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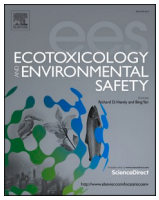

Impact of salinities, metals and organic compounds found in saline oil & gas produced water on microalgae and cyanobacteria

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ABSTRACT

This work evaluates the impact of salinity and the toxicity of some metals and organic compounds commonly found in produced waters on the growth of model photosynthetic organisms. Five strains of marine microalgae and one cyanobacteria (i.e. *Dunaliella salina*, *Nannochloropsis oceanica*, *Tetraselmis suecica*, *Picochlorum costavermella*, *Coccomyxa simplex* and *Synechococcus rubescens*) were tested in microplates as well as the freshwater *Chlorella vulgaris* selected as reference. Results revealed that *D.salina* was able to growth at high salinity (up to 135 $g{\cdot}L^{-1}$). Copper was the most toxic metal for all strains (half maximal effective concentration between 0.1 and 10 $mg{\cdot}L^{-1}$) except for *D.salina* and *C.simplex*. These two strains were the most resistant to all metals tested. All organic compounds presented half maximal effective concentration above 10 $mg{\cdot}L^{-1}$, none of them being very toxic for the studied microorganisms. *P.costavermella* and *C.simplex* were the most resistant strains to organic compounds. Looking at tolerance to salinity, metals and organic compounds, *D.salina* appeared to be the best choice for biomass production in produced waters. In addition, growths in 80% artificial produced water supplemented with *f* medium confirm the feasibility to use this medium to produce biomass.

1. Introduction

Since few decades, microalgae have gained interest for their ability to fix carbon dioxide (CO_2) and produce biomass through their autotrophic metabolism. They are considered as a promising feedstock for several renewable energy production processes (Berberoglu et al., 2009; Gerbens-Leenes et al., 2013; Linares et al., 2017; Duran et al., 2021) and also for the production of valuable molecules in pharmaceutic, cosmetic, food and feed sectors. These organisms are found in a large variety of habitats, like rivers, lakes, oceans, soil, forests, urban area and some extreme environments such as hypersaline lakes, deserts, Antarctic soils or the outflow of geothermal springs (Barsanti et al., 2008; Seckbach and Oren, 2007).

Microalgae cultivation needs a large amount of water (Borowitzka and Moheimani, 2013), which can contribute to environmental problems like the freshwater scarcity. Thus, a solution can be the use of seawater supplemented with industrial wastewaters, available in large quantities, providing nutrients necessary for microalgae growth (Abdel-Raouf et al., 2012; Pires et al., 2013; Gonçalves et al., 2017). Fortunately, depending of the species and their natural habitats, microalgae can grow at various range of salinity (Borowitzka and Moheimani, 2013). Within the fossil energy industry, the production of oil and gas leads to the generation of produced waters (PW) (Govorushko, 2013), characterized by a complex chemical composition, depending of the origin of the geological reservoir (Veil et al., 2004). PW are known to be a serious source of pollution, which affects ecosystems in the receptive environment. PW are mainly composed of inorganic salts with total dissolved solids varying from 100 $mg{\cdot}L^{-1}$ to 400 $g{\cdot}L^{-1}$ (Al-Ghouti et al., 2019). They contained metals (e.g. iron, copper, aluminum, zinc), and organic molecules such as aliphatic hydrocarbons, polycyclic aromatics hydrocarbons, phenols and derivative compounds, benzene, toluene, ethylbenzene, xylene (BTEX), or organic acids. PW also contain chemical additives, added to enhance oil extraction and to facilitate oil, gas and water separation processes (Al-Ghouti et al., 2019; Brendehaug et al., 1992), but the impact of these compounds is not investigated in this study. Therefore, PW need to be treated before

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discharge to prevent environmental issues.

Few studies investigated the growth of photosynthetic microorganisms in PW to produce biomass and possibly recycle and/or bioremediate the wastewater. Arriada and Abreu, 2014 (Arriada and Abreu, 2014), tested the viability of growing marine microalgae *Nannochloropsis.oculata* in $f_{/2}$ culture medium mixed with 0%, 50% and 100% v/v of PW at final salinity of 25 g·L^{-1}. They measured growth rates from 0.06 to 0.13 d^{-1}. After a first growth in 100% v/v PW, higher growth rates were measured at 50% and 100% v/v of PW loading (0.22 and 0.09 d^{-1}, respectively), indicating a possible acclimation of microalgae. Ammar et al., 2018 (Ammar et al., 2018), conducted successive growth experiments with *N.oculata* using BG-11 culture medium mixed with 10%, 25% and 50% v/v of saline PW (138 g·L^{-1} of salinity). Best growth rates were obtained with 25% and 10% v/v (0.18 d^{-1}). These higher growth rates than those measured by Arriada and Abreu, 2014 (Arriada and Abreu, 2014), could be explained by a different PW composition, possibly in terms of salinity, and/or by the higher nutrient concentrations in BG-11 than in $f_{/2}$ medium. Authors also showed that *N.oculata* was also able to remove oil and chemical oxygen demand (COD) from PW, with higher removal efficiency at low PW loading, with more than 65% of oil removal regardless of the PW loading, and 90% and 55% of COD removal at PW loading of 10% and 50% v/v, respectively. The decrease in COD removal can be due to the higher salinity at 50% v/v than at 10% v/v PW loading, bioremediation efficiency of bacteria and microalgae being reduce at high salinities (Lefebvre and Moletta, 2006). Parsy et al., (2020), investigated the growth of *N.oculata* in a mix of seawater, saline PW (114 g·L^{-1} of salinity) and liquid digestate from anaerobic digestion process. Successive stage cultures were conducted by varying the PW loading, from 0 up to 50% v/v. *N.oculata* was able to grow in PW from 10 up to 30% v/v, salinity being too high at higher PW loading. Maximal growth rates obtained were 0.35, 0.27 and 0.16 d^{-1} for 10%, 20% and 30% v/v of PW, respectively. *N.oculata* was able to remove nitrogen (around 100%), organic carbon (approximately 40% after one step of acclimation), whatever the PW loading. According to these data, microalgae seemed able to remediate PW regarding nitrogen and COD, even at high PW loading. However, growth rates drop with the increase in PW load, suggesting a possible toxic effect from one or several compounds. In the literature, the toxicity of metals and organic molecules on microalgae has been investigated. However, the majority of these studies concerned strains from the genus *Chlorella* grown in freshwater. Only a few studies worked with marine and halophilic strains (Duan et al., 2017; Ertürk and Saçan, 2012; Ebenezer and Ki, 2013).

In this context, the objective of this study was to estimate the salinity tolerance and the toxicity of some compounds found in PW, like metals and metalloid (i.e. iron, copper, zinc, chromium, barium and arsenic) and organic molecules (i.e. acetate, benzene, naphthalene and phenol) on five microalgae strains (i.e. *Dunaliella salina*, *Nannochloropsis oceanica*, *Tetraselmis suecica, Picochlorum costavermella, Coccomyxa simplex*) and a cyanobacterium (i.e. *Synechococcus rubescens*). Freshwater *Chlorella vulgaris* was also studied to compare results with literature data. Culture in artificial PW were also performed to estimate the growth parameters of these strains in this complex medium and potential toxicity due to cocktail effects.

2. Materials and methods

2.1. Strains and cultivation for inoculum preparation

A freshwater microalga, (*Chlorella* sp. CA104), five halotolerant microalgae (*Dunaliella* sp. CA113, *Nannochloropsis* sp. CA101, *Tetraselmis* sp. CA106, *Picochlorum* sp. RCC4223 and *Coccomyxa* sp. RCC537) and a halotolerant cyanobacterium (*Synechococcus* sp. RCC752) have been selected for this study. Strains identified "CA" and "RCC" were purchased from Greensea (Mèze, France) and from the Roscoff Culture Collection (Roscoff, France), respectively. Strains have been previously identified thanks to 18S rDNA sequencing for microalgae (Díez et al., 2001; Zimmermann et al., 2011) and 16 rDNA sequencing for the cyanobacterium (Marchesi et al., 1998). They are referred as strains *Chlorella vulgaris* CA104, *Dunaliella salina* CA113, *Nannochloropsis oceanica* CA101, *Tetraselmis suecica* CA106, *Picochlorum costavermella* RCC4223, *Coccomyxa simplex* RCC537 and *Synechococcus rubescens* RCC752.

Each halotolerant strain was inoculated in 500 mL glass bottles (500 mL of working volume) constituted of a sterile *f* medium (Guillard and Ryther, 1962) at 30 g·L^{-1} of salinity in a simulated seawater environment (Instant Ocean salts (Capel, 1999), Aquarium Systems, France). *f* medium was prepared in stock solution concentrated 50 times and diluted in the seawater to prepare the medium. For stock solution preparation, copper, cobalt, manganese, molybdenum, zinc and cyanocobalamin were dissolved in stock solution concentrated 10,000 times. Biotin was prepared in stock solution concentrated 2000 times. Nitrate, phosphate, iron, thiamin and EDTA were prepared in stock solution concentrated 10 times. All solutions were added to hot water with volume corresponding to their concentration (except vitamins which were added after cooling to prevent degradation) to facilitate mixing and prevent precipitation. Small precipitation may occur during preparation, precipitates were removed during sterilization using 0.2 μm filter. *C.vulgaris* CA104 was inoculated in the same medium without artificial seawater. Reactor mixing was ensured by air bubbling, while the pH was maintained at 8.0 thanks to regular injections of CO_2. Light was provided with 14/10 h light/dark periods, by 3 LED lamps (CorePro LED-tube, 23 W, 2700 lm, 6500 K, Philips, Netherlands). Photosynthetically active radiation (PAR) was adjusted from 50 to 150 $\mu mol_{photons} \cdot m^{-2} \cdot s^{-1}$, during cellular growth to avoid photoinhibition or photolimitation. Finally, to limit bacterial growth, ampicillin, streptomycin and gentamycin antibiotics (final concentrations: 100, 25 and 25 mg·L^{-1}, respectively) were added to each culture medium, except for cyanobacteria *S.rubescens*. At the end of each batch, cultivated cells were withdrawn and re-inoculated with an initial optical density at 680 nm (OD_{680}) of 0.3 to follow the growth.

2.2. Salinity tolerance assessment

Tests were conducted by varying salinity (from 13.5 to 135 g·L^{-1} with a step of 13.5 g·L^{-1}). For the strain *D.salina* CA113, 7 additional salinities were tested, from 149.5 to 231 g·L^{-1} with a step of 13.5 g·L^{-1}. Salinity tests were not conducted with the freshwater *C.vulgaris* CA104. Experiments were conducted in 96-well plates (working volume of 1 mL). Peripheral wells were filled with demineralized water to limit side effects and evaporation; thus 6 replicates were performed for each salinity. Each well was spiked with: 20 μL of *f* medium (concentrated at 50X) to bring nutrients and oligoelements for microalgae growth; 33.6 μL of $NaHCO_3$ solution at 50 g·L^{-1} (final concentration of 20 mM) to bring inorganic carbon for autotrophic growth, hypersaline water (solution of Instant Ocean salts at 290 g·L^{-1}) to reach the desired salinity and demineralized water to make up 1 mL.

Microalgae were inoculated at an initial OD_{680} of 0.2 (UV–Vis spectrophotometer (Thermoscientific Evolution 201 UV–visible, USA)). Initial pH was 8.0. Plates were incubated until stationary phase at 22 °C in a Multitron (Infors AG, Switzerland). Light was provided, by assuring a photoperiod of 14/10 h light/dark and a PAR of 36 $\mu mol_{photon} \cdot m^{-2} \cdot s^{-1}$. OD_{680} was monitored to follow cell growth with a Synergy HTX Multi-Mode Microplate Reader (Biotek Instruments, USA).

2.3. Toxicity tests on metals and organic compounds

According to PW composition field data, 6 metals and metalloid (Copper Cu^{2+}, Barium Ba^{2+}, Iron Fe^{2+}, Chromium Cr^{3+}, Zinc Zn^{2+} and Arsenate AsO_2^-) and 4 organic compounds (benzene, naphthalene, phenol and acetate) were selected. Ranges of concentration tested were 3.91–1000 mg·L^{-1} for Ba(II), 0.156–40 mg·L^{-1} for Fe(III), 0.117–30 for

mg·L^{-1} Cu(II), 1.56–400 mg·L^{-1} for Zn(II), 0.780–200 mg·L^{-1} for Cr (III), 0.390–100 mg·L^{-1} for As(III), 6.25–1600 mg·L^{-1} for acetate, 2–513 mg·L^{-1} for benzene, 0.115–30 mg·L^{-1} for naphthalene and 1.96–500 mg·L^{-1} for phenol.

Tests were performed in 96-well plates (working volume of 1 mL, one well plate for each compound and strain). Peripheral wells were filled with demineralized water to limit side effects and evaporation. 9 concentrations were tested, with 6 replicates for each. The last column of the microplate was used as control (no compounds were added). Stock solutions of the 6 metals and 4 organic compounds (except naphthalene) were prepared with concentration four times higher than the highest tested concentrations. Test being performed in 1 mL working volume, 250 µL of the stock solution were added in wells of the first column containing 250 µL of demineralized water. Then 250 µL of each well of the column were transferred to the next column and mixed with 250 µL of demineralized water. 2-fold dilutions were performed in series until the lowest concentration tested was reached. Then, each well was spiked with: 20 µL of *f* medium (concentrated at 50X) to bring nutrients and oligoelements for microalgae growth; 33.6 µL of $NaHCO_3$ solution at 50 g·L^{-1} (final concentration of 20 mM) to bring inorganic carbon for autotrophic growth; hypersaline water (solution of Instant Ocean salts at 290 g·L^{-1}) to reach the final salinity of 30 g·L^{-1} (except for tests with freshwater *C.vulgaris* for which no hypersaline water was added); 151 µL of microalgae or cyanobacteria suspension and demineralized water to make up 1 mL. A salinity of 30 g·L^{-1} was selected as it allows a good microalgae and cyanobacteria growth according to the salinity tolerance assessment. In addition, for arsenate test the plates were sealed with plastic foils and cell growth was followed by OD_{680}. Concerning naphthalene, naphthalene crystal were directly added to the wells of the first column and concentration was considered saturated concentration (approximately 30 mg·L^{-1}). 2-fold dilutions were performed as for other compound to obtain the desired concentration range.

Tests were started with initial OD_{680} of 0.2 (UV–Vis spectrophotometer (Thermoscientific Evolution 201 UV–visible, USA)). Initial pH was 8.0. Plates were incubated 7 days in an Economic Lux Chamber (Snijders Scientific, Netherlands) at 20 °C. Light was provided by maintaining a photoperiod of 14/10 h light/dark and a PAR of 36 $\mu mol_{photon} \cdot m^{-2} \cdot s^{-1}$. OD_{680} as well as chlorophyll *a* fluorescence (except for arsenate) (excitation: 485/20 nm, emission: 645/40 nm) (Zhao et al., 2018) were monitored after 96 h to estimate half maximal effective concentration with a Synergy HTX Multi-Mode Microplate Reader (Biotek Instruments, USA).

2.4. Growth in artificial produced water

Selected strains of microalgae and cyanobacteria were grown in an artificial produced water (aPW). The composition was based on the composition of a real PW from a TotalEnergies operating site described by Sambusiti et al. (2020) and is detailed in Table 1. All organic compounds were prepared concentrated at 10000X in pure ethanol (>99%), then added to aPW via injection using a micro syringe to maximise dispersion and dissolution of the organic phase. Final total organic carbon concentration of aPW was 80.3 mg·L^{-1}.

Cultures in aPW were conducted in 96-well plates (working volume of 250 µL) with the 6 halophilic strains. Peripheral wells were filled with demineralized water to limit side effects and evaporation. For each strain, culture in *f* medium and culture in *f* medium with 80% v/v of aPW were performed with 12 replicates. Each well of culture in *f* medium was spiked with: 20 µL of *f* medium (concentrated at 50X) to bring nutrients and oligoelements for microalgae growth; 33.6 µL of $NaHCO_3$ solution at 50 g·L^{-1} (final concentration of 20 mM) to bring inorganic carbon for autotrophic growth, hypersaline water (solution of Instant Ocean salts at 290 g·L^{-1}) to reach the final salinity of 30 g·L^{-1} and demineralized water to make up 250 µL. For wells of culture in *f* medium with 80% v/v of aPW, volume of demineralized water had been reduced to add 200 µL of aPW, to reach 80% v/v.

Table 1
Composition of artificial produced water.

Compound	Concentration (mg·L^{-1})
Na	9222[a]
Cl	16570[a]
SO_4	2279[a]
Ca	341[a]
Mg	1129[a]
K	345[a]
Li	0.15[a]
B	5.1[a]
Al	<0.04[a]
As	<0.0002[a]
Cd	<0.02[a]
Co	<0.05[a]
Mo	<0.01[a]
Ni	<0.04[a]
Pb	<0.005[a]
Sr	7.4[a]
Mn	<0.01[a]
N (NH_4Cl)	26.7
P ($NaH_2PO_4 \cdot H_2O$)	2
Fe ($FeCl_3 \cdot 6H_2O$)	1
Ba ($BaCl_2 \cdot 2H_2O$)	1.2
Cr ($CrCl_3 \cdot 6H_2O$)	0.06
Cu ($CuSO_4 \cdot 5H_2O$)	0.02
Zn ($ZnSO_4 \cdot 7H_2O$)	0.18
Acetate	62
Phenol	11.26
Benzene	8
Toluene	7.5
Ethylbenzene	5
o-Xylene	1.081
m-Xylene	2.876
Naphthalene	0.246
Phenanthrene	0.044
Benzo(a)pyrene	0.0002
Acenaphthylene	0.0105
Acenaphtene	0.0135
Fluorene	0.021
Anthracene	0.006
Fluoranthene	0.001
Pyrene	0.005
Ethanol	45.6

[a] Elements brought by Instant Ocean salts (30 g·L^{-1}).

Tests were started with initial OD_{680} of 0.2 (UV–Vis spectrophotometer (Thermoscientific Evolution 201 UV–visible, USA)). Initial pH was 8.0. Plates were incubated until stationary phase in an Economic Lux Chamber (Snijders Scientific, Netherlands) at 22 °C. Light was provided by maintaining a photoperiod of 14/10 h light/dark and a PAR of 36 $\mu mol_{photon} \cdot m^{-2} \cdot s^{-1}$. OD_{680} was monitored to follow cell growth with a Synergy HTX Multi-Mode Microplate Reader (Biotek Instruments, USA).

2.5. Data processing

Growth rates were determined using Eq. (1):

$$\mu = \frac{\ln(OD2) - \ln(OD1)}{t2 - t1} \quad (1)$$

(Arriada and Abreu, 2014)Where: µ is the specific growth rate, OD_1 and OD_2 are the optical densities at 680 nm at time t_1 and t_2, respectively.

Doubling time t $_D$, were $OD_2 = 2 * OD_1$, was determined using Eq. (2):

$$t_D = \frac{\ln(2)}{\mu} \quad (2)$$

Where: µ is the specific growth rate, t_D is the doubling time.

To determine optimal salinities for each species, both growth rates and final cellular concentrations reached during stationary phases were

considered. Optimums were determined when both growth rates and final OD_{680} values where higher than 80% of the maximum values measured for each species.

To determine the half maximal effective concentration (EC_{50}) of the different compounds, a four-parameter dose-response curve (Hill equation, Eq. 3) (Gadagkar and Call, 2015) was fitted with OD_{680} (for arsenate) or chlorophyll *a* fluorescence values measured after 96 h of incubation, in accordance with several toxicity publications to be able to compared the data (Duan et al., 2017; Ertürk and Saçan, 2012; Zhao et al., 2018; Shigeoka et al., 1988; Shitanda et al., 2005; Fujiwara et al., 2008; Subramaniyam et al., 2016; Levy et al., 2005). Effective concentrations generating an effect response of 10% of the tested population (EC_{10}) were also estimated thanks to Hill equation.

Fits were realized with Regtox macro in Microsoft Excel (Microsoft, USA):

$$Y(C) = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{(1 + 10\text{^}((\text{LogEC50} - \text{LogC})*\text{HillSlope}))} \quad (3)$$

(Gadagkar and Call, 2015)Where: Y is the fluorescence or the OD_{680} (expressed as % relative to a control without the compound tested), C is the variable concentration of the compound ($mg{\cdot}L^{-1}$), EC_{50} is the half maximal effective concentration ($mg{\cdot}L^{-1}$), Top and Bottom are maximum and minimum responses (%), fixed to 100% and 0%, respectively, HillSlope describes the steepness of the family of curves (no unit).

Concerning the fit of the data, when applicable Hill model always had R^2 values > 0.80. When non applicable, an interval was given to estimate the EC50 according to inhibition observed.

For tests about growth in aPW, a *t*-test for paired data was performed to evaluate differences in growth rates of each species between the test conditions (*f* medium and *f* medium with 80% v/v of aPW). A confidence level of 95% (significance α level of 0.05) was considered. Thus, *p*-values < 0.05 were deemed to be statistically significant.

3. Results and discussion

3.1. Optimal salinity assessment

For this study, a taxonomic diversity of six halotolerant microalgae and cyanobacteria was chosen to assess the impact of salinities, metals and organic compounds found in PW. Fig. 1 shows the evolution of growth rates obtained at different salinities for each halotolerant microorganism strain.

All strains had a maximal growth rate between 14 and 40 $g{\cdot}L^{-1}$ with the exception of *D.salina*, which had its maximal growth rate between 68 and 95 $g{\cdot}L^{-1}$. Growth rates tended to decrease with increasing in salinity. Moreover, each strain showed a maximal salinity tolerance beyond which no growth could be observed, corresponding to 230, 135, 122, 95, 95 and 81 $g{\cdot}L^{-1}$ for *D.salina*, *C.simplex*, *T.suecica*, *P.costavermella*, *S.rubescens* and *N.oceanica*, respectively.

As expected, *D.salina* was the most halotolerant strain, being the only species growing significantly above 122 $g{\cdot}L^{-1}$, confirming the feasibility of growing *D.salina* in high saline PW. This result is in agreement with previous studies which noticed that strains of *D.salina* could grow up to 290 $g{\cdot}L^{-1}$ (Ahmed et al., 2017; Chen et al., 2009). *T.suecica* in turn could grow up to 95 $g{\cdot}L^{-1}$ with good growth rates. Results concerning other strains suggest that 68 $g{\cdot}L^{-1}$ is the maximal salinity for which their growth could be viable for large scale production, with doubling time of less than 10 days.

According to growth rates calculated and final OD_{680} values reached during stationary phases (data not shown), optimal salinities have been determined for each strain, corresponding to 81–95 $g{\cdot}L^{-1}$ for *D.salina*, 41–68 $g{\cdot}L^{-1}$ for *T.suecica*, 14–54 $g{\cdot}L^{-1}$ for *P.costavermella* and *S.rubescens* and 14–41 $g{\cdot}L^{-1}$ for *N.oceanica* and *C.simplex*. Therefore, *P.costavermella*, *S.rubescens* and *C.simplex* appeared to be truly marine strains, while *D.salina* halophilic. Moreover, *N.oceanica* seemed to be more adapted to brackish waters, whereas *T.suecica* was slightly halophilic, exhibiting growth until 110 $g{\cdot}L^{-1}$.

3.2. Toxicity of metals

Toxicity tests were conducted on barium, iron, copper, chromium, zinc, arsenate, benzene, naphthalene, phenol and acetate to detect their potential effect on cell growths. Freshwater *Chlorella vulgaris* was also investigated and considered as reference for literature comparison purposes. Growth rates were calculated for each concentration. Each strain had a different growth rate observed for the control, corresponding to $0.57 \pm 0.05\ d^{-1}$ for *C.vulgaris*, $0.26 \pm 0.02\ d^{-1}$ for *D.salina*, $0.44 \pm 0.09\ d^{-1}$ for *N.oceanica*, $0.45 \pm 0.11\ d^{-1}$ for *T.suecica*, $0.38 \pm 0.03\ d^{-1}$ for *P.costavermella*, $0.29 \pm 0.03\ d^{-1}$ for *S.rubescens* and $0.04 \pm 0.04\ d^{-1}$ for *C.simplex*. For each metal or metalloid and for each strain, Hill equation (Eq. 3) was used to fit the inhibition data and determined the EC_{50} and EC_{10} at 96 h. Hill fitting for each metal and metalloid are provided in Fig. 2 while EC_{50} and EC_{10} for each strain are summarized in Table 2.

Concerning barium and iron, selected microorganisms did not exhibit any 50% impact after 96 h whatever the concentration used. For these two compounds, EC_{50} are superior to the maximal tested concentrations (1000 and 40 $mg{\cdot}L^{-1}$, respectively). Concerning zinc, no EC_{50} could be determined for halotolerant strains as maximum

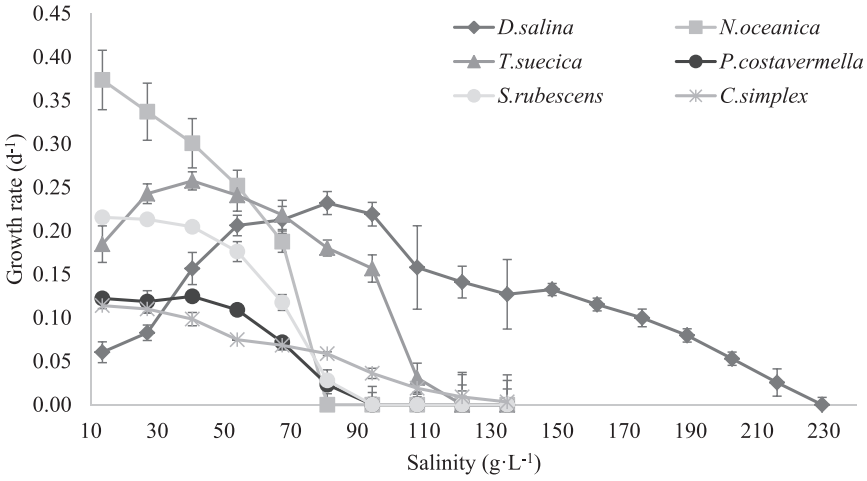

Fig. 1. Growth rates obtained at different salinities (as Total Dissolved Solids in $g{\cdot}L^{-1}$) for each halotolerant microalgal and cyanobacterial strain. Values correspond to mean ± standard deviation, n = 6.

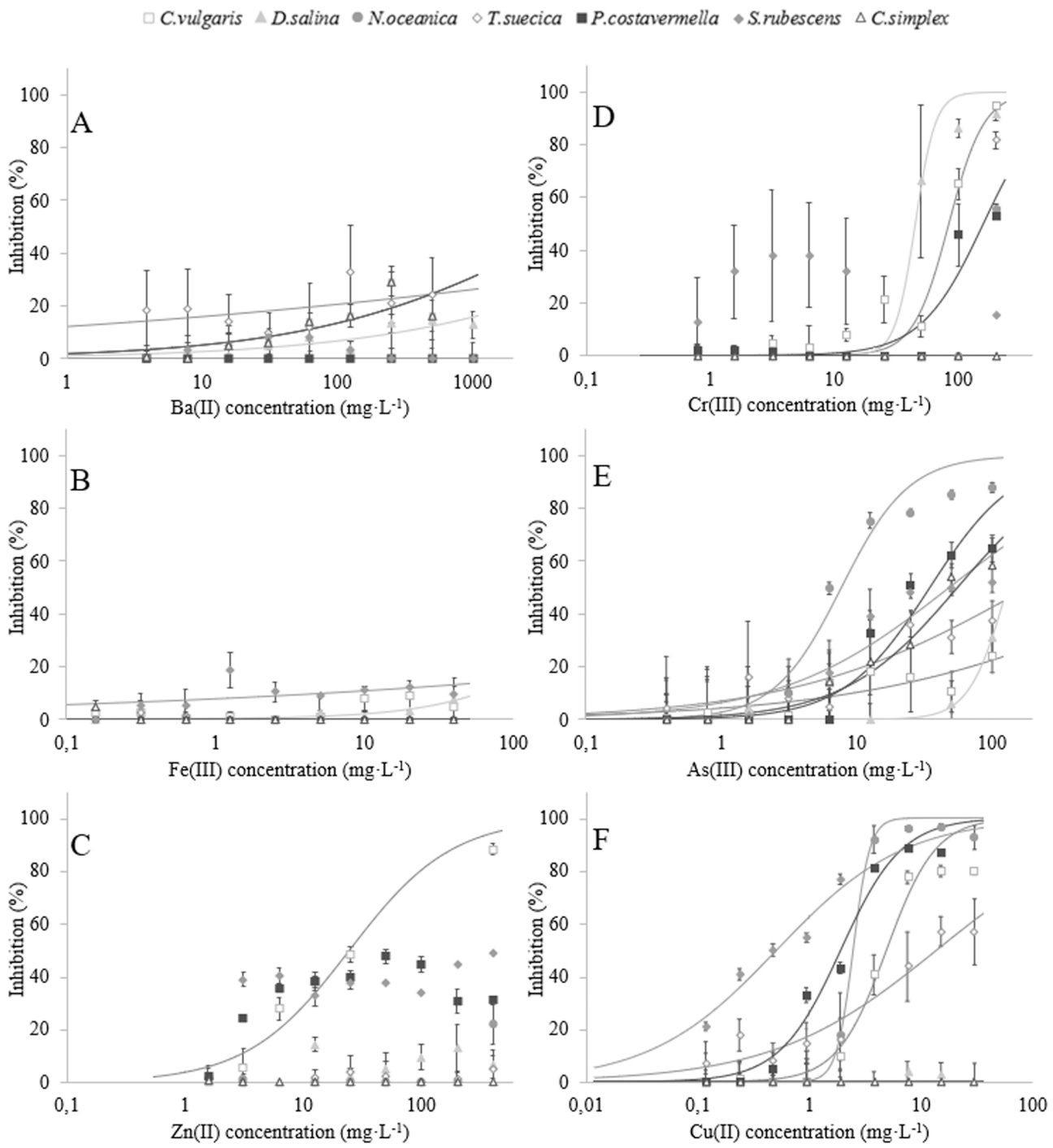

Fig. 2. Hill model fitting of inhibition data for each strain. A: Ba(II); B: Cr(III); C: Fe(III); D: As(III); E: Zn(II)) and F: Cu(II). Values correspond to mean ± standard deviation, n = 6. Lines correspond to Hill model fitting.

concentrations were not high enough to detect a 50% effect. Only freshwater *C.vulgaris* was enough sensitive to determine an EC_{50} of 24.6 mg·L^{-1}. However, 10% effects could be observed and EC_{10} went down to few mg·L^{-1} for *P.costavermella* and even lower for *S.rubescens*. Chromium and arsenic were generally more toxic than barium, iron and zinc. EC_{50} for chromium was between 44 and 200 mg·L^{-1} except for *S. rubescens* and *C.simplex*. However, while the EC_{10} remains close to the EC_{50} for all microalgae, the EC_{10} drops from more than 200 to a few mg·L^{-1} for *S.rubescens*. Arsenic was even more toxic, with EC_{50} between 7 and 56 mg·L^{-1} for all strains except *C.vulgaris*, *D.salina* and *T.suecica*. EC_{10} were observed at 9.3 mg·L^{-1}, 63.8 mg·L^{-1} and between 6.25 and 12.5 mg·L^{-1} for these strains, respectively. Finally, copper was the most toxic metal, with EC_{50} between 0.5 and 14 mg·L^{-1} for all strains, except for *D.salina* and *C.simplex* for which no 50% and 10% effects could be observed.

Concerning barium and iron, EC_{50} from 10 to 240 mg·L^{-1} and from 1.83 to 4.57 mg·L^{-1} can be found in literature data, respectively, for *Chlorella* sp. (Subramaniyam et al., 2016; Golding et al., 2018). *C.vulgaris* CA104 and halotolerant strains of this study appeared to be more resistant. About zinc, EC_{50} determined for *C.vulgaris* (24.6 mg·L^{-1}) is close to the EC_{50} found in the literature, from 6.6 to 110 mg·L^{-1} for other *Chlorella* strains (Fujiwara et al., 2008; Johnson et al., 2007). For chromium, Vignati et al. (2010) performed similar tests using microplate method for the toxicity bioassay, and found EC_{50} from 89 to 110 mg·L^{-1}

Table 2
EC_{50} and EC_{10} of selected metals and metalloids on studied microorganisms at 96 h. EC_{50} and EC_{10} were determined with Hill equation and are expressed in $mg{\cdot}L^{-1}$. Values in brackets correspond to EC_{10}. Values correspond to mean ± standard deviation, n = 6.

	C.vulgaris	*D.salina*	*N.oceanica*	*T.suecica*	*P.costavermella*	*S.rubescens*	*C.simplex*
Ba(II)	> 1000 (48.1)	> 1000 (289)	> 1000 (> 1000)	> 1000 (< 3.91)	> 1000 (> 1000)	> 1000 (> 1000)	> 1000 (48)
Fe(III)	> 40 (> 40)	> 40 (> 40)	> 40 (> 40)	> 40 (> 40)	> 40 (> 40)	> 40 (5.5)	> 40 (> 40)
Zn(II)	24.6 (2.8)	> 400 (> 400)	> 400 (200–400)	> 400 (> 400)	> 400 (1.56–3.13)	> 400 (< 3.25)	> 400 (> 400)
Cr(III)	83.5 (40.7)	44.8 (29.7)	198 (100–200)	100–200 (100–200)	160 (54.1)	> 200 (< 1.56)	> 200 (> 200)
As(III)	> 100 (9.3)	> 100 (63.8)	7.7 (2.2)	> 100 (6.25–12.5)	34.6 (6.9)	48.1 (2.0)	55.3 (6.6)
Cu(II)	4.9 (1.6)	> 30 (> 30)	2.5 (1.7)	13.8 (0.34)	1.9 (0.48)	0.51 (0.03)	> 30 (> 30)

for a freshwater *Chlorella* strain, being very close to values found in this study for *C.vulgaris* (83.5 $mg{\cdot}L^{-1}$). About arsenic, EC_{50} determined are in accordance with EC_{50} found in literature for *Chlorella* and *Nannochloropsis* strains, from 1 to 27 $mg{\cdot}L^{-1}$. Levy et al. (2005), and (Rahman et al. (2014), reported EC_{50} of 25.2 and 27 $mg{\cdot}L^{-1}$ respectively, showing that studied *C.vulgaris* strain was more resistant (EC_{50} > 100 $mg{\cdot}L^{-1}$) to arsenic too. Finally, copper is widely studied in literature and data are available for *Nannochloropsis gaditana*, *Tetraselmis chuii* and *T.suecica* and various *Chlorella* strains. Debelius et al. (2009), reported copper EC_{50} of 0.137 and 0.33 $mg{\cdot}L^{-1}$ for *N.gaditana* and *T.chuii* respectively, these strains seem to be more sensitive than those selected in this study. Ebenezer and Ki (2013), measured an EC_{50} at 72 h of 10.9 $mg{\cdot}L^{-1}$ for *T. suecica*, which is very close to the value for *T.suecica* strain of this study (13.8 $mg{\cdot}L^{-1}$). Concerning *Chlorella* sp., literature EC_{50} vary a lot depending on the strain tested, from 0.200 to 7.3 $mg{\cdot}L^{-1}$ (Johnson et al., 2007; Qian et al., 2009).

In conclusion, considering only metals, *D.salina* CA113 and *C.simplex* RCC537 were the most resistant organisms, with EC_{50} superior to the maximum concentrations tested for almost all compounds and to maximum concentration in PW for all compounds, except for Fe(III) as its concentrations in PW can go up to 4.5 $g{\cdot}L^{-1}$ (Dórea et al., 2007). Among the strain tested, they are the most promising strains for growth

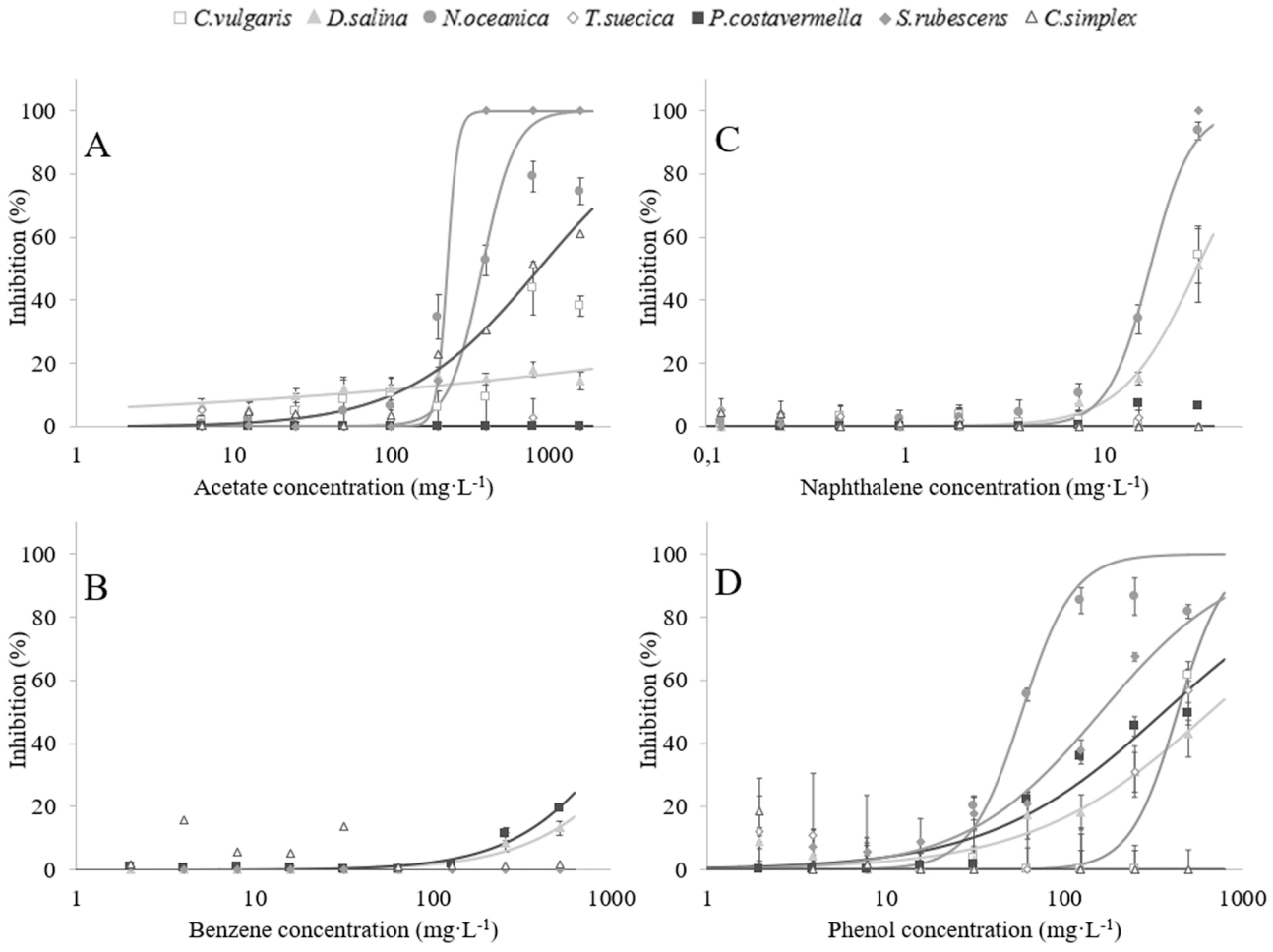

Fig. 3. Hill model fitting of inhibition data for each strain. A: acetate; B: naphthalene; C: benzene and D: phenol. Values correspond to mean ± standard deviation, n = 6. Lines correspond to Hill model fitting.

in waters contaminated by metals. Concerning reference strain *C.vulgaris* CA104, it appeared to be more resistant than *Chlorella* sp. strains tested in literature, as the EC_{50} calculated are higher than those than found in other studies, at least for Ba(II), Fe(III) and As(III). EC_{50} for other metals were in the range of literature data. It can be hypothesized that *C.vulgaris* CA104 is more resistant to metals as the strain is able to grow at 30 $g{\cdot}L^{-1}$ of salinity (data not shown). Moreover, it must be noted that these results concern the toxicity of individual compounds. Toxicity may occur at lower concentrations if several of them are present simultaneously, by cocktail effect.

3.3. Toxicity of organic compounds

In addition to metals and metalloid, toxicity tests were conducted on benzene, naphthalene, phenol and acetate, representatives of the different families of organic compound found in PW. As for metals, EC_{50} and EC_{10} at 96 h were estimated thanks to Eq. 3. Hill fitting for each organic compound are shown in Fig. 3 while EC_{50} and EC_{10} for each strains are summarized in Table 3.

Among organic molecules, acetate was toxic to *N.oceanica*, *S.rubescens* and *C.simplex* with EC_{50} of 382, 229 and 881 $mg{\cdot}L^{-1}$, respectively. No toxic effect was observed for the other strains, suggesting that they could grow in PW containing up to 1600 $mg{\cdot}L^{-1}$ of acetate. Growth of *P. costavermella* was higher with acetate, with growth rate 20% higher with 800 $mg{\cdot}L^{-1}$, suggesting that this strain consumes acetate. Concerning benzene, selected microorganisms were not impacted after 96 h whatever the concentration used. Due to the high volatility of this compound, it was not possible to concluded that benzene concentrations were constant in the wells of the microplates and that EC_{50} are really superior to 513 $mg{\cdot}L^{-1}$. However, the high volatilisation of benzene is a phenomenon that will also occurred in larger scale PW cultures with stirred bioreactors, suggesting that it is unlikely to be a problem for microalgae growth even at higher concentration. Naphthalene appears to be the most toxic for *C.vulgaris*, *D.salina*, *N.oceanica* and *S.rubescens* with EC_{50} at 96 h below 30 $mg{\cdot}L^{-1}$. It was not possible to study toxicity at higher concentrations for this compound, as solubility of naphthalene is 30 $mg{\cdot}L^{-1}$ in water. Naphthalene toxicity was investigated with two *Chlorella* strains by Hutchinson et al. (1980), and Kong et al. (2011). Both have shown that naphthalene is fast acting, with a 50% effect measured after 3 h and 24 h expositions with concentrations of 19.2 and 11 $mg{\cdot}L^{-1}$, respectively. For phenol, EC_{50} from 58 to 500 $mg{\cdot}L^{-1}$ were estimated except for *D.salina* and *C.simplex*, which were more resistant to this compound than the other strains. Phenol impacts on *Dunaliella* sp. strains have already been studied. Authors report EC_{50} between 72 and 187 $mg{\cdot}L^{-1}$ (Duan et al., 2017; Ertürk and Saçan, 2012), suggesting that *D.salina* CA113 is more resistant. Phenol impacts on *Chlorella* strains (Shigeoka et al., 1988) have also been studied, with EC_{50} of approximately 370 $mg{\cdot}L^{-1}$, being in line with the value found in this study for *C. vulgaris*. Considering organic molecules, *P.costavermella* and *C.simplex* are the most tolerant strains. However, all strains have high EC_{50} concerning organic compounds, suggesting they could all be good candidates to grow in PW.

Among all the publications listed in both metals and organic compounds parts, a large majority studied toxicity at 96 h, but none used a microplate culture method for toxicity tests, the most common reactor being the stirred glass flask, from 10 to 500 mL. It can be hypothesized that differences between 1: strains and 2: growth methods and protocol, explained EC_{50} variations observed for some compounds. However, majority of EC_{50} are consistent with literature values, with values in the same concentration ranges. The use of microplates and photosynthetic microorganisms fed with *f* medium and $NaHCO_3$ therefore appears to be an effective method for studying compounds toxicity.

3.4. Produced water as a source of saline water for marine microalgae and cyanobacteria

Regarding PW common composition found in literature and calculated EC_{50} and EC_{10}, if PW is used to cultivate microalgae, it can be hypothesized that a 10–50% toxic effect could be observed due to copper, arsenate and acetate if the PW is particularly charged with one of these compounds (without considering potential cocktail effects). *P. costavermella* and *S.rubescens* being more sensible to zinc, at least 10% effect could also be observed with these strains. Concerning benzene and iron, no 10% and 50% toxic effect should be observed in PW containing less than 513 and 40 $mg{\cdot}L^{-1}$, respectively. No conclusion can be made for PW with higher concentrations of benzene and iron as no tests were conducted above these concentrations. Concerning other compounds, no 10–50% toxic effect should be observed if PW is used as part of culture media for marine microalgae or cyanobacteria growth. However, given the wide variety of PW, with very different compositions depending on their origin, growth tests will have to be performed each time a PW have to be used, on a case-by-case basis.

In this regard, marine microalgae and cyanobacteria were grown at 30 $g{\cdot}L^{-1}$ in *f* medium and *f* medium complemented with 80%v/v of aPW. These tests were conducted to evaluate the potential toxicity of aPW. According to its composition and EC_{50} and EC_{10} showed before, a 10% toxic effect could be predicted for *D.salina* due to acetate and for *S. rubescens* due to copper. However, higher inhibition could be observed due to the presence of several compounds simultaneously. Growth rates calculated in each medium are shown in Fig. 4.

Growth rates were similar in both conditions for *N.oceanica*. For *T. suecica* and *P.costavermella*, growth rate were approximately 20% higher in *f* medium complemented with 80%v/v aPW than in *f* medium alone. For *D.salina*, *S.rubescens* and *C.simplex*, growth rate were 270%, 90% and 170% higher, respectively. Considering these results, 80%v/v of aPW has no toxic effects on the selected marine microorganisms. Moreover, its effect was positive since biostimulation was observed. Regarding the results for *N.oceanica*, a potential cocktail effect could be hypothesised as no biostimulation was observed as for the other strains. Nonetheless, growth rates were similar to those observed for *N.oculata* by Arriada and Abreu, (2014), Ammar et al. (2018) and Parsy et al. (2020), from 0.12 to 0.35 d^{-1} at PW loading from 10% to 50%.

As growth rates were higher in *f* medium with 80%v/v of aPW than in *f* medium, it can be concluded that aPW has no toxic effects on the selected marine microorganisms. Moreover, it can be hypothesized that microorganisms used nitrogen (21.4 $mg{\cdot}L^{-1}$) and phosphorus

Table 3
EC_{50} and EC_{10} of selected organic compounds on studied microorganisms at 96 h. EC_{50} and EC_{10} were determined with Hill equation and are expressed in $mg{\cdot}L^{-1}$. Values in brackets correspond to EC_{10}. Values correspond to mean ± standard deviation, n = 6. n.d.: not determined.

	C.vulgaris	*D.salina*	*N.oceanica*	*T.suecica*	*P.costavermella*	*S.rubescens*	*C.simplex*
Acetate	> 1600 (400–800)	> 1600 (34.7)	382 (72.0)	> 1600 (> 1600)	> 1600 (> 1600)	229 (193)	881 (102)
Benzene	n.d. (n.d.)	> 513 (387)	n.d. (n.d.)	n.d. (n.d.)	> 513 (128–257)	> 513 (> 513)	> 513 (> 513)
Naphthalene	29.8 (15–30)	29.7 (11.5)	17.2 (10.1)	> 30 (15–30)	> 30 (> 30)	15–30 (15–30)	> 30 (> 30)
Phenol	250–500 (250–500)	> 500 (51.5)	58.1 (27.9)	435 (221)	364 (30.2)	161 (23.7)	> 500 (> 500)

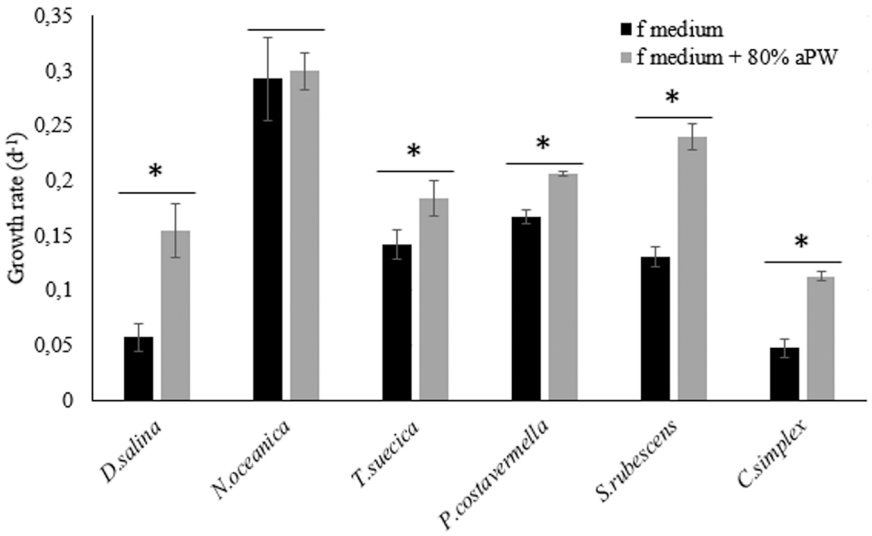

Fig. 4. Growth rate of halophilic microalgae and cyanobacteria in *f* medium and in *f* medium with 80% v/v artificial produced water (9 days cultures). Values correspond to mean ± standard deviation, n = 12. * , Significant difference (p-value lower than 5%) between the two growth rates measured for each species.

(1.6 $mg{\cdot}L^{-1}$) available from aPW to grow, resulting in biostimulation. As no micro-organisms showed any growth enhancement in the presence of acetate during the toxicity tests (except *P.costavermella*), it cannot be concluded that the increase in growth rates is due to its presence in aPW (62 $mg{\cdot}L^{-1}$).

It is noteworthy that these results were obtained with our synthetic water which is relatively low in metals (real PW used as a model being low in concentration). Culture tests should be carried out when a new PW or other strains are used, and the use of microplate method is easy and reliable. In addition, PW often contains additives that were not considered in this study. In the case of particularly toxic water used for the cultivation of marine micro-organisms, the water may be diluted with seawater or wastewater to lower the concentrations of toxic compounds. Moreover, dilution will be also necessary if the water is extremely salty, as only few strains are able to grow at higher salinity than 80 $g{\cdot}L^{-1}$. Further studies should be conducted to investigate growth in PW in more detail, and to find an alternative source of nutrients, such as anaerobic digestion effluents, to replace synthetic culture media.

4. Conclusion

This study investigated the salinity tolerance and the toxicity of several metals and organic molecules found in PW on various halotolerant photosynthetic microorganisms. Results allowed us to conclude that *D.salina* CA113 was the most tolerant strain to high salinity, growing up to 216 $g{\cdot}L^{-1}$ (other strains could grow up to 80–108 $g{\cdot}L^{-1}$), positioning it as the best candidate for growth in saline PW. Concerning metals and metalloids, Cu(II) was the most toxic, followed by As(III) then Cr(III). *D.salina* CA113 and *C.simplex* RCC537 were the most resistant strains to all metals tested. All organic compounds presented various EC_{50} according to the micro-organisms, however, none were highly toxic as EC_{50} were never below 10 $mg{\cdot}L^{-1}$. *P.costavermella* RCC4223 and *C.simplex* RCC537 were the most resistant strains to organic compounds, but all strains are good candidates to grow in PW. Comparing to PW concentrations (salinity, metals and organic compounds), *D.salina* CA113 is the best choice for biomass production in PW. Finally, aPW shows biostimulating effects on the different strains. In the perspective of using PW as part of culture media for cyanobacteria and microalgae, additional growth tests should be carried out to understand the impact on the micro-organisms. Moreover, PW containing low amounts of nutrients (nitrogen, phosphorus), its use should be complemented with a nutrient rich source, such as liquid digestate from anaerobic digestion process or wastewater treatment plant influent.

CRediT authorship contribution statement

Aurelien Parsy: Conceptualization (Ideas; formulation or evolution of overarching research goals and aims), Investigation (Conducting a research and investigation process, specifically performing the experiments, or data/evidence collection), Formal analysis (Application of statistical, mathematical, computational, or other formal techniques to analyze or synthesize study data), Writing – original draft (Preparation, creation and/or presentation of the published work, specifically writing the initial draft (including substantive translation)). **Rémy Guyoneaud:** Conceptualization (Ideas; formulation or evolution of overarching research goals and aims), Supervision (Oversight and leadership responsibility for the research activity planning and execution, including mentorship external to the core team), Writing – review & editing (Preparation, creation and/or presentation of the published work by those from the original research group, specifically critical review, commentary or revision – including pre-or postpublication stages). **Marie-Claire Lot:** Formal analysis (Application of statistical, mathematical, computational, or, other formal techniques to analyze or synthesize study data), Writing – review & editing (Preparation, creation and/or presentation of the published work by those from the original research group, specifically critical review, commentary or revision – including pre-or postpublication stages). **Patrick Baldoni-andrey:** Writing – review & editing (Preparation, creation and/or presentation of the published work by those from the original research group, specifically critical review, commentary or revision – including pre-or postpublication stages). **Frederic Perie:** Funding acquisition (Acquisition of the financial support for the project leading to this publication), Writing – review & editing (Preparation, creation and/or presentation of the published work by those from the original research group, specifically critical review, commentary or revision – including pre-or postpublication stages). **Cecilia Sambusiti:** Conceptualization (Ideas; formulation or evolution of overarching research goals and aims), Supervision (Oversight and leadership responsibility for the research activity planning and execution, including mentorship external to the core team), Writing – review & editing (Preparation, creation and/or presentation of the published work by those from the original research group, specifically critical review, commentary or revision – including pre-or postpublication stages).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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