested on a two-stage process. Besides, mutants specialized in assimilate nitrates have been also isolated. The isolation of this strains was carried out by growing the fungus different strains under laboratory conditions and analyzing the change in color of the culture substrate. When *Mucor* preferably consumes nitrate, the substrate pre-mixed with methyl orange changes its color from red to yellow. Finally, from the tested Petri dish, spores are isolated from the mutant *Mucor* where this change of color and preferably nitrate consume is observed (*Figure 1*).

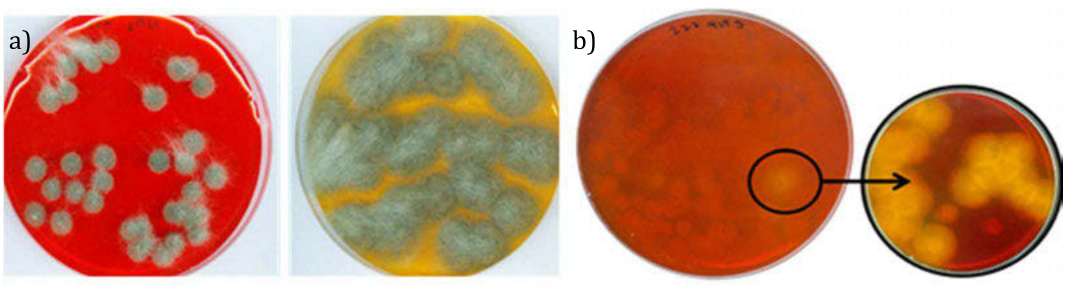

Figure 1. a) Mucor fungus culture showing the specialized nitrate consumption mutant. b) Isolation process of the nitrate specialized fungus.

By needs of the wastewater treatment process, the fungus may be preserved during long time ready to be dosed into the biological reactor (MBBR). With that aim, multiple *Mucor* candidates were kept in laboratory being periodically put in contact with aliquots of wastewater, confirming its survival and growth performance.

The pilot plant is located at Murcia Este WWTP and the influent wastewater is the same for both plants. Composites samples of the influent wastewater are collected daily automatically, whereas individual samples are collected manually every 48 from the different stages of the pilot plant (MBBR, settlers, anoxic reactor and effluent). The analysis of these sampling points was monitored during the 18 months of continuous evaluation of the pilot plant. The inlet flow was kept between 120-150 liter/hour, and the retention time between 6 and 10 hours. The average treated volume has been 3.6 m^3/day.

The control parameters during the project development consisted of: pH, conductivity, redox, temperature, chemical oxygen demand (COD), biochemical oxygen demand (BOD5), total nitrogen (TN), ammonia (NH_4^+), nitrates (NO_3^-), mixed liquor suspended solids (MLSS) and settleability (V30 and sludge volumetric index, SVI). Sporadically, it was also analyzed solid content (dried matter, DM) and volatile content (VM) from the mixed liquor and sludge. The analytical methods applied are the conventional for wastewater analysis.

The acceptance criteria used for the treated wastewater are the ones defined by the government regulatory entity to Murcia Este WWTP: (i) COD< 150 mg/l, (ii) TN<150 mg/l, (iii) NH_4^+<15 mg/l y (iv) NO_3^- <15 mg/l.

The anaerobic digestion of the sludge was tested, firstly, by biomethane potential test (BMP) using a “Automatic Methane Potential Test System (AMPTS) from Bioprocess Control” commercial equipment, and, secondly, by a laboratory scale anaerobic digestion process (10 liters reactors).

Emerging contaminants have been analyzed by an external accredited laboratory and methods using high pressure liquid chromatography (HPLC).

3 RESULTS AND DISCUSSION

3.1 Pilot plant design and operation

In a previous study [9] several mutants of the *Mucor circinelloides* were proven as good candidates for wastewater treatment, eliminating organic matter and ammonia but remaining a high concentration of nitrate in the effluent water. Biofungus project has been focused on the isolation of strains of the same *Mucor circinelloides* fungus specialized on the consumption of nitrate. The chosen fungus for the validation of the process at the pilot plant has shown nitrate consumption preference over the ammonia, though it is able to assimilate both sources of nitrogen.

For the validation of the process a two-stage pilot plant was design and built, it is presented in the *Figure 2 a)*. The plant is divided into two stages: i) a MBBR reactor (25% carrier volume), equipped with fine bubble diffusers, where the fungus responsible of the degradation of organic matter and ammonia is dosed (from now on called “aerobic fungus”) and ii) a secondary anoxic reactor where the nitrate specialized *Mucor* strain is dosed (called “anoxic fungus”). The used of polymeric supports (also known as “carriers”) allows the biomass (bacterial or fungal cultures) to growth attached to it and preserve a high concentration at the reactor. The plant has also decanters after the aerobic and anoxic stages, for the recovery of the biomass and its recirculation and purge. Moreover, for the in-situ growth of the fungus, two cultivation tanks were incorporated to the plant, both with pH and temperature control and nutrient solution dosing.

a)

b)

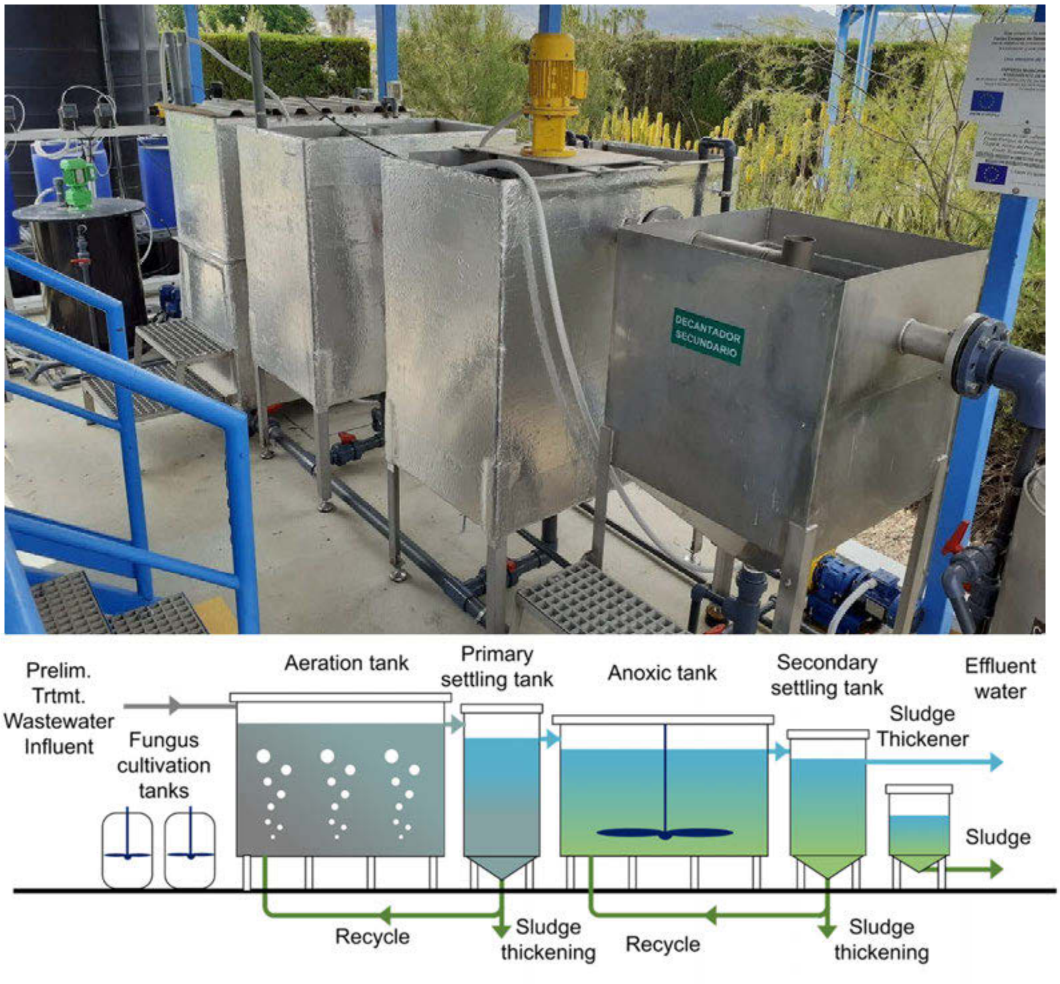

Figure 2. a) Photography and b) schematic of the Biofungus pilot plant located at Murcia Este WWTP.

The working flow of the pilot plant *Figure 2 b)* consist of: the influent wastewater, after passing through the pre-treatment, enters the aerobic reactor where remains in contact with the fungal biomass until the organic matter and ammonia is removed. 2ppm O_2 is used as default value but it was increased up to 4-5ppm for punctual high COD periods. After the aerobic treatment, the biomass is recovered and recirculated to the aerobic reactor or purged if is required. The clarified water enters the anoxic reactor where the nitrate consumption specialized fungus is dosed at a concentration of 1-2% in volume per day and the denitrification process is carried out. Both aerobic and anoxic reactors have 1000 liters capacity. After the anoxic reactor another decanter is used for last clarification and recovery of the biomass. Primary and secondary settlers have 600 and 300 liters of capacity respectively. The purged sludge is sent to a concentration tank where water and sludge are separated. The plant is additionally equipped with a control system for timing every equipment and data logger allowing to register all sensor parameters.

Some microscopy photographs from the *Mucor* hypha structure and mycelium attached to the carrier substrate are shown in the *Figure 3 a* and *b* respectively. Image *c) shows Mucor* biofilm attached to the carrier and picture *d)* shows its growth after 48 hours.

During the project development, one of the objectives was to optimize the fungus growth conditions from spores to a ready-to-use mycelium solution and to increase its lifespan. The optimum conditions for the in-situ culture were first established at laboratory. Parameters like composition, dosing, frequency of feeding, pH and temperature were identified. Urban wastewater and residual water with high COD content from food industry were tested as culture media. *Mucor* fungus showed the same growth performance in artificial media than in presence of both food industry residual water and wastewater.

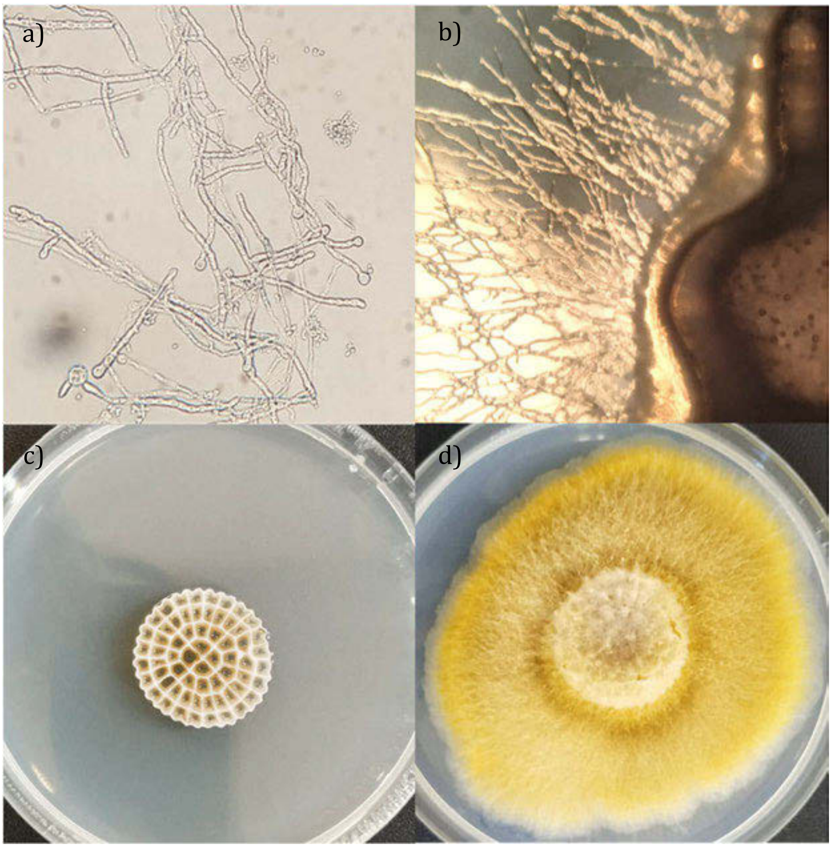

Figure 3. Microscopy images of the Mucor a) hypha structure and b) mycelium attached to the carrier. c) Pilot plant carrier with fungal biofilm and d) its growth after 48 hours.

The established conditions were later validated during the pilot plant validation and all parameters where slightly adjusted. Cultures of 100-200 liters mycelium solutions were periodically prepared for the aerobic and anoxic fungus. For 100 liters of culture media 2 kg of glucose supplemented with 0.2 kg of yeast nitrogen base (YNB) were mixed with water. The mycelium solution was ready after less than 24 hours of adding 50ml of the spore solution. Growth time mainly depends on the temperature of the tank and the age of the spores. It has been observed that preserving several weeks the spores ready at 4 or -20°C led to a delay of 12-24 additional hours. The optimum culture temperature was 26°C and pH between 4-5. In practice, the culture pH drops to 3.5-4 when the spores are mixed with the culture media and stays at 4-5 range, indicative of a good development of the fungus growth.

Among the difficulties faced during the development of the project, avoiding bacterial contamination of the fungus and nutrient solutions have been one of the priorities. One of the proposed solutions was to pasteurize the nutrients solution, increasing its temperature periodically to 60°C for 20 minutes. Although it eliminates the bacterial contamination it has a high energy cost. In practice, using solid nutrients instead of nutrients in solution avoid part of the contamination. The fungus solution last in the cultivation tank between 1 and 1.5 months before the *Mucor* culture is inhibited. The presence and concentration of the *Mucor* was confirmed by microscopy technique every 1-2 weeks.

The pilot plant was operated in continuous mode between January 2021 and June 2022. Four validation processes (stages) were carried out. Every stage lasts between two-four months and real conditions such us rainy, cold or very warm weather periods were tested, besides peaks of influent load (COD) o unprogrammed stops or failures. The results obtained during one of these stages is presented in the *Figure 4*.

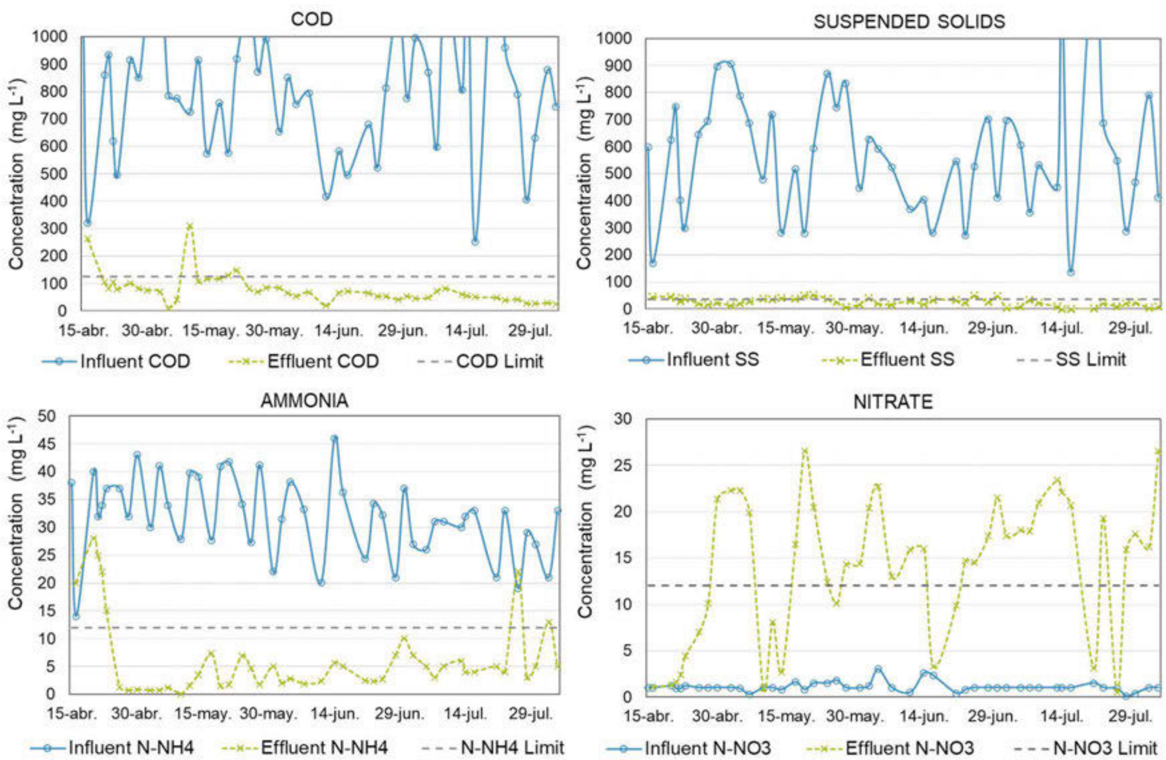

Figure 4. Influent and effluent concentrations of the main parameters monitored during the continuous operation of the Biofungus pilot plant.

Influent and effluent daily concentrations during three months of operation are shown in *Figure 4*. High performance eliminating the COD and suspended solids are observed since the first week of operation, 87% and 96% on average, respectively. On the contrary, the total nitrogen removed is in the range of 37%. Although the ammonia removal is 80% on average, the fungal sludge has very poor settleability hindering a proper mixed inside the anoxic reactor and avoiding the continuous elimination of the nitrate. The poor settleability of the fungal sludge is attributed to the nature itself of the fungus. *Mucor* fungus is an oleaginous and filamentous organism and both characteristics have negative impact on settling properties. Besides, probably related to the glucose content of the fungus solution, it has been observed that the sludge present in the anoxic reaction suffers some kind of fermentation/foaming. During several stages of the project different approaches have been tested but still the poor settleability prevent achieving a stable concentration and mixture inside the anoxic reaction. An alternative configuration for the anoxic reaction that prevent the escapement of the biomass by flotation must be investigated.

Although, with this pilot plant configuration was not possible to achieve continuous elimination of nitrate, several control experiments validating the fungus nitrate consumption were performed. In the *Figure 5* one of several control experiments is presented. The anoxic fungus characterized previously at laboratory showed its preference for the nitrate over the ammonia. Same behavior is observed when fungus is dosed at the pilot plant in presence of both compounds. Reduction rates between 10-30 mgN-NO_3/h for the nitrate and 1,5-2 mgN-NH_4/h for ammonia were obtained. First, 100% of nitrate was removed in less than an hour from the anoxic tank for initial concentration of 15mg/l. After its complete consumption, 100% ammonia was eliminated in the next 12 hours. Similar analytical results were obtained by standard laboratory test (dotted line) and by the multiparametric probe for NH_4 and NO_3 installed in the pilot plant (continuous line) (see *Figure 5*).

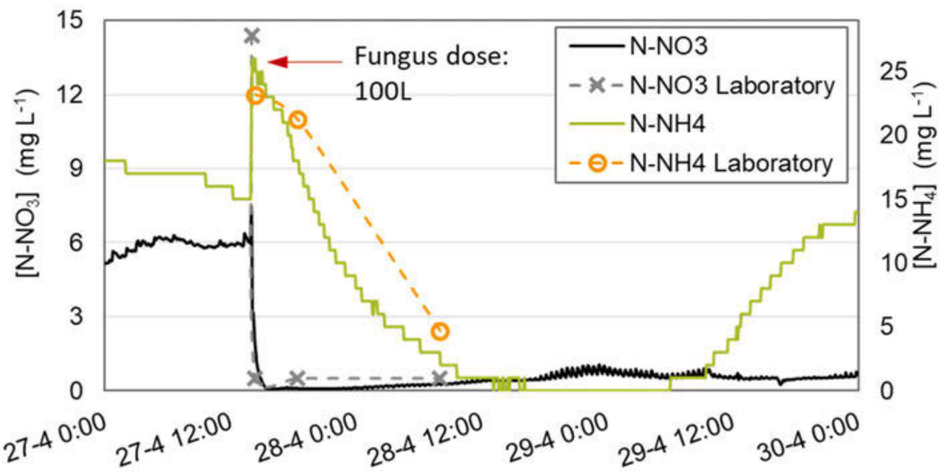

Figure 5. Control experiment validating nitrate and ammonia consumption after dosing the Mucor fungus.

Bacteria and fungus coexist in the pilot plant. The biofilm growth in the MBBR carriers, besides a high fungus content visible by microscopy (see images from *Figure 2* c and d), has the typical bacteria colonies from a conventional wastewater treatment process. This has been proven by fluorescence in-situ hybridization (FISH) technique where some fluorescent probes are attached to specific nitrifying bacteria genetic DNA sequences or chromosomes. The bacteria populations are then directly observed by fluorescence microscopy. The presence of the fungus is also confirmed for every stage of the process by adding aliquots of wastewater or sludge to Petri dishes and cultivating them for 24-48 h. This coexistence between both organisms also presents a synergistic effect for the process. The fungus provides fast response of the process and toxicity resistance whereas the conventional bacteria is still working in parallel.

During the continuous operation of the pilot plant, it has been noted the low production of obtained sludge, values between 0.3-0.6 kgDM/m^3. This low production is a great advantage of the process due to the importance of the sludge management in a conventional WWTP.

3.2 Contaminants of emerging concern: fungus resistance and concentration removal

Wastewater treatment process and fungus resistance to contaminants of emerging concern were a key point to study for the Biofungus project. Influence and interaction of contaminants for both *Mucor* strains were analyzed under laboratory conditions and pilot plant conditions. Several contaminants were chosen according to literature [10], but also, to previous influent analysis showing the presence of certain compounds in the wastewater. Three different experiments were proposed: 1) validating if the contaminants produce some evidence of toxicity over the fungus, inhibiting its normal growth. 2) to measure concentrations variations of contaminants in presence of the fungus. 3) evaluating the Biofungus pilot plant performance after the ingress of a combination of contaminants.

Different drugs, pesticides, herbicides, fungicides and hormones were added to the culture media of the *Mucor* fungus, and its growth performance was compared to a blank sample. The list of tested contaminants, all with purity between 97%-99%, are indicated in the *Figure 6 a*.

The first experiment consisted of the comparison between the mycelium growth mass after 72h in presence of the contaminant and compared to a control sample without contaminants. The test was conducted with every individual contaminant at a time, and with multiple concentrations: 20 µg/l, 200 µg/l y 20 mg/l. The results concluded that the growth performance was not clearly hindered by the presence of the contaminants. A small decrease was measured (between 2-5%) compared to the blank sample for every contaminant except for the sulfamethoxazole, that led to a mass 10% lower compared with the control sample.

For the second test, the concentration of the contaminants were measured after 72h in contact with the fungus. The difference between initial and final concentration is presented in *Figure 6 a*, and the performance of removal (by assimilation or degradation) is showed. The elimination was measured for every contaminant and, on average, 90% and 71% was removed for initial concentrations of 20 µg/l y 200 µg/l respectively. Further experiments are needed to discern whether the contaminants are assimilated or degraded by the fungus. Typical contaminants elimination processes during the wastewater treatment, such as evaporation (volatile components), oxidation reactions due to aeration or degradation by other chemical agents, are discarded.

The third and final test was performed to evaluate the Biofungus pilot plant performance when contaminants are present in the influent water. A solution of the indicated contaminants was prepared at a concentration of 200 µg/l and directly poured into the MBBR reactor. The days after the contaminants enter the plant, daily COD and ammonia analysis were conducted. As can be observed in the graph of the *Figure 6 b* the treatment performances were not affected by the contaminants, and the concentration of selected representative contaminant was zero after the third day.

These three experiments not only confirm the great resistance of the *Mucor* fungus to the selection of contaminants of emerging concern if not that it is a robust process much more resilient than conventional processes.

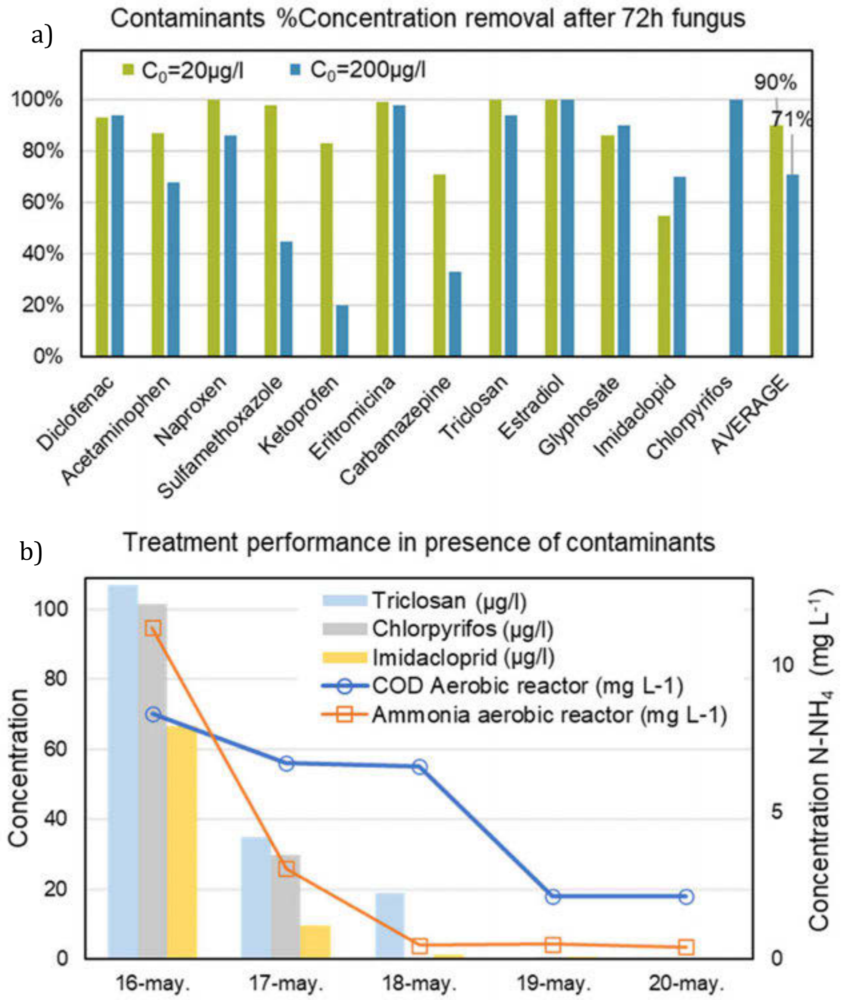

Figure 6. a) Contaminants %concentration removal after 72 hours in presence of Mucor fungus. b) Pilot plant wastewater treatment performance after adding a mixture of 13 contaminants at a concentration of 200 µg/l.

3.3 Biofungus sludge biomethane potential

Biofungus sludge was also characterized by BMP during different stages of the process. The results were in line with the sludge age of the plant. Due to the low production of sludge very few purges were needed. The biomethane production evolves accordingly with the sludge age, showing relation with the measured initial volatile content of the samples.

BMP results during the first month of operation were very promising resulting into values two-fold the ones obtained with conventional WWTP sludge. On the contrary, after 2 months of operation, biomethane productions were equal for both samples or slightly lower for the fungal sludge. The reduction in biomethane production with the sludge age was confirmed and therefore, some representative experiments of stabilized Biofungus sludge compared with the WWTP sludge are presented in *Figure 7*.

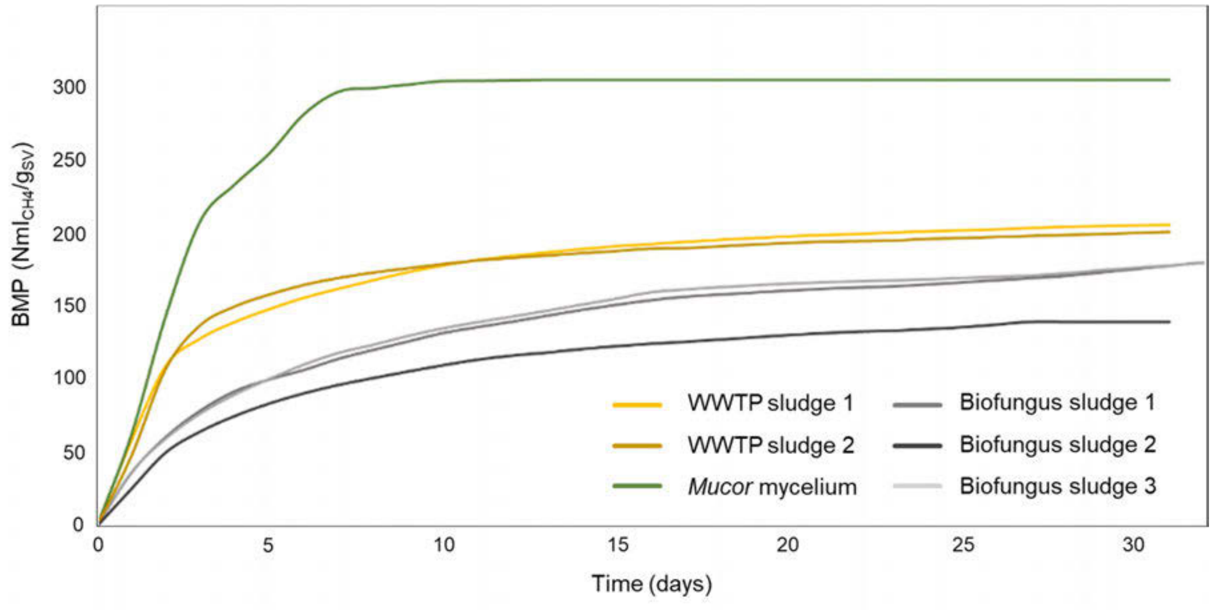

Figure 7. Average values of Biofungus pilot plant sludge samples compared with conventional WWTP sludge and the pure Mucor mycelium.

Figure 7 shows, respectively, the evolution over time of the biomethane potential of sludges produced in the Biofungus plant (from the last stage, anoxic decanter), compared with fungus mycelium and with the conventional WWTP sludge. As can be seen, BMP of mycelium is almost 50% higher than that of conventional sludge. However, the sludge generated during the daily operation using these mycelia as inoculum in the reactors has a methane production potential much lower than that of conventional sludge. It is also less that the half of the BMP of its corresponding mycelia.

Nevertheless, it is proven that Biofungus sludge is completely valid for its treatment as co-digestate by anaerobic digestion.

4 CONCLUSIONS

The development of the Biofungus project has proven that *Mucor circinelloides* is good candidate for the wastewater treatment application, not only for the aerobic treatment, achieving similar performance to that obtained in conventional treatments, but also for denitrifying process. Although, technical difficulties in the design of this plant have hindered the continuous elimination of nitrate, the elimination of 100% of ammonia and nitrate has been proved with high elimination rates.

The in-situ culture of two different strains of *Mucor* fungus has been carried out. Food industry residues have been used as culture media, being this a good initiative for residues circularity.

The high floatability observed for the fungus sludge has been related to the oleaginous and filamentous nature of this fungus. The continuous operation of the pilot plant was hampered since the beginning of the project, resulting on the escapement of the anoxic fungus from this stage and making the continuous denitrification almost impossible. No solution has been found with the proposed plant design and some alternatives considering the sludge flotability must be consider in the future.

BMP Biofungus sludge characterization was performed since the beginning of the project. High biomethane production was observe at low sludge ages, but due to the small amount of sludge produce by this process, the conventional sludge of the plant resulted on high ages and lower volatile content. The fungal sludge results, on average, on lower biomethane production compared with conventional WWTP sludge, but still can be perfectly used as co-digestate at anaerobic digestion treatment.

Finally, the *Mucor* fungus have show very high resistance to contaminant of emerging concern, included very different categories such as drug, pesticides, herbicides, fungicides or hormones, up to concentrations as high as 20mg/l. High removal efficiency of these contaminants was also measured, but still further investigation is needed to know if contaminants are assimilated o degraded. The robustness and resilience of the process was also proven in presence of contaminant after a discharge containing 200 µg/l of thirteen contaminants.

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