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Ecotoxicity assessment in aquaculture system using the test organism *Pseudokirchneriella subcapitata* (Chlorophyceae)

Luciana Miashiro*, Julio Vicente Lombardi and Cacilda Thais Janson Mercante

*Instituto de Pesca, Rod. Washington Luis, km 445, Cx. Postal 1052, 15025-970, São José do Rio Preto, São Paulo, Brazil. *Author for correspondence. E-mail: lumiashiro@yahoo.com.br*

ABSTRACT. The purpose of this study is to perform toxicity tests with microalgae *Pseudokirchneriella subcapitata* (Chlorophyceae) using inland water samples to evaluate the impact caused by aquaculture. Six field samples were collected ranged November 2006 to March 2007, at Experimental Station, Pindamonhangaba, State of São Paulo. Abiotic factors pointed out to the mesotrophic and eutrophic characteristics already observed at the fish pond and its effluent. The results of ecotoxicological tests carried out with the microalgae *Pseudokirchneriella subcapitata* showed that the fish pond effluent was potential enough to stimulate the algal growth, therefore eutrophication, to the extent that it is naturally diluted to at least 12.5% of its initial concentration. This type of test can be a tool to be used by environmental managers in attempts to measure the extents of the impacts of effluent discharges from fish farming and to propose treatments based on qualitative and quantitative information.

Keywords: Nile tilapia, effluent, eutrophication, microalgae.

Avaliação da ecotoxicidade em sistema de aquicultura utilizando o organismo-teste *Pseudokirchneriella subcapitata* (Chlorophyceae)

RESUMO. O objetivo deste trabalho foi realizar testes ecotoxicológicos com a microalga *Pseudokirchneriella subcapitata* (Chlorophyceae) em amostras de água de piscicultura continental, para avaliar impactos gerados pela atividade. Seis campanhas amostrais foram realizadas entre novembro de 2006 e março de 2007 na piscicultura experimental, Pindamonhangaba, Estado de São Paulo. Os fatores abióticos apontaram para características mesotróficas e eutróficas do viveiro e do efluente. Os testes com amostras brutas indicaram que mesmo após o encontro do efluente com o corpo de água receptor, a carga orgânica do viveiro foi capaz de estimular crescimento algáceo. Os resultados obtidos nos ensaios ecotoxicológicos realizados com a microalga *Pseudokirchneriella subcapitata* demonstraram que o efluente do viveiro de piscicultura exerceu um risco de eutrofização do meio até o ponto em que o mesmo estiver naturalmente diluído a 12,5% da sua concentração no corpo hídrico receptor. Ficou demonstrado que este tipo de ensaio pode ser uma ferramenta passível de utilização por gestores ambientais nas tentativas de mensurar as extensões dos impactos dos lançamentos de efluentes de piscicultura e de propor tratamentos com base em informações qualitativas e quantitativas.

Palavras-chave: tilápia do Nilo, efluente, eutrofização, microalga.

Introduction

The development of aquaculture and the increasing degradation of water resources have raised some concern about the effluents related to this activity. Due to the presence of high nutrients loading, such effluents when abandoned without treatment have been considered as one of the main reasons for the eutrophication of the receiving streams (STEPHENS; FARRIS, 2004).

Intending to optimize the yield at fish farms, ration and fertilizers are cast to the water. The portion not consumed, added to the dejection of cultivated organisms, is accumulated in the environment, thus altering the level of dissolved oxygen and the quantity of suspended solids, what leads to the production of phytoplankton in excess as a consequence of the high level of nutrients, mainly nitrogen and phosphorus (SIPAÚBA-TAVARES et al., 1999).

Together with organic matter, effluents may contain chemical substances that are carelessly used in many instances to control pathogens and parasites. There is no specific legislation about the use of drugs in aquaculture and likewise no legalized products for that purpose. These products are evaluated by Agriculture, Livestock Sector and Supply Ministry without involvement of health and environmental public authorities, meaning that no

appraisal of the impact of such substances to human health and to the environment is available. Consequently, the lack of products and specific regulations facilitate the abuse and the inappropriate use (MAXIMIANO et al., 2005).

According to Castro et al. (2006), in most of the fish farms situated in São Paulo, pond effluents are not treated before the outflow and so they present levels of organic loads and toxic substances out of the pattern established by the Environment National Council (CONAMA). Chapter IV of Resolution number 357 dated March 17, 2005 from CONAMA, about conditions and patterns for effluents discharge establishes that the effluent must not cause, or even may be able to cause, toxic effects to aquatic organisms in the receiving streams, in accordance with the toxicity criteria established by the related environmental public office. These criteria are to be based on results of standard eco-toxicological tests with aquatic organisms from the effluent (BRASIL, 2005).

The present study is part of the Project named 'Eco-toxicological analysis of effluents at continental aquaculture' (Process FAPESP 2005/05180-0). Therefore, the purpose of this study was to analyze the quality of the water collected from a semi-intensive fish farm during the Nile tilapia (*Oreochromis niloticus*) rearing period, by means of eco-toxicological tests with microalgae *Pseudokirchneriella subcapitata* (Chlorophyceae), in order to evaluate the possible impacts caused to environment by the activity effluents as well as to subsidize further monitoring and/or treatment measures.

Material and methods

The sampling area was the experimental fish farm located at Agribusiness Technological Development Regional Site at Vale do Paraiba – APTA Regional – Agribusiness Technology Office in São Paulo (SAA-SP), in Pindamonhangaba, a country town located 147 km far from the capital of São Paulo State, altitude between 530 and 550 meters, coordinates 22°55'50"S 45°27'22"W.

The chosen fish pond was operated in a semi-intensive system and was used for Nile tilapia (*O. niloticus*) fattening. Structural characteristics of the fish pond are 1.08 m deep (average), area 1,500 m^2, volume 1,620 m^3, and 2.7 L s^{-1} average outflow; 7-day residence interval, and an individual, constant and non-mechanic ventilation water renewal system. The pond was populated with 3,750 young fish, Nile tilapia (*O. niloticus*) monosex "♂". The organisms were fed with extruded ration containing 28% crude protein, twice a day, ratio: up to 3% live weight.

Six field samples were collected between November 2006 and March 2007, always in the morning. The dates were identified as: Nov./2006, Dec./2006, Jan./2007, Feb./2007, Mar. I/2007 and Mar. II/2007, respectively. The sampling covered all the Nile tilapia (*O. niloticus*) fattening period.

Six sampling sites were previously delimited as to cover all the water flow along the system, as per Figure 1: Site 1 (affluent); Site 2 (fish pond); Site 3 (effluent); Site 4 (mixing zone); Site 5 (mixing zone upstream; receiving stream site, 11 meters before joining the fish pond effluent); Site 6 (mixing zone downstream; receiving stream site, 13 meters after joining the fish pond effluent).

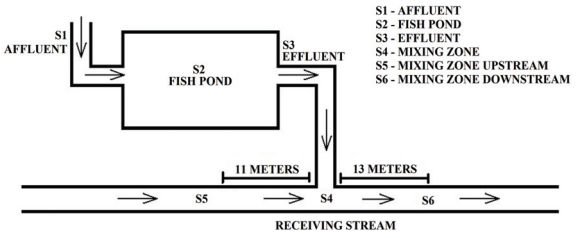

Figure 1. Diagram of sampling site location (the arrows indicate the direction of water flow). Drawing is not to scale.

Analysis of abiotic variables and Chlorophyll *a*: The following parameters were measured in all sites at collection time using the multi-parameter sounder Horiba® U-22: water temperature (°C), dissolved oxygen (mg L^{-1}), potential hydrogen (pH).

The limnological analysis was performed by Laboratory of Physical and Chemical Analysis of Water from Limnology Reference Laboratory Unit – Research and Development Unit for Water Resources from the Fishing Institute (SP).

The analysis for determination of total phosphorus (mg L^{-1}) and total nitrogen (mg L^{-1}) was carried out simultaneously according to the techniques described at Valderrana (1981). As per total ammoniac nitrogen (mg L^{-1}) Nessler's technique was adopted as described at APHA (2005).

All data mentioned were based on the protocol of recommended analyses at the document Standard Methods for the Examination for Water and Wastewater (APHA, 2005).

The determination of chlorophyll *a* (μg L^{-1}) was achieved through the pigment extraction technique, according to Marker et al. (1980) and Sartory and Grobellar (1984), using ethanol 90% as organic solvent.

The results of the analyses involving total nitrogen, total phosphorus, total ammoniacal nitrogen, orthophosphate, pH, dissolved oxygen, % of O_2 saturation, water temperature and

chlorophyll *a* were submitted to multi-variation analyses considering the Principal Components Analysis (GOODALL, 1954 apud VALENTIN, 2000). This procedure was carried out to verify the relation of the abiotic variables results with the sampling sites space distribution and sampling time distribution. It was applied the covariance matrix and data was transformed by the 'ranging' variation amplitude ($[(x-x_{min.})/(x_{max}-x_{min.})]$).

Pearson and Kendall's correlation coefficient calculation (r) made it possible the assessment of the relation between the ordination values (sampling units axis position) and individual variables (abiotics) applied to the ordination composition (McCUNE; MEFFORD, 1997). Data were transformed with program FITOPAC and The multi-variation analyses were performed with program PC-ORD release 3.1 for Windows (McCUNE; MEFFORD, 1997).

Ecotoxicological tests with *P. subcapitata*: Toxicity tests were performed at the Water Ecotoxicology Laboratory of the Fishing Institute (SP), in line with recommendations from the Brazilian Association for Technical Procedures (ABNT, 2005).

The selected species was *P. subcapitata* (Korschikov) Hindak, formerly known as *Selenastrum capricornutum*. The culture medium adopted was L. C. Oligo, prepared according to the procedure described by ABNT (2005). The method applied for the test was the static one. The algae remained in an incubator, under constant 175 rpm shaking, illumination at ± 4,500 lux intense, and temperature at ± 25ºC during the exposure period of 72 hours.

Six series of tests were performed, each one corresponding to one sampling campaign (Nov./2006, Dec./2006, Jan./2007, Feb./2007, Mar. I/2007 and Mar. II/2007). Each series of tests considered six samples, in three replicates, each one corresponding to one sampling site.

A pre-culture environment was prepared three days before each series of tests, so that the cells exposed to the samples could be in their exponential growth phase. The pre-culture was prepared with 1,000 mL of culture medium L. C. Oligo and 50 mL of *P. subcapitata* in suspension, maintained in a 2-Liter Erlenmeyer, under conditions similar to the tests (temperature ± 25ºC, illumination ± 4,500 lux and constant ventilation).

On the first test day, the inoculum culture (algae cells exposed to the samples) was prepared with 100 mL pre-culture centrifugation, at 1,500 rpm, for 15 minutes. The floating material was discarded and the sediment was re-suspended into 100 mL of culture medium. This procedure was repeated and the cell density into the resulting suspension was determined with the Neubauer counting chamber.

The inoculum was prepared as from this suspension so that it always had 1 x 10^5 cells and volume ranged to 0.1 and 1 mL, according to the formula: Vi = (Vf x Ci) / N; where: Vi = inoculum volume (mL); Vf = test solution final volume (mL); Ci = initial concentration of test recipient (cells mL^{-1}); N = number of cells in suspension (cells mL^{-1})

For Sites 1, 2, 4, 5 and 6 the test recipients were prepared mixing inoculum (1 x 10^5 cells) to 50 mL of original sample. For Site 3, the inoculum was added to sample dilutions (3.1, 6.2, 12.5, 25, 50 and 100%), according to ABNT (2005).

All test recipients were 125 mL Erlenmeyer, containing 50 mL of test-solution (sample + inoculum), in three replicates, covered with plastic film and taken to the incubator immediately after the preparation. The cell density of each sample was determined by Neubauer counting chamber after 72-hour exposition.

Test validation was performed according to ABNT (2005) recommendation. It was validated the test which: a) after 72-hour exposition achieved an average control algal biomass 16 times higher than the original biomass; and b) presented by the end of the test a coefficient algal biomass variation for the control repetitions lower or equal to 20%.

Results of Neubauer counting chamber were statistically analyzed using program Toxtat 3.3 (GULLEY et al., 1991). All of them were approved at normality and variance homogeneity tests, what permitted the application of Tukey test to verify the existence of significant differences ($p < 0.05$) between the treatments.

Tukey test was also applied to the results of tests with original samples in order to check the occurrence of major differences among the results of tests with samples collected the same month (e.g. among results of Sites 1, 2, 3, 4, 5 and 6 in November).

Results of tests with the effluent (S3) dilution were submitted to Tukey test as well, in order to observe significant differences between the samples collected each month and the control sample, i.e. among concentrations 3.1, 6.2, 12.5, 25, 50 and 100% and the control sample of the same month.

The results of tests with the effluent (S3) dilution were expressed in OEC (observed effect concentration), considering the lowest concentration of the sample that presented a significant difference in comparison with the control group, and NOEC (non-observed effect concentration), considering the higher concentration of the sample that did not present a significant difference when compared to the control group.

Results and discussion

The Principal Components Analysis (PCA) is illustrated with Figure 2 that includes the relation of the following aspects: water temperature, pH, dissolved oxygen, % of O_2 saturation, total phosphorus, orthophosphate, total nitrogen, total ammoniacal nitrogen and chlorophyll *a*, with the samples space (Axis 1) and time (Axis 2) distribution.

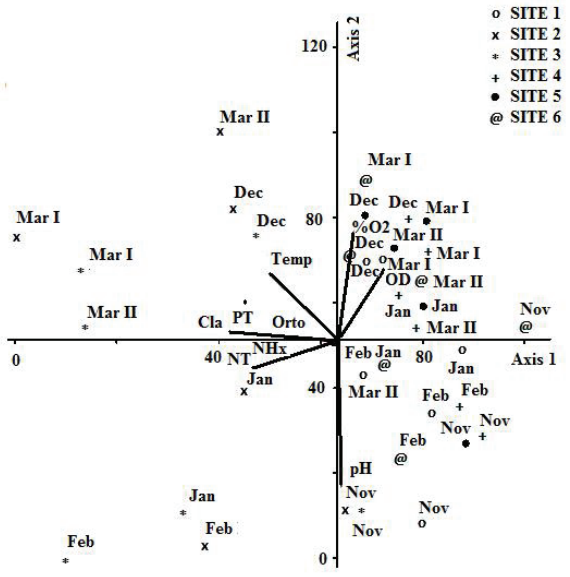

Figure 2. PCA concept applied to biplot ordination of sample units and abiotic variables. Sample units are identified according to sample sites. Vector correlation as per Table 1.

Axis 1 accounted for 36% of joint data variability, while Axis 2 showed 25% (Table 1). It means that 36% of the variation of results related to the factors above is explained by the space distribution of the samples (location of the sampling sites), and 25% refers to time distribution.

As illustrated at Figure 2, PCA showed that water conditions at sites 2 and 3 were similar, positioning most of the samples from these sites to the negative side of Axis 1, associated with the higher figures for total nitrogen, total ammoniacal nitrogen, total phosphorus, orthophosphate, chlorophyll *a* and water temperature.

Pearson and Kendall's correlation coefficient (Table 1) showed that total nitrogen, total ammoniacal nitrogen, total phosphorus, orthophosphate and chlorophyll *a* were strongly associated with the samples space distribution, as represented by Axis 1. This is related to the kind of management adopted and to the organisms' dejection rate that interfered in the variation of such results, thus differentiating the water quality at sampling sites S2 and S3 from the other collection sites.

Table 1. Coefficients of Pearson and Kendall's correlation among the main abiotic variables and the first two ordination axis (N = 36).

Abiotic Variables	Abbreviations	Correlation	
		Axis 1	Axis 2
Total nitrogen	NT	-0.711	-0.358
Total phosphorus	PT	-0.802	0.186
Total ammoniac nitrogen	NHx	-0.602	-0.092
Orthophosphate	Orto	-0.611	0.020
Chlorophyll *a*	Cla	-0.835	-0.002
pH	pH	0.153	-0.820
Dissolved oxygen	OD	0.535	0.579
% of O_2 saturation	$\%O_2$	0.306	0.703
Water temperature	Temp	-0.639	0.549
Explained variation		36%	25%

Samples from S2 and S3 sites collected in November were the only ones positioned at the positive side of Axis 1, thus presenting similarity with the other samples placed at the same side. This is because November is the beginning of the cultivation cycle and the management interference was not so intense. The portion of ration supplied, the organisms' excretion rate and the organic matter decomposed were not enough yet to differentiate the water quality of S2 and S3 from the other stations in terms of total nitrogen, total ammoniacal nitrogen, total phosphorus, orthophosphate and chlorophyll *a*.

PCA also showed that water quality at sites 1, 4, 5 and 6 are similar, thus positioning all samples belonging to these places at the positive side of Axis 1. Figure 2 shows that the vectors related to dissolved oxygen, % of O2 saturation and pH are associated to the samples that registered the higher values.

Pearson and Kendall's correlation coefficient regarding pH and % of O_2 saturation revealed that the samples space distribution, as represented by Axis 1, had little influence on the results variation. On the other hand, it also pointed out the significant relation of these data with the samples time distribution, thus positioning the vectors close to Axis 2.

The results achieved with PCA demonstrate that water is different at sites 2 and 3 from the other sampling sites due to the elevated rates of total nitrogen, total ammoniacal nitrogen, total phosphorus, orthophosphate and chlorophyll *a* registered at these sampling places. Together with the ration, fertilizers and fish dejection, the management of the fish pond directly interfered in such factors what explains why the location of the collection sites (Axis 1) presented the higher rate (36%) for the variation of results.

Studies realized at the same place and time have clearly evidenced the effect of the management over the water quality presenting results of apparent feeding conversion that ranged from (1.2:1) through (2.3:1), and a fish production of 15,500 kg ha^{-1}. According to Boyd and Tucker (1998) from the nitrogen and

phosphorus used in the fish ponds not more than 25 to 30% are reverted into biomass. As observed by Boyd (2004), the feeding input should not exceed the capacity of the fish pond to assimilate the residues. Water deterioration may cause a fall in the productive performance of the fish pond as well as provoke fish mortality, thus affecting production and consequently reducing profitability (BOYD; TUCKER, 1998; PESTANA et al., 2007). Diet and fertilizers are the major reasons for the load of nitrogen, phosphorus and organic substances into the fish ponds dedicated to the production of fish and shrimps (BOYD, 2003). Standing out that phosphorus is one of the main nutrients for the artificial euthrophication (HENRY-SILVA et al., 2006). For Cole and Boyd, (1986), it is observed an increase in the high concentrations of these nutrients and a probability of low levels of dissolved oxygen at night and in the beginning of the morning as a consequence of an increment of the feeding rate at the fish ponds. Considering that the characteristics of the effluents can vary according to the cultivated species, the intensity of production, the feeding management and the level of technology used in the cultivation (BOYD, 2003).

Schneck et al. (2007), analyzing the structure of a community of diatomaceous in a stream impacted by fish pond effluents, also observed that the more relevant physical and chemical factors were related to the management of the fish pond, i.e. total phosphorus, nitrogen and chlorophyll *a*. The authors concluded that the fish pond effluent affected directly the structure of the diatomaceous community, thus causing the replacement of species typical of oligotrophic environment for species typical of eutrophic environment.

Opposite to the expected results of ecotoxicological tests carried out with *P. subcapitata*, it was observed a phenomenon of growth of the algae culture submitted to the tests. As observed at Figure 3, Tukey test revealed that in general the significant differences, in comparison with the control group, were concentrated at sites 2 and 3 (fish pond and effluent). The reason for that is the high concentration of nutrients at the fish pond water that is favorable to the algae growth when *P. subcapitata* was exposed to such samples.

Significant differences were verified at March I samples only at sites 3, 4 and 6, when compared to the control. This sampling did not show any significant difference at site 5, compared to the control, indicating that the aspects that contributed to the algal growth observed at site 3 had also contributed to the results obtained at sites 4 and 6. It means that at this sampling the potential of the effluent to stimulate the algal growth was present in the water even after joining the receiving stream.

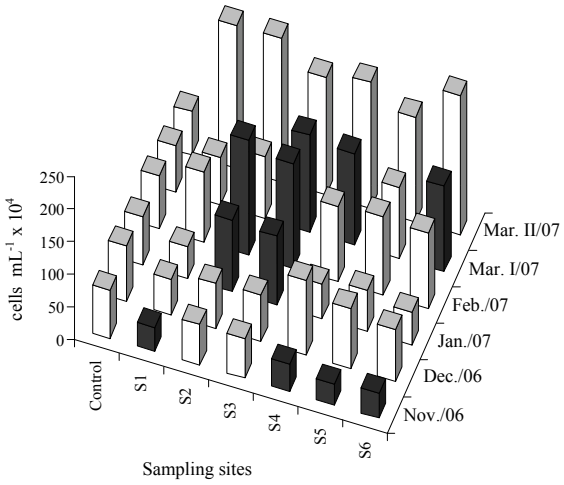

Figure 3. Algal growth average (cells mL^{-1} x 10^4) during the tests with original samples. Dark bars indicate significant difference in the results of tests with each sampling campaign in comparison with the control.

The results of tests with effluent dilution are illustrated at Figure 4. When performing Tukey test with different samplings these results show a significant difference compared to the control group only at 25, 50 and 100% concentrations. In November 25% of OEC (observed effect concentration) was obtained against 12.5% of NOEC (non-observed effect concentration); in January and March I, OEC = 50% and NOEC = 25%; and in February and March II, OEC =100% and NOEC = 50%.

Nevertheless, in order to assure a broader safe margin, it was considered OEC 25% and NOEC 12.5%. It means that at the analyzed system it is supposed that the plume that exerts a stimulating effect on the primary production of the effluent can be extended to where this effluent is naturally diluted in 12.5% of its initial concentration.

In the majority of studies realized with *P. subcapitata*, the authors observed an inhibition of growth (DELLAMATRICE; MONTEIRO, 2006; GUÉGUEN et al., 2004; IVANOVA; GROUDEVA, 2006; MA et al., 2006; RODRIGUES et al., 2003). However, the results found in the present study pointed out to stimulation not inhibition of growth. A possible reason for that is the high concentration of nutrients (nitrogen and phosphorus) in the samples contents. Ration, fish dejection, and decomposition of organic matter promoted the raising of nutrients concentration in the water. The exposure of *P. subcapitata* to samples from the fish pond and from the effluent stimulated the algal growth instead of inhibiting it.

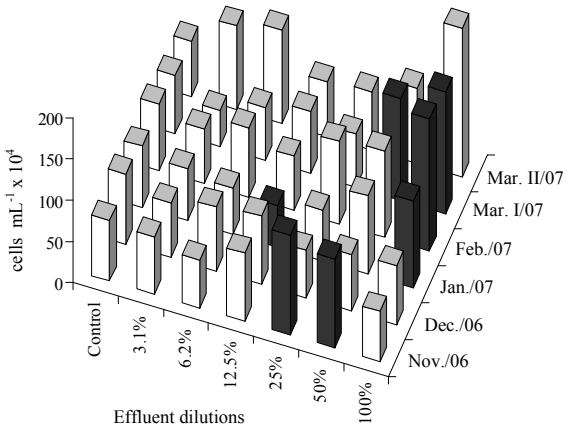

Figure 4. Average of algal growth (cells mL^{-1} x 10^4) in tests with effluent dilution. Dark bars indicate significant difference in the results of tests at each sampling campaign compared to the control.

Marques et al. (2008) performed tests with *Chlorella vulgaris* and *P. subcapitata* in surface waters of a rice field. Besides concluding that *P. subcapitata* was more sensible than *C. vulgaris*, in some samples it was also obtained stimulation to algal growth instead of inhibition. The authors assumed that the growth is due to the higher concentrations of phosphates and nitrates in the fertilizers of the plantations. The same way in the present study the nutrients presented in the samples stimulated the algal growth during the tests, thus overlapping any possibility of inhibition effect.

Bazante-Yamaguishi et al. (2009) performed eco-toxicological essays with *Ceriodaphina dubia* using the same samples object of this study. In the analysis with the crude samples, the authors observed acute and chronic toxicity at all sampling stations. This indicated that the studies with *P. subcapitata* were more efficient to appoint the increment of nutrients as the major modifying factor along the water flow in the system, when compared to the results of studies with *C. dubia* that were similar at all stations.

At the same time, ecotoxicological tests with original samples were valid to demonstrate that the management of the fish pond contributes decisively to the algal growth, and this reflected in higher values and significant differences for the samples from sites 2 and 3 when compared to control. Tests also demonstrated that the effects of addition of nutrients to the water of the fish pond could be detected still in the receiving stream, as observed in the sampling of March I.

Conclusion

This study permitted to conclude that the fish pond effluent was potential enough to stimulate the algal growth, therefore eutrophication, to the extent that it is naturally diluted to at least 12.5% of its initial concentration. This type of test can be a tool to be used by environmental managers in attempts to measure the extents of the impacts of effluent discharges from fish farming and to propose treatments based on qualitative and quantitative information.

It is recommended the adoption of an environmentally sustainable production management in order to minimize the impact caused by the activity.

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