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Ecotoxicological Bioassays with the microalga *Pseudokirchneriella subcapitata* in lotic ecosystems and impact of metals using confocal fluorescence microscopy techniques

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Abstract

A rapid and simple ecotoxicological bioassay allows a reliable estimation of state of the lotic ecosystems from Camana, Majes and Colca watershed located in region Arequipa (Peru) in six sampling points (Taparza, Grande, Majes1, Majes2, Camana1 and Camana2) by studying growth inhibition of the microalga *Pseudokirchneriella subcapitata* at 24, 48 and 72 hours and Mean Effective Concentration (EC_{50}), at 72 hours compared to National Environmental Quality Standards (EQSs) and water quality guidelines of the World Health Organization (WHO). Observing that in the sampling points of Majes1 and Majes2 surpassed the values of thermotolerant coliforms, Aluminum, Manganese, Iron and Total Suspended Solids (TSS), compared to the values of EQSs and water quality guidelines of the WHO, with an EC_{50} in the Sampling stations of Majes1 and Majes2 categorizing them as moderately toxic. In this article, fluorescence confocal microscopy techniques were used to evaluate the impact of metals that exceeded the EQSs and WHO, proposing a model as the application of these microscopic techniques, opening wide perspectives, for future studies of metal ecotoxicity.
Key Words: Bioassays, growth, fluorescence, *Pseudokirchneriella subcapitata.*

Bioensayos Ecotoxicológicos con la microalga *Pseudokirchneriella subcapitata* para medir el impacto de los metales en ecosistemas lóticos utilizando técnicas de microscopía confocal de fluorescencia

Resumen

Un bioensayo ecotoxicológico rápido y sencillo permite una estimación confiable del estado de los ecosistemas lóticos de las cuencas de Camaná, Majes y Colca localizados en la Región en Arequipa-Perú en seis estaciones de muestreo (Taparza, Grande, Majes1, Majes2, Camaná1 y Camaná2), mediante la inhibición del crecimiento de la microalga *Pseudokirchneriella subcapitata* a las 24, 48 y 72 horas y la Concentración Efectiva Media (CE_{50}), a las 72 horas en comparación con los Estándares peruanos de Calidad Ambiental (ECA) y las directrices de la calidad del agua de la Organización Mundial de la Salud (OMS). Se observó que en los puntos de muestreo de Majes1 y Majes2 se superaron los valores de coliformes termotolerantes al aluminio, manganeso, hierro y sólidos suspendidos totales (SST) comparados con los valores de ECA y directrices de calidad del agua de la OMS, con una CE_{50} categorizándolos como moderadamente tóxicos. En este artículo, se utilizaron técnicas de microscopía confocal de fluorescencia para observar el impacto de los estándares de la EQS y los de la OMS, proponiendo como modelo la aplicación de técnicas microscópicas, con amplias perspectivas para futuros estudios de ecotoxicidad por metales.
Palabras Clave: Bioensayos, crecimiento, fluorescencia, *Pseudokirchneriella subcapitata.*

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Introduction

The use of bioassays with standard test organisms represents a fundamental approach in the definition of ecological risk in the aquatic environment for chemicals products (Smiglak *et al.*, 2006). The microbial growth rate decreases with increased toxicity (Pardos, Benninghoff & Thomas, 1998; Gabrielson *et al.*, 2002) and to determine and to evaluate the presence of toxic substances in the aquatic environment, the micro-algae *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum*), have been used (Wells & Coombe, 2006; Cho, Pham, Jeon & Yun, 2008). Aquatic organisms that may be affected by pollutants are primary producers, which are essential for the maintenance of the structure and function of aquatic ecosystems, so any negative effect on them will affect the primary trophic levels. Highly sensitive microalgae to a high variety of toxic substances as *P. subcapitata* are used as model systems for toxicity bioassays (Castillo, Vila & Neild, 2000; O'Farrel, Lombardo, Tezanos & Loez, 2002).

P. subcapitata is a planktonic species that lives in freshwater ponds, lakes and rivers. Cells in cultures are solitary except during cell division. The cells are helical, usually semi-circular curved in vegetative phase. The diameter of the arc (154 - 360°) oscillates between 4.8 and 10.8 µm, width ratio of 1.6-4.4 µm. The chloroplast is parietal and lacks in pyrenoide. The reproduction is by division of the stem cell in 2, 4 or 8 autosporas (Nygaard, Komárek, Kristiansen & Skulberg, 1986).

The strains of *Pseudokirchneriella subcapitata* used in laboratories around the world have been isolated from the Nidelva River, Akershus, Norway, by Olav Skulberg in 1959 (Norwegian Institute for Water Research (NIVA) - CHL 1) (Aruoja**,** 2011).

According to aquatic ecosystems classification, there are ecosystems of type lotic (rivers, ravines), lentic (lakes) and lentic-lotic temporal (flooded jungle during river overflow). The velocity of the current, the permanence of the water and the temporary flood of each type determine aspects such as the ease of establishment of aquatic communities, contributions and washes of nutrients and productivity, among others (Pinilla, 2005). The lotic ecosystems present a constant movement of water with hydrological, chemical and biological characteristics determined by the climate, geology and vegetation of the watershed (Payne, 1986; Allan, 1995).

Many developed and developing countries face serious ecological and toxicological problems derivative of the release of complex effluents and toxic substances to the environment. To contend with this problem a wide range of biological tests are used with fish and other aquatic organisms of various trophic levels for biological monitoring and evaluation of toxicity (Bringmann & Kuhn, 1980; Cairns, Dickson & Westlake, 1976; Little, 1978; Maciorowski, Sims, Little & Gerrard, 1981).

The lotic ecosystems of the rivers of the watershed of Camana and Majes are located in southern Peru; their area of influence is mainly the Arequipa Region, but it also includes a part of the Cusco and Puno regions. Along the watershed of Camana and Majes the following activities are carried out: economic, agricultural-livestock farming, mining and fishing; as a result of such human activities multiple pollutants are discharged into the environment, under that consideration the sampling stations were selected (Taparza, Grande, Majes1, Majes2, Camana1 and Camana2), from the watershed of Camana and Majes which are located in the provinces such as Castilla, Caylloma, Camana, Condesuyos and Arequipa in the Peru's Arequipa region (ANA, 2012).

This research proposes to determine the reliability of fast and simple ecotoxicological bioassays in lotic ecosystems such as the rivers of Taparza, Grande and Camana - Majes (Arequipa, Peru), using the Mean Effective Concentration (EC_{50}), categorizing it according to the toxicity range and growth inhibition of *Pseudokirchneriella subcapitata* and compared to physico-chemical analyzes that exceed the values of the National Environmental Quality Standards (EQSs) and water quality guidelines of the World Health Organization (WHO).

Materials and methods

Test organism

The microalga *Pseudokirchneriella subcapitata* (Korshikov) Hindak (formerly named *Raphidocelis subcapitata* Korshikov and *Selenastrum capricornutum* Printz), was the species used to carry out ecotoxicological bioassays. They are easily available species (from crop collections) and maintained in the laboratory under controlled conditions and currently approved for regulatory purposes (Nalewajko & Olaveson, 1998; Lewis, 1990; Weyers, Sokull-Kluttgen, Baraibar.Fentanes & Vollmer, 2000).

The microalga, kindly provided by NEC (National Environment Center) of the University of Chile, Metropolitan Region, Santiago, Chile, was kept in the Microbiology and Biotechnology Laboratory of the National University of San Agustin (UNSA), Arequipa, Peru, from June to August 2016. It was cultured in 250 mL Erlenmeyer flasks, with 100 mL of culture medium adding one milliliter of macronutrients and micronutrients in approximately 900 mL of purified water (MILLI-Qs, Millipore, Billerica, MA, USA) according the EPA manual (USEPA, 1994). The culture of *P. subcapitata* was maintained in a lighting chamber using an air pump at 2000 lux continuous illumination and temperature at 24 - 25°C; Medium's pH was adjusted to = 7.5 $\pm$ 0.1 with 1N of NaOH or HCl. At the end of the culture, an inoculum of 2.5 x 10^6 cells/mL was normalized by counting the Neubauer chamber from

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which 2 mL were diluted in 18 mL of fresh culture medium in order to obtain an initial concentration of 10^4 cells/mL in each test vial for ecotoxicological bioassays.

Study area

To evaluate the selected area of the lotic ecosystems of the Arequipa, Peru region with the bioassays of the microalga *P. subcapitata*, the identification of contaminant sources; the classification of natural bodies of surface water and the parameters established in the National Environmental Quality Standards (EQSs) was taken into account for water where the sampling station E1 = Taparza river (791888 UTM East, 8333378 UTM North at 1544 meters above sea level), located at 20 meters before the confluence with the Tipan river, this place was chosen by the existing thermal waters, like the thermal baths of Taparza, to about 10 Km bathed by a stream that descends of the skirts of Coropuna, where are the springs of sulphurous thermal water. The thermal water of Taparza in different points has cracks, it is a sandstone rock and something metamorphic, giving off a strong smell of sulfuric gas.

In that spring, the water at the exit of the land has the temperature of 46.6 °C and in the circular pool that serves as a bath reaches 44 °C. E2 = Grande river (UTM East, 8238815 UTM North at 1386 meters above sea level), located at the height of the Huario Bridge, passing through the Chuquibamba district of the province of Condesuyos, which has important economic activities such as agriculture and cattle. E3 = Majes1 river (769459 UTM Este, 8220997 UTM Norte at 619 meters above sea level, located at the height of the Huancarqui Bridge in the district of Huancarqui and the E4 = Majes2 river (772525 UTM Este, 8198706 UTM Norte at 373 meters above sea level), of the bridge Punta Colorada of the district of Corire; between the E3 and E4 are the district of Aplao, Huancarqui, Uraca and Corire unloading their dumps to the Majes river, also agriculture predominates. E5 = The Camana1 river (745343 UTM East, 8169611 UTM North at 68 meters above sea level), located at the height of the Bocatoma El Brazo, place chosen for sampling because it is located before all the discharges of Camana. E6 = Camana2 river (740355 UTM Este, 8162246 UTM Norte at 17 meters above sea level), located in the Montes Nuevos sector after all the discharges of Camana and before reaching the sea (Figure 1) (COPASA, 2012; ANA, 2012; UNSA, 2001).

Physicochemical analysis

A analyzes of pH, dissolved oxygen (DO), chemical oxygen demand (COD), biochemical oxygen demand (BOD), total suspended solids (TSS), electrical conductivity, oils and fats, cyanide, Sulphides, Phosphates and Nitrates, of the water samples, were determined according to the methodology described by APHA (2000).

Metal levels were determined by emission spectroscopy with source of inductively coupled plasma ICP (Ocampo-Duque, Sierra, Ferré-Huguet, Schuhmacher & Domingo, 2008). The physicochemical data are shown in Table I.

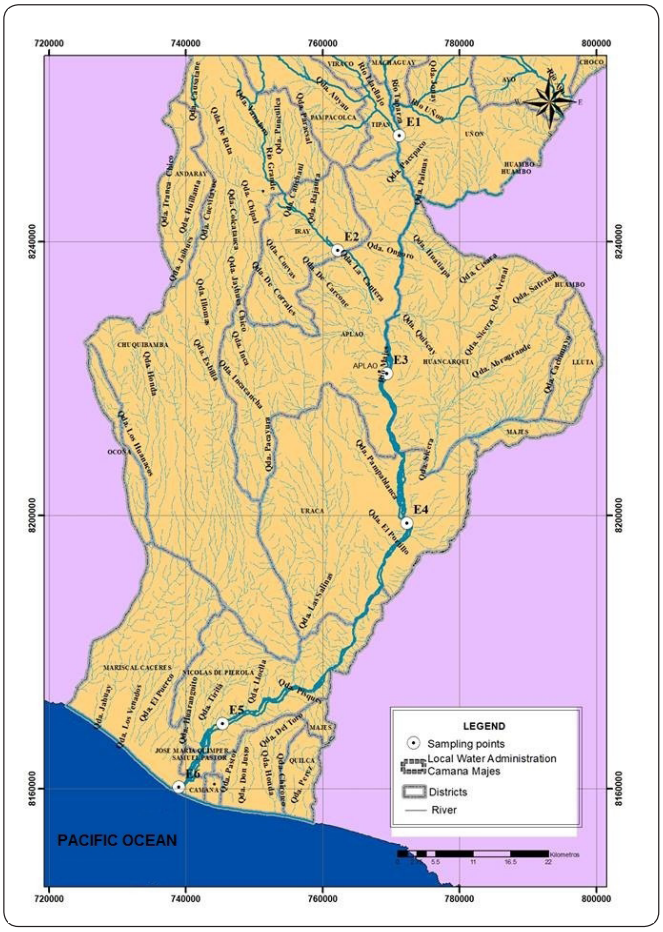

Figure 1. Map of the sampling stations of the watershed Camana, Majes and Colca; Arequipa - Peru : E1 = Taparza; E2 = Grande; E3 = Majes1; E4 = Majes2; E5 = Camana1; E6 = Camana2.

Ecotoxicological Bioassays

Ecotoxicological bioassays with *P. subcapitata* were carried out on samples of water from the watershed of Majes, Camana and Colca at the sampling stations: E1, E2, E3, E4, E5, E6 of static type, with the following design: four concentrations per Bioassay; three replicates, 10^4 cell/mL exposure in each sampling unit. The concentrations were expressed in percentages of 50%; 25%; 12.5% and 6.25% in relation to a positive control consisting in water of a problem sample and a negative control consisting of a buffer solution of 15 mg/L of $NaHCO_3$. The sample unit for the bioassays was test tubes containing 2.5 mL of solution. Growth and inhibition of cell growth were evaluated at the same concentrations and expressed as volume percentages of water. The cell density (N) was determined by direct count in a microscope using the Neubauer camera of bright-line at 24, 48 and 72 h of exposure and the growth rate (μ) in number of divisions (ISO, 1989).

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Table I. Physicochemical analyzes of the sampling stations E1, E2, E3, E4, E5 and E6 of the Camana Majes, Colca watershed, Arequipa, Peru 2016 and comparison with the "Peruvian of Environmental Quality Standards (EQSs)" with categories 3 and 4 and water quality guidelines of the World Health Organization (WHO).

									(EQS) Category 3*	(EQS) Category 4		WHO
Parameter	**Analysis**	**Unity**	**E1**	**E2**	**E3**	**E4**	**E5**	**E6**	**Irrigation of low and high steam vegetables**	**Animal drink**	**Conservation of the aquatic environmental**	**Guidelines**
Flow	in situ	m3/s	1.3	5	154.6	154.2	149.2	149.03				
Temperature	in situ	(°C)	20.6	21.6	18.75	18.45	18.5	18.75				
pH	in situ	-	8.6	8.75	8.2	8.06	8.41	8.41	6,5- 8,5	6,5- 8,5	6,5- 8,5	6,5- 8,5
Conductivity	in situ	µS/cm	885.5	1172	561.6	996.1	650.5	655.2	<2000	≤5000	-	n.a
Dissolved Oxygen	in situ	mg/L	6.42	6.78	7.08	6.75	8.24	8.32	≥4	> 5	≥4	n.a
N-NO3-	Laboratory	mg/L	<0.030	<0.030	0.231	0.363	0.341	0.396	10	50	10	50
PO43-	Laboratory	mg/L	<0.030	<0.030	<0.030	<0.030	0.031	0.036	1	-	0.5	n.a
N-NH3	Laboratory	mg/L	0.021	<0.020	0.034	0.103	0.056	0.045	-	-	0.02	1.5 y 35
DBO5	Laboratory	mg/L	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00	15	≤15	<10	n.a
DQO	Laboratory	mg/L	<10.0	<10.0	<10.0	<10.0	<10.0	<10.0	40	40	-	n.a
TC	Laboratory	NMP /100mLa	////	490	79	7900a	23	49	1000	1000	2000	n.a
Oils and fats	Laboratory	mg/L	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00	1	1	-	n.a
Total Nitrogen	Laboratory	mg/L	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00	-	-	1.6	n.a
Sulfide	Laboratory	mg/L	<0.002	<0.002	<0.002	<0.002	<0.002	<0.010	0.05	0.05	0.002	n.a
Wad Cyanide	Laboratory	mg/L	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006	0,1	0,1	-	n.a
B	Laboratory	mg/L	0.553	1.385	0.36	0.384	0.477	0.47	0,5 - 6	5	-	2.4
Na	Laboratory	mg/L	185.94	301.44	226.65	194.83	171.33	232.74	200	-	-	50
Al	Laboratory	mg/L	0.55	0.02	18.05a	19.64a	1.68	1.68	5	5	-	0.2
Ti	Laboratory	mg/L	0.0158	0.0052	0.091	0.087	0.038	0.0393	-	-	-	n.a
Cr	Laboratory	mg/L	0.006	0.005	0.0098	0.0136	<0.0004	<0.0004	-	-	-	0,05
Mn	Laboratory	mg/L	0.0202	0.0424	0.7426a	0.8144a	0.1583a	0.1528a	0,2	0,2	-	0.1
Cu	Laboratory	mg/L	0.0202	0.0012	0.0363	0.0407	0.0044	0.0039	0,2	0,05	0,02	2
Zn	Laboratory	mg/L	0.008	0.005	0.019	0.163	0.053	0.026	2	24	0,03	3
As	Laboratory	mg/L	0.003	0.062	0.019	0.017	0.014	0.012	0,05	0.1	0,05	0.01
Se	Laboratory	mg/L	0.005	<0.003	<0.003	<0.003	<0.003	<0.003	0,05	0,05	-	n.a

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Table I. Physicochemical analyzes of the sampling stations E1, E2, E3, E4, E5 and E6 of the Camana Majes, Colca watershed, Arequipa, Peru 2016 and comparison with the "Peruvian of Environmental Quality Standards (EQSs)" with categories 3 and 4 and water quality guidelines of the World Health Organization (WHO) (*continuation*).

									(EQS) Category 3*	(EQS) Category 4		WHO
Parameter	**Analysis**	**Unity**	**E1**	**E2**	**E3**	**E4**	**E5**	**E6**	**Irrigation of low and high steam vegetables**	**Animal drink**	**Conservation of the aquatic environmental**	**Guidelines**
Ag	Laboratory	mg/L	<0.0005	<0.0005	<0.0005	<0.0005	<0.0005	<0.0005	0,05	0,05	-	n.a
Cd	Laboratory	mg/L	<0.0004	<0.0004	0.0018	0.0023	<0.0004	<0.0004	0,005	0.01	0,004	0.003
Sn	Laboratory	mg/L	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	-	-	-	-
Sb	Laboratory	mg/L	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002	-	-	-	n.a
Ba Total	Laboratory	mg/L	0.025	0.046	0.298	0.293	0.064	0.063	0.7	-	0.7	0.7
Hg	Laboratory	mg/L	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0,001	0,001	0,001	0.006
Pb	Laboratory	mg/L	0.0102	0.0095	0.0413	0.046	0.0113	0.0109	0,05	0,05	0,001	0.01
Fe	Laboratory	mg/L	0.382	0.026	15.120a	19.510a	1.9a	1.893a	1	1	-	n.a
P Total	Laboratory	mg/L	0.023	0.014	1.049	1.348	0.173	0.168	-	-	-	n.a
Be Total	Laboratory	mg/L	<0.0002	0.0002	0.0009	0.0011	<0.0002	<0.0002	-	0.1	-	n.a
Ca Total	Laboratory	mg/L	<0.0002	0.0002	0.0009	0.0011	<0.0002	<0.0002	200	-	-	200
Ce	Laboratory	mg/L	0.006	0.003	0.054	0.05	0.006	0.008	-	-	-	n.a
Co Total	Laboratory	mg/L	<0.0003	<0.0003	0.0108	0.0132	<0.0003	<0.0003	0,05	1	-	n.a
K Total	Laboratory	mg/L	9.33	20.19	8.03	9.9	5.89	5.85	-	-	-	n.a
Li Total	Laboratory	mg/L	0.027	0.177	0.128	0.157	0.109	0.108	2,5	2,5	-	n.a
Mg Total	Laboratory	mg/L	34.15	28.46	24.39	39.5	13.25	12.97	150	150	-	n.a
Mo Total	Laboratory	mg/L	<0.002	0.002	<0.002	<0.002	<0.002	<0.002	-	-	-	n.a
Ni Total	Laboratory	mg/L	<0.0004	<0.0004	0.012	0.0206	<0.0004	<0.0004	0.2	0.2	0.025	0.07
STS	Laboratory	mg/L	24.94	<3.00	1315a	1493a	106.4a	114a	-	-	≤25-100	n.a
Free cyanide	Laboratory	mg/L	<0.004	<0.004	<0.004	<0.004	<0.004	<0.004	-	-	0,022	n.a

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Toxicity Tests

The results of the toxicity test were quantified in terms of average growth of *P. subcapitata* by calculating the 10, 15, 20, 25 and 50% of Effective Concentration (EC) values determined by the dose-response curve (Nyholm, Sorensen, Kusk & Christensen, 1992). At the same time, the toxicity units (TU) for each sampling point in the growth of *P. subcapitata* were calculated according to the Sprague equation (Sprague & Ramsay, 1965):

$$UT = (EC_{50})^{-1} \times 100$$

Based on the toxicity values obtained in each bioassay for each mean Effective Concentration (EC_{50}) a toxicological category was attributed, following the guidelines of Bulich (1982) and Coleman & Quershi (1985) (Table II).

EC_{50}	Toxicity Category	Rank	Toxicity Units
< 25	Very Toxic	1	>4
25 - 50	Toxic	2	2 - 3.99
51 - 75	Moderately Toxic	3	1.33 - 1.99
76-100	Slightly Toxic	4	1.01-1.32
>100	Not Toxic	5	< 1

Table II. Toxicological categories of the aqueous samples according to the results obtained using the ecotoxicological bioassays with *Pseudokirchneriella subcapitata* (Bulich 1982; Coleman & Quershi, 1985).

Determination of pigment

The extraction and spectrophotometric determination of algal pigments were based on APHA (1998). These measurements were made on basis of protocols described by Rowan, (1989) with the following formulas to calculate chlorophyll:

$$\text{Chlorophyll a } (mgL^{-1}) = \frac{2.67(664_b - 665_a)V_1}{V_2 L}$$

$$\text{Chlorophyll b } (mgL^{-1}) = \frac{[21.03(OD_{647}) - 5.43(OD_{664}) - 2.66(OD_{630})]\,V_1}{V_2}$$

Where V_1 is the extract volume (1); V_2 is the volume of the sample (l); L the length of the cuvette; 664a and 665b the optical density of 90% Acetone before and after of the acidification at 664 and 665 nm; to OD647, OD664 and OD630 the optical density is 647, 664 and 630 nm; C_a and C_b of chlorophylls a and b, respectively (mg/L) (Rai, Singh & Mallick, 1990).

Digital image analysis

Samples of the microalgae *P. subcapitata* from cultures grown stained with acridine orange and mounted on slides with Dako to visualized in Leica TCS-SP8 spectral confocal microscope (Wavelength, 425-520 nm) at Institute of Antofagasta, Chile, for measuring the mean fluorescence intensity (MFI) of data sets xy, using the software Leica confocal (Leica Microsystems CMS GmbH) with the regions of interest (ROI.001) as a function of the software. Digital image analyzes were performed using the free ImageJ 1.29 software, downloadable from the http://rsb.info.nih.gov/ij site.

Analysis of data

The water samples were taken following the "National Protocol for Monitoring the Quality of Natural Surface Water Bodies" (ANA, 2011).

For the evaluation of the surface water quality of the sampling stations, the National Environmental Quality Standards (EQSs) for Water, established in Supreme Decree N° 002-2008-MINAM, were used; and water quality guidelines for the World Health Organization (WHO) (WHO, 1996; WHO, 2011) in order to establish the level of concentration or the degree of elements, substances or physical, chemical and biological, parameters present in water, in its condition of receptor body and basic component of aquatic ecosystems, which do not represent significant risk to human health neither the environment. The parameters that were evaluated *in situ* correspond to the measurement of pH, Dissolved Oxygen (DO), temperature and electrical conductivity, using the Multiparameter Equipment, according to the application of the "Peruvian national protocol for monitoring the quality of natural bodies of surface water".

A multivariate Principal Component Analysis (PCA) was used to relate and order the physicochemical analyzes and sampling points of the watershed of Camana and Majes with the statistical program Minitab 17 (http://www.minitab.com).

Data processing was performed using an Analysis of Variance ANOVA to evaluate the growth of *P. subcapitata* by effect of different ecotoxicological bioassays and concentrations every 24 hours for three days and a Tukey multiple contrast test was used to evaluate the Growth groups to bioassays and concentrations using the statistical program SPSS vers. 20.0 (http://www-01.ibm.com).

To correlate the growth inhibition of *P. subcapitata* and physicochemical analyzes of the sampling stations E1, E2, E3, E4, E5 and E6, a Student's t test of bivariate relation was used from the statistical program SPSS vers. 20.0 (http://www-01.ibm.com).

At the end of the biological tests, the concentration % of the sample, which produces 50% of the specified effect (Mean Effective Concentration, EC_{50}), was determined using the statistical program Regtox_EV7.0.7 (http://eric.vindimian.9online.fr).

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Results

Physical-chemical analysis of lotic systems

The results of the physicochemical analysis of samples collected in July 2016, in the different monitored stations and their comparison with "Peruvian of Environmental Quality Standards (EQSs)", in categories 3 and 4 and water quality guidelines of the World Health Organization (WHO) are shown in the Table I.

Figure 2 shows the graph of Principal Component Analysis (PCA) performed between physicochemical analyzes and sampling points of the watershed Majes, Camana and Colca Arequipa- Peru, 2016. The Principal Component 1 (PC) presented 78.9% of the variance and Principal Component 2 (PC) presented 20.2% of the variance. The six sampling stations: E1; E2; E3; E4; E5 and E6 were found to be associated with the first principal component with positive correlation. The stations E1, E2, E5 and E6 had a negative correlation to the second principal component.

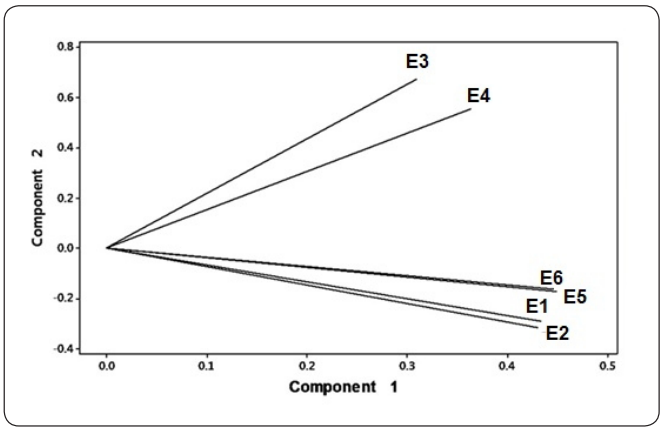

Figure 2. Graph of Principal Component Analysis (PCA) between the physicochemical analyzes and the six sampling points in the Taparza, Grande and Camana-Majes rivers (Arequipa, Peru). E1 = Taparza; E2 = Grande; E3 = Majes1; E4 = Majes2; E5 = Camana1 and E6 = Camana2.

Evaluation of the growth rate of *Pseudokirchneriella subcapitata*

In bioassays of 72 hours of exposure was observed a higher growth rate of *P. subcapitata* in the sampling points of E2 = Grande river at concentrations of 6.25%, and E2 = Grande river, E1 = Tapas river, E3 = Majes1 river at concentrations of 12.5%.

In concentrations of 50 and 100% the bioassays of E4 = Majes2 river and E3 = Majes1 river, have a growth rate below zero, respectively (Figure 3a).

Ecotoxicity

The results indicated that at 72 h the values of the mean Effective Concentration (EC_{50}) for the growth of *P. subcapitata* at the six sampling points presented the following decreasing order E1 (Taparza) = E6 (Camana2) > E2 (Grande) > E5 (Camana1) > E3 (Majes1) > E4 (Majes2), where the lowest EC_{50} are observed at sampling points E4 with 57.43% and E3 with 60.7%, being concentrations for growth of half of the population of *P. subcapitata* (Figure 3b, c, d, e, f, g and Table III).

Besides, the values of the Effective Concentration (EC) at different percentages (EC_5, EC_{10}, EC_{15}, EC_{25}, EC_{50}) in the growth of *P. subcapitata* were calculated to compare the relative toxicity of the river water using the Toxicity Units (TU). According to the data shown in the Table III, the sampling points E1 and E5 were categorized as non-toxic; E2 as slightly toxic, and; E3 and E4 as moderately toxic.

Sensitivity of *P. subcapitata* to exposure of metals that exceeded the limits of EQSs and WHO

The sensitivity of the microalga was evaluated by determination of pigments (chlorophyll a and b), growth rate, EC_{50} and use of confocal fluorescence microscopy, together considered as a versatile, reliable and fast method with multi-parametric optical detection having defined advantages in bioassays of growth inhibition.

Sampling points	EC_5	EC_{10}	EC_{15}	EC_{20}	EC_{25}	EC_{50}	TU_{50}	TC
E1=Taparza	23.97	36.31	48.05	60.04	72.68	157.14	0.64	5
E2=Grande	2.59	5.59	9.39	14.18	20.2	84.2	1.19	4
E3=Majes1	27.27	32.54	36.66	40.3	43.72	60.7	1.65	3
E4=Majes2	14.57	19.72	24.2	28.47	32.72	57.43	1.74	3
E5=Camana1	8.79	13.66	18.38	23.27	28.49	64.5	1.5	3
E6=Camana2	89.07	93.88	96.99	99.39	>100	109.57	0.91	5

TC.: Toxicological Categories; 5 = Non-toxic; 4 = Slightly Toxic; 3 = Moderately toxic, Toxicity Units: TU.

Table III. Results of the toxicity test (EC50 = Mean Effective Concentration in percentage) with the growth inhibition of *P. subcapitata* at 72 hours.

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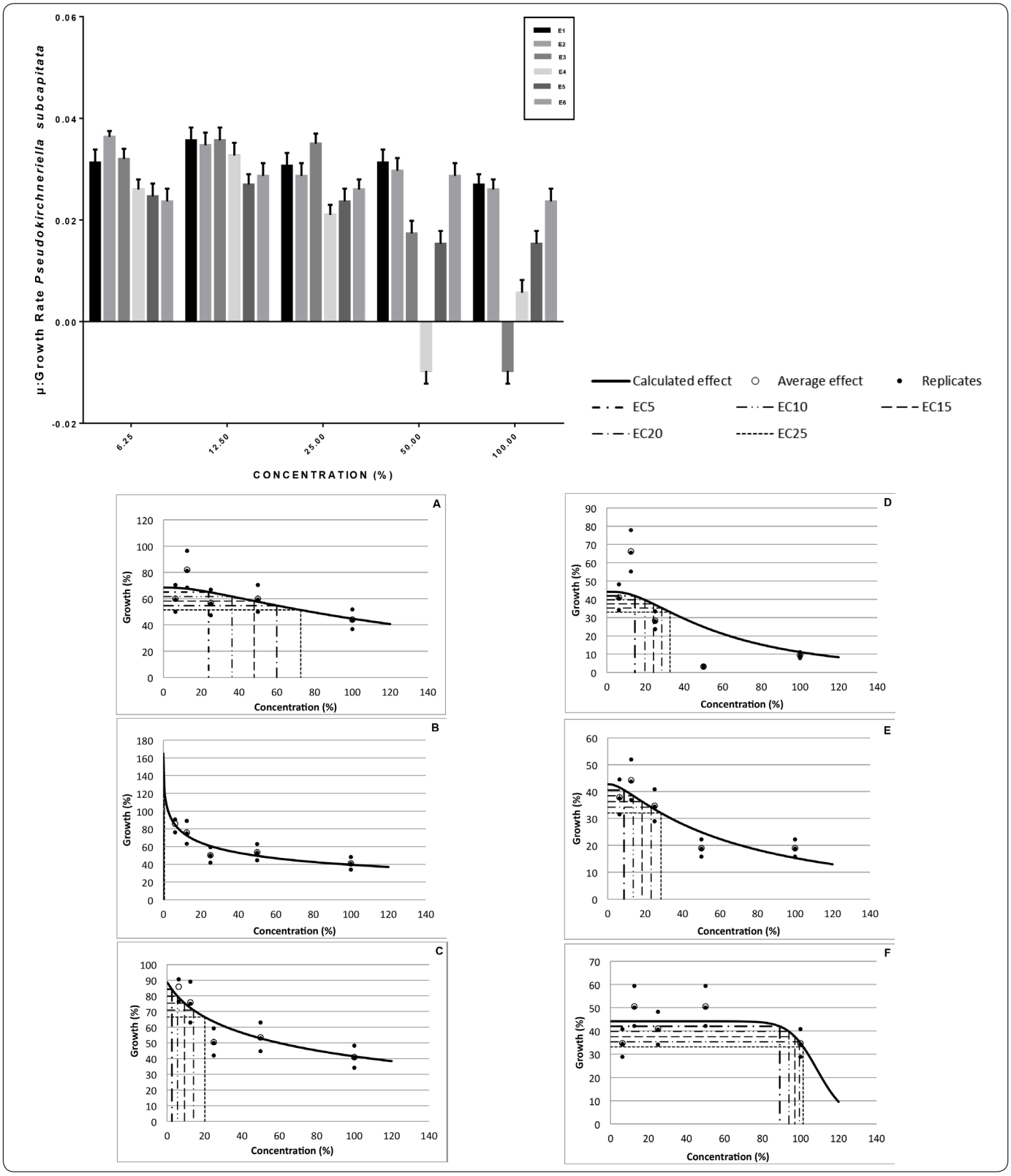

Figure 3. Rate growth and concentration vs. growth (%). a. Growth rate of *Pseudokirchneriella subcapitata* according to zones of the watershed of Camana and Majes (Arequipa, Peru) at 72 hours of the bioassay. Graphs of the Concentration-Response showing the effects of the bioassay of the sampling point with the growth of *P. subcapitata* with Effective Concentration (EC) at different percentages at 72 hours. b. E1 = Taparza; c. E2 = Grande; d. E3 = Majes1; e. E4 = Majes2; f. E5 = Camana1; g. E6 = Camana2.

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There was a decrease in the growth rate of *P. subcapitata* as the concentrations of Al, Fe and Mn increased, which was accompanied with a decrease in concentrations of chlorophyll a and chlorophyll b. The lowest growth rates of *P. subcapitata* were observed in bioassays with high concentrations of Al and Fe. The concentrations of chlorophyll "a" were higher than the concentrations of chlorophyll "b" in the bioassays with Al, Fe and Mn with EC_{50} in the growth inhibition of the microalga at 72 hours of exposure of 5.875 mg/L to Al; 0.548 mg/L to Fe; and 0.375 mg/L to Mn showing statistically highly significant differences ($p < 0.01$) (Figure 4a, b, c).

In Figure 4d, bacterial-microalga interactions were evaluated in laboratory conditions simulating freshwater environments, where an antagonistic interaction between growth of microalgal *P. subcapitata* and bacterial growth of *Escherichia coli*, was observed; that is, a decrease in the growth of *P. subcapitata* and an increase in the growth of *E. coli* as the dilutions of LB liquid medium with *E. coli* decrease.

The fluorescence detected in cultures of *P. subcapitata* corresponding to an optical section "x" and "y" is shown in Figures 5a, b, c, d, e, f, g, h, i, j.

In addition, the mean fluorescence intensities (MFI) at different wavelengths of algae cultures grown at different concentrations of Al (Figure 5k), Fe (Figure 5l) and Mn (Figure 5m) were plotted. In such graphs it is shown that:

(A) The maximum fluorescence (F) peak in the presence of aluminum corresponds to 518.6 nm in any element concentration (Figure 5k). That peak of fluorescence decreases while the concentration of Al increases, and all the data showed statistically significant differences at $P < 0.05$ ($F = 125.3$).

(B) The fluorescence at different concentrations of Fe (0, 1, 10 and 20 mg/L; Figure 5l) and Mn (0; 0.4; 0.8 and 1 mg/L; Figure 5m), also showed a decrease as the concentration

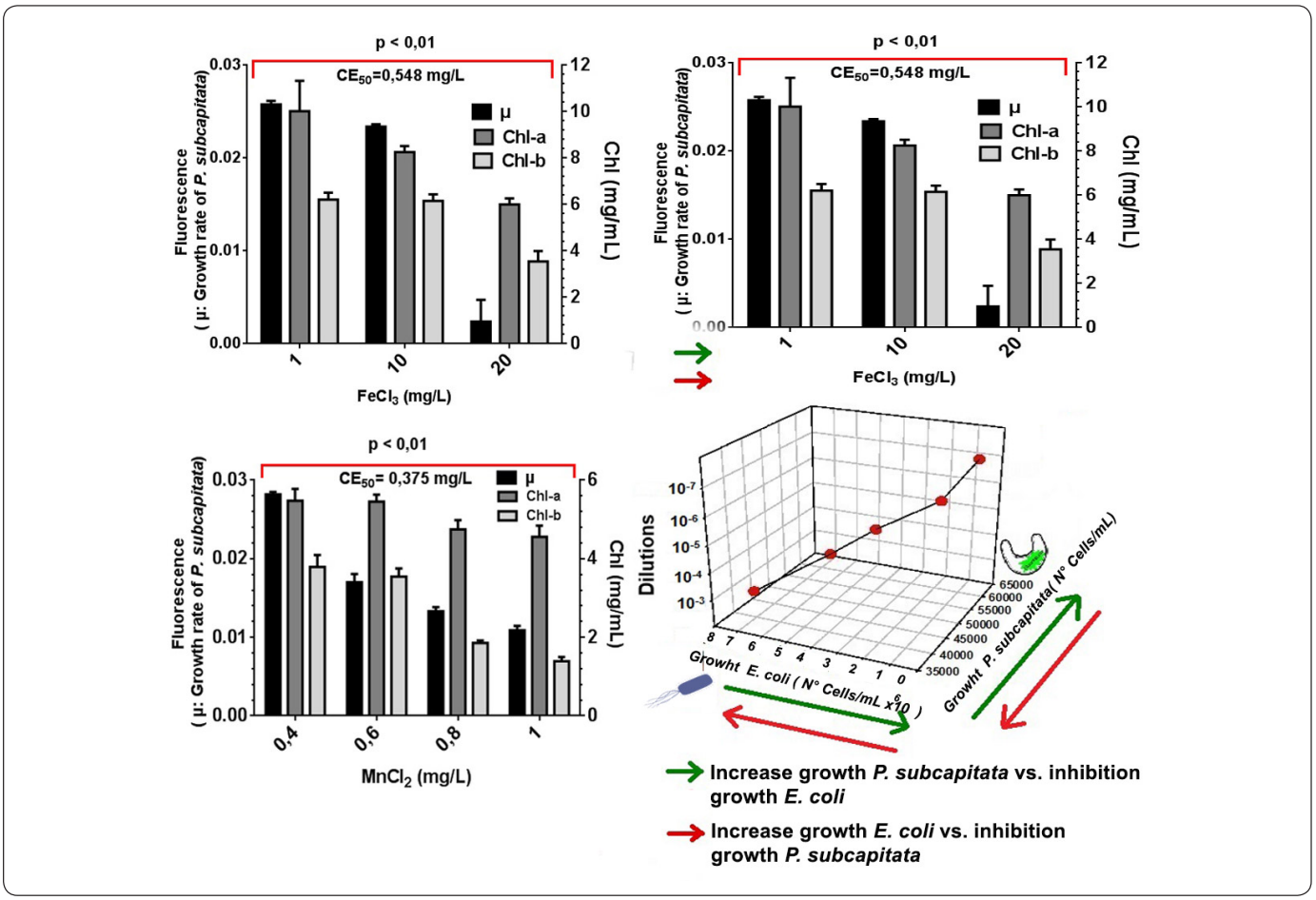

Figure 4. Effect of the concentration of Al, Fe and Mn on the microalga *Pseudokirchneriella subcapitata* based in the growth rate, concentration of chlorophyll a and b (Chl-a and Chl-b) and mean effective concentration (EC_{50}) at 72 hrs of exposure. (a) Toxicity of $Al_2(SO_4)_3$, (b) Toxicity of $FeCl_3$, (c) Toxicity of $MnCl_2$ in *P. subcapitata* and (d) Graph in 3D with antagonistic interaction between the bacterial growth of *E. coli* and microalgal growth *P. subcapitata*.

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of the elements increases, showing the maximum peak of fluorescence to 518 nm. To both elements also statistically significant differences were found at $P < 0.05$ Fe, $F = 391{,}333$; Mn, $F = 19{,}896$.

Discussion

We studied the ecotoxicological bioassays with *Pseudokirchneriella subcapitata* in lotic ecosystems of the Arequipa Peruvian Region, like the stations E1, E2, E3, E4, E5 and

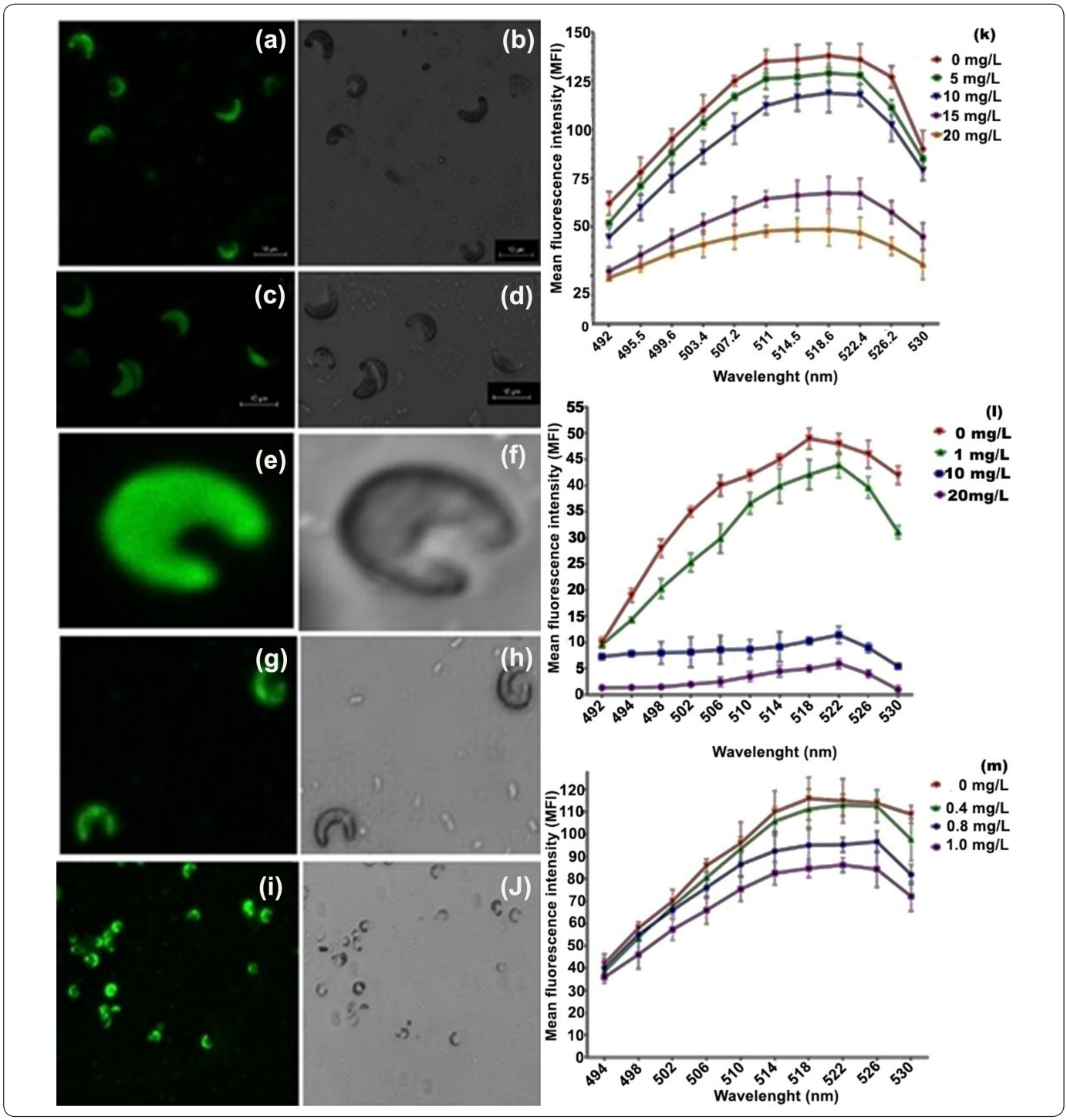

Figure 5. Images of *Pseudokirchneriella subcapitata* (a, c, e, g, i). Representative confocal microscopy images of *P. subcapitata* (63X, 40X) (b, d, f, h, j). Representative phase contrast microscopy images of *P. subcapitata* (63X, 40X) Bar scale 10 µm. MFI: emission ("y" axis) to several wavelengths, ("x" axis); obtained from algae cultures of *P. subcapitata* grown to different concentrations of Al (k), Fe (l) and Mn (m).

E6 compared to National Environmental Quality Standards (EQSs) and guidelines of the water quality of the World Health Organization (WHO), that will allow a rapid estimation of the effects of pollutants on the growth of *P. subcapitata*. This method involves three steps: (1) Exposure of *P. subcapitata* to water samples from lotic ecosystems; (2) Bioassays of 72 hours; and (3) Measurement of growth inhibition every 24 hours.

This study has two important results. First, the intensity of the most polluted points in lotic ecosystems through inhibition the growth of *P. subcapitata* compared to the National Environmental Quality Standards (EQSs) and guidelines of the water quality of the World Health Organization (WHO). Second, the growth of *P. subcapitata*, Mean Effective Concentration (EC_{50}) and Toxicity Units (TU) at 72 hours in bioassays at the sampling points were compared to toxicological categories established by Bulich (1982) and Coleman & Quershi (1985). The studies about the evaluation of the growth rate of *P. subcapitata* in bioassays with samples from the watershed of Camana, Majes and Colca, Arequipa, Peru were also reported by Huarachi *et al.* (2014), who evaluated the toxicity in different points of the watershed.

Figure 3a presents the growth rate of *P. subcapitata* to the water exposure of the different stations: E1, E2, E3, E4, E5 and E6 where the lowest growth rate is reached at E3 with an EC_{50} = 60.7% and E4 with an EC_{50} = 57.43% categorized as moderately toxic according to Coleman & Qureshi (1985), due to the presence of thermotolerant coliforms, aluminum, manganese and iron surpassing the limit values of the EQSs for the districts of Aplao, Huancarqui, Uraca and Corire unloading their dumping to the river of Majes where a population dump was identified by a national company of sewage service located in Majes; which there are collapsed oxidation ponds, an industrial dump was also identified by a dairy company located in Majes that does not have a treatment system to their effluent (ANA, 2012). After exposure to the lotic ecosystems of the waters of the Limache estuary (central Chile), it shows a lower growth rate of *P. subcapitata* in stations with greater anthropic activity and in the discharge zone of the effluent of a water treatment plant (Córdova, Gaete, Aránguiz & Figueroa, 2009). The investigation of acute toxicity in *P. subcapitata* in industrial effluents in the Delta region of the Pearl River in China showed sensitivity to effluents from factories of electroplating and electronic (Fang *et al.,* 2012). In the lotic ecosystem, Delta of the Ebro river, Spain the microalga *P. subcapitata* presented sensitivity to herbicides such as diuron, simazine and terbuthylazine (36%, 26% and 17% of toxicity, respectively), (Köck *et al.,* 2010). Along the Sava river in the countries of Slovenia, Croatia, Bosnia and Herzegovina and Serbia, the sampling positions were chosen to cover the Sava River watershed Majes, Camana and Colca Arequipa- Peruvian, 2016, considering the impact of the Sava River's pollution by their principal effluents (Savinja, Krka, Kolpa, Una, Vrbas, Bosna and Drina), with 14 sampling stations where the Lukavec station presents a growth rate of *P. subcapitata* in 40% being the most toxic sample causing a growth inhibition of 20% (Källqvist *et al.,* 2008). In the Vistula river upstream of Cracovia, Poland with six sampling stations (Lipowiec, Gora, Chelmek, Bobrek, Metkow and Tyniec), the high toxicity with *P. subcapitata* is observed in Chelmek and Teniec stations with high concentrations of Zn (Guéguen, Gilbin, Pardos & Dominik, 2004). The Wangyang river in northern of China recorded the presence of sixteen antibiotics and the ecotoxicological risk with *P. subcapitata* showed sensitivity with Sulfadiazine, Ofloxacin, Roxithromycin and Erythromycin (Jiang *et al.,* 2014). The toxicity tests of water on a small urban river (Store Vejleå, Denmark), which receives discharges from urban runoff; a sample taken from this river was analyzed by a combination of toxicity tests and chemical analysis, the tests with *P. subcapitata* showed that the toxicity was due to the presence of copper (25 mg/L), (Christensen, Nakajima & Baun, 2006).

In the north of Morocco, the Sebou river and its affluent the Fez river, the highest toxic effects are obtained with the inhibition of growth using the microalga *P. subcapitata* being observed in a point of the Fez river where the limit for ammonium and chromium is exceeded in comparison with the guidelines of water quality of the World Health Organization's (Koukal *et al.,* 2004). The effect of dissolved organic matter on the growth of the microalga *P. subcapitata* in the lakes of Korea, where the results demonstrated a high growth rate of *P. subcapitata*, correlated with the dissolved organic matter hydrophobic in five lakes under controlled conditions of nutrients (Lee *et al.,* 2009). In the growth test with *P. subcapitata* by leaching water from the hot pile of PCM - Plovdiv, significant growth inhibition was observed from 24 to 72 hours of exposure (Ivanova & Groudeva, 2006). In an in situ bioassay with *P. subcapitata,* for freshwater environments, growth inhibition was observed in impacted sites, demonstrating the sensitivity of the test, where the nearest site was impacted by the discharge of effluents and the farther downstream was moderately impacted (Moreira, Soares & Ribeiro, 2004). A rapid and simple ecotoxicological analysis allowed a reliable estimation of the effects of Simazine (CAT) or 3,5-dichlorophenol (3,5 DCP) on the growth of *P. subcapitata* with the results of inhibition tests of the standard growth with 72 hours of exposure (Katsumata, Koike, Nishikawa, Kazumura & Tsuchiya, 2006).

In the evaluation of impact of metals that exceeded the EQSs and WHO, in bioassays of *P. subcapitata* with Al the $CE_{50\text{-}96}$ = 5.875 mg/L compared to the results of Satizabal, Andrade & Zuñiga (1999), with an $EC_{50\text{-}48}$ = 5.51 mg/L in bioassays with *Daphnia magna.*

In the exposure to different concentrations of Fe in *P. subcapitata* the $EC_{50\text{-}96}$ = 0.548 mg/L compared to the result of Shuhaimi-Othman, Nadzifah, Nur-Amalina & Umirah 2012 with $EC_{50\text{-}96}$ =

0.75 mg/L in aquatic organisms and in bioassays with Mn EC_{50-96} = 0.375 mg/L compared to the value of EC_{50-96} = 8.3 mg/L in *P. subcapitata* according to Reimer (1999), being greater its found value. In interaction bacterium-microalga according to Riquelme & Avendaño-Herrera (2003), the mechanisms of these interactions are poorly understood; future research should be directed at understanding the mode of action of bacterium-microalga interactions at the molecular level.

In this work, was evaluated the fluorescence in the microalgae *P. subcapitata* in response to different concentrations of $Al_2(SO_4)_3$, $FeCl_3$ and $MnCl_2$ for a lapse of 72 hours. The toxic effect of the metals on microalgal cells was evaluated using the orange acridine fluorochrome where a decrease in fluorescence is observed as the concentration of metals increases.

Conclusions

The physicochemical analyzes present thermotolerant coliforms, Manganese and total suspended solids in the Majes River that surpass the National Environmental Quality Standards (EQSs) and water quality guidelines of the World Health Organization (WHO), compared to a higher inhibition of growth of *P. subcapitata* and a Mean Effective Concentration (EC_{50}) categorized with moderate toxicity. The principal advantages of these test systems are simple, cheap, sensitive and reproducible, providing profitable results in a fast way. Considering all the results presented in this work, along with other investigators (Huarachi *et al.*,2004; Moreira *et al.*, 2004), we evaluated the impact of metals that exceeded the EQSs and WHO, so in the diversity, the ecological importance of the metallic contamination could be applied with techniques of confocal microscopy of fluorescence contributing with a model of broad perspectives in future studies of the ecotoxicity of metals. Besides, our results, according to Machado & Soares (2015), support a fluorescence-based approach being useful to detect disturbance of cellular characteristics. Therefore, fluorescent probes are useful diagnostic tools for assessing the impact of toxins on specific targets of microalgae cells of *P. subcapitata.*

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References

Allan, D. (1995). Stream ecology. Structure and Function of Running Waters. Londres: Chapman & Hall.

ANA (Autoridad Nacional del Agua) (2012). Informe de la identificación de fuentes contaminantes en la cuenca de los ríos Camaná – Majes – Colca". Informe Técnico Nº 029–2012–ANA–DGCRH/MPPC-JLFZ.

ANA (Autoridad Nacional del Agua) (2011). Protocolo nacional de monitoreo de la calidad de los cuerpos naturales de agua superficial. En: www.ana.gob.pe/media/355522/protocolo_182.pdf

APHA (American Public Health Association) (1998). American Water Works Association (AWWA), Water Environment Federation (WEF), In: Clesceri LS, Greenberg AE, Eaton AD (Eds.), Standard Methods for the Examination of Water and Wastewater, 20th ed. United Book Press, Baltimore, MD, USA

APHA (American Public Health Association) (2000). Standard Methods for Examination of Water and Wastewater, 21st ed. Washington, DC, USA.

Aruoja, V. (2011). Algae *Pseudokirchneriella subcapitata* in environmental hazard evaluation of chemicals and synthetic nanoparticles. Thesis for applying for the degree of Doctor of Philosophy in Environmental Protection. Eesti Maaülikool/ Estonian University of Life Sciences. República de Estonia.

Bringmann, G. & Kuhn, R. (1980). Comparisons of the toxicity thresholds of water pollutants to bacteria, algae and protozoa in the cell multiplication inhibition tests. *Water Res*., **14**, 231-241. DOI:10.1016/0043-1354(80)90093-7

Bulich, A. (1982). A practical and reliable method for monitoring the toxicity of aquatic samples. *Process Biochem.*, **17**, 45-47.

Cairns, J., Dickson, JrKL. & Westlake, G.F. (1976). (eds) Biological monitoring of water and effluent quality. ASTM STP 607. Amer. Soc. for Test Mat., Philadelphia, 246 pp.

Castillo, G.C., Vila, I.C. & Neild, E. (2000). Ecotoxicity assessment of metals and wastewater using multitrophic assays. *Environ. Toxicol.,* **15**, 370-375. DOI: 10.1002/1522-7278(2000)15:5<370::AID-TOX3>3.0.CO;2-S

Cho, C.W., Pham, T.P.T., Jeon, Y.C. & Yun, Y.S. (2008). Influence of anions on the toxic effects of ionic liquids to a phytoplankton *Selenastrum capricornutum Green Chemistry*, **10**, 67-72. DOI: 10.1039/B705520J

Christensen, A.M., Nakajima, F. & Baun, A. (2006). Toxicity of water and sediment in a small urban river (Store Vejleå, Denmark). *Environmental Pollution*, **144,** 621-625. DOI:10.1016/j.envpol.2006.01.032

Coleman, R.N. & Qureshi, A.A. (1985). Microtox and Spirillum volutans test for assessing toxicity of environmental samples. *Bull. Environ. Contam. Toxicol.*, **35,** 443-451. DOI: 10.1007/BF01636536

COPASA (Cooperación para el proceso de Autodesarrollo Sostenible de Arequipa) (2012). Estudio Socioeconómico de Microcuencas en la Provincia de Castilla y Condesuyo. In: Cap. 4. Análisis de Castilla media. http://copasa.gob.pe/proyectos/pr_ejecutados/pgrd/estudio_sistem/informefinal_cap4.pdf

Córdova, S., Gaete, H., Aránguiz, F. & Figueroa, R. (2009). Evaluación de la calidad de las aguas del estero Limache (Chile central), mediante bioindicadores y bioensayos. *Lat. Am. J. Aquat.* Res., **37**, 199-209. DOI: 10.3856/vol37-issue2-fulltext-7

Fang, Y.X., Ying, G.G., Zhang, L.J., Zhao, J.L., Su, H.C., Yang, B. & Liu, S. (2012). Use of TIE techniques to characterize industrial effluents in the Pearl River Delta region. *Ecotoxicology and Environmental Safet.*, **76**, 143-152. DOI:10.1016/j.ecoenv.2011.10.003

Gabrielson, J., Hart, M., Jarelöv, A., Kühn, L., McKenzie, D. & Möllby, R. (2002). Evaluation of redox indicators and the use of digital

scanners and spectrophotometer for quantification of microbial growth in microplates. *J. Microbiol Methods,* **50(1)**, 63-73. DOI: 10.1016/S0167-7012(02)00011-8

Guéguen, C., Gilbin, R., Pardos, M. & Dominik, J. (2004). Water toxicity and metal contamination assessment of a polluted river: the Upper Vistula River (Poland). *Applied Geochemistry*, **19**, 153-162. DOI: 10.1016/S0883-2927(03)00110-0

Huarachi, R., Dueñas, A., Mendoza, G., Yapo, U., Alatrista, G. & Gonzalez R. (2014). Ecotoxicological assessment with *Pseudokirchneriella subcapitata* (Chlorophyta) and physicochemical parameters in the upper-middle part of Camaná-Majes-Colca basin (Arequipa-Peru). *The Biologist (Lima),* **12(2)**, 169-180.

ISO (International Standards Organization). (1989). Water Quality Fresh water algal growth inhibition test with *Scenedesmus subspicatus* and *Selenastrum capricornutum*. ISO 8692.

Ivanova, I. & Groudeva, V. (2006). Use of *Selenastrum capricornutum* growth inhibition test for testing toxicity of metal ions in soil and water. *Biotechnology and Biotechnological Equipment.* **20**, 178-183. DOI:10.1080/13102818.2006.10817329

Jiang, Y., Li, M., Guo, C., An, D., Xu, J., Zhang, Y. & Xi, B. (2014). Distribution and ecological risk of antibiotics in a typical effluent–receiving river (Wangyang river) in north China. *Chemosphere.* **112**, 267-274. DOI:10.1016/j.chemosphere.2014.04.075

Källqvist, T., Milačič, R., Smital, T., Thomas, K., Vranes, S. & Tollefsen, K.E. (2008). Chronic toxicity of the Sava River (SE Europe) sediments and river water to the algae *Pseudokirchneriella subcapitata. Water Research*, **42**, 2146-2156. DOI:10.1016/j.watres.2007.11.026

Katsumata, M., Koike, T., Nishikawa, M., Kazumura, K. & Tsuchiya, H. (2006). Rapid ecotoxicological bioassay using delayed fluorescence in the green alga *Pseudokirchneriella subcapitata. Water Research*, **40**, 3393 - 3400. DOI:10.1016/j.watres.2006.07.016

Köck, M., Farré, M., Martínez, E., Schrantz, K.G., Ginebreda, A., Navarro, A., López de Alda, M. & Barceló, D. (2010). Integrated ecotoxicological and chemical approach for the assessment of pesticide pollution in the Ebro River delta (Spain). *Journal of Hydrolog*,. **383**, 73-82. DOI:10.1016/j.jhydrol.2009.12.029

Koukal, B., Dominik, J., Vignati, D., Arpagaus, P., Santiago, S., Ouddane, B. & Benaabidate, L. (2004). Assessment of water quality and toxicity of polluted Rivers Fez and Sebou in the region of Fez (Morocco). *Environmental Pollution*, **131**, 163 - 172. DOI:10.1016/j.envpol.2004.01.014

Lee, J., Park, J.H., Shin, Y.S., Lee, B.C., Chang, N.I., Cho, J. & Kim, S.D. (2009). Effect of dissolved organic matter on the growth of algae, *Pseudokirchneriella subcapitata*, in Korean lakes: The importance of complexation reactions. *Ecotoxicology and Environmental Safety*m, **72**, 335-343. DOI:10.1016/j.ecoenv.2008.01.013

Lewis, M.A. (1990). Are laboratory-derived toxicity data for freshwater algae worth the effort? *Environ. Toxicol. Chem.*, **9**, 1279-1284. DOI: 10.1002/etc.5620091006

Little, L.W. (1978). Bioassays - procedures and results. *J. Water Poll Control Fed.*, **50**, 1852-1868.

Machado, M.D. & Soares, E.V. (2015). Use of a fluorescence-based approach to assess short-term responses of the alga *Pseudokirchneriella subcapitata* to metal stress. *Journal of Applied Phycology,* **27(2)**, 805-813. DOI: 10.1007/s10811-014-0351-1

Maciorowski, A.F., Sims, J.L., Little, L.W. & Gerrard, E.D. (1981). Bioassays - procedures and results. *J. Water Poll Control Fed*,. **56**, 974-993.

Moreira, M., Soares, A. & Ribeiro, R. (2004). An in situ bioassay for freshwater environments with the microalga *Pseudokirchneriella subcapitata. Ecotoxicology and Environmental Safety*, **59**, 164-173. DOI:10.1016/j.ecoenv.2003.07.004

Nalewajko, C. & Olaveson, M.M. (1998). Ecophysiological considerations in microalgal toxicity tests. In: Wells PG, Lee K, Blaise C. (Eds.), Microscale Aquatic Toxicology, Advances, Techniques and Practice. Lewis, Boca Raton, FL, pp. 289-309.

Nygaard, G., Komárek, J., Kristiansen, J. & Skulberg O.M. (1986). Taxonomic designations the biossay alga NIVA-CHL 1 (*Selenastrum capricornutum*) and some related strains. *Opera Bot.,* **90**, 1- 46.

Nyholm, N., Sørensen, P.S., Kusk, K.O. & Christensen, E.R. (1992). Statistical treatment of data from microbial toxicity tests. *Environmental and Toxicological Chemistry*, **11**, 157-167. DOI: 10.1002/etc.5620110204

O'Farrel, L., Lombardo, R., de Tezanos P. & Loez, C. (2002). The assessment of water quality in the Lower Luján river (Buenos Aires, Argentina): phytoplankton and algal bioassays. *Environ. Pollut.,* **120**, 207-218. DOI: 10.1016/S0269-7491(02)00136-7

Ocampo-Duque, W., Sierra, J., Ferré-Huguet, N., Schuhmacher, M. & Domingo, J.L. (2008). Estimating the environmental impact of micro-pollutants in the low Ebro River (Spain): An approach based on screening toxicity with *Vibrio fischeri. Chemosphere*, **72**, 715-721. DOI: 10.1016/j.chemosphere.2008.03.055

Pardos, M., Benninghoff, C. & Thomas, R.L. (1998). Photosynthetic and population growth response of the test alga *Selenastrum capricornutum* Printz to zinc, cadmium and suspended sediment elutriates. *Journal of Applied Phycology*, **10**, 145 - 151. DOI: 10.1023/A:1008043931094

Payne, A.L. (1986). The ecology of tropical lakes and rivers. Londres: John Wiley & Sons.

Pinilla, G. (2005). Ecología del fitoplancton en un lago amazónico de aguas claras (Lago Boa, Caquetá Medio, República de Colombia). Universidad de Bogotá Jorge Tadeo Lozano. 258 pp.

Rai, L.C., Singh, A.K. & Mallick, N. (1990). Employment of CEPEX enclosures for monitoring toxicity of Hg and Zn on in situ structural and functional characteristics of algal communities of river Ganga in Varanasi, India. *Ecotoxicol. Environ. Saf.*, **20**, 211-221.

Reimer, P.S. (1999). Environmental effects of manganese and proposed freshwater guidelines to protect aquatic life in British Columbia [MSc thesis]. Vancouver, B.C., University of British Columbia.

Riquelme, C.E. & Avendaño-Herrera, R.E. (2003). Interacción bacteria-microalga en el ambiente marino y uso potencial en acuicultura. *Revista Chilena de Historia Natural,* **76(4)**, 725-773. DOI: 10.4067/S0716-078X2003000400014

Rowan, KS. (1989). Photosynthetic Pigments of Algae. Cambridge University Press, Cambridge.

Satizabal, A., Andrade, M. & Zuñiga, M.C. (1999). Toxicidad aguda del aluminio sobre *Daphnia magna* en aguas con diferentes niveles de dureza. *Actual Biol*., **21**, 131-142. DOI:10.17533/udea.acbi

Shuhaimi-Othman, M., Nadzifah, Y., Nur-Amalina, R. & Umirah, N.S. (2012). Deriving freshwater quality criteria for iron, lead, nickel, and zinc for protection of aquatic life in Malaysia. The Scientific World Journal. Article ID 861576, 7 pages. DOI:10.1100/2012/861576

Smiglak, M., Reichert, W.M., Holbrey, J.D., Wilkes, J.S., Sun, L., Thrasher, J.T., Kirichenko, K., Singh, S., Katrinsky, A.R. & Rogers R.D. (2006). Combustible ionic liquids by design: is laboratory

safety another ionic liquid myth?. *Chemical Communications*, **24**, 2554-2556. DOI: 10.1039/B602086K

Sprague, J.B. & Ramsay, B.A. (1965). Lethal effects of mixed copper and zinc solutions for juvenile salmon. *Journal of the Fisheries Research Board of Canada*, **22**, 425-432. DOI: 10.1139/f65-042

UNSA [Universidad Nacional de San Agustín de Arequipa] (2001). CONVENIO UNSA-INDECI (Instituto Nacional de Defensa Civil) Proyecto PER 98/018 PNUD (Programa de las Naciones Unidas para el Desarrollo)-INDECI. In: Evaluación de peligros de Chuquibamba. http://www.bvcooperacion.pe/biblioteca/bitstream/123456789/6493/1/BVCI0006701.pdf

USEPA [United States Environmental Protection Agency] (1994). Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, third ed., EPA/600/4-91/002.

Wells, A.S. & Coombe, V.T. (2006). On the fresh water ecotoxicity and biodegradation properties of some common ionic liquids. *Organic Process Research & Development*, **10**, 794-798. DOI: 10.1021/op060048i

Weyers, A., Sokull-Klüttgen, B., Baraibar-Fentanes, J. & Vollmer, G. (2000). Acute toxicity data: a comprehensive comparison of results of fish, Daphnia, and algae tests with new substances notified in the European Union. *Environ. Toxicol. Chem.*, **19**, 1931-1933. DOI: 10.1002/etc.5620190731

WHO [World Health Organization] (1996). Health criteria and other supporting information, second ed. Guidelines for drinking-water quality, vol 2. World Health Organization, Geneva, Switzerland.

WHO [World Health Organization] (2011). Guidelines for drinking-water quality. Fourth ed. World Health Organization. Geneva. Switzerland.