UNIVERSIDADE FEDERAL DO RIO GRANDE DO NORTE - UFRN
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Effect of Okadaic Acid treated with pulse ultraviolet in phytoplankton using a modified miniaturized test

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Natal, 18 novembro de 2016

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Monografia apresentada como pré-requisito para a conclusão do curso de graduação em Ecologia pela Universidade Federal do Rio Grande do Norte

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Dedicatory

To my loved ones and to the scientific community.

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SUMARY

Resumo

Ácido Okadáico (OA) é uma biotoxina produzida por dinoflagelados de dois gêneros, *Dinophysis* e *Prorocentrum*. Acumula-se facilmente em filtradores causando envenenamento diarréico por moluscos (EDM) em humanos. Os sintomas incluem diarreia, náusea, vomito, dor abdominal e possível desidratação. Moluscos com níveis de OA superiores a 160 mg/kg não podem ser comercializados em países Europeus (Regulamento (CE) no 853/2004). Representando um forte impacto economico na aquicultura. O Pulso ultravioleta (PUV) é frequentemente utilizado para efeitos germicidas, no entanto seu uso para degradação de toxinas é pouco relatado na literatura. Nossa hipótese, é que a exposição ao PUV irá degradar o OA, reduzindo sua toxicidade. Técnicas eficientes são cada vez mais necessárias, em ordem de maximizar os resultados dos testes de toxicidade. *Pseudokirchneriella subcapitata* bioteste é frequentemente usado em testes multi tróficos. O clássico alga bioteste descrito no (ISO 8692:2012), entretanto, requer grandes quantidades de vidraria e substâncias. Por isso, um teste miniaturizado foi desenvolvido para substituir o ensaio clássico, usando-se 96 microplacas ao invés de frascos de erlenmyer. As vantagens da miniaturização incluem menos espaço, resultados mais rápidos e automatização, pois utiliza um leitor de placas e reduz o volume de substâncias caras, como o OA. Neste trabalho, testamos o teste miniaturizado para acessar o efeito do ácido okadáico tratado com PUV em *Pseudokirchneriella subcapitata*. Nossos resultados demonstram que, o teste miniaturizado e clássico alcançaram coeficientes de correlação maiores que 94% para cromato de potássio e 3,5- Diclorofenol (r^2 =0.9797 e 0.9455, respectivamente). Concluímos, que o teste miniaturizado é tão eficiente quanto o clássico, podendo ser usado como substituto devido as vantagens apresentadas. OA inibiu o crescimento de *P. subcapitata* no entanto, em relação ao PUV, o seu efeito não foi estaticamente significante.

Keywords: *Pseudokirchneriella subcapitata,* alga bioteste, ISO standard, ácido okadáico, pulso ultravioleta, envenenamento diarréico por moluscos, degradação.

Abstract

Okadaic acid (OA) is a biotoxin produced by dinoflagellate from two genera *Dinophysis* and *Prorocentrum*. It is easily accumulated in filter feeding leading to Diarrhetic shellfish poisoning (DSP) in humans. The symptoms include diarrehetic, nausea, vomiting, abdominal pain and possible dehydration. Shellfish with OA toxins levels exceed 160 mg/kg cannot be marked in European countries (Regulation (CE) no 853/2004) representing an impact in European Aquaculture. Pulse UV (PUV) technology is often used for germicidal propose. However, the use for toxin degradation is not well described in literature. Our hypothesis is, the expose to PUV would degraded OA reducing its toxicity. Efficient methodologies are even more required nowadays, in order to maximise higher throughput toxicity. *Pseudokirchneriella subcapitata* algal test is frequently used in a multi trophic test battery. The classic algal phytotoxicity flask assay (ISO 8692:2012) requires large amount of glassware and chemicals. The miniaturization of phyto-toxicity test was modified to replace the classic test, using 96 microwell plates instead of Erlenmeyer flasks. The advantages of miniaturization include less space, faster results, automation using a plate counter and reduced volumes of expensive analytes, as OA. In this project we assessed the effect of Okadaic Acid treated with PUV on *Pseudokirchneriella subcapitata* using the miniaturized test. We concluded that the miniaturized and classic test are efficient. The miniaturized could replace the classic based on the advantages and the effect of PUV on OA was not observed in this work

Keywords: *Pseudokirchneriella subcapitata* , algal bioassay, ISO standard, Okadaic acid, pulse ultraviolet, Diarrhetic shellfish poisoning, degradation.

1. Introduction

Harmful algal blooms (HAB) are a global issue with negative effects on ecosystem services, public health and economy. Their occurrences range has recently, colonizing new areas, not previously described in literature (Hoagland and Scatasta, 2006; Johansson et al. 2016). This phenomenon appears as result of excessive proliferation of certain toxic species or microalgae, generally after nutrient input, leading to poisoning of marine organisms (Kalaitzis et al. 2010). Those toxins enter food web through filter feeding, transferring the toxin to shellfish, where they can accumulate concomitant with shellfish poisoning syndromes, following human consumption (Nielsen et al. 2016).

Diarrhetic shellfish poisoning (DSP) is caused by ingestion of Okadaic Acid (OA) and its analogues, such as dinophysistoxins (DTX) as well as pectenotoxins (PTX). They are produced by species of the two marine dinoflagellate genera *Dinophysis* and *Prorocentrum* (Miles et al., 2006a; Hackett et al., 2009; Fux et al., 2011; Nielsen et al., 2013; Reguera et al., 2014). OA is responsible for a major problem for European Aquaculture. Shellfish cannot be marketed in EU countries when OA toxins levels exceed 160 mg OA per kg/shellfish meat (Regulation (CE) no 853/2004).

DSP symptoms occurs within 30 min to 4 h after eating contaminated shellfish, including diarrhea, followed by nausea, vomiting, abdominal cramps and possible dehydration (Grattan, Holobaugh e Morris Jr, 2016). OA inhibits the protein phosphatase types 1 (PP1) and 2A (PP2A). These phosphatases are responsible for dephosphorylate serine and threonine residues and regulate many cellular processes in mammalian cells (Le Hegarat et al., 2006). OA is also related with tumour formation (Munday & Reeve 2013).

Nowadays, the focus of diverse research projects are new technologies to degrade toxins present in the environment. Pulse of Ultraviolet Light (PUV) are quick pulses of light with a high peak power and a broad spectrum used for water disinfection. It is known by far, that natural UV radiation, mostly UV-B (280-315 nm), interacts with organic matter in aquatic ecosystems promoting its degradation through photolysis (Kumar et al. 2016; Tedetti & Sempéré 2005). Under PUV irradiation, organic compounds are excited to excited states and then react with dissolved oxygen, or homolysis of organic compounds forms radicals and then react with dissolved oxygen (Y. Liu et al. 2016; Legrini et al. 1993). Recent research's had described UV as an efficient methodology to degraded organic compounds, including the biotoxin Microcystin (S. Liu et al. 2016); contaminants: 1,4 dioxane (solvent-stabilizer), n-nitrosodimethylamine (NDMA) (nitrosamine), tris-2-chloroethyl phosphate (TCEP) (flame retardant), gemfibrozil (pharmaceutical), and 17β estradiol (hormone)(Alvarez-Corena et al. 2016), the herbicide atrazine (Moreira et al. 2016).

Pulse of Ultraviolet Light has already been described in the literature, for inactivation of food pathogens and prevent degradation by microorganisms (Kasahara et al. 2015). In addition, these newer technologies allow the emission of UV light as pulses of short-duration, high-energy UV-C-rich light, resulting in a highly germicidal effect on products, such as water and liquid foods (Smith et al., 2002; Takeshita et al., 2003; Elmnasser et al., 2007; Hosseini et al., 2011;(Kasahara et al. 2015).

Taking into account the photodegradation effect of UV radiation on organic substances we hypothesized that pulse UV will reduce the toxicity of okadaic acid in phytoplankton. This hypothesis was tested by using the *Pseudokirchneriella subcapitata* algal bioassay to detect the effect of OA treated and non treated with PUV on microalgae. We aimed to detect if OA toxicity would be decreased after exposition the toxin to UV pulses. *Pseudokirchneriella subcapitata* algal bioassay is a widely used ecotoxicology test, recommend by regulatory agencies to assess aquatic toxicity of chemicals (OECD guidelines 201, 2002). However, the classics phytotoxicity flask assay described in ISO 8692:2012 and 6341:2012 requires large amount of glassware and chemicals. In order to increase throughput toxicity testing, the miniaturization of phytotoxicity test was modified to replace the classic test, using microplates instead of Erlenmeyer flasks. The advantages of the miniaturized test include less lab space requirement, reduced volume of analytes and faster results.

In this context, the aim of the present project is to assess the effect of UV-treated and non treated Okadaic Acid on *Pseudokirchneriella subcapitata* using the modified, miniaturized test. We expect to contribute to the knowledge of food treatment in order to promote OA control. Also, we provide an improvement in the *Pseudokirchneriella subcapitata* algal bioassay.

2. Methodology

2.1 Phytoplankton culture

Pseudokirchneriella subcapitata culture was initiated through a strain from Culture Collection of Algae and Protozoa (CCAP 278/4). The culture was maintained in ISO medium (ISO 8692 2000) in a 500mL Erlenmeyers, under continuous cool white light luminescent tubes (6,000–10,000 lux), in axenic conditions and continuously shaken (100–200 rpm) at 23 ± 2°C in an orbital shaking incubator. Generally, a 3-day algal culture was used for the inoculation of tested concentrations and controls (Paixão et al. 2008).

2.2 Experiment of validation for miniaturazed bioassay

The classic format requires large amount of glassware (erlenmeyer flask), chemicals and manual cell counting. While the miniaturized format reduce the amount of glassware (microplates), chemical and automatized cell counting using a microplate reader. The green algae *Pseudokirchneriella subcapitata* was exposed to five concentrations (0.1, 0.5, 1, 5 and 10 ppm) of 3,5-Dichlorophenol and Potassium Dichromate. The classic test was carried out as per ISO 8692:2012 guidelines. A volume of 1mL from the aliquot of *Pseudokirchneriella subcapitata* (1x104 cells/mL) was inoculated in 50 mL erlenmeyer flask. Thus, 9mL from each concentration tested were added to each flask. The experiment duration was three days. Cells concentration were measured twice (0 and 72h) using hemocytometer.

For the microplate bioassay, 100 µL from the same aliquot of *Pseudokirchneriella subcapitata* used for the classic test, were inoculated into each well of a 96 well flat bottom microplate. A volume of 200 µL from each concentration of analyte was also added to each well of treatment. For cell concentration, the absorbance of the plates were measured (0 and 72 hours) using a multi plate reader at wavelength @630 nm.

In both tests: Jaworski medium was used as diluent. The control corresponded to the algal aliquot and the medium only. The treatments had 3 replicates while controls had 6 replicates. The median inhibition growth rates were determined. The IC_{50} values were within 95% confidence limits stipulated in the ISO standard (ISO 8692:2012). Same laboratory conditions of the validation experiment were applied in this section.

2.3 Effect of Okadaic acid pulsed ultraviolet

The miniaturized test was performed using the methodology described above. Some modifications were done to precede the MicroPlate bioassay. *Pseudokirchneriella subcapitata* was exposed to nine concentrations (0.1, 0.5, 1, 2, 3, 4, 5, 7.5 and 10 ppm) of Okadaic Acid

ammonium salt standards (Sigma Aldrich). The treatments was exposed to OA treated with PUV (300 x 900 volt pulses at 1 pulse / sec) using Samtech ™ and the control was exposed to OA non-treated with pUV. Treatment and control had triplicates. Same measurement and laboratory conditions of the validation experiment were applied in this section.

2.4 Data Analyses

The specific growth rate (μ) was determined for each test and control batch replicate, as per ISO 8692:2012. Following the formula:

$$\mu = \frac{\ln nL - \ln n0}{t_L - t_0}$$

Where, n_0 and n_L are the initial cell density and at time L, respectively. The cell density is the absorbance. t0 and tL are time of the test start and time of the test termination. After, the percentage inhibition was calculated using the follow formula:

$$I_{\mu i} = \frac{\mu_c - \mu_i}{\mu_c} \times 100$$

Where, μ_i is the growth rate for the treatments and μ_c is the mean growth rate for the control. A Pearson correlation was used to compare classic and miniaturized test. Statistical analysis were performed using prism graphpad 7.0 and R.

3. Results

3.1 Experiment of validation for miniaturazed bioassay

The classical and miniaturized test protocols yielded 72 hours IC_{50} values within the 95% confidence limits stipulated in ISO 8692:2012 guidelines for 3,5-Dichlorophenol and Potassium Dichromate (Table 1). Classic and miniaturized format were correlated with correlation coefficient greater than 94% for 3,5-Dichlorophenol and Potassium Dichromate (r^2 =0.9797 and 0.9455, respectively). Positive significant concordance was found among the tests for both substances tested ($K_2Cr_2O_7$, $p < 0.01$ and $C_6H_4Cl_2O$, $p < 0.01$); Figures 1 and 2. Therefore, the correlation was stronger for $C_6H_4Cl_2O$ (r^2 =0.9797; $K_2Cr_2O_7$ r^2 =0.9455).

Table 1: IC_{50} *values* of 3,5-Dichlorophenol and Potassium Dichromate for both classic and miniaturized test

Chemical Name	Type of Test	IC_{50} Values (ppm)
3,5-Dichlorophenol	ISO guidelines	3.38 ppm +/- 1.30 ppm
3,5-Dichlorophenol	Classic (Erlenmeyer Flask)	2.753
3,5-Dichlorophenol	Miniaturized (96 microwell)	3.877
Potassium Dichromate	ISO guidelines	1.19 ppm +/- 0.27 ppm
Potassium Dichromate	Classic (Erlenmeyer Flask)	1.250
Potassium Dichromate	Miniaturized (96 microwell)	1.450

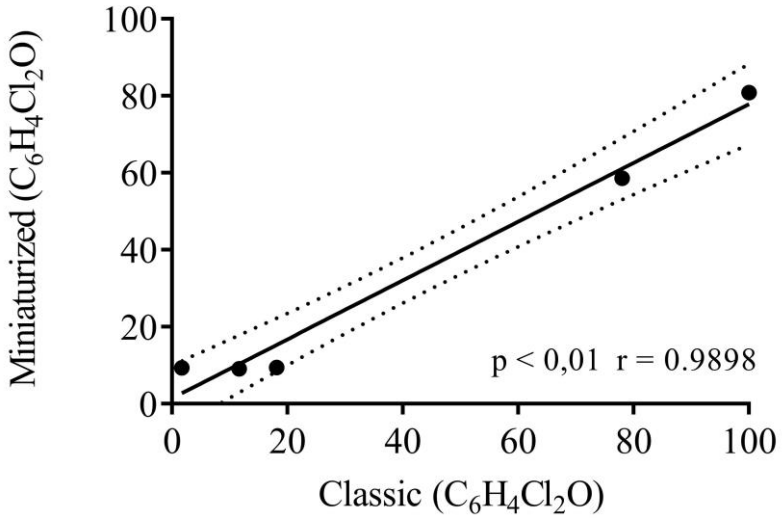

Figure 1: Correlation between results from miniaturized and classic test (% inhibition growth of *P. subcapitata*) expose to 3,5-Dichlorophenol ($p < 0.01$; r =0.9898; r2 =0.9797; 95% confidence bands).

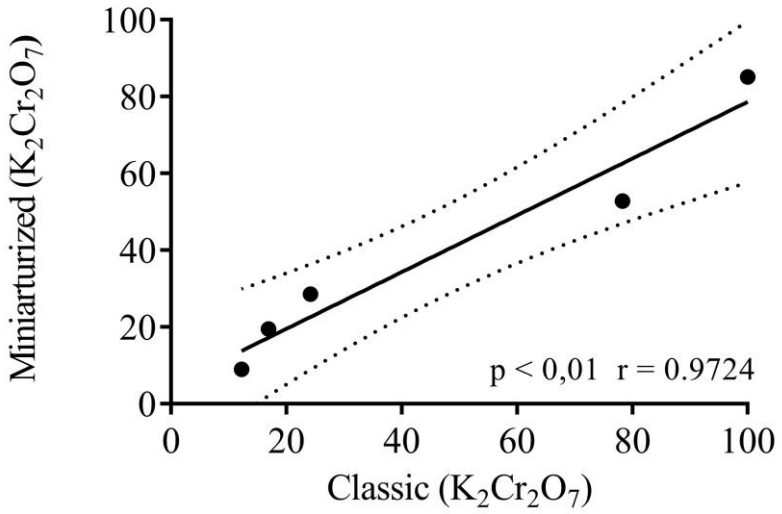

Figure 2: Correlation between results from miniaturized and classic test (% inhibition growth of *P. subcapitata*) expose to potassium dichromate ($p < 0.01$; $r = 0.9724$; $r2 = 0.9455$; 95% confidence bands).

3.2 Effect of Okadaic acid pulsed ultraviolet

The cell growth in the control increased by a factor higher than 67 in 72 h, as recommend on ISO 8692.2012, in the validity criteria section (Unshown data). The effect of OA on culture cell density of *Pseudokirchneriella subcapitata* was demonstrated in a dose response curve (Fig.3). Both culture had their growth inhibited in all concentrations tested (0.1, 0.5, 1, 2, 3, 4, 5, 7.5 and 10ppm), independently of pulse UV. Even at the smallest concentration of OA (0.1ppm) the cell growth of *P. subcapitata* was reduced. The percentage of the inhibition growth did not pass of 70%, even with the highest concentration tested. The exponential rise of inhibition growth in response of the increase in the concentration of OA was slightly more accentuated for unpulsed than pulsed UV (blue and red line, Figure 3). Based on statistic analyses, the difference was not significant. However, the pulse UV decreased almost by half of the IC_{50} for pulsed UV compare with unpulsed UV treatment (IC_{50} unpulsed = 3,915; IC_{50} pulsed = 6,668).

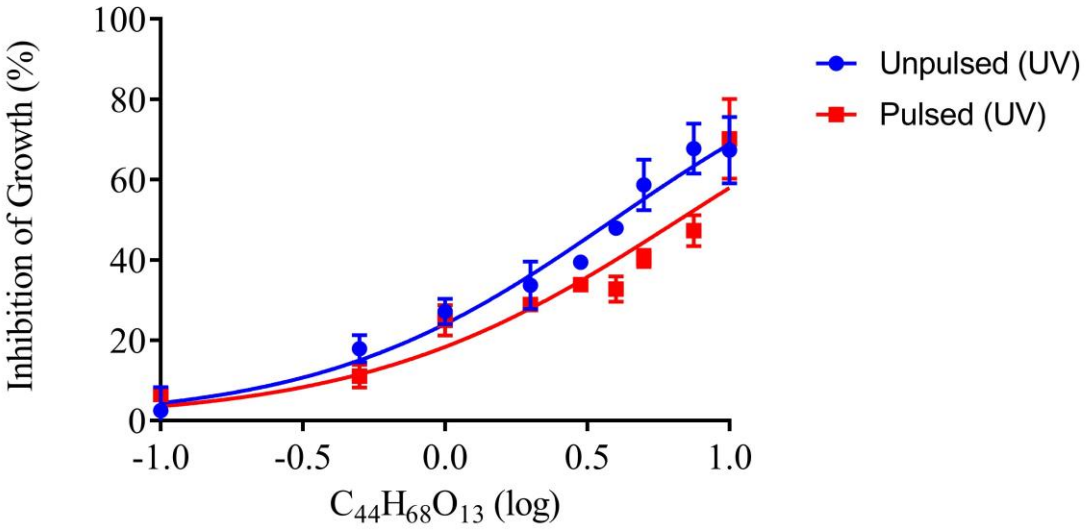

Figure 3: Dose response curve from miniaturized test (74h), concentration of Okadaic Acid in ppm versus % inhibition growth for both treatments: Unpulsed UV (blue) IC_{50} = 3,915; Pulsed UV (red) IC_{50} = 6,668

4. Discussion

Algae are often used for risk impact assessment in the ecosystems. They are key primary produces and are basis in mostly of food web in aquatic environment, thus, exposure to pollutants at this level could cause a bottom-up effect (Grzesiuk et al. 2016; Hylland and Vethaak 2011).

The correlation between the classic and miniaturized test using *Pseudokirchneriella subcapitata* indicated that the miniaturized test is efficient as the classic test described on ISO 8692:2012. The coefficient of correlation was greater than 94% for both substances tested, indicating a strong correlation among tests. *Pseudokirchneriella subcapitata* is the most common green algae specie used in toxicity tests, which are required for registration of a new chemical in European Union countries (OECD 1998; USEPA 2000). The miniaturization version presents more advantages compared with the classic test, including less glassware and space requirements, faster results, because a microplate reader automatizes the process and reduced volumes of expensive analytes. Okadaic acid has an average cost of €194 / 10µg (Sigma Aldrich). Thus, miniaturized test is a powerful tool to increase the number of tested samples, especially for regulation of new chemical substances. As ecotoxicity tests becomes even more necessary nowadays, in order to access the effect of toxins in the environment, a more efficient and cheaper toxicity essay has a major importance.

We have shown a clear negative effect of okadaic acid on *Pseudokirchneriella subcapitata* growth (Fig 3). Currently, the lack of studies of OA on *Pseudokirchneriella subcapitata* occur probably because OA is a marine toxin and *P. subcapitata* is a freshwater organism. Still, *P. subcapitata* may be a model organism for studies on the effect of OA.

Most ecological studies of PUV focus on the microbial inactivation and the safety of this methodology for aquatic species. Ecotoxicological multi trophic tests have proven that PUV reduce microbial viability in water samples and that, those samples are environmentally safe to be released into the environment, Including for primary producer, primary and secondary consumer, decomposer (Garvey et al. 2015). Our study adds another function for PUV. The PUV altering the toxicity of toxic water-soluble compounds. Therefore, our result could not prove this. There was a difference among the IC_{50} for unpulsed and pulse OA. Despite, this difference was not related with PUV.

PUV irradiation technology had already been applied for degradation of toxic compounds. Microcystins is a toxin produced by cyanobacteria in freshwater eutrophic environment and undergoes photolysis upon UV radiation (Zhang et al. 2016; Tsuji et al. 1995). Microcystins degradation is widely discussed because is the most common cyanotoxin in

contaminated aquatic systems. Sublethal doses of it, in drinking water can lead to liver damage and tumor formation. Also it is considered one of the key factors in high occurrence of primary liver cancer (Y. Liu et al. 2016). Under PUV irradiation, organic compounds are excited to excited states and then react with dissolved oxygen, or homolysis of organic compounds forms radicals and the generated radicals react with dissolved oxygen (S. Liu et al. 2016; Legrini et al. 1993).

Based on literature review, there is a gap in the literature about the effect of PUV on marine toxins degradation. Exposure to these toxins can have a negative impact on marine wildlife and coastal species of fish, birds, mammals, as serious human health consequences (Khan et al. 2010; Hallegraeff, 1995). The use of PUV on marine toxins, as okadaic acid is crucial. The degradation of okadaic acid would protect marine wildlife and human health. OA inhibits the activity of serine/threonine protein phosphatases. Bioaccumulation of it causes DSP syndrome. It symptoms include resulting in vomiting, diarrhea and abdominal pain in humans (Prego-Faraldo et al. 2016; Bialojan & Takai 1988).

Multi-trophic bioassays are required to characterise the environmental risk assessment. It is important to establish a marine biological test set including organisms belonging to different trophic levels, such as bacteria, algae, crustaceans and fish, because they have different sensitivity to the tested matrix (Pane et al. 2008). For this reason, a multi-trophic bioassay is required to access the effect of pUV.

We repeat the experiment using *Daphnia Magna* as test organism instead of *Pseudokirchneriella subcapitata*, to access the effect of OA treated with PUV on zooplankton. The difference of IC_{50} for non-pulsed and pulsed UV was more distinct when used *Daphnia Magna* as test specie. The pulsed OA is 4.6 times less toxic to *Daphnia Magna* than no pulsed OA. It demonstrates a relation between pUV and reduction of toxicity of Okadaic acid for zooplankton (Murray, Ian. 2013 unpublished raw data). A comparison between Okadaic acid treated and no treated with pUV (Fig. 3) reveals a difference among IC50 (IC_{50} unpulsed = 3,915; IC_{50} pulsed = 6,668). However, this difference was not pronounced as *Daphnia Magna* results. Furthermore, the mechanism for competition are different of consumers, lower bioaccessibility rates associated with a certain species indicate that consumers are less exposed to toxins, for this reason they can be more sensitive (Braga et al. 2016).

Lastly, future studies are needed to repeat the experiment proposed by the present study using species from different trophic levels, in order to observe if the result will persist. For further understanding, a range of wavelength and irradiation time should be applied to observe efficiency on OA degradation. Application of liquid chromatography mass spectrometry to analyse the degradation pathway of OA is reccomended. Additionality, we suggested for next studies the

examination of others parameters beyond inhibition growth, such as morphological responses, in order to observe if PUV would affect it.

5. Conclusion

We concluded that the classic and miniaturized ecotoxicological test using *P. subcapitata* are equally efficient, under the tested experimental conditions *Pseudokirchneriella subcapitata* had their growth inhibited by OA pulsed and non-pulsed with PUV since the smallest concentration range tested (0,1 ppm). Even with the highest concentration of the range tested (10 ppm) the inhibition growth did not yield 100%. The treatment pulsed with UV had the IC_{50} decreased almost by half compare with unpulsed UV (IC_{50} unpulsed = 3,915; IC_{50} pulsed = 6,668). However, this difference was not statistically significant. Other pulse parameters should be tested in order to optimize the technique for OA remediation.

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