ELIMINATION OF MT FROM AQUACULTURE MASCULINIZATION SYSTEMS: USE OF CATALYSIS WITH TITANIUM DIOXIDE AND BACTERIAL DEGRADATION

Mitigating Negative Environmental Impacts/ Experiment/ 07MNE06UA

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INTRODUCTION

Agricultural and industrial activities generate a significant number of pollutants that are released to the environment through sewage water, which reach the superficial and underground water reservoirs. Many of these substances are highly toxic and do not degrade easily in nature. As a result of years of negligence, the levels of these substances in rural and industrial areas in Latin America are dramatically high. In order to reduce risks to human health and the environment, water treatment systems have been implemented (Valladares, 1995). Water treatments aim at 1) eliminating waste, floating fats and oils, sand, and all other coarse elements water may contain; 2) eliminating decantable materials, both organic and inorganic; 3) eliminating biodegradable organic matter dissolved in water; and 4) stabilizing and disposing mud extracted as a result of those processes (Crites and Tchobanoglous, 2000).

Traditional technologies used to separate organic substances from water use activated charcoal absorption or air hauling. However, these processes only transfer pollutants from water to other environmental media, which perpetuates the problem. At present there is a group of technologies based on pollutant destruction processes through chemicals known as hydroxyl radicals, which are highly oxidative. In these technologies, called "Advanced Oxidation Processes" (AOP), radicals react with pollutants and transform them into

environmentally harmless compounds, mostly CO2 and water. Implementation of these technologies has begun in North America, Europe, and Japan.

Among the materials used as catalysts are TiO2, ZnO, CdS, iron oxides, WO3, and ZnS, among others. These chemicals are financially affordable, easily traceable in nature, and can excite with low-energy light, absorbing part of the sun's spectrum radiation that hits the Earth's surface ($\lambda > 310$ nm). In particular, photocatalytic degradation based in the use crystallized titanium dioxide (TiO2,) and low-energy (320-390 nm) ultra-violet (UV) sunlight is interesting for water treatment purposes. This method has been tested in laboratories since the mid-80's for hydrocarbons such as chlorate and phosphate compounds contained in pesticides, herbicides, colorants and surfactants. The technique consists in generating hydroxyl radicals which then oxidate organic compounds. Recently, interest in this technology has increased due to its potential solar energy applications, as 5% of the sunlight reaching the troposphere provides enough energy to activate titanium dioxide (Valladares, 1995).

Aquaculture is an area in which AOPs could be applied to effluent treatment. This industry uses steroidal hormones to manipulate phonotypical sex (Yamamoto, 1969). If these chemicals are adequately treated, their use is beneficial and has no adverse environmental effects. However, there seems to be grounds for concern regarding water overuse. In particular, a legitimate international concern is the lack of up-to-date quantitative information regarding the chemicals being used and the lack of efforts oriented to studying and treating the polluting by-products generated by this industry --steroids being one of them. Hormones used in aquaculture are among a list of compounds which environmental effects and possible treatments have been prioritized for research by GESAMP (FAO's Joint Group of Experts in the Scientific Aspects of Marine Environmental Protection).

Recent innovations in hormone applications in aquaculture include sex control, thus producing the so-called mono-sex lines, which result in significant production improvement (GESAMP, 1997). In particular, the molecule 17 α-Methyltestosterone (MT) has been widely used as androgenic agent in masculinization of tilapia (Contreras-Sánchez, 2001). After the hormonal treatment (28 days), the hormone is released to the water through urine and feces and becomes a powerful pollutant capable of producing harmful effects in wild species when effluents are released. In recent studies (Contreras-Sánchez, 2001), we found that masculinization of fry through dietary treatment with MT resulted in the accumulation of MT in sediments which produced both intersex fish and females with altered ovarian development. In systems where substrate was not present, there were higher concentrations of MT in the water and lower (sometimes null) masculinization rates than in systems with either soil or gravel. We found that charcoal filtration of water from systems where substrate was not present lowered the amount of MT in water to almost background levels and the treatment resulted in almost complete masculinization of all three broods tested (100, 98 and 100% males, respectively). Apparently, the recommended dose of MT for masculinizing tilapia is higher than needed and a significant portion of it separates from the food and remains either in suspension in the water for the short term or persists in the sediments over the long term (Contreras-Sánchez, 2001). In the cited study, we recommended the use of filtration systems to eliminate excess MT to increase masculinization, and to prevent potential risks to humans of unintended exposure to MT due to contamination of water and soils in farms. In this sense, research has been published on photocalytic hormone elimination (Tanizaki, 2002). However, the published studies have focused on sewage water estrogen elimination due to the harmful biological effects of the presence of small levels of steroidal estrogens in water (Ike, 2002; Coleman et al., 2000 ; Carballa, 2004). Nevertheless, the elimination of MT in aquaculture using advanced oxidation processes have not been addressed.

Another possible approach to eliminating MT from masculinization systems involves the use of bacterial degradation since it has been reported that some bacteria are capable of degrading steroids (Voishvillo et al, 2004). From this information and results from the previous investigations we hypothesized that MT was being eliminated from water by solar irradiation and/or bacterial degradation within the filtration system. In our latest experiments (unpublished data) we have detected specific bacteria colonies growing in the biological filter of MT-treatment tanks, but not in the control tanks (showing mainly algae). In this particular study, the ultimate goal of our research was to isolate, characterize and cultivate the species of bacteria responsible for degradation of steroids and determine if the titanium dioxide or bacteria present in biofilters are capable of degrading MT.

MATERIALS AND METHODS

Experiment 1. Use of TiO2, UV light and air. To conduct this experiment, a rectangular 30x30x20 cm experimental reactor was used. Glass plates (the size of the reactor's base) were prepared with immobilized titanium oxide. Water flow was recirculated with a peristaltic pump. The volume of water in the reactor was 3,000 ml. High-pressure mercury light was set over the reactor to serve as a source of UV light (Luck, 1997).

Fixation of TiO2 in glass plate. Four consecutive layers of a TiO 25% m/v ethanol suspension were applied with a foamy rubber roller. Following application of each layer, the panels were sun-dry and once dry, heated at 200° C for one hour in a laboratory oven to eliminate any remaining organic matter and contribute to the cohesion of the semiconductor. The panels were washed with distilled water in order to eliminate any semiconductor trace that did not adhere after the thermal treatment. After applying the last layer, the panels were heated at 280 °C for 2 hours (Byrne, 1998).

Solutions. A stock solution of 17 α-Methyl testosterone (Argent Labs) diluted in ethanol was prepared with a final concentration of 1 ug/L. A working solution of 3 L of water containing MT was prepared to assure a concentration of 1 ng/ml. The titanium oxide used was TiO2 P25 (Degussa, Corp.) in distilled water (Colleman, 2004).

Experimental design. This experiment was conduced at the Laboratory of tropical Aquaculture at UJAT, Tabasco, México. The experiment was a 2 x 2 x 3 factorial experiment where factor A is the air effect, factor B is the effect of UV light and factor C is the type of titanium oxide delivery method (attached to a glass plate, in suspension and absent). All treatments were evaluated in duplicate. Treatments were as follows:

FACTOR A (AIR FLOW)	FACTOR B (UV LIGTH)	FACTOR C (TiO2)	TREATMENT CODE
YES	NO	ABSENT	T1
		IMMOBILIZED	T2
		IN SUSPENSION	T3
	YES	ABSENT	T4
		IMMOBILIZED	T5
		IN SUSPENSION	T6
NO	NO	ABSENT	T7
		IMMOBILIZED	T8
		IN SUSPENSION	T9
	YES	ABSENT	T10
		IMMOBILIZED	T11
		IN SUSPENSION	T12

Three liters of water (control or MT-treated) were placed in the reactor and were recirculated using a peristaltic pump. Water was exposed to treatments for 90 min. Water samples were collected at 0, 30, 60, and 90 minutes.

Samples were diluted in 40 % methanol (Cromasolv™ Sigma-Aldrich) and the MT concentration was obtained by Metiltestosterone EIA Ridascreen™ determination kit (R3601, r-Biopharm) and data were processed by RIDA®SOFT Win software to obtain the final concentration.

Experiment 2. Use of bacterial degradation. This experiment was conducted at Tropical Aquaculture and Microbiology Laboratories at UJAT.

For bacterial isolation, heterotrophic bacterial colonies were obtained from biological filter units present in our masculization tanks. These colonies where isolated from inoculation of microbiological culture media with materials from the filter (sediments and fouling). Samples for inoculation were obtained from a traditional 28-day MT-masculinization process (60 mg/kg of food feeding regime). Samples were collected on days 7, 11, 20 and 28. Culture media used for this phase were nutritive agar, methylene blue and eosin based agar. Only the best growing and most abundant colonies where selected. Bacteria identification was performed using the identification system API WEB (Biomérieux™) and dichotomy based keys (Koneman et al., 1999).

To evaluate the MT degradation capacity based on its use as carbon source, five best-growing species were cultured on MT enriched mineral media (chemical composition shown in table 1) based on Perez et al., (2006) containing different 17 α-Methyl testosterone (Argent Labs) concentrations for each 100 mL of culture media (1 μg; 10 and 40 mg MT). Best growing conditions (based on the MT concentration) were used for the degradation capacity experiment. Based on this, a complete randomized; one factor experimental design was constructed, consisting of six treatments: 1) Control: No bacterial colonies added. 2) BC1: *Pseudomonas aeruginosa*. 3) BC2: *Pseudomonas fluorescens*. 4) BC3: *Bacillus subtilis*. 5) BC4: *Bacillus ceresus*. 6) BC5: *Serratia marcescens*. All treatments were run using mineral culture media enriched with 40 mg/100 mL of MT and evaluated in triplicate.

Erlenmeyer flasks containing 100 mL of culture media were inoculated with 2 mL of bacterial suspension containing 15 x 10-8 CFU/ 100 ml (0.5 Mc Farland turbidimetric units). Flasks were placed in a temperature controlled shaker bath and maintained to constant temperature and agitation (30° C @ 175 rpm) during a culture period of 26 days. Daily bacterial growth was checked by plate counting microbiological procedures at 30°C to establish the growing stages. Samples for MT determination were collected depending on bacterial culture lifetime.

General sampling days were for all treatments on day: 0 (before bacteria added), 2, 6, 10, 16 and 20. All samples were frozen (–20°C) and preserved until processing. Samples were diluted in 40 % methanol (Cromasolv™ Sigma-Aldrich) and the MT concentration was obtained by Methyltestosterone EIA Ridascreen™ determination kit (R3601, r-Biopharm) and data were processed by RIDA®SOFT Win software to obtain the final concentration.

Colony formation units for each species were compared using a Kruskal-Wallis test. Final MT concentrations among treatments were evaluated by means of One-way ANCOVA using the initial MT concentration as covariate. Differences between initial and final MT quantity were analyzed by Kruskal-Wallis test.

Experiment 3. Use of bacterial degradation of MT in masculinizing systems.
This experiment was conducted at UJAT laboratories, the Microbiology lab for bacterial biomass and biofilm-biosphere production and Tropical Aquaculture for biofilter inoculation and fish masculinization.

The experiment was based on one factor with 2 treatments. All treatments were evaluated in triplicate. Treatments were as follows: 1) Fry fed with MT at 60 mg/kg of food for 28 days; water recirculated through biofilter without bacterial inoculation (BA). 2) Fry fed with MT at 60 mg/kg of food for 28 days; water recirculated through biofilter with bacterial inoculation (BP).

Pseudomonas aeuriginosa was selected for running this experiment, since was the most abundant species. Mass production was achieved using a laboratory-scale bioreactor. Starting cultures were prepared in a two 250 mL Erlenmeyer flasks containing 100 mL of liquid media (two-fold concentration); cultures were performed in a static system for 12 to 24 h at 35°C in a temperature-controlled oven.

Starting culture initiated containing 3.27 E+07 *P. aeuriginosa* cells/mL, which were transferred to 2,000 mL Kettler Jar bioreactor (1100 mL working volume). The jar system is equipped with an air inlet and a high pressure valve. Media agitation and filtered air was supplied using an aquarium compressor connected to a 0.45 µm Millipore™ membrane air filter. FCU counts were obtained by normal plate culture count procedure at 30°C. Polyethylene floating biospheres (450 units) were disinfected by immersion during 48 h in a 10 % (v/v) solution prepared with commercial sodium hypochlorite and washed with distilled water. Biospheres were transferred to a glass container containing 10 L of similar starting culture media and then inoculated with 5.7 E+12 cells/mL and maintained for a period of 15 days with one culture media renovation at day 7.

Experimental units consisted of 8,000 L concrete tanks, equipped with a ½ HP centrifugal water pump (10 gpm, Aquapack AP-5X ™) for water recirculation. Water was pumped to a 200 L vertical tank containing the biofiltration unit. The biofilter consisted of a 35 cm bottom layer made of coarse river gravel (~1" diameter) and an upper layer containing polyethylene biospheres (Aquatic Ecosystems). Water pumped from the masculinization tank was supplied to the biofilter by a water inlet at the top of the filter spraying the water over the biospheres, then, by gravity, water passed by the biospheres and gravel to return to the masculinization tank by a water outlet located near the biofilter bottom. Each tank was stocked with 5,700 tilapia fry (*Oreochromis niloticus*) obtained from the Tilapia Farm "Pucté del Usumacinta" located in the county of Emiliano Zapata and then fed for 28 days with feed enriched with MT at 60 mg/kg food.

After 7 days of fish treatment, bacterial biomass (liquid suspension with biospheres) was inoculated into the biofilters for treatment BP, in case of no bacterial inoculation only biospheres were added. Air was supplied to each biofilter for a three day-period and then water recirculation was restarted. Water samples for MT determination were collected the day of inoculation and at days 14 and 28 of MT treatment. All samples were preserved frozen (-20°C) until processing. Samples were processed directly or diluted in 40 % methanol -- depending on MT concentration-- (Cromasolv™; Sigma-Aldrich).

MT concentrations by date, and final growth for total length and weight were compared using a Kruskal-Wallis test. Sex ratios and final survival were compared by a Chi-square test to determine efficacy of MT treatment and possible bacterial effects on survival.

RESULTS

Experiment 1. Use of TiO2, UV light and air. The EIA had a consistent 35.05% (± 2.9) MT detection efficiency in water samples. However, only detected values are presented in this report. MT data were bouncing back and forth in all treatments. Some data are non detectable even at time cero, before initiating treatments, indicating errors in the entire experiment. Statistical analysis show effects for the factor Air at T_0 (Multifactor ANOVA, *p=0.01*), all other factors did not show significant effects at that time (p>0.5). However these results must be taken with caution. MT elimination was observed when air was not supplied to the reactor. For all another exposition times evaluated (30, 60 and 90 minutes) no effects were observed for any factor and treatment. MT concentrations for all treatments at each exposition time are shown in table 2. Some high values for MT concentration were observed for treatments 4 and 7 at 60 minutes of exposition time.

Experiment 2. Use of bacterial degradation. Fifteen species were present in the 140 selected colonies from the sediment and fouling samples. Best growing results were obtained when 40 mg of MT/100 mL of enriched culture media was used. Species *Pseudomonas aeuriginosa, Pseudomonas fluorescens, Bacillus ceresus, Bacillus subtilis* and *Serratia marcescens* showed the best adaptation pattern and growing rates for the culture media used. Bacterial growth obtained for the other MT concentrations used (10 µg &10 mg MT/100 mL) was not adequate for our experimental goals. The MT sampling protocol was re-defined according with the growing phases obtained for each species.

Statistical differences where observed for MT enriched media adaptation (KW, p<0.001). *P. aeuriginosa* grew faster and with the higher number of microorganisms obtained in a 24 h culture period, followed of *P. fluorescens* with initial culture growth at 48 h. *Bacillus ceresus* started at 72 h, while *Bacillus subtilis* and *Serratia marcescens* showed the lowest speed growth initiating at 96 and 144 h (Figs. 1 and 2).

The EIA had a consistent 23.98% (± 1.4) MT detection efficiency in bacteria culture media. However, only detected values are presented in this report. MT initial concentration (as detectable by the EIA) in the culture media ranged from 6.5 to 11.6 mg MT/100 mL. MT degradation was observed in cultures with all experimented species, but not statistical differences were observed among species capacities (ANCOVA, p=0.33). Tendencies were observed but no differences among MT quantity degradation (KW, p= 0.30). *Pseudomonas fluorescens* showed the higher tendency for degradation capacity showing only 0.54 mg MT/l00 mL at the end of the culture period, followed by *P. aeruginosa* showing at day 16, 2.17 mg MT/100 mL. Final MT concentrations for all the other species were: 3.12 mg MT/100 mL for *Serratia marcescens,* while *Bacillus ceresus* showed a final concentration of 0.64 mg MT/100 mL and 7.50 mg MT /100 mL for *Bacillus subtilis* (Figs. 3 and 4).

Experiment 3. Use of bacterial degradation of MT in masculinizing systems. No statistical differences were observed in MT concentration among treatments for the three days sampled (KW, p=0.3; Fig 5). An infestation of dragonfly larvae caused high mortality in all tanks. Statistical differences were observed, with better results in survival when bacteria biomass was present; however, this result should be taken with caution (Table 3). One hundred percent males were obtained as a result of MT feeding in both treatments.

Statistical significant differences were found for final weight among treatments (KW, p= 0.00). Best results were obtained in the BP treatment with 0.26 (± 0.38 g), while in the BA treatment the final weight was 0.18 (± 0.35 g). No significant differences were observed in total length (KW, p=0.56). Final length for BP treatment was 23.00 (± 7.40 mm) and 22.65 (± 7.74 mm) for BA treatment.

DISCUSSION

The experiment with titanium dioxide provided confusing results. We have several doubts regarding the quality of MT used, since our stock was old (provided by Argent, Thailand). Since MT is restricted in Mexico, making difficult obtaining the chemical. Despite this, in several tests, we found a consistent, but low detection of MT (35% approximately). The amount used in the assay was 1 ng/ml (sufficient for EIA detection) but data are inconsistent. Little can be said about this technique until more experiments are conducted.

In the experiments with bacteria, we found good adaptation of *Pseudomonas aeuriginosa, Pseudomonas fluorescens, Bacillus subtilis, Bacillus ceresus* and *Serratia marcescens* to culture media containing MT as the only source of carbon and energy. The microorganism have been identified as part of the microbiota composing bacterial biofilms in natural, clinical and industrial wet environments, characterized by a minimal presence of nutrients (Piera, 2003; Echeverri, 2002; Escalante, 2002). Biotransformation of steroids by microorganisms has been reported by several authors (Akherem y Titov, 1972) using the steroid molecule as the only source of carbon, degrading it to carbon dioxide and water. Despite having good adaptation results with the five species studied, we consider that *Pseudomonas aeuriginosa* showed the best adaptation to culture media enriched with high amounts of MT. The genus *Pseudomonas* has been isolated from soils contaminated with polycyclic aromatic hydrocarbons. It is considered a group capable of utilizing an enormous variety of organic compounds as substrates to grow; this capability allows them to colonize inhospitable niches and microenvironments with a limited amount of nutrients that other microorganisms are not capable of using (Martínez and Martínez, 2003). This versatility is reinforced by the fact that two species of *Pseudomonas* were abundantly found in this investigation, showing great potential for MT degradation.

In this study, we found the highest biodegradation activity in the treatment containing *P. fluorescens*, removing up to 97% of the total amount of MT after 16 days of culture and 99% at the end of the experiment (20 days). *B. ceresus* also eliminated 99% of MT by the end of the experiment (day 20). *P. aeruginosa* degraded 90% of MT by day 10 and 97% by day 16. The last sample at day 20 was lost due to contamination. *Bacillus subtilis* degraded Only 10% of the MT present by day 10 and 26% by day 20, while *Serratia marcescens* utilized 10% by day 10 and 40% by day 16, at the end of the experiment we found 40% of the total amount of the MT used, present in the culture media. These results indicate that the genus *Pseudomonas* and *Bacillus* can efficiently remove MT from culture media. The highest biodegradation effect was observed during the exponential phase of the bacterial culture. It is well known that at this stage the number of microorganisms and the microbial activity increase rapidly. We infer that at this stage bacteria actively take carbon from MT to incorporate it as source of energy during development (Madigan and Martinko. 2004). The most important bacteria genus used in this study have been used in other studies to amply biodegrade benzene, kerosene and biodiesel reaching up to 80-90% degradation of these compounds. One of the characteristics of the genus *Pseudomonas* is the ability to catabolize aromatic and aliphatic hydrocarbons; this characteristic was mainly codified in plasmids (Atlas y Bartha, 2002; Pineda *et al.,* 2002; Hans, 2005; Pérez *et al.,* 2006). *Pseudomonas sp.* and *Bacillus sp.* have been utilized as bacterial consortiums in hydrocarbon degradation. These groups show and efficient elimination rate and they have been recommended for bioremediation processes

(Mohamad *et al.,* 2004). This condition maybe valid for future experiments were both groups could be tested to demonstrate degradation capacity of MT. *Serratia marcescens* has also been used in studies related to biodegradation of oils, diesel, and 2, 4, 6-trinitrotoluene, showing biodegradation of these compounds similar to those found in the current study with MT (69-70%; Mohanan *et al.,* 2007; Rajasekar *et al.,* 2007; Montpas *et al.,* 1997).

Bacillus subtillus y B. ceresus are Gram positive microorganisms that form high-temperature resistant endospores. These endospores have the capacity of remaining latent during several days, even years. The genus *Bacillus* has been isolated and studied for its degrading capabilities of Trinitrotoluene, N-Hexadecane and crude oil, among other chemicals. Studies have reported that this genus is capable of removing up to 80% of these compounds (Kurinenko *et al.,* 2003; Patil 2009 *et al*., 2009). Those results also coincide with the biodegradation of MT reported in this study.

Our results strengthen the idea that biodegradation of 17 α-Metyltestosterone can be effectively achieved with bacterial biofiltration using the species isolated in our masculinization systems. All species tested (*Pseudomonas fluorescens, Pseudomonas aeruginosa*, *Bacillus subtillus, Bacillus ceresus* y *Serratia marcescens*) showed significant biodegradation of MT, both in culture media as well as in the water. In the masculinization trial, bacteria appeared not to have a significant impact on MT concentration; however, it is possible to see that there is a clear tendency for MT loss with time once bacteria were added. Lee and Liu (2002) have acknowledged that bacterial activity can be used for degrading substances such as 17 B estradiol, present in most effluents. Therefore, our results support that bacterial biofiltration can be used effectively for removing steroids from the water. More research is needed regarding timing, use of multiple species, optimal conditions and maturation of the biofilter, in order to achieve better results.

Another important fact is that all bacteria analyzed are very common in al fish culture systems. In some cases, they have been considered as probiotic organisms. Gram et al., (1999) reported *P. fluorescens* as benefic against the action of *Vibrio anguillarum* and Bly et al., (2003) reported it beneficial against *Saprolegnia*. Despite the high mortality found in our experiment, the fact that the fish in the tanks with bacteria grew more than those in the tanks without bacteria (with lower final density), may point out that bacteria is acting as probiotic, enhancing growth.

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Table 1. Chemical composition of mineral media used for bacteria culture isolated from MT based masculinization system.

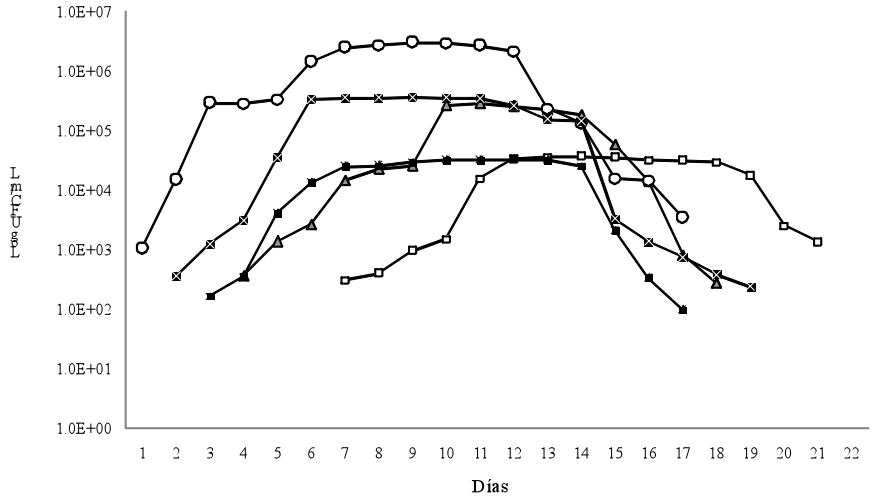
Reagent	Concentration (g/L) used by genus		
	Pseudomonas	*Bacillus*	*Serratia*
$(NH4)_2SO_4$	-	1	2.38
NH_4Cl	0.1	-	-
$CaCl_2$	0.001	0.1	14.7
$MgSO_4$.$7H_2O$	0.05	0.1	0.246
$K_2HPO_4.3H_2O$	0.1	6.3	-
KH_2PO_4 .$7H_2O$	0.05	1.83	1.36
$FeSO_4.7H_2O$	0.001	0.1	0.003
KCL	0.01	-	-
$Na_2HPO_4.7H_2O$	-	-	2.68

Table 2. Average MT concentration (pg/L) for treatments at different exposition times.

	Exposition time (min)			
Treatment	**T_0**	**T_{30}**	**T_{60}**	**T_{90}**
1	5.24	0	6.23	7.94
2	3.23	2.6	0.16	0
3	0	0	0	0
4	0	43.63	3558.00	12.31
5	58.03	55.41	2.180	91.78
6	0.12	2.91	108.30	406.17
7	0	211.37	4261.94	104.26
8	237.85	176.5	171.50	290.64
9	213.60	406.18	149.82	210.33
10	164.55	204.39	358.42	193.00
11	133.32	160.08	494.45	98.62
12	209.47	250.93	241.78	150.81
13	0.067	0.092	0.0005	0

Table 3. Final results from masculinized tilapia fry after 28 days of treatment concrete tanks inoculated (BP) or not inoculated (BA) with bacterial biofilms of *Pseudomonas aeuriginosa* induced in polyethylene biospheres.

Treatment	Survival (%)	Males (%)
Bacteria Present (BP)	20.6 ± 5.06^a	100
Bacteria Absent (BA)	16.8 ± 2.71^b	100



Pseudomonas aeruginosa	*Pseudomonas fluorescens*	*Bacillus ceresus*	*Bacillus subtilis*	*Serratia marcescens*

Figure 1. Microbial growing kinetic in mineral culture media enriched with 17α-Metiltestosterone (MT) for the selected strains isolated from a masculinization system.

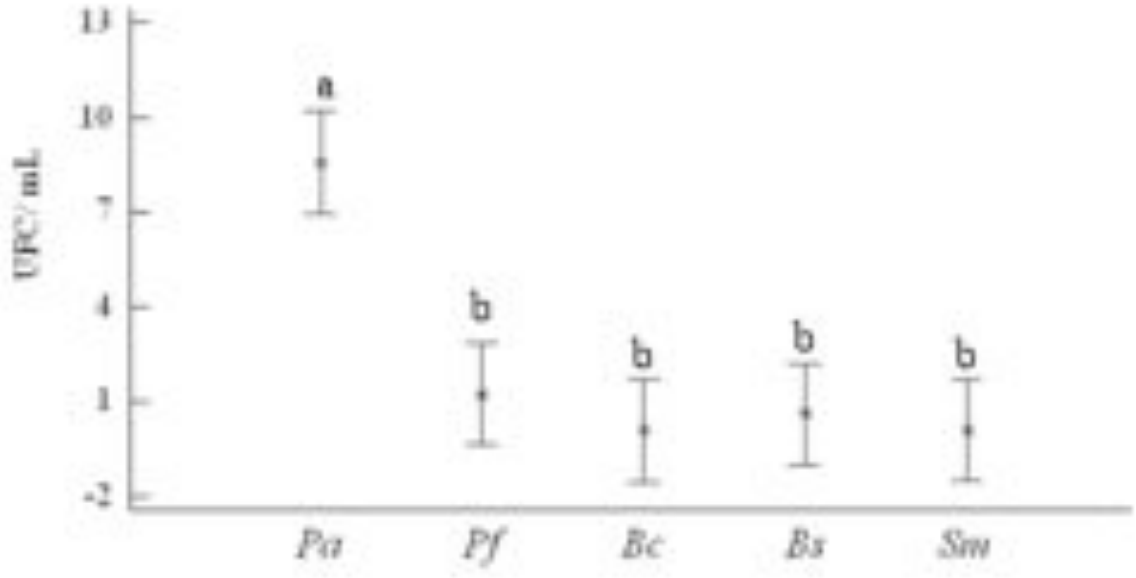


Figure 2. Average (±SE) of total microorganism concentration observed at day 22 of culture in mineral media enriched with 40 mg/100 mL of MT. Different letters indicate statistical significant differences. *Pa*) *Pseudomonas aeruginosa; Pf) Pseudomonas fluorescens; Bc) Bacillus ceresus; Bs)Bacillus subtilis, Sm) Serratia marcescens.*

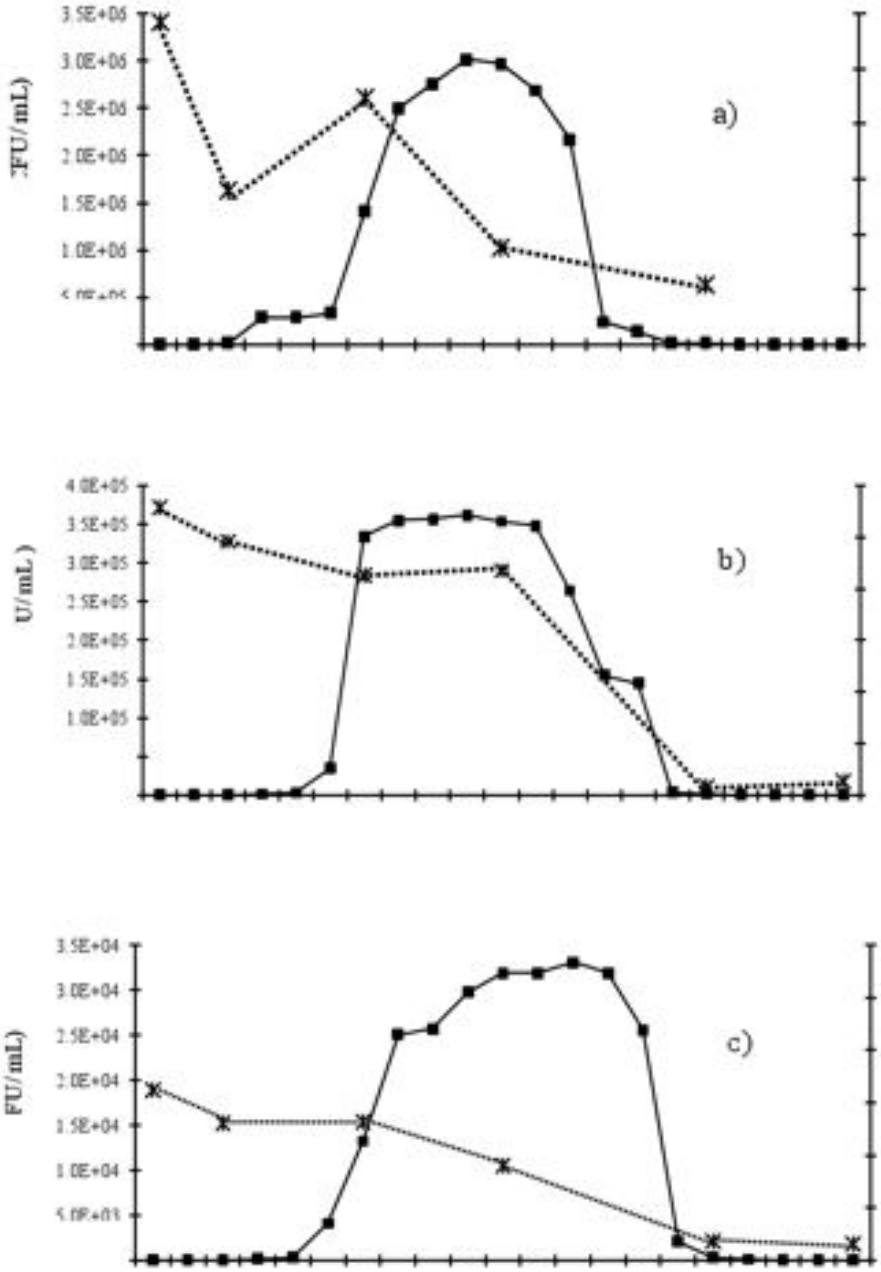


Figure 3. Microbial growing kinetic (continuous line) and MT concentration (dotted line) for selected bacterial strains. a) *Pseudomonas aeruginosa*)*;* b) *Pseudomonas fluorescens;* c) *Bacillus ceresus* in mineral culture media enriched with MT.

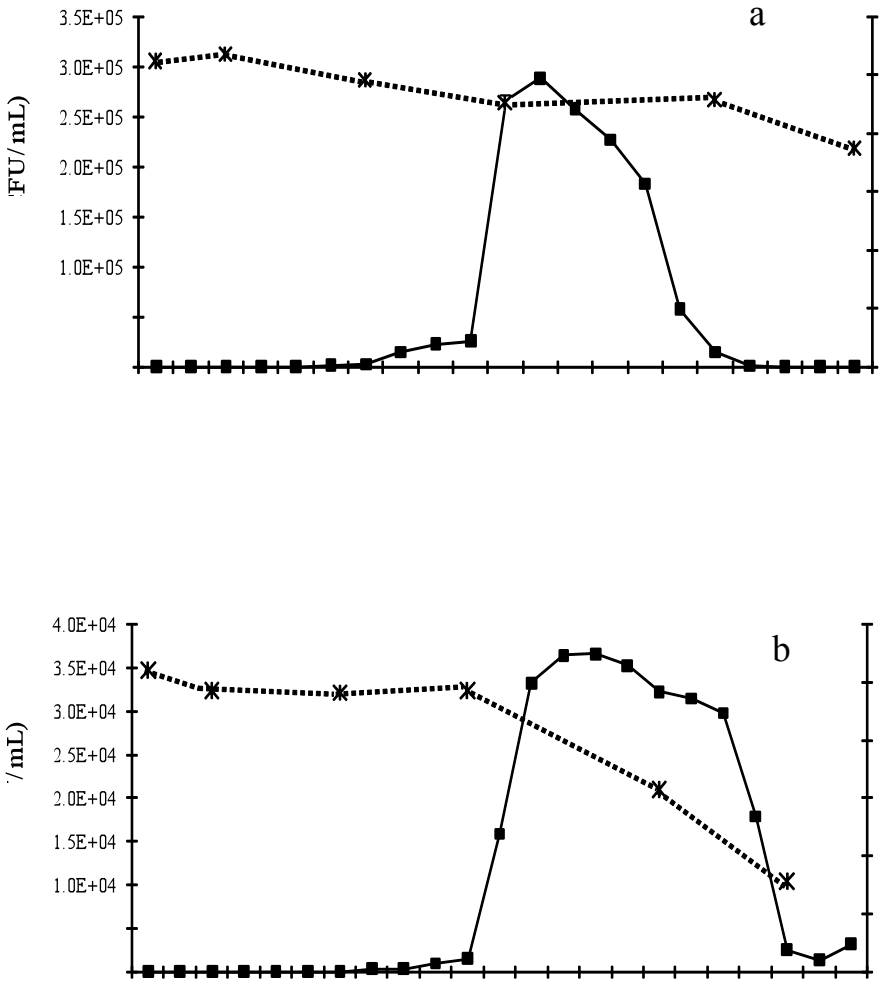


Figure 4. Microbial growing kinetic (continuous line) and MT concentration (dotted line) for selected bacterial strain of a) *Bacillus subtilis* and b) *Serratia marcescens* from masculinizing h MT.

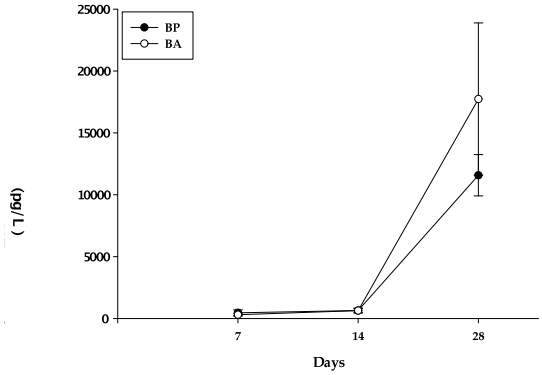


Figure 5. Average MT concentration in water from concrete tanks used for tilapia masculinization inoculated (BP) or not inoculated (BA) with bacterial biofilms of *Pseudomonas aeuriginosa* adhered to polyethylene biospheres.