REPORT

Impact of microbial and chemical pollution in Cuban freshwater ecosystems: strategies for environmental recovery

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ABSTRACT

Contamination of water resources requires of systematic environmental actions, based on integral researches which allow proposing biological process as ecological and economical alternatives in order to decrease its negative impact and to guarantee the integrity of aquatic ecosystems. The objetives of this paper were to determine the microbial and chemical water pollution in Almendares river, and to evaluate the microbial capacity for decrease or eliminate chemical contaminants present on wastewaters, natural ecosystems and industrial wastes. The microbiological and chemical pollution of the Almendares basin exceeded maximum permissible values for recreational waters according to Cuban standards, respect to total and faecal coliforms, to the presence of clinical significance multiresistant microorganisms against antimicrobial agents, and to nitrates, ammonium, phosphates and heavy metals concentrations. It was demonstrated the potential of monocultures and microbial consortiums for the elimination of heavy metals and the capacity of White Rot Fungi to degrade industrial textil colorants. The results indicate the contamination degree in freshwater systems in Cuba, a threat for biodiversity and a risk for human health. Therefore, these results permit to dispose of a microbial collection which represents potential candidates for biotechnological applications, which contribute to the protection of the environment and the preservation of waters as a valuable natural resource.

Keywords: Freshwaters ecosystems, microorganisms, heavy metals, xenobiotic compounds

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RESUMEN

Impacto de la contaminación microbiana y química en sistema dulceacuícolas cubanos: estrategias para la recuperación ambiental. La contaminación de los recursos hídricos requiere de una sistemática gestión ambiental, basado en investigaciones integrales que permitan proponer procesos biológicos como alternativas ecológicas y económicas para disminuir su impacto negativo y asegurar la integridad de los ecosistemas acuáticos. Los objetivos de este trabajo fueron determinar la contaminación microbiana y química de las aguas del río Almendares y evaluar la capacidad microbiana de reducir o eliminar contaminantes químicos presentes en aguas residuales, ecosistemas naturales y residuales industriales. La contaminación microbiológica y química de la cuenca Almendares excedió los valores máximos permisibles para aguas con uso recreativo según las normas cubanas, por la concentración de coliformes totales y fecales, a la presencia de microorganismos de importancia clínica con multirresistencia a agentes antimicrobianos, a las concentraciones de nitratos, amonios, fosfatos y de metales pesados. Se demostró las potencialidades de monocultivos y consorcios microbianos, para la eliminación de cinc y cadmio. Se comprobó la capacidad que presentan hongos de la Podredumbre Blanca, para degradar colorantes textiles industriales. Los resultados indican el grado de contaminación en sistemas dulceacuícolas del país, lo que constituyen una amenaza para la biodiversidad y un riesgo para la salud humana. Además, permiten disponer de una colección de microorganismos candidatos potenciales para aplicaciones biotecnológicas que tributen a la protección del medio ambiente y a la preservación de ese valioso recurso natural que es el agua.

Palabras clave: Ecosistemas dulceacuícolas, microorganismos, metales pesados, compuestos xenobióticos

*I*ntroduction

Nowadays the contamination of aquatic ecosystems with not treated or insuficient treated wastewaters constitute one of the most dangerous environmental problems. A large population in developing countries uses surface waters for irrigation, recreational, drinking and other domestic purposes [1]. In Cuba wastewaters treatment are not efficient and the urban rivers recieve high load of untreated sewage, industrial pollutans and domestic wastewaters. The pollution of natural waters by biological agents and toxic chemical compounds decreases the self-cleaning capacity of the recipient ecosystems, which results in the accumulation of contaminants to high and damaging levels. The increasing deterioration of this natural resource is a serious worldwide problem that the international community has to face up due to the high demand of water. Havana is the most affected Cuban province because of the industrial development and the population density, which have lead to the contamination of different rivers. The large urban settlings as well as the installations of public and recreative services near the Almendares river, contributes to increase the volume of waste in this hydrographic basin, resulting in a target for microbial and chemical contamination.

A solution for this environmental problem could be the use of microorganisms with potential capacity for bioremediation of this ecosystem as a more ecological and economic method. Also the implementation

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of wastewater treatment plants for cleaning up and recovery contaminated waters contributes to the protection of the environment [2, 3].

This research approached to an integral study in one of the most important aquatic ecosystem in Havana city, with the purpose of determine the microbial and chemical contamination and the ability of microbial biomass to decrease or eliminate efficiently toxic chemical pollutants such as heavy metals and recalcitrants and persistent compounds.

Results and discussion

The integral characterization of Almendares river through sampling during a 4 years period in 14 stations, showed the persistence of high concentrations of microbial contaminants (Figure 1). The DQO, indicator of organic matter contamination, exceeded more than 10 times the reference values of Cuban standards [4]. This river showed a high faecal contamination, as indicated in the Figure 1B; total coliforms (TC) and faecal coliforms (FC) concentrations exceed the maximum permissible values for waters of recreational use according to Cuban standards [5], by this reason this water can not be used for recreative activities and crop irrigation. In this investigation were isolated different microorganisms of clinical importance, such as: *Escherichia coli, Pseudomonas aeruginosa*, *Staphylococcus lentus*, *Enterobacter*, *Citrobacter* y *Enterococcus.* In addition, one hundred and thirteen *E. coli* isolates were serotyped and screened for virulence determinants (LT, ST, Stx, bfpA and eaeA genes) by PCR. HEp-2 cells adherence assays were also performed in these strains. In this study, 42 O serogroups and 63 O:H serotypes were determined, and 50% of these isolates belongs to one of serogroups O1, O8, O16, O23, O25, O30, O45, O64, O75, O117, O169 and O174. PCR assays showed that 23% of the isolates carried at least one of the virulence markers tested. Diffuse (DA), aggregative (AA), chain-like adherence (CLA) and variants of the AA pattern were found in 25 strains. Moreover, the 23% of pathogenic strains showed resistance against the different antimicrobials evaluated, mainly against ampicilline and trimetropin-sulfametoxazole. Both antimicrobials are used in the treatment of infections caused by these microorganisms. These results support the impossibility of the use of this water by the population. The higher diversity and number of serogroups, serotypes, virulence factors and adherence pattern found in *E. coli* strains isolated from Almendares river might be related to the sources of contamination in this reservoir as has been reported in other freshwater ecosystems [6].

These results allow to identify the major risk factors inherent to infectious agents present in the water, with an insufficient performance (37.1%) of the established requirements for its utilization, but the level of risk was classified as low despite deficiencies detected. However, the results indicate that the measures that contribute to gradual cleaning up of this river are necessary due to the biological risk implicated in the use of this water by the population. A solution to this microbial contamination is the remotion of coliforms bacteria in natural treatment plants, which can constitute an alternative to this problem. Determining of efficiency degree in samplings carried out in the

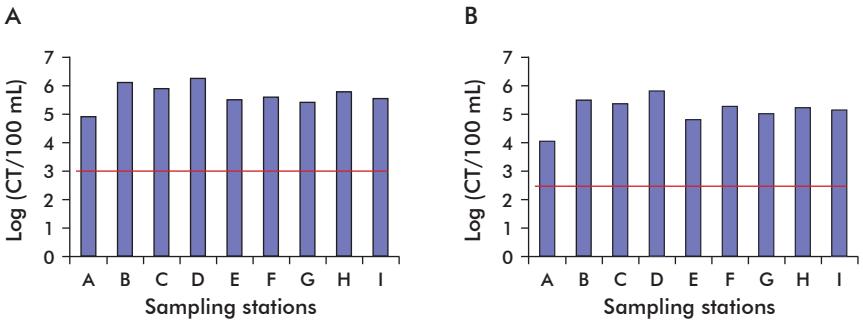

Figure 1. Means of concentration logarithms of total and faecal coliforms in Almendares river. A) Total coliforms (TC); B) Faecal coliforms (FC). The sampling stations were: A- Río Cristal; B- María del Carmen; C- Coppelia Factory; D- 100 y Boyeros; E- Puentes Grandes; F- Papelera; G- Puente de Piedra; H- Puente Almendares; I- Puente de Hierro. The red line shows the value of the NC 22, 1999 for each case.

Palatino and Cubeco wastewater treatment plants, indicated a decreasing of two logarithmic units in the concentration of coliforms, accompanied of a great decreasing in other parameters, such as the DBO_5 and the DQO, which isn't sufficient for the ecosystems protection. The establishment of new methodologies based in advanced technology, with a multidisciplinary approach, which don't have been used until now in tropical climates, allowed the evaluation of freshwater ecosystems, which support its extension to other ecosystems of interest.

The determination of chemical contamination in the ecosystem, referred to Almendares river showed phosphate (PO_4) values range from 0 to 4 ppm, which exceed 0.1 ppm, the recommended maximal concentration in flowing water to discourage excessive growth of aquatic plants. For the nutrients involved in the nitrogen cycle, a balance between the reduced forms is observed in the river and its tributaries. The concentrations of nitrates (NO_3) vary between 0 and 3 ppm while ammonium (NH_4) and nitrites (NO_2) concentrations vary between 1 and 12 ppm and 1 and 20 ppm, respectively. The nutrients involved in the nitrogen cycle and the phosphates were found in concentrations over the limits that guarantee the health of natural freshwater systems. The presence of high ammonium concentrations indicates the presence of faecal contamination. These results are comparable with others obtained in the case of polluted rivers in temperate regions [7]. The concentration of heavy metals were high for Mn^{2+}, Zn^{2+} (10.05-11.51 µg/L), Pb^{2+} (2.86-73.29 µg/L), Cu^{2+} (0.05-0.49 µg/L), Cr^{6+} (0.14-0.20 µg/L), Ni^{2+} (1.90-5.17 µg/L), Co^{2+} (0.96-3.17 µg/L) and Cd^{2+} (0.12-0.81 µg/L). Besides, it was demonstrated the accumulation of metals in different organisms such as fishes and plants, which bioaccumulate a higher spectrum and quantity of heavy metals, specially in roots, so that can be used as biologic indicators.

In contrast with these results, the water of tourist complex "Las Terrazas" didn't show a high pollution degree. The FC values were slightly higher to those established by standards, but much lower than those found in Almendares river. However, the *E. coli* values where higher that the maximum limit established by Cuban standards for recreational water and irrigation. There was not linear correlation between the concentrations of these indicators of faecal con-

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tamination, with an average value of 0.46 for the ratio *E. coli*/faecal coliform. These results show the good microbiological water quality of Las Terrazas and increase the information about this ecosystem located in Sierra del Rosario (Biosphere Reserve).

According to that, it is necessary the development of new approaches and technologies to decrease the negative environmental impact of the metals and to preserve the waters for assure the integrity of aquatic ecosystems and to recover the metals. The selection of collection´s microorganisms *Pseudomonas mendocina* (Ps-1), *Anabaena* sp. PCC 7120, *Saccharomyces cerevisiae* (10), *Chaetoceros ceratosphorus* (1P) and the native isolates (bacteria A6, CBM4 and microalgae CMM6), from sources of contaminated waters with metals, that reached capture levels of Zn^{2+} and Cd^{2+} from aqueous solutions between 16 and 34 mg/g, shows the diversity of physiological and genetic answers of microorganisms, which permit them to activate mechanisms of uptake these chemical contaminants, converting them in potential tools for bioremediation process, as ecological, effective and economical solutions for this environmental problem [8]. The study of factors associated to removal of metals, permitted to define that physiological age of culture, pH of biomass-metal suspension and the initial concentration of metal in solution, increased the capture of Zn^{2+} and Cd^{2+} for each microorganism. The application of physical and chemical treatments to selected microbial biomass, allowed to verify that inactivated cells by dry hot treatment increased the biosortive capacity of ions by all the microorganisms. This treatment increased the extracellular capture of zinc until 4.4 times and the capture of cadmium until 8.3 times more, in relation to non-treated cells. The pretreatments could modify the surface characteristics/groups either by removing or masking the groups or by exposing more metal binding sites. Thus nonviable cells would offer a larger available surface area and expose the intracellular components and more surface binding sites because of the destruction of the cell membranes [9]. On the other hand, nonviable cells offers the advantages of their conservation at room temperature for long periods of time, that don't depend of metal toxicity, neither requires nutrient supply and constitutes a low risk for environment.

Molecular biotechnology is a potent tool to construct engineered organisms with higher biosorption capacity and selectivity for the objective metal ions. The application of genetic engineering techniques provided the genetic modification of *Anabaena* sp. PCC 7120. This transgenic cyanobacterium expressed *smtA* gene which codify for metallothioneins in the citosol of *Synechococcus* sp. PCC 7940. This result was verified by the molecular size of the 1700 bp band corresponding to the porine-*smt*A-porine fragment, amplified with designed oligonucleotides (291 + 315), using the 1500 bp molecular marker. By this form it was verified the presence in the asp. PCC 7120 and permitted corroborate the positive results of the triparental conjugation fulfilled. This clone was identified as PIM 141-7. The application of these techniques, that might contribute to enhance metal biosorption capacities in the microorganisms, has been reported in other investigations about the peptides and proteins expression with uptake capacities of metallic ions [10].

The effectivity of this gene fusioned to a protein of external membrane in the capture of metal was corroborated by the double biosortive capacity of cadmium ions in the transformed strain. The transgenic *Anabaena* sp. PCC 7120 strain PIM 141-7 was able to uptake 33.76 ± 0.95 mg of cadmium per gram from the aqueous solution. That amount was significantly higher (as evidenced by a one-way ANOVA Test of the mean of three replicates, followed by Tukey´s test, $p < 0.05$) than that achieved with the wild strain either untransformed or bearing the mock plasmid pRL 277 (16.67 ± 2.1 and 15.98 ± 1.25 mg/g, respectively). From the total metal captured\ by transgenic strain PIM 141-7, 56.3% was adsorbed to cellular surface, percentage that differs with statistical signification from the percentage of extracellular metal linked by the wild strain. Previous results referred the increase in metal uptake by eukaryote metallothionein expression in *E. coli* [11].

From the different procedures evaluated were combined the variants with best percentages of metal remotion. The results showed that with 10 h of contact biomass-metal, the monocultures of *Pseudomonas mendocina* (Ps-1) and *Saccharomyces cerevisiae*-10, reached efficiency percentages in the biosortion of Zn^{2+} superior to 97%, this result demonstrate the use of these strains in liquid waste shocked with this metal. Two microbial consortiums increased the cadmium biosortion efficiency to 85.4 and 96.9% (Table).

These biosorbents have metal-sequestering property and can be used to decrease the concentration of heavy metal ions in solution. It can effectively sequester dissolved metal ions out of dilute complex solutions quickly and with high efficiency which correspond with the report for other microbial species [12].

The rhizosphere microorganisms, in particular, have an important contribution to the removal of pollutants. *From* 58 rhizosphere bacterial isolates obtained from the rhizosphere of the hydrophyte plant, *Typha dominguensis*, in Cuban natural wetlands the interaction with organic matter, nitrogen, phosphorus and heavy metals was studied. Thirteen isolates were selected by their potentialities as bioremediating agents, the 13 isolates showed resistance to more than one metal. It was found that some strains removed metals, 4 of them can be used in a consortium for chromium and lead removal, from simulated effluents. The results obtained are important, considering that metal concentrations studied (1.0, 2.0 and 3.0 mM) are above the allowed maximum concentrations regulated in water, according to Cuban Standard [4] and other international standards. Also, the selection of strains with resistance and remotion capacity of more

Table. Biosortion of cadmium by microbial consortiums §

Microbial consortiums	Cd^{2+} q (mg/g)	Efficiency E (%)
MECIIMA / 08 *Pseudomonas mendocina* (Ps-1); *Saccharomyces cerevisiae* (10); microalgae CMM6; transgenic strains (PIM 141-7)	197.1 ± 3.09[a]	85.4 ± 1.3[a]
ISMEC / 08 *Pseudomonas mendocina* (Ps-1); microalgae CMM6	145.4 ± 2.8[b]	96.9± 1.9[a]

§ Data presented as mean ± standard deviation from 3 repetitions by microorganisms. An ANOVA test of simple classification to the analyzed microorganisms by metals was used. Different letters in the table indicates significant differences between the microorganisms; q represent values for $p < 0.05$, by a statistic Tukey´s test.

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than one metal is important because in the ecosystems all the elements are in interaction. Some authors have reported the capacity of different bacterial strains for metal remotion [13, 3], but the majority of these results are related with remotion of less harmful compounds. On the other hand, many strains showed remotion of 50% of organic matter and phosphate from synthetic wastewater, which are pollutants also present in Almendares river, some strains present remotion of 100% and it was observed remotion of nitrogen too, but with lower values. The remotion percentages were similar or superior to those obtained with other microorganisms [14, 15]. These results contribute to the future application of the bacterial isolates in water treatment technologies.

Other way of biotechnological application, especially in bioremediation, was the use of white rot fungi (WRF) to degrade a wide range of recalcitrant organic compounds, including pesticides, polycyclic aromatic hydrocarbons, dyes and textile effluents. These compounds have complex structures and cause pollution problems that seriously affect ecosystems, because are chemicals toxic, mutagenic or carcinogenic and are often released into natural environments from industrial operations.

The high capacity of lignin degradation by 33 WRF strains, collected from woody material present in several ecosystems of Havana city and Sancti Spíritus, allowed the biotransformation of 12 kinds of textil dyes which are toxic and chemically diverse. From them, *Ganoderma* aff. *zonatum* (B-18) and *Trametes maxima* (MUCL 44155) showed the higher decolorizing capacity (Figure 2A). These strains of HPB removed textile dyes in superior values to that obtained with conventional treatments. The enzymatic crudes of *T. maxima* MUCL 44155, were stable for more than 7 days to pH between 4.5 and 7.0, at 30 ^{o}C. This result makes its industrial use easier. A simple and economic medium with molasse-urea and mineral salts which removed 100% of dye at the fourth day of treatment with *G.* aff. *zonatum* (B-18) was obtained.

The studied strains showed a high biodegradative capacity upon the DDT (1, 1, 1-trichloro-2,2-bis (4- clorophenil) etane) with values of 99% and 73% respectively (Figure 2B). *T. maxima* MUCL 44155 presents the lacase enzyme as the only involved in the degradation of the dye model acid blue 62. Only in the cultures of *G.* aff. *zonatum* was detected the presence of intermediary metabolites of degradation, so both strains can be considered adequate for use in the biological treatment of textile effluents and in the remotion of the pesticide DDT. The values of dyes decolorisation and DDT degradation achieved by these strains are comparable to the results reported by others authors using promising WRF strains [16].

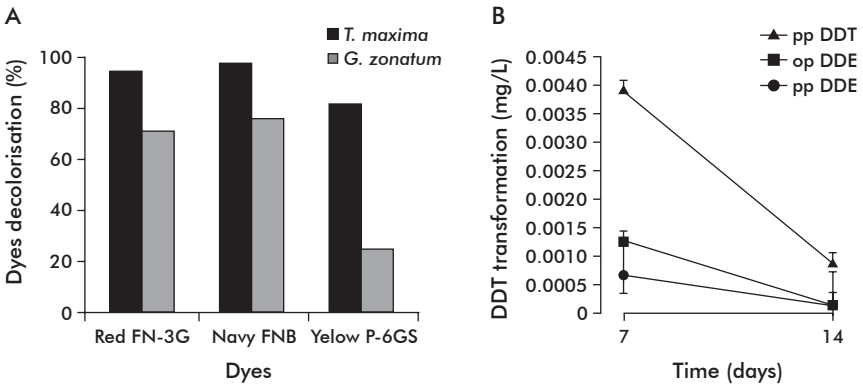

Figure 2. Decolorisation of three different dyes (A) and degradation of DDT (B) in submerged culture at 30 oC and 50 r/min during seven days. Batch fermentation processes were developed in Kimura medium supplemented with 0.05% of dyes or 0.01 mg/L of DDT. DDT: 1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane. DDE: 1,1-dichloro-2,2-bis (4- chlorophenyl) ethylene as ortho para (opDDE) and para para (ppDDE) conformers.

The control of microbiological and chemical water quality is of primary importance in developing tropical countries, as the high levels of this contamination of waters constitute a potential risk for biodiversity and human health. By this reason the exploitation of local biodiversity in tropical area appears as a potentially productive approach for identifying promising microbial strains for biotechnological use.

Conclusions

The methods used in this study are suitable for the evaluation of microbiological and chemical quality in the tropical environment featured and contribute to improve the evaluation of the level of contamination in Cuban freshwater ecosystems. This investigation demonstrated that biosorption is a useful alternative to the conventional systems for the removal of heavy metal ions from aqueous solution. The expression of metallotioneins in bacterial cell provides an improvement in accumulation of metals and offers a promissory strategy for the development of microbial biosorbents in metals bioremediation of polluted water. The use of WRF presented high degradative capacity on different dyes and DDT. These strains could be considered very promising candidates for developing a biotechnological treatment of industrial wastewaters with xenobiotic compounds.

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