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Original article:

ANALYSIS OF CONCENTRATION-DEPENDENT EFFECTS OF COPPER AND PCB ON DIFFERENT *CHATTONELLA SPP.* MICROALGAE (RAPHIDOPHYCEAE) CULTIVATED IN ARTIFICIAL SEAWATER MEDIUM

Jeanette Niestroy[1*], Alfonso Bárbara Martínez[2], Christine J. Band-Schmidt[1]

[1] Instituto Politécnico Nacional, Centro Interdisciplinario de Ciencias Marinas (CICIMAR-IPN), Departamento de Plancton y Ecología Marina, P.B. 592, La Paz, B.C.S. 23000, México
[2] Instituto Politécnico Nacional (IPN), Av. Luis Enrique Erro S/N, Unidad Profesional Adolfo López Mateos, Zacatenco, Delegación Gustavo A. Madero, C.P. 07738, México, Distrito Federal
* Corresponding author: jeanette.niestroy@yahoo.com

ABSTRACT

In the present study, the effect on the chlorophyll a and the total protein content as well as the *Chattonella spp.* cell viability were examined after concentration-dependent exposure to $CuCl_2$ and Aroclor 1242. The comparison between various raphidophyte strains provides an insight into the different susceptibilities to contaminants of *Chattonella subsalsa* (CSNAV-1), *C. marina* var. *marina* (CMCV-1) and *C. marina* var. *ovata* (COPV-2). The microalgae were cultivated in artificial seawater medium. Exponentially growing microalgae (8-10 days in culture) were used for exposure experiments. We observed in all three raphidophyte species cytotoxicity-mediated modifications beginning at concentrations of 150 and 200 µM of the heavy metal copper after 24 hours exposure. But interestingly, the three strains exhibited only slight differences in their susceptibility to $CuCl_2$. *C. subsalsa* and *C. marina* var. *marina* cells were first affected at the chlorophyll a level and in cell viability. The total protein amount was reduced significantly only after exposure to 300 µM of $CuCl_2$. However, *C. marina* var. *ovata* microalgae showed similar reduction curves for all three analysed cytotoxicity endpoints after heavy metal exposure. On the other hand, after Aroclor 1242 incubation the cytotoxic modification pattern indicated clearly the different susceptibilities of the three raphidophyte strains. *C. subsalsa* cells noticeably exhibited a decrease in the analysed pigment amount (30-20 % compared to that of the control) already after 0.007 mg/L PCB exposure. In contrast, cell viability and total protein content were slightly reduced and fell below the 50 % threshold after 0.7 and 3.3 mg/L of Aroclor 1242, respectively. Interestingly, *C. marina* var. *ovata* showed almost no cytotoxic modification caused by the PCB mixture. Only the concentration of 0.7 mg/L Aroclor 1242 clearly affected the cell viability. As opposed to that we observed a concentration-dependent decrease of cell viability and chlorophyll a amount in CMCV-1 microalgae. These observations confirmed that the susceptibility of the raphidophytes strains CSNAV-1, CMCV-1 and COPV-2 is contaminant-dependent. We showed differences even between two variants of Chattonella (*Chattonella marina* var. *marina* and *C. marina* var. *ovata*). Furthermore, we were able to show the different mode of action of two common pollutants by simple cytotoxic parameters like total protein and chlorophyll a content as well as by cell counting analysis.

Keywords: Chattonella spp., cytotoxicity, copper, PCB, total protein content, chlorophyll a

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INTRODUCTION

Ecotoxicological analyses are often focused on terrestrial or freshwater organisms. Although more than 70 % of the earth's surface is covered by the oceans and phytoplankton is the base of marine food chain, toxicological studies are rarely conducted with marine microalgae. Even though, little is known about the magnitude and the impact of environmental contaminants on organisms in third world countries like Mexico. It was demonstrated, however, that the contamination by heavy metals like mercury, arsenic, plumb and copper is a serious problem in the peninsula of southern Baja California (Sánchez-Rodríguez et al., 2001; Huerta-Diaz et al., 2007; Leal-Acosta et al., 2010; Riosmena-Rodríguez et al., 2010). Copper is an essential micronutrient for plants and algae. It is used in cells as a component of several proteins/enzymes involved in a variety of metabolic pathways. In higher concentration copper ion may generate oxidative stress by inducing production of reactive oxygen species (ROS), via its toxic effects on photosynthesis and hence cause serious damages to macromolecules. Thus plants, algae and fungi developed an active detoxification mechanism by means of glutathione and glutathione-related peptides to avoid heavy metal poisoning (Morelli and Scarano, 2004). Another way to prevent cytotoxic damages is the degradation and biotransformation of xenobiotics. These defense mechanisms are often related to an increase of diverse proteins like enzymes and transcription factors (Manimaran et al., 2012). Cell growth also is affected by higher copper concentrations as shown in many studies. In addition, copper inhibits the process of cell division independently of any effect on the production of new cell material (Stauber and Florence, 1989). Disruption or delay of mitosis and cytokinesis in the first cell cycle were also already demonstrated by persistent organic pollutants like PCB. Even after 2–4 days exposure cell growth can be slowed or arrested in fucoid algae (Hable and Nguyen, 2013). Persistent organic pollutants like polychlorinated biphenyls (PCB) are already detected in the environment of southern Baja California (Yunuén et al., 2011). Furthermore, the bioaccumulation of PCBs is demonstrated in marine and terrestrial animals (Gutiérrez Galindo and Cajal Medrano, 1981; Jiménez et al., 2005; Richardson et al., 2010; Yordy et al., 2010; Rosales Ledezma et al., 2011). In general adverse effects of pollutants on molecular level are rarely investigated in wild animals (Richardson et al., 2010) and even less in plants compared to the toxicological data based on human analysis and cell line *in vitro* studies. Nevertheless, concentration- and time-dependent analyses of some heavy metals and organochlorine compounds are already studied in several marine micro- and macroalgae (Cid et al., 1995; Abalde et al., 1995; Ritter et al., 2008; Ebenezer and Ki, 2012). Since researches conduct cytotoxic analysis using supplemented sterile seawater the observed results cannot be explained only by the examined substance. The amount and the types of pollutants which are already present in the seawater are generally not known. Furthermore, possible synergistic effects of substances present in sterile seawater media cannot be excluded. Because of this reason we decided to cultivate our microalgae model *Chattonella spp.* in an artificial seawater medium to minimise this source of error. Our studies are geared to the OECD Principles of Good Laboratory Practice (GLP) and the manual of methods in aquatic environment research of FAO to determine toxicological factors like NOAEC/LOAEC/EC_{50} in respect to cell growth/survival, total protein and chlorophyll a content. Furthermore, the present study shows the different susceptibilities to $CuCl_2$ and Aroclor 1242 of the three analysed raphidophyte strains.

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MATERIAL AND METHODS

Chemicals and material

All laboratory chemicals were purchased in p.a. quality from Sigma-Aldrich, (Toluca, México), Fermont (Monterrey, México), Mallinckrodt AR (Phillipsburg, USA) or Faga Lab (Sinaloa, México). All glass or plastic culture flasks, tubes and cells were purchased from Pyrex or VWR México.

Methods

Cell culture, growth rate and light microscope pictures

All strains were collected and isolated by C. Band-Schmidt as described in Band-Schmidt et al. (2012). For the exposure analysis, *Chattonella subsalsa* (CSNAV-1), *Chattonella marina* var. *marina* (CMCV-1) and *Chattonella marina* var. *ovata* (COPV-2) were cultivated in artificial seawater medium (ASW) as described by Imai et al. (2004). To evaluate more clearly the ability of the ASW medium as a common microalgae culture medium each raphidophyte strain was also cultivated in the modified sterile seawater medium f/2 (modified as described in Band-Schmidt et al. (2012); Guillard and Ryther (1962)) and GSe (Blackburn et al., 1989). In each culture media cells were preadapted at least three generations. The microalgae were grown in 250 ml polycarbonate culture tissue flasks and maintained at 23 °C with a 12 h:12 h light–dark cycle at 150 µmol photons m^{-2} s^{-1} illumination. The cell growth rates of each stain cultivated in the different medium were determinated. Every second day, subsamples were counted in a 1 ml Sedgwick-Rafter counting slide under an optical light microscope (Axioskop 40 Zeiss). Cell density was used to calculate exponential growth rates according to Guillard (1973). Light microscope pictures (using a Konus Digital Camera with the ScopePhoto 3.1.475 program) were taken to demonstrate copper-/PCB-induced morphological changes.

For chlorophyll a and protein analyses, cultures were harvested at the late exponential growth phase. The supernatant of each culture solution was apportioned for all three endpoint analyses. Microalgae which were already at the bottom of the culture tube were not considered. All measurements were repeated three times (n=3).

Determination of the total protein concentration

$4x10^5$ cells of microalgae per sample were used to analyse the modifying effect by contaminant. 24 hours after seeding, the cells were exposed to different concentrations of $CuCl_2$ (0, 0.1, 0.3, 1, 3, 10, 30, 100, 150, 200, 250 and 300 µM) or Aroclor 1242 (0, 0.007, 0.03, 0.07, 0.3, 0.7, 3.3 mg/L) for further 24 hours. Aroclor 1242 was dissolved in DMSO and final exposure concentrations of DMSO did not exceed 0.7 %. The total cell-lysate protein concentration was determined by modified Lowry assay (Lowry et al., 1951) as described by Gerhardt et al. (1994). In brief, cells were harvested by centrifugation at 3,000 rpm for 10 minutes. The supernatant was removed and the pellet was resuspended in 1 ml distilled water and 1 ml Lowry solution. After brief vortexing the samples were incubated for 10 minutes in the dark at room temperature. 100 µl Folin Reagent (1N) were added to each sample and incubated for further 30 minutes at room temperature in the dark. Bovine serum albumin was used as protein standard. The protein content was measured at 750 nm using a Spectronic ®Genesys Z Spectrophotometer. The copper and PCB-induced changes of the total protein content are expressed as percentage related to the control value. The Student t-test was employed to compare total protein content changes. The statistical probability of $p < 0.05$ was considered significant.

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Determination of chlorophyll a, b and c concentration

The chlorophyll a, b and c content of cells were determined by photospectometry according to Strickland and Parsons (1972) and Hongjie et al. (2013). In brief, the cells were harvested by centrifugation at 3,000 rpm for 10 minutes. The supernatant was removed and the pellet was resuspended in 2 ml 90 % acetone and stored for at least 12 h. The chlorophyll content was measured at 665 nm, 645 nm, 630 nm and 750 nm. The spectrophotometer (Spectronic ®Genesys Z) was adjusted to zero with 90 % acetone. In addition, the absorption of chlorophyll was zeroed to the 750 nm absorption of each sample to correct the turbidity and contaminating coloured compounds of the cell solution. The copper and PCB-induced changes in the chlorophyll a content are expressed as percentage related to the control value. The Student t-test was used and the statistical probability of $p < 0.05$ was considered significant.

RESULTS

Culture medium comparison

The raphidophytes *C. subsalsa*, *C. marina var. marina* and *C. marina var. ovata* were cultivated for around 3 weeks in f/2, GSe and ASW medium and in each cultivating method the growth rate was determined. The microalgae were cultivated at least for three passages in the analyzing media to adapt the cell culture to its new environment.

C. subsalsa grown in GSe and ASW medium incipiently showed a similar exponential proliferation up to 8 days. The growth curve of the cells cultivated in ASW medium reached a plateau of $6x10^4$ cells up to 15 days and started to show a decline on the 17 day. Due to this, this raphidophyte strain cultivated in ASW medium has a growth rate (r; shown in Table 1.) of 0.36. In contrast, *C. subsalsa* cultivated in GSe medium exhibited an exponential growth up to 18 days and a growth rate of 0.2. On the other hand, the microalga grown in f/2 medium showed also a growth phase of 12 days, indicated a growth rate of 0.27 and reached a maximal cell number of $7x10^4$. After 20 days the growth curve did not pass into a dead phase (Figure 1a).

Table 1: Growth curve comparison of the three used raphidophyte strains cultivated in GSe, f/2 and ASW medium. Determination of cell growth rate (r), divisions per day (k), and cell doubling time (T2) at the exponential growth phase (expressed in (dt) delta time)

	CSNAV-1			COPV-1			CMCV-1		
	GSe	ASW	f/2	GSe	ASW	f/2	GSe	ASW	f/2
time period [days]	6-15	5-9	7-13	4-12	4-11	4-13	9-18	5-13	7-12
dt	9	4	6	8	7	9	9	8	5
r	0.20	0.36	0.27	0.25	0.37	0.30	0.17	0.29	0.30
k	0.28	0.53	0.39	0.36	0.54	0.43	0.25	0.41	0.43
T2	3.55	1.90	2.53	2.78	1.86	2.34	4.04	2.42	2.33

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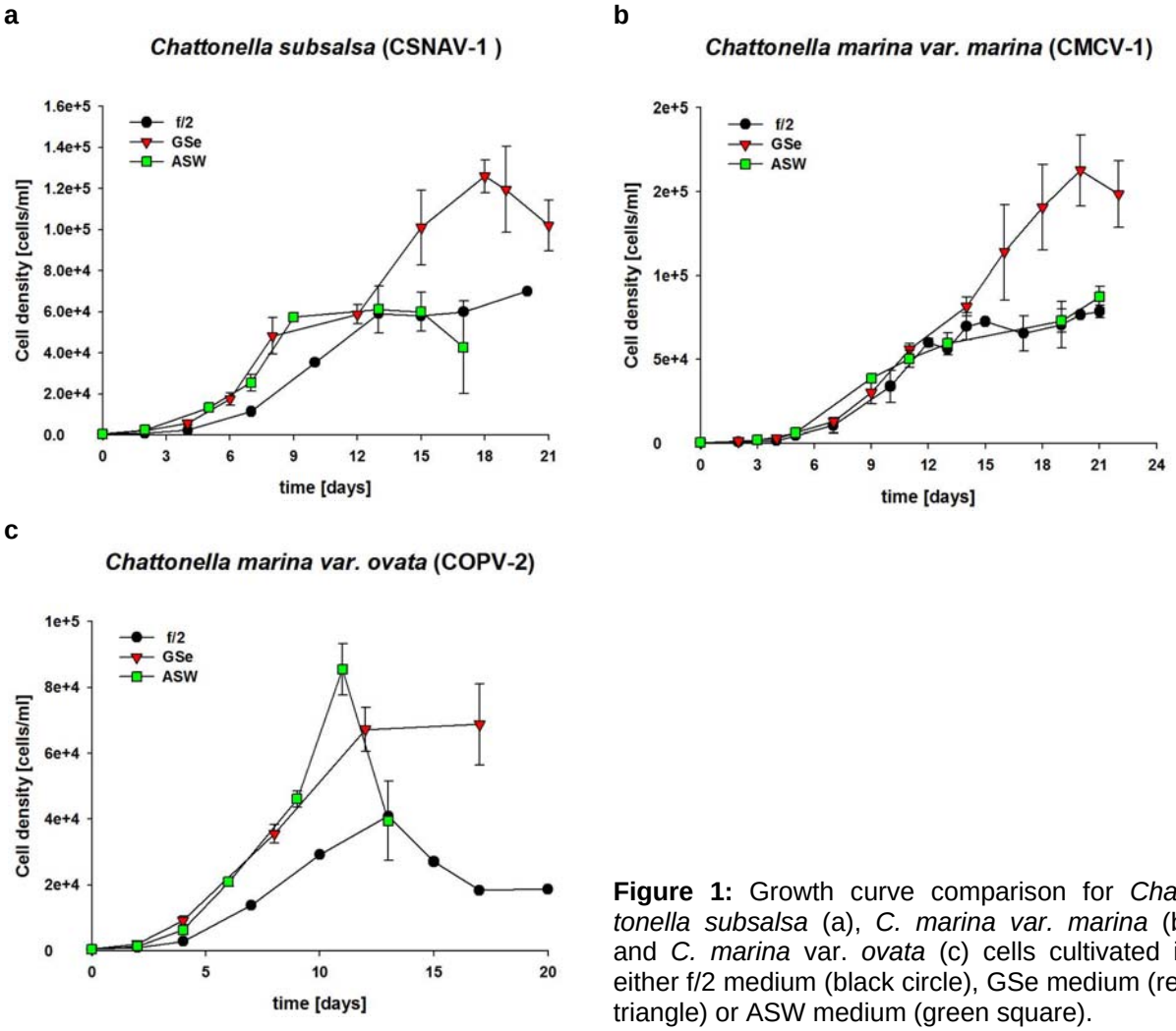

Figure 1: Growth curve comparison for *Chattonella subsalsa* (a), *C. marina var. marina* (b) and *C. marina* var. *ovata* (c) cells cultivated in either f/2 medium (black circle), GSe medium (red triangle) or ASW medium (green square).

C. marina var. marina cultivated in GSe medium exhibited the highest number of cells with around $1.6x10^5$ cells (r=0.17) after 20 days in culture. Likewise to *C. subsalsa* and *C. ovata*, also *C. marina* presented in ASW and f/2 medium a similar growth curve and maximal cell number of $8.7x10^4$ (r= 0.3) and $8.1x10^4$ (r= 0.3), respectively. Both growth curves reached the moderate proliferation phase after 13/14 days in culture (Figure 1b).

C. ovata showed a similar exponential proliferation up to 8 days in GSe and ASW medium as already observed for the growth curve of *C. subsalsa*. The growth curve of the cells cultivated in ASW medium reached a maximum of $8.6x10^4$ cells up to 11 days and showed a rapid decline of $4x10^4$ cells after 13 days in culture. *C. ovata* presented a growth rate (r) of 0.37 in ASW medium. However, the cells cultivated in GSe medium exhibited an exponential growth up to 12 days (r=0.25). In addition, the growth rate in f/2 medium was determinated by 0.3 even though the growth phase lasted for 8 days, too. The maximal amount was $4.1x10^4$ cells. After 15 days in culture the microalgae started the death rate to growth up (Figure 1c).

All *Chattonella spp.* stains cultivated in ASW medium show curve progression and growth rates similar to *Chattonella spp.* cells cultivated in GSe or f/2 medium. Interestingly, all used raphidophyte strains possessed different growth characteristics in all three media. These results indicate that the three strains require different nutrimental conditions to grow. Nevertheless,

the observations reassure that the artificial seawater medium is a suitable alternative to sterile seawater and can be used for toxicological studies.

Effects of copper exposure

As already described by Band-Schmidt et al. (2012) the main pigment in the three raphidophyte strains was chlorophyll a while chlorophyll b and c were detected in all three strains in precursory experiments, but the absorption was low corresponding to one fifth of that of chlorophyll a. Because of this reason we decided to focus the cytotoxic analysis on the chlorophyll a pigment of the microalgae. We used a least $2x10^5$ cells per measurement to ensure a clear absorption result. 24 hours after seeding the cells were exposed to different concentrations of copper as Cu^{2+} for further 24 hours.

Chattonella subsalsa (CSNAV-1) presented significant changes in the chlorophyll a content, cell amount and total protein content after exposure to 150, 200 and 300 µM of $CuCl_2$, respectively (Figure 2a). These copper concentrations can be considered as the LOAEL values for each analysis parameter after 24 hours incubation. Notably, the chlorophyll a content of *C. subsalsa* was already significantly reduced by 150 µM $CuCl_2$ (46 ± 27.7 %) and dropped to only 25.3 ± 14.7 % chlorophyll a content compared to the control after 300 µM of $CuCl_2$ treatment. The next higher concentration of the heavy metal (200 µM) caused a strong significant reduction of the microalgae viability by more than 50 %, i.e. 39.3 ± 16.8 %. Remarkably, the viability curve of *C. subsalsa* cells ran parallel to that of the chlorophyll a curve whereupon the pigment of the microalgae showed a stronger effect caused by copper. However, at the concentration of 300 µM of copper the cell number dropped to 11.6 ± 6.8 % compared to the control and showed thus a stronger toxic effect than in the pigment analysis. Even though we measured a significant reduction in the total protein content caused by 300 µM of $CuCl_2$ compared to the control algae, the 50 % value marker was not exceeded (59.6 ± 30.3 %). We can conclude that the concentration of 100 µM $CuCl_2$ caused no observed effect (NOAEL value) in the raphidophyte strain *C. subsalsa* after 24 hours of incubation.

Also the strains of *Chattonella marina*, i.e. *var. marina* and *var. ovata* (Figure 2b. and 2c, respectively) showed clear changes in the amount of total protein, chlorophyll a and in the cell amount in response to high concentrated copper exposure. Nevertheless, the two raphidophyte variants exhibited different cytotoxic responsiveness. However, *C. marina* var. *marina* (CMCV-1) exhibited a similar curve progression as *C. subsalsa*. Interestingly, $CuCl_2$ concentrations of 0.1, 0.3 and 1 µM caused significant increases of the total protein content to (111.4 ± 7.0 %, 122.9 ± 14.0 % and 117.8 ± 9.1 %, respectively). The cell and chlorophyll a amount of the microalgae also tended to increase. On the other hand, 200 µM of $CuCl_2$ caused a significant decrease of chlorophyll a and of cell content lower than 50 % compared to the control (40.9 ± 15.9 % and 42.7 ± 7.5 %, respectively). Because of this we can determine 200 µM of copper as the LOAEL concentration in *C. marina* var. *marina* cells after 24 hours of exposure. At the highest $CuCl_2$ concentration of 300 µM, the chlorophyll a concentration fell to 25.9 ± 27.6 % and the cell number was reduced to 8.3 ± 5.4 % compared to the control. Likewise observed in *C. subsalsa*–treated microalgae, *C. marina* var. *marina* cells exhibit no significant decrease in the total protein content compared to the control even after incubation with 300 µM $CuCl_2$ (66.4 ± 29.5 %). In this respect, the NOAEL concentration of copper after 24 hours incubation in *C. marina* var. *marina* microalgae was 150 µM.

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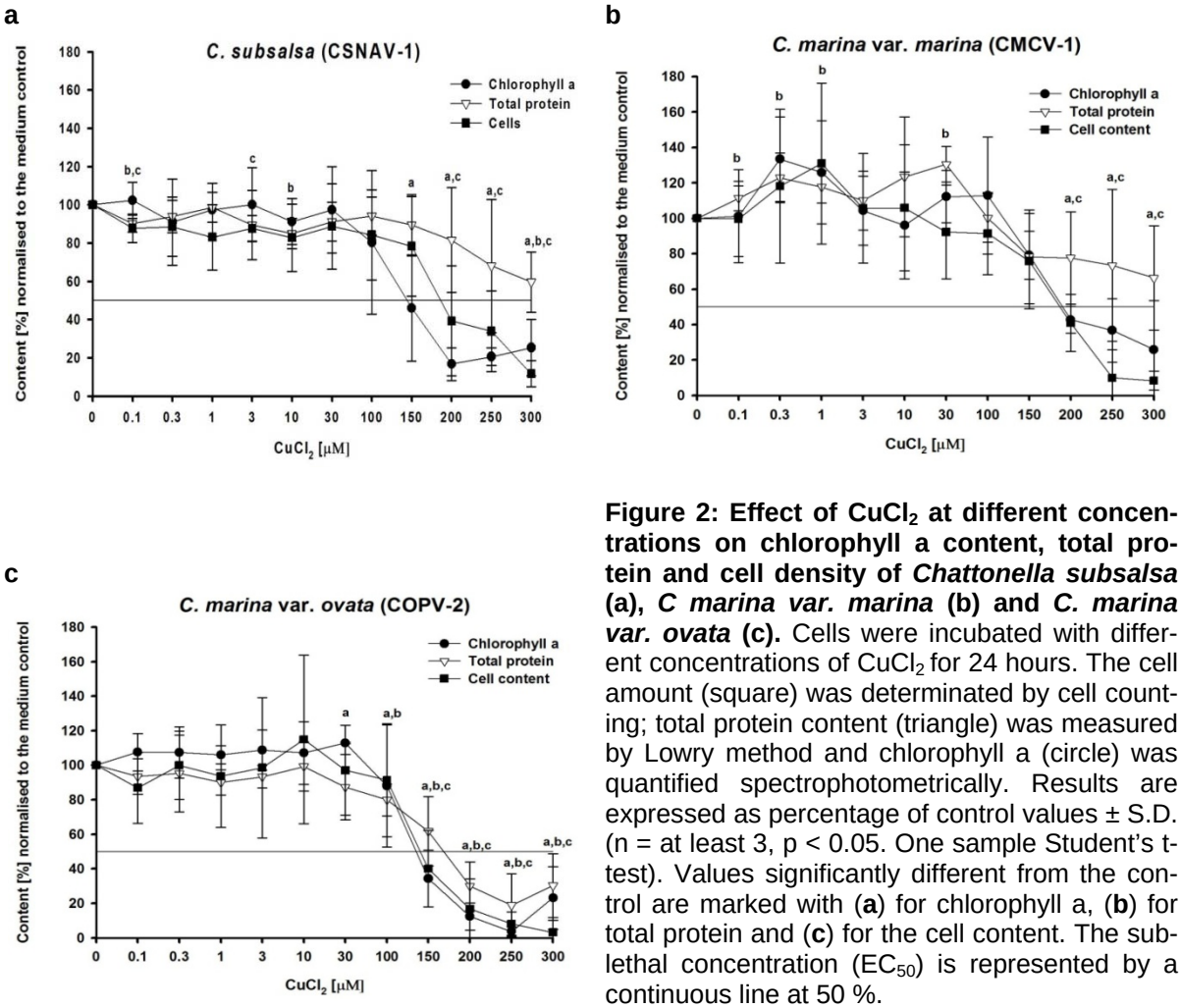

Figure 2: Effect of $CuCl_2$ at different concentrations on chlorophyll a content, total protein and cell density of *Chattonella subsalsa* (a), *C marina var. marina* (b) and *C. marina var. ovata* (c). Cells were incubated with different concentrations of $CuCl_2$ for 24 hours. The cell amount (square) was determinated by cell counting; total protein content (triangle) was measured by Lowry method and chlorophyll a (circle) was quantified spectrophotometrically. Results are expressed as percentage of control values ± S.D. (n = at least 3, $p < 0.05$. One sample Student's t-test). Values significantly different from the control are marked with (**a**) for chlorophyll a, (**b**) for total protein and (**c**) for the cell content. The sublethal concentration (EC_{50}) is represented by a continuous line at 50 %.

Unlike to the previous observations, *Chattonella marina* var. *ovata* (COPV-2) showed basically the same curve progression in all three analysed endpoints after copper exposure. Interestingly, after the exposure to 30 µM of $CuCl_2$ the chlorophyll a amount was significantly increased to 112.3 ± 0.5 % compared to the control cells. In contrast, total protein and cell viability were not affected. However, a significant reduction of all three cytotoxic endpoints was detected after 150 µM of the heavy metal. Thereby, the pigment (34.4 ± 16.4 %) and the cell amount (40.1 ± 22.2 %) fell below 50 %. Similarly to the already described observations above, at this concentration also *C. marina* var. *ovata* cells exhibited still higher protein content (61.8 ± 20.1 % compared to the control cells). But in contrast to *C. subsalsa* and *C. marina* var. *marina* microalgae, in the *C. marina* var. *ovata* cells the protein content decreased significantly in a copper-concentration dependent manner. With a concentration of 200 µM of $CuCl_2$ the chlorophyll a content fell to 12.4 ± 7.8 %, the cell amount decreased to 16.8 ± 17.3 % and the total protein was reduced to 30 ± 14 % compared to the medium control. Interestingly, some curve progressions exhibited a "hockey stick" dose response curve as already observed in the pigment curve of the CSNAV-1 strain. At the highest concentration of 300 µM of copper the chlorophyll a and the protein content increased again to 23.1 ± 18.1 % and 30.3 ± 18.5 % compared to the control, respectively. However, the amount of alive *C. marina* var. *ovata* cells

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was reduced to 3.2 ± 6.9 % compared to the control.

Effects of PCB (Aroclor 1242) exposure

The three raphidophyte strains were also exposed to different concentrations of the polychlorinated biphenyl mixture Aroclor 1242. Its effect on the chlorophyll a and total protein content as well as the viability of the microalgae strains was determined after 24 hours of exposure. Interestingly, the three PCB-treated microalgae exhibited different alterations compared to that of the $CuCl_2$-treated cultures. Furthermore, strain-specific response patterns upon Aroclor exposure were observed for the used raphidophyte strains.

The microalga *Chattonella subsalsa* showed a clear significant reduction of chlorophyll a (54.5 ± 9.9 % compared to the DMSO control, Figure 3a) caused by the lowest concentration of Aroclor 1242 (0.007 mg/L). The amount of the measured pigment was dose-dependently increased and fell below the 50 % line (24.1 ± 9.1 %) after incubation for 24 hours with 0.03 mg/L of the PCB mixture. Also the curves of alive cells and total protein amount showed a tendency of a dose-dependent decrease. However, the total protein amount was significantly reduced after the treatment of 0.3 mg/L Aroclor 1242 (67.8 ± 5.9 % compared to the control). In contrast, the number of *C. subsalsa* cells were significantly affected by 0.7 mg/L of the PCB solution and fell below the 50 % threshold (24.6 ± 8.6 compared to the control cells). Even though all three measured endpoints were significantly reduced after exposure with 0.7 mg/L Aroclor 1242, the total protein amount did not reach a reduction lower than 50 %. Only at the highest concentration of the PCB mixture all three analysis endpoints were decreased below 50 %.

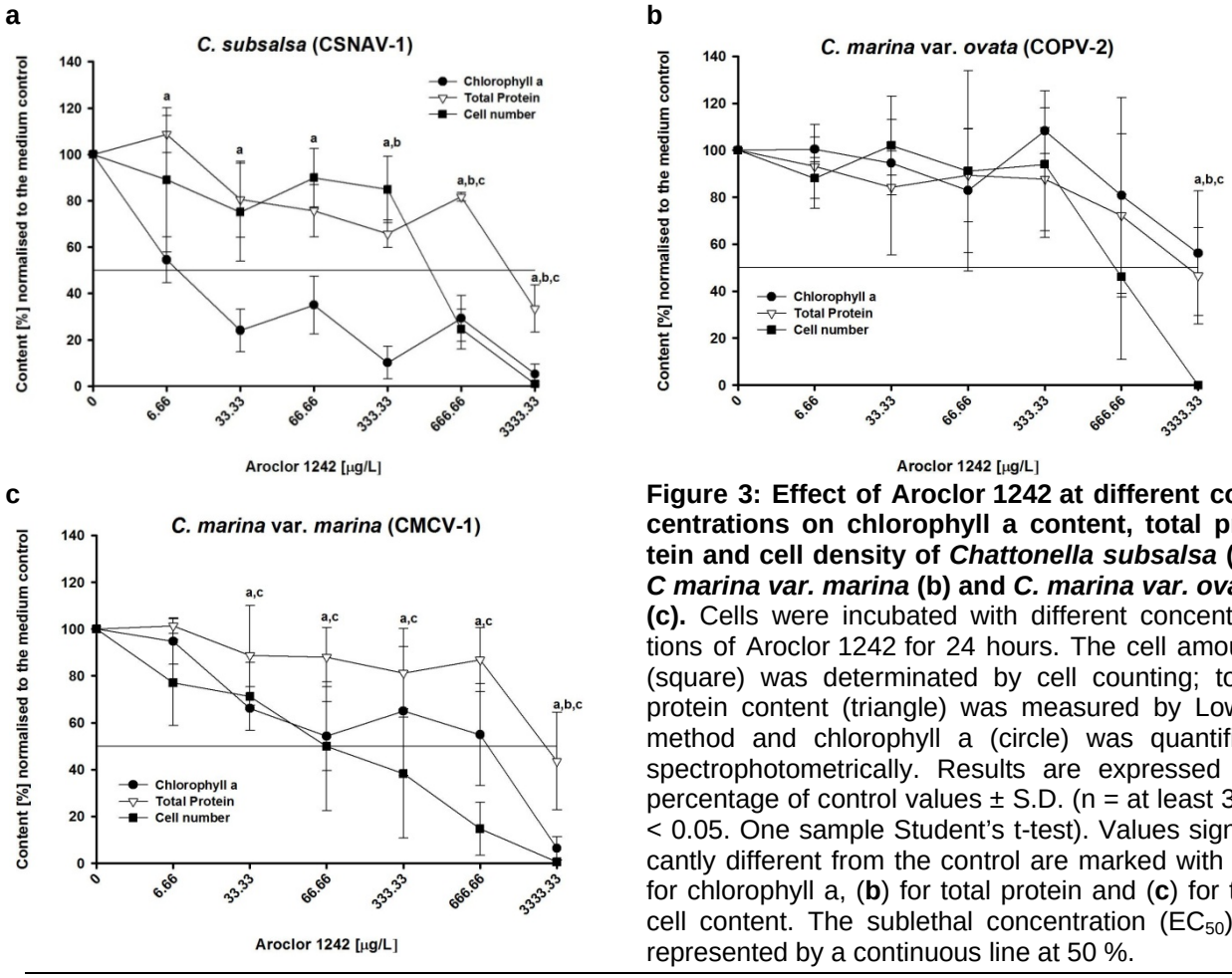

Figure 3: Effect of Aroclor 1242 at different concentrations on chlorophyll a content, total protein and cell density of *Chattonella subsalsa* (a), *C marina var. marina* (b) and *C. marina var. ovata* (c). Cells were incubated with different concentrations of Aroclor 1242 for 24 hours. The cell amount (square) was determinated by cell counting; total protein content (triangle) was measured by Lowry method and chlorophyll a (circle) was quantified spectrophotometrically. Results are expressed as percentage of control values ± S.D. (n = at least 3, $p < 0.05$. One sample Student's t-test). Values significantly different from the control are marked with (**a**) for chlorophyll a, (**b**) for total protein and (**c**) for the cell content. The sublethal concentration (EC_{50}) is represented by a continuous line at 50 %.

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Notably, the raphidophyte strain COPV-2 possessed a high resistance to polychlorinated biphenyls (Figure 3b). Even though the analysis exhibit high standard error bars (mainly in the cell viability analysis) the tendency of all three measured endpoints did not indicate a cytotoxic effect caused by 24 hours Aroclor 1242 exposure up to a concentration of 333 µg/L. At higher Aroclor 1242 concentrations of about 700 µg/L the number of live cells seemed to be reduced (not significantly). The chlorophyll a, total protein and cell amount were significantly affected by 3.3 mg/L Aroclor 1242 (56.1 ± 26.6 %, 46.6 ± 20.5 % and 0 ± 0 % compared to the DMSO control, respectively).

After the 24 hours Aroclor 1242 exposure *Chattonella marina* var. *marina* showed a clear dose-dependent decrease in cell viability (Figure 3c). Also the pigment amount was concomitantly reduced in a similar manner, which indicates that this reduction was related to the cell amount. Even the analysed total protein amount exhibited an equal tendency. Nevertheless, the reduction was significant at the highest concentration of the PCB mixture (43.6 ± 20.8 % compared to the DMSO sample). However, the chlorophyll a and the number of cells were already significantly decreased after the 0.03 mg/L PCB exposure (66.1 ± 9.3 % and 71.1 ± 14.5 %, respectively). The amount of alive cells fell below 50 % threshold after 0.07 mg/L of Aroclor 1242 (49.9 ± 27.4 % compared to the control); however, the chlorophyll a analyses fell clearly below this threshold after the incubation with 3.3 mg/L of Aroclor 1242 (6.4 ± 5 % compared to the control).

Morphologic modification and algae viability after contaminant exposure

After 24 hours incubation with different concentrations of copper or the PCB mixture Aroclor 1242, also morphological alterations of the microalgae such as formation of temporary cysts were analysed microscopically. These observations referred to the supernatant of the culture solution (as shown with an arrow in Figure 4a). Microalgae which were already at the bottom of the culture tube were not considered. The three different microalgae exhibited similar morphological modifications after copper or PCB exposure. At concentrations of 250 µM $CuCl_2$ and 0.7 mg/L Aroclor 1242 we observed an increase in cyst formation in all used raphidophyte cultures (examples are shown in Figure 4c, f and i). The highest used $CuCl_2$ and Aroclor 1242 concentration caused necrotic cell death in all raphidophyte strain (as shown in Figure 4d, g and j).

DISCUSSION

Artificial microalgae medium assessment

The necessity to use artificial algal culture medium as a comparable and reproducible medium was already clarified by some investigators like Price et al. (1988), Tortell and Price (1996) and Gagneux Moreaux et al. (2007). In their review, Berges et al. (2001) enumerate and compare developed artificial seawater media including their own formulation. Using artificial seawater allows maintaining consistent conditions from one experiment to another and permits comparable physiological studies of microalgae. Nevertheless, each medium must be adapted to the needs of the studied organism. Because of this, the use of artificial seawater medium in toxicological analysis is rare. On the other hand, we are able to learn and understand more about marine phytoplanktonic species by optimising culture media composition e.g. by adjusting the concentrations of nutrients for each investigated microalgae.

Our results show that ASW medium as described by Imai et al. (2004) is a good option compared to the sterile filtered seawater media like GSe and f/2. The exponential proliferation of *Chattonella spp.* was alike to that in GSe cultivated microalgae in the first week. Since the compounds like metals and vitamins were given in cer-

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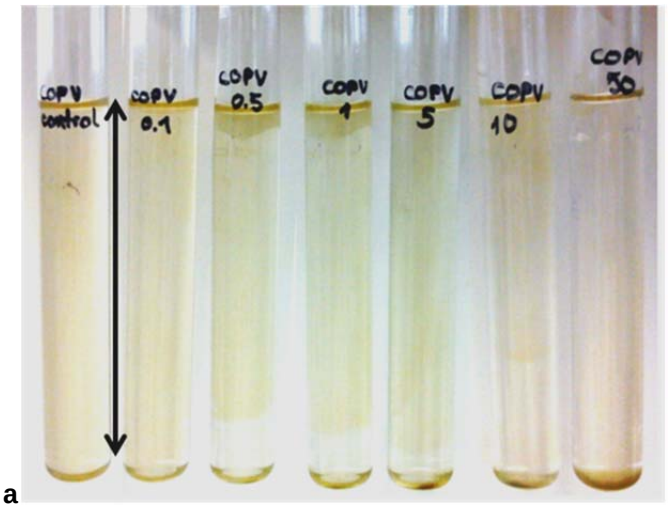

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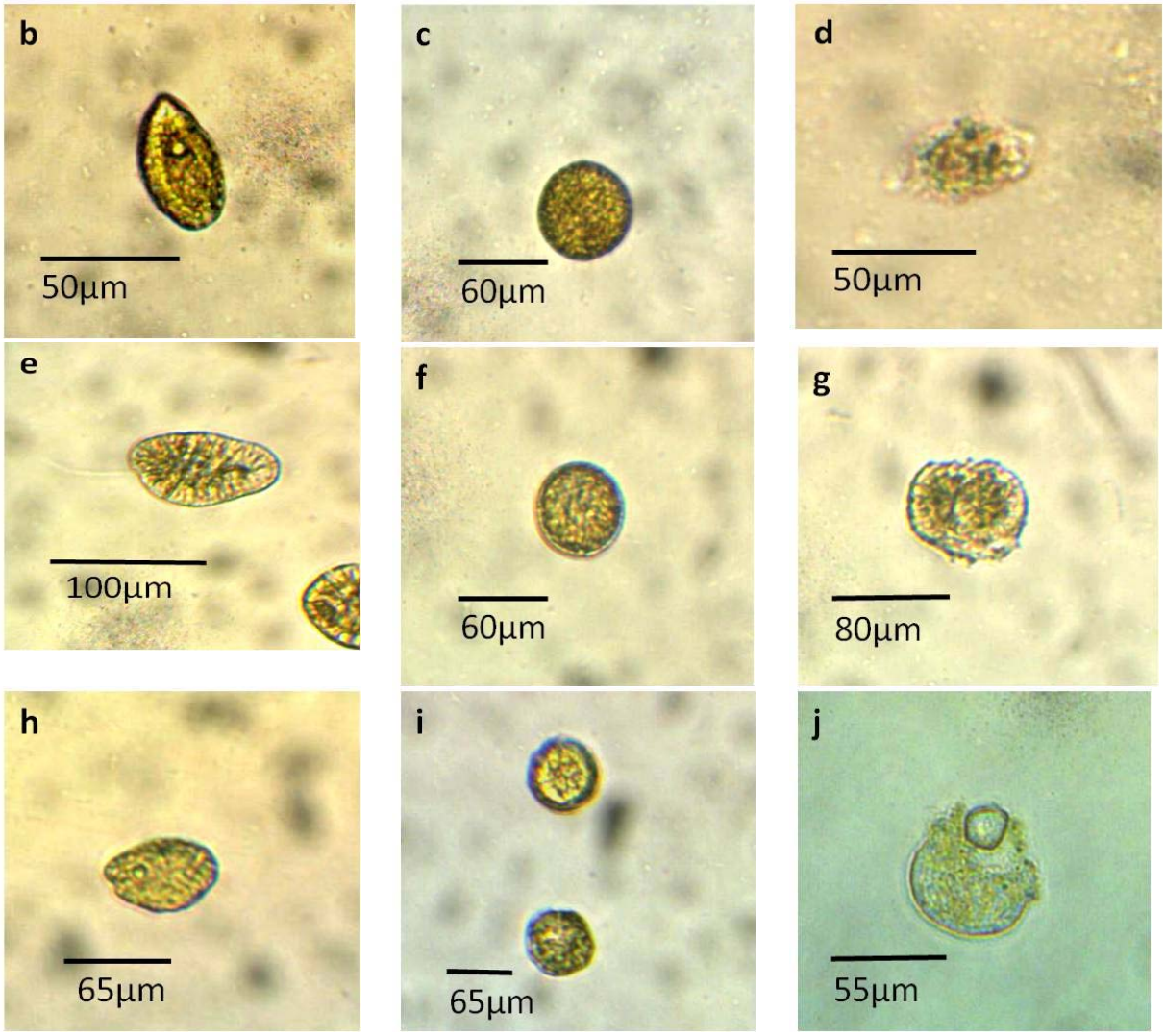

Figure 4: Morphologic modification of microalgae upon exposure to $CuCl_2$ or Aroclor 1242. *Chattonella spp.* cultivated in culture tubes were exposed to different concentrations of $CuCl_2$ or Aroclor 1242 for 24 hours (a). Cell viability and morphological changes were analysed by microscope. For that, 1 ml aliquot of each cell solution (total cell solution volume shown by the arrow) was used. The typical normal cells shape, cyst formation and necrotic cells of CSNAV-1 cells (4b, c and d), COPV-2 (4e, f and g) and CMCV-1 (4h, i and j) are shown.

tain amounts this could cause a limitation in the microalgae proliferation. The growth curve of *C. marina* var. *ovata* (in ASW cultivated) showed a sudden drop in the exponential phase after 11 days of growth. This seems to be an indication of missing nutrients. In addition, *C. subsalsa* and *C. marina* var. *marina* grown in ASW medium reached only 50 % of maximal cell number as in GSe cultivated cell. But they present-

ed in ASW medium similar proliferation curves as cells cultivated in f/2 medium. Nevertheless, both microalgae did not show the same sudden decline as observed in the growth curve of *C. marina* var. *ovata*. Moreover, both microalgae strains possessed a stationary phase enduring 5 days. These results alluded that *C. subsalsa* and *C. marina* var. *marina* present different growth traits in comparison to *C. marina* var. *ovata*. Furthermore, these results indicate that ASW medium as described by Imai et al. (2004) can be used as a growth medium for the *Chattonella spp*. However, we still have to optimise the medium composition to get the same growth properties as seen in the growth curve of cells cultivated in GSe medium.

Cytotoxic analysis of Chattonella spp. microalgae induced by copper or PCB exposure

Many cytotoxic analyses are performed with fresh water organism, few are done in marine microalgae. Heavy metals are one of the most studied toxic substances in phytoplankton. One of the essential elements for all living organisms is the heavy metal copper. It is part of enzymes, works like cofactor and is a key participant in several metabolic pathways like photosynthesis, chlorophyll synthesis, fatty acid metabolism and carbohydrate synthesis. But at elevated concentrations it becomes toxic as already demonstrated by several investigators like Clijsters and Van Assche (1985), Gledhill et al. (1997) and Pinto et al. (2003).

Abalde et al. (1995) incubated the chlorophyceae *Dunaliella tertiolecta* for 0.5, 1, 2 and 4 hours with different concentrations of copper. They already observed a decrease in carbon assimilation after 0.5 hour of incubation with 8 mg/L of copper. And the chlorophyll a and carotenoid contents were reduced by 16 mg/L of copper after 24 hours. Furthermore, the growth rate was lowered by 12 mg/L of the heavy metal. Also Ebenezer and Ki (2012) showed that copper reduced the cell growth rate of another marine microalga. They exposed *Cochlodinium polykrikoides* to different concentrations of copper for 72 hours and determined 12.7 mg/L of copper as the sublethal concentration (EC_{50}) in the tested dinophyceae. Also Ritter et al. (2008) intoxicated the brown alga *Laminaria digitata* and observed an increased free fatty acid release and oxylipin synthesis induced by copper stress (300 µg/L; equivalent to approx. 4.7 µM). These results showed that the toxicity of copper differs extremely depending on the used concentrations, time exposure, examined algal model, type of seawater medium, differing metal uptake rates and detoxification pathways of the model organism and the focused modified endpoints. Nonetheless, our results resembled that of Ebenezer and Ki (2012). They observed that 12.7 mg/L (equivalent to 200 µM) of copper seemed to be the sublethal concentration (EC_{50}) for *C. polykrikoides*. 100 µM of copper slightly affects some analysed endpoints in our studies with *Chattonella spp*. However, exposure to higher concentrations like 200 µM of copper significantly reduced the chlorophyll a content and even more the total protein content in all strains. Noticeably, the cell viability is greatly reduced by this copper concentration, too. Formation of temporary cysts as observed in our copper and PCB exposure experiments were already described as a defense mechanism of phytoplankton (Anderson et al., 2003). Furthermore, Morelli and Scarano (2004) and Manimaran and coworkers (2012) demonstrated that microalgae possessed defense mechanism based on enzymatic degradation and biotransformation. This was related to strong increase of proteins and enzymes such as phytochelatins, nitrate reductase, superoxide dismutase, catalase and peroxidase, glutathione-related peptides and transcription factors. These observations could explain the increased protein level after high concentration exposure in our studies. In addition, high doses of copper or PCBs also caused necrotic cell death. Because of

this proteins or protein fragments (peptides) could be released or could be embedded in damaged cell membrane fragments. Further protein quantification assays of the culture supernatant after exposure could provide more evidences. Moreover it should be noted that cell counting of live raphidophyte algae in a Sedgwick-Rafter counting slide was difficult and is an important reason of higher error bars. Because Lugol's fixation as shown by Band-Schmidt et al. (2012) caused deformations or even destroys the cells, other fixation methods have to be developed to minimise this error source.

Unlike to heavy metals very few exposure studies are done in marine organisms with PCB. Bioaccumulation, kinetics and dynamics as well as bioavailability in phytoplankton and their grazer were examined in some studies (Ko et al., 2012; Magnusson and Tiselius, 2010; Braune et al., 2005; Keil et al., 1971). However, cytotoxic effects of PCB (mostly focused on the cell growth) were rarely done in marine phytoplankton (Ebenezer and Ki, 2012; Biggs et al., 1979). Nevertheless, our results show the same cytotoxic effects as already observed by Keil and co-workers (1971) with diatoms. Their results exhibited that the marine diatom *Cylindrotheca closterium* after exposure to 0.1 ppm Aroclor 1242 for two weeks significantly reduced the chlorophyll a content, the RNA level and cell growth. Though, Ebenezer and Ki (2012) determinated similar to our results a high EC_{50} concentration (1.07 mg/L) after PCB incubation for 72 hours in *Cochlodinium polykrikoide*. Furthermore, the analysed marine dinoflagellate did not survive exposure to 5 mg/L of Aroclor 1016. On the other hand, Biggs et al. (1979) demonstrated that 50 µg/L Aroclor 1254 reduced the growth rate of *Thalassiosira pseudonana* co-cultivated with *Dunaliella tertiolecta* after 48/72 hours exposure. However, *D. tertiolecta* was not affected by the PCB pollutant. Once more, all these results confirm the species- and time-dependence of cytotoxic pollutant effects. Because of this, it is necessary to examine contaminants under different experimental conditions (e.g. acute, sub-chronic and chronic exposure) to determinate the environmental impact of the pollutant. Furthermore, the knowledge of the defense mechanisms as well as of the physiology of rarely studied marine phytoplankton organism will be increased. Likewise, our results demonstrated that the three used raphidophyte strains exhibited equally cytotoxic modification patterns after $CuCl_2$ exposure. However, after Aroclor 1242 incubation the species/strain-dependent differences were clearly demonstrated. Even within the same *Chattonella* species (*Chattonella marina* var. *marina* and *C. marina* var. *ovata*) we observed quite different cytotoxic responses to the same pollutant. The different fatty acid composition of the three *Chattonella spp.* as demonstrated by Band-Schmidt et al. (2012) could be one of the reasons of the different susceptibilities. Because PCBs are very lipophylic substances they tend to pass through membranes and to bioaccumulate in organism. However, Jabusch and Swackhamer (2004) already investigated in the green alga *Chlamydomonas reinhardtii* subcellular accumulation of 13 PCB congeners. They showed that thylakoids are only one of subcellular lipid pools of PCB accumulation. But also they clarified that the K_{ow} value of the analysed PCB congeners could not explain the bioaccumulation in the green alga. They postulated a mechanism involving cellular lipid compartments of different accessibility, with operationally defined peripheral and internal lipid pools. Our results could serve as a further indication for species-dependent compartment accumulation as a defense mechanism to environmental pollutants.

In conclusion, we can predicate that *Chattonella spp*. cultivation in ASW is a "seawater free" alternative to the generally used algal culture media and suitable to realise cytotoxic analyses avoiding synergistic effects of comprised pollutants. Furthermore, we demonstrated that all three

Chattonella spp. possessed different susceptibilities to PCB mixture Aroclor 1242. While *C. marina* var. *ovata* did not show cytotoxic changes after 24 hours of PCB exposure, the raphidophytes *C. subsalsa* and *C. marina* var. *marina* were affected significantly. Interestingly, the microalga *C. subsalsa* only exhibited a high susceptibility of the pigment chlorophyll a to the PCB solution. On the other hand, chlorophyll a and the cell survival were reduced at once in exposed *C. marina* var. *marina* cells. However, the heavy metal copper caused cytotoxic modification pattern in the used raphidophytes with respect to the three analysed endpoints. After acute exposure of 200 µM $CuCl_2$ *C. subsalsa* and *C. marina* var. *marina* exhibited a significant reduced cell survival and chlorophyll a content. Nevertheless, the total protein content did not fall under the 50 % threshold in both microalgae strains. Unlike to these observations, *C. marina* var. *ovata* showed a dose-dependent decrease of the cell viability, chlorophyll a and total protein content. These results demonstrate that even a genetic similarity like that of *C. marina* var. *marina* and *C. marina* var. *ovata* does not mean that both microalgae have the same physiological characteristics. To understand these physiological differences of the three strains further examinations are needed. For example, analysis of specific molecular components e.g. of certain signal pathways after low concentration of PCBs or copper exposure could provide further information about the different susceptibilities of *Chattonella spp*.

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