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Selection of photosynthetic microorganisms grown in artificial saline industrial effluents with liquid digestate: from screening to consortium cultures

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Abstract

The objective of this study was to determine the feasibility of using saline industrial streams as a culture medium to grow microalgae and cyanobacteria. Experiments were performed to determine the extent of the growth in artificial saline produced water and aquifer water supplemented with liquid digestate. Tests were performed in 96-wells microplates. Media were composed with different proportion of saline artificial produced water or aquifer water supplemented with 5 %v/v liquid digestate (final concentrations: 149-195 mgN·L^{-1}, 1.5-2.7 mgP·L^{-1}). Media were completed to 100% with artificial seawater, corresponding to final salinities of 40, 70 and 100 g·L^{-1}. *D.salina, N.oceanica* and *T.suecica* showed the best growth rates. They were selected to perform mixed cultures in 80 mL tubes in the same culture media. Population evolutions were followed for 19 days. Depending on salinity and industrial effluent used, different species became predominant over the two others *(N.oceanica, T.suecica* and *D.salina*. at 40, 70 and 100 g·L^{-1}, respectively). It appears that mixed culture is a good solution to have a biomass production during a culture process where the culture media will evolve in terms of salinity and composition.

Keywords: aquifer water, cyanobacteria, liquid digestate, microalgae, produced water

Abbreviations: aAW: artificial aquifer water; AW: aquifer water; aPW: artificial produced water; PW: produced water; COD: chemical oxygen demand; PAR: photosynthetically active radiation; PCA principal component analysis; TDS: total dissolved solids; TOC: total organic carbon; TSS: total suspended solids.

I Introduction

In the last ten years, aerobic photosynthetic organisms, and among them microalgae, have gain interest in wastewater treatment and biomass production [1]. Their ability to fix carbon dioxide (CO_2) through their autotrophic metabolism and organic carbon through their hetero/mixotrophic metabolism is well studied. They are considered as a promising feedstock for several renewable energy production processes [2–5] and also for the production of valuable molecules in pharmaceutic, cosmetic, food and feed sectors.

Within the fossil energy industry, oil and gas production leads to large quantity of produced waters (PW) [6]. PWs have complex chemical composition, depending on the origin of the geological reservoir [7]. PWs are a source of pollution and need to be treated before discharge to prevent environmental issues. PWs are mainly composed of inorganic salts with total dissolved solids (TDS) varying from 100 $mg \cdot L^{-1}$ to 400 $g \cdot L^{-1}$ [8]. They contained metals (*e.g.* iron, copper, aluminum, zinc), organic molecules (0 – 600 $mgC \cdot L^{-1}$) such as aliphatic hydrocarbons, polycyclic aromatics hydrocarbons, phenols and derivative compounds, benzene, toluene, ethylbenzene, xylene (BTEX), or organic acids, and some nitrogen (0 – 190 $mgN \cdot L^{-1}$) [9]. PWs also contain chemical additives (corrosion inhibitors, biocides, emulsion breakers, wax inhibitors, asphaltene inhibitors, H_2S scavengers), added to enhance oil extraction and to facilitate oil, gas and water separation processes [8,10]. In the CO_2 storage sector, saline waters, named aquifer water (AW), are extracted during CO_2 sequestration in

saline aquifers, as it is a promising process to stock and reduce atmospheric CO_2 [11,12]. AWs are mainly composed of inorganic salts, depending on the geological history of the reservoir. AWs usually have TDS from 1 $g \cdot L^{-1}$ to 150 $g \cdot L^{-1}$ and may contain heavy metals (*e.g.* manganese, barium, iron) [13]. They can also contain carbon and nitrogen in low concentration (0 – 400 $mgC \cdot L^{-1}$, 0 – 165 $mgN \cdot L^{-1}$) [13]. Groundwaters such as PWs and AWs can be athalossohaline depending on their origins, having a different ion composition than seawater and potentially impacting the growth of marine microalgae. Cultivation of microalgae and cyanobacteria in wastewaters are interesting to recycle water and nutrients. The microorganisms are already used to treat industrial wastewaters to produce biomass and remediate effluents [14]. Groundwaters potentially being highly saline, the use of halotolerant and halophile is necessary to obtain interesting biomass production.

Industrial saline wastewaters such as PWs and AWs do not contain enough nutrients for photosynthetic biomass production. Among available nutrient sources, digestate from anaerobic digestion process are suitable for microalgae and cyanobacteria cultivation. The microorganisms use the nitrogen and phosphorous nutrients and can also in some conditions reduce the chemical oxygen demand [14]. The use of digestate, PWs or AWs, reduces the costs of biomass production, as they are produced in large quantities. However, their use often limits the possible ways to valorize the biomass, limiting it to the energy sector. Indeed, they can bring some compounds unsuitable for other sectors (food, cosmetics), and requiring too expensive processes to remove them.

Very few studies about microalgae consortia culture in industrial saline water supplemented with anaerobic digestate are available in literature, as most studies focus strains grown individually. Racharacks *et al*. [15] worked with *Nannochloropsis* sp. *and Dunaliella* sp. in flowback water from shale gas exploration (TDS = 42 $g \cdot L^{-1}$) supplemented with liquid digestate. Authors reported productivities up to 240 $mg \cdot L^{-1} \cdot d^{-1}$ during individual culture at

lab-scale at salinity of 40 $g \cdot L^{-1}$, with growth rates up to 0.3 d^{-1} (batch tests at lab-scale). In a previous study, Parsy *et al*. [16] investigated the growth of *Nannochloropsis* sp. in a mix of seawater, saline PW (114 $g \cdot L^{-1}$ of salinity) and liquid digestate. This strain was able to grow in PW with growth rates between 0.2 and 0.3 d^{-1} (batch tests at lab-scale).

The objective of this study was to select the best performing strains among six halotolerant photosynthetic microorganisms (*Dunaliella salina*, *Nannochloropsis oceanica*, *Tetraselmis suecica*, *Picochlorum costavermella*, *Coccomyxa simplex* and *Synechococcus rubescens*). The growth rates in different culture media were compared. First f medium and digestate were compared at various salinities (40, 70, 100 $g \cdot L^{-1}$) to identify the best candidates. Then, medium composed of saline industrial effluents supplemented with liquid digestate were compared. The three best performing strains were selected to make an upscaling with mixed cultures, to follow the microalgal population evolution in the different medium. The novelty of this research lies in the selection of a microalgae consortium able to tolerate and adapt to a wide range of salinity in complex saline effluents. Halotolerant strains were first selected using microplate techniques according to their growth rates in the culture media, then cultivated together to observe the consortium evolution for scale-up applications.

II Materials and methods

1. Strains and inoculum preparation

Five halotolerant or halophilic microalgae and one halotolerant cyanobacterium have been selected for this study. These microorganisms have been identified in a previous work [17] and are referred as strains *Dunaliella salina* CA113, *Nannochloropsis oceanica* CA101, *Tetraselmis suecica* CA106, *Picochlorum costavermella* RCC4223, *Coccomyxa simplex* RCC537 and *Synechococcus rubescens* RCC752. Strains identified “CA” and “RCC” were

purchased from Greensea (Mèze, France) and from the Roscoff Culture Collection (Roscoff, France), respectively.

Each strain was cultivated in glass bottles (500 mL of working volume) constituted of a sterile *f* medium [18] at a salinity of 30 $g \cdot L^{-1}$ in a simulated seawater environment (Instant Ocean salts, Aquarium Systems, France). Reactor mixing was ensured by air bubbling, while the pH was maintained at 8.0 ± 0.5 thanks to regular injections of CO_2. Light was provided with 14/10 h light/dark periods, by 3 LED lamps (CorePro LEDtube, 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. Growth was monitored by spectrophotometry (Thermoscientific Evolution 201 UV–visible spectrophotometer, USA). 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.

2. Culture experiments

2.1. Liquid digestate as nutrient source

Liquid digestate from an industrial biogas plant (Aire-sur-Adour, France) was used to grow microalgae and cyanobacteria at various salinities in *f* medium. Three conditions have been tested at 40 and 70 $g \cdot L^{-1}$ salinity: *f* medium, *f* medium with 5% v/v digestate, 5% v/v digestate. These conditions were also tested at 100 $g \cdot L^{-1}$ salinity with the microalgae *D.salina* CA113.

Tests were performed in 96-well plates (working volume 250 µL). Peripheral wells were filled with water to limit side effects/evaporation and plates were sealed with plastic foils to limit evaporation. Each condition was performed with 12 replicates. Each well was spiked with: 20 µL of *f* medium (concentrated at 50X), 12.5 µL of liquid digestate for corresponding conditions; 33.6 µL of $NaHCO_3$ solution at 50 $g \cdot L^{-1}$ (final concentration of 20 mM) to bring

inorganic carbon for autotrophic growth, hypersaline water (solution of Instant Ocean salts at 290 $g \cdot L^{-1}$) to adjust the final salinity and artificial seawater (solution of Instant Ocean salts at 35 $g \cdot L^{-1}$) to make up 250 µL. Tests were started with initial OD_{680} of 0.2 (Thermoscientific Evolution 201 UV–visible spectrophotometer, USA). Initial pH was 8.0. Plates were incubated 3 weeks 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} was monitored each 2 days to follow the growth with a Synergy HTX Multi-Mode Microplate Reader (Biotek Instruments, USA). Plastic foils used to limit evaporation were removed for OD measurements and replaced afterwards.

2.2. Artificial produced water and artificial aquifer water

Two synthetic waters were used as part of the media to grow microalgae and cyanobacteria. Compositions of artificial produced water (aPW) and artificial aquifer water (aAW) are presented in Table 1. aPW composition was based on the composition of real produced water from a TotalEnergies operating site described by Sambusiti *et al.*, 2020 [19]. In order to simplify the composition of aPW, no chemical additives (inhibitors, biocides) were considered in this study. aAW composition was based on the composition of real aquifer water from Chaunoy (Paris basin, France). Synthetic waters were made using chemical products purchased from Sigma-Aldrich.

Table 1: Composition of artificial seawater, aPW and aAW.

Compound	artificial seawater ($mg \cdot L^{-1}$)	aPW ($mg \cdot L^{-1}$)	aAW ($mg \cdot L^{-1}$)
Total Dissolved Solids (Instant Ocean)	35000*	150000*	122000
Na (NaCl)	10757*	46101*	37909
Cl (NaCl)	19251*	82504*	73549

SO_4 (Na_2SO_4)	2659*	11396*	681
Ca ($CaCl_2$)	398*	1706*	6448
Mg ($MgCl_2$)	1317*	5644*	1098
K (KCl)	402*	1723*	969
Sr ($SrCl_2$-$6H_2O$)	8.6*	7.4*	325
Li (LiOH)	0.18*	0.77*	40.9
B (H_3BO_3)	1.2*	5.1*	51.9
Br (NaBr)	2.3*	9.9*	688.8
As ($NaAsO_2$)	< 0.0002*	< 0.0002*	4.0
Fe ($FeCl_3$ – 6 H_2O)	< 0.03*	1	11.3
Ba ($BaCl_2$ – 2 H_2O)	< 0.05*	1.2	8.2
Cr ($CrCl_3$ – 6 H_2O)	< 0.006*	0.06	8.8
Cu ($CuSO_4$ – 5 H_2O)	< 0.03*	0.02	0.003
Zn ($ZnSO_4$ – 7 H_2O)	< 0.02*	0.18	2.0
N (NH_4Cl)	-	26.7	49
P (NaH_2PO_4 - H_2O)	-	2	0
Total Organic Carbon	-	80.3**	0

(*): Elements brought by Instant Ocean salts.
(**): Total organic carbon composed of (expressed in mgC·L^{-1}): acetate: 25.22; ethanol: 23.16; phenol: 8.62; benzene: 7.38; toluene: 6.85; ethylbenzene: 4.53; o-xylene: 0.98; m-xylene: 2.61; naphthalene: 0.229; phenanthrene: 0.046; benzo(a)pyrene: 0.0003; acenaphtylene: 0.0134; acenaphtene: 0.0118; fluorene: 0.020; anthracene: 0.008; fluoranthene: 0.001; pyrene: 0.007.

Two conditions have been tested at 40 and 70 g·L^{-1} salinity: aPW with 5% v/v digestate and aAW with 5% v/v digestate. These conditions were also tested at 100 g·L^{-1} salinity with microalgae *D.salina* CA113.

Tests were performed in 96-well plates as described in part 2.1 except that a part of artificial seawater used to complete the volume to 250 µL was replace by aPW and aAW for the corresponding conditions: 5% v/v aPW & 7% v/v aAW at 40 g·L^{-1}, 31% v/v aPW & 41% v/v aAW at 70 g·L^{-1} and 57% v/v aPW & 75% v/v aAW at 100 g·L^{-1}.

2.3. Growth in supplemented saline effluents with a microalgal consortium

D.salina, *N.oceanica* and *T.suecica* strains were selected to perform additional growth tests in artificial produced water and aquifer water at a larger scale. Tests were conducted in test tubes with 80 mL working volume. The three stains have been cultivated in media containing aPW with 5% v/v digestate, aPW without organic compounds with 5% v/v digestate and aAW with 5% v/v digestate. A condition with aPW without organic compounds was added to see the

impacts of such compounds on the microalgae consortia. These conditions were studied at 40, 70 and 100 $g \cdot L^{-1}$ salinity. Loads of aPW and aAW were the same as described in part 2.2 depending on salinity. Culture media were completed to final volume with artificial seawater (solution of Instant Ocean salts at 35 $g \cdot L^{-1}$). Tests were performed in the same conditions as described in part 1. pH was maintained at 8.3 ± 0.6 thanks to regular injections of CO_2. PAR was maintained at 50 $\mu mol_{photons} \cdot m^{-2} \cdot s^{-1}$ with a 14/10h light/dark periods. Each condition was performed with 3 replicates. Tests were started with an OD_{680} of 0.3, with 0.1 of OD_{680} from each microalga, corresponding to initial cellular concentration of 0.2 ± 0.1, 3.0 ± 0.9 and 0.2 ± 0.1 10^6 cells$\cdot mL^{-1}$ for *D.salina*, *N.oceanica* and *T.suecica* strains, respectively.

Samples were taken each 2 days and population evolution was followed by cell counting using Malassez cell counting chamber (Herka, France). Mobile strains (*D.salina* and *T.suecica*) were immobilized during cell counting by mixing them with commercial lugol's iodine solution (50/50 proportion) 10 minutes before counting. Mixing with lugol solution was also helpful to distinguished *D.salina* and *T.suecica* cells. pH was also measured by using pH-meter ProfiLine pH 1970i (Xylem Analytics) to adjust frequency of CO_2 injections used to regulate pH. Theoretical Total Suspended Solids (TSS_{theo}, expressed in $g \cdot L^{-1}$) were estimated for each microalga according to cell counting of each species using correlation coefficients estimated with pure strain (data not shown). Linear correlation between TSS_{theo} and cellular concentration for each strain were (R^2: coefficient of determination):

D.salina: $TSS_{theo} = 5.09 \cdot 10^{-10}$ * cellular concentration ($R^2 = 0.8570$)

N.oceanica: $TSS_{theo} = 1.67 \cdot 10^{-11}$ * cellular concentration ($R^2 = 0.9692$)

T.suecica: $TSS_{theo} = 8.57 \cdot 10^{-10}$ * cellular concentration ($R^2 = 0.9464$)

With TSS_{theo} expressed in $g \cdot L^{-1}$, cellular concentration expressed in cells$\cdot L^{-1}$ and correlation coefficient expressed in $g \cdot cell^{-1}$.

2.4. Data processing

Concerning growth in 96-wells microplates, growth rates were determined between day 0 and day 9 using equation 1 [20]:

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

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

To determine statistical differences between growth rates monitored in each medium and at each salinity, one-way analyses of variance were performed using Rstudio software. Then, *t*-tests for paired data were performed to evaluate differences in growth rates between each condition for each species. For statistical tests, a confidence level of 95% (significance α level of 0.05) was considered. Thus, *p*-values < 0.05 were deemed to be statistically significant.

To visualize the influence of culture media parameters (salinity, TOC, NH_4^+ and PO_4^{3-}) on growth rates measured, Principal Component Analysis (PCA) was performed also using Rstudio software, using package "Hmisc", "FactoMineR", "Rcpp", "missMDA" and "factoextra".

3. Analytical methods

Liquid digestate was characterized in terms of physico-chemical composition. pH was measured by using pH-meter ProfiLine pH 1970i (Xylem Analytics). Turbidity was measured by a 2100Qis portable turbidimeter (Hach Company, Loveland, Colorado, USA). Total Solids (TS), Total Dissolved Solids (TDS) and Total Suspended Solids (TSS) were measure according to standard methods [21]. Liquid digestate was filtered using glass fiber filters of 0.45 µm. Permeate was used to monitor dissolved nutrients and organic/inorganic carbon.

Total carbon (TC), inorganic carbon (IC) and total organic carbon (TOC) were analyzed by a TOC-meter (Shimadzu TOC-L, Japan). Chemical oxygen demand (COD), ammonium (NH_4^+) and phosphate (PO_4^{3-}) concentrations were evaluated by spectrophotometric methods with LCK1014, 1414 kits (for COD), LCK303 kit (for NH_4^+) and LCK348 (for PO_4^{3-}) (Hach Company, USA). Nitrate (NO_3^-) concentration was measured by Ionic liquid chromatography (Dionex ICS1000, column AS9-HC, 4 ∗ 250 mm, Thermoelectro, USA). Volatile fatty acids (VFA) present in liquid digestate were analyzed after centrifugation at 10000g during 10 min and 0.2 μm filtration, using a gas chromatograph (GC-7090 B, Agilent, USA) equipped with a CP 8400 sampler, a FFAP ECTM 1000 column and a flame ionization detector (FID). Acetic (C2), propionic (C3), butyric and iso-butyric (C4 and iC4), valeric and iso-valeric (C5 and iC5) and caproic and iso-caproic (C6 and iC6) acids standards were purchased from Sigma Aldrich (USA).

III Results and discussion

1. Liquid digestate as source of nutrients

Physico-chemical composition of liquid digestate used in this study is reported in Table 2.

Table 2: Chemical composition of liquid digestate (n.d.: not detected). Values correspond to mean ± standard deviation, n=3.

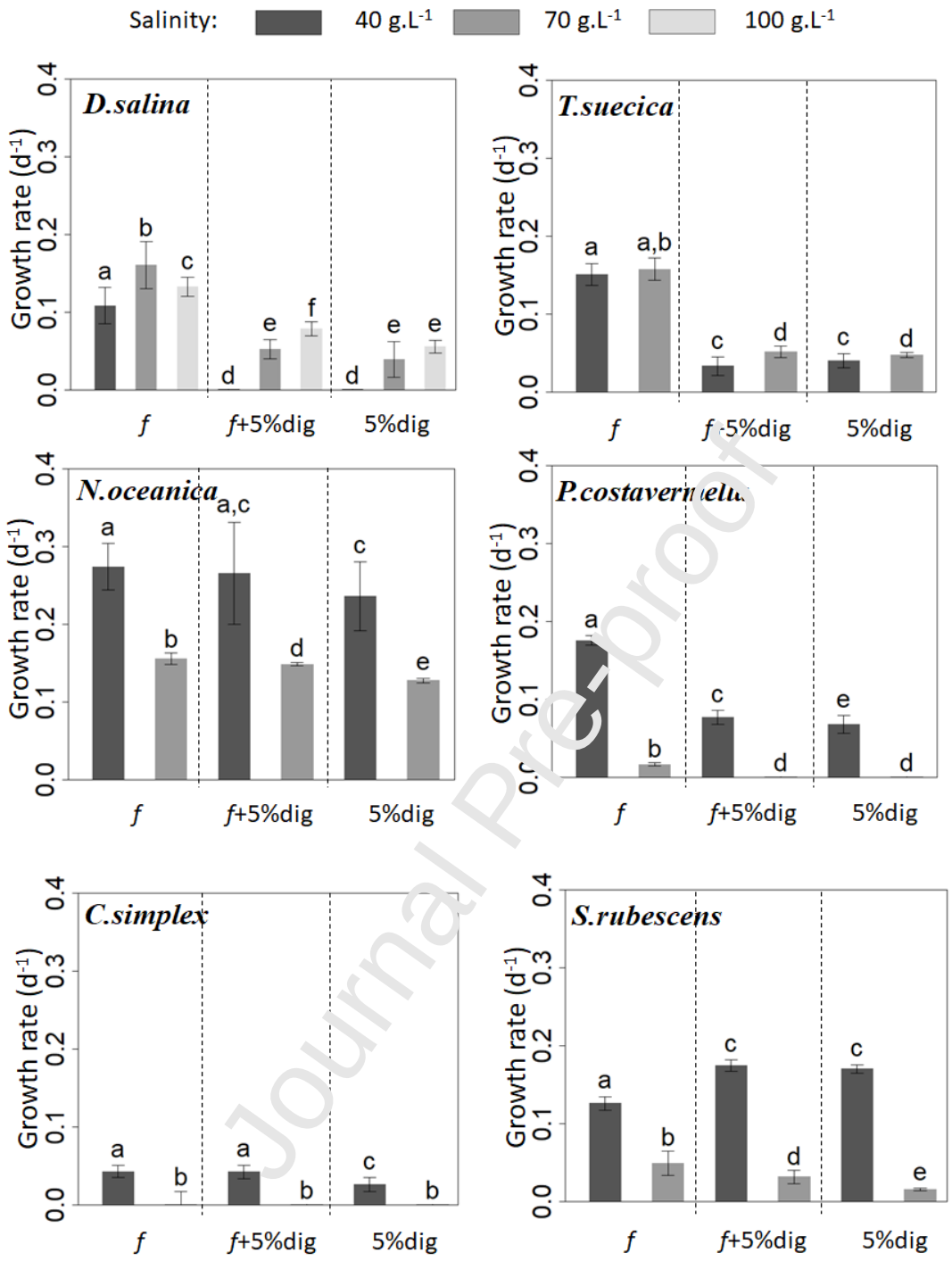
	Liquid digestate
Turbidity (NTU)	825 ± 8
pH	8.4 ± 0.1
TS ($g \cdot L^{-1}$)	9.6 ± 0.1
TDS ($g \cdot L^{-1}$)	9.0 ± 0.1
TSS ($g \cdot L^{-1}$)	0.4 ± 0.0
TC ($mgC \cdot L^{-1}$)	2987 ± 33
IC ($mgC \cdot L^{-1}$)	1912 ± 5
TOC ($mgC \cdot L^{-1}$)	1074 ± 29
COD ($mg \cdot L^{-1}$)	2524 ± 80
NH_4^+ ($mg \cdot L^{-1}$)	2664 ± 39
NO_3^- ($mg \cdot L^{-1}$)	n.d.
PO_4^{3-} ($mg \cdot L^{-1}$)	94.3 ± 0.6

VFA ($mgCOD \cdot L^{-1}$)	489.5 ± 15.5
VFA ($mgC \cdot L^{-1}$)	174.8 ± 5.7

Composition of liquid digestate was similar to literature data described by Racharacks *et al*., 2015 [15], and Parsy *et al*., 2020 [16], in terms of TDS (7.3-9.3 $g \cdot L^{-1}$), pH (8.3-8.6), NO_3^- (0.6-1.3 $mg \cdot L^{-1}$) and PO_4^{3-} (20-333 $mg \cdot L^{-1}$). NH_4^+ concentration was higher compared to literature data (1275-1860 $mg \cdot L^{-1}$). Lower COD concentration was also observed compared to literature data (concentration range from 4 to 90 $gCOD \cdot L^{-1}$) [22], probably due to the filtration of liquid digestate (0.45 µm filter) applied to perform the analysis. It is well known that digestate composition can by highly variable and depends on the inputs used for the anaerobic digestion process [23]. VFA represented approximately 16.3 ± 0.5 % of TOC, and were composed of 77% of C2 acids (i.e. acetate), 12% of C3 acids (i.e. propionate), 6% of C4 acids (i.e. butyrate and iso-butyrate) and 5% of C5 acids (i.e. valerate and iso-valerate). VFA with longer molecular chains (6 atoms of carbon) were not detected. Due to high turbidity and ammonium concentration, liquid digestate have to be diluted to allow the passage of light through the microalgae culture and to reduce ammonium concentration, potentially toxic for microalgae at concentration above 150-200 $mg \cdot L^{-1}$ [15,24,25]. Therefore, only 5% v/v of digestate loading was used during the experiments, to have a final ammonium concentration of 133 $mg \cdot L^{-1}$ and a turbidity of 41 NTU. Previous work has shown that interesting growth rates (0.4 d^{-1}) could be achieved in this range of turbidity [16].

Growth rates monitored for each microorganism after 9 days of growth in *f* medium, *f* medium with 5% v/v digestate, 5% v/v digestate are shown in Figure 1. Daily volumetric productivities were also calculated for the same period of time and follow the same trends as growth rates (data not shown).

Figure 1: Growth rates of tested microorganism after 9 days of growth in *f* medium, *f* medium with 5% v/v digestate, 5% v/v digestate. Final salinity was adjusted using Instant Ocean salts. Values correspond to mean ± standard deviation, n=12. Same letter above bars in each microalgae histograms indicates no significant difference between the tests (p-value > 0.05).



Regarding results in *f* medium at the different salinities, all strains except *D.salina* and *T.suecica* exhibited higher growth rates at 40 g·L^{-1} than at 70 g·L^{-1}. For *T.suecica*, there was no major difference between 40 g·L^{-1} and 70 g·L^{-1}, whereas *D.salina* exhibited higher growth rates at 70 g·L^{-1} or 100 g·L^{-1} than at 40 g·L^{-1}. Previous work [17] investigated the impact of salinity on these 6 strains of microalgae and cyanobacteria. Results shown in Figure 1 are

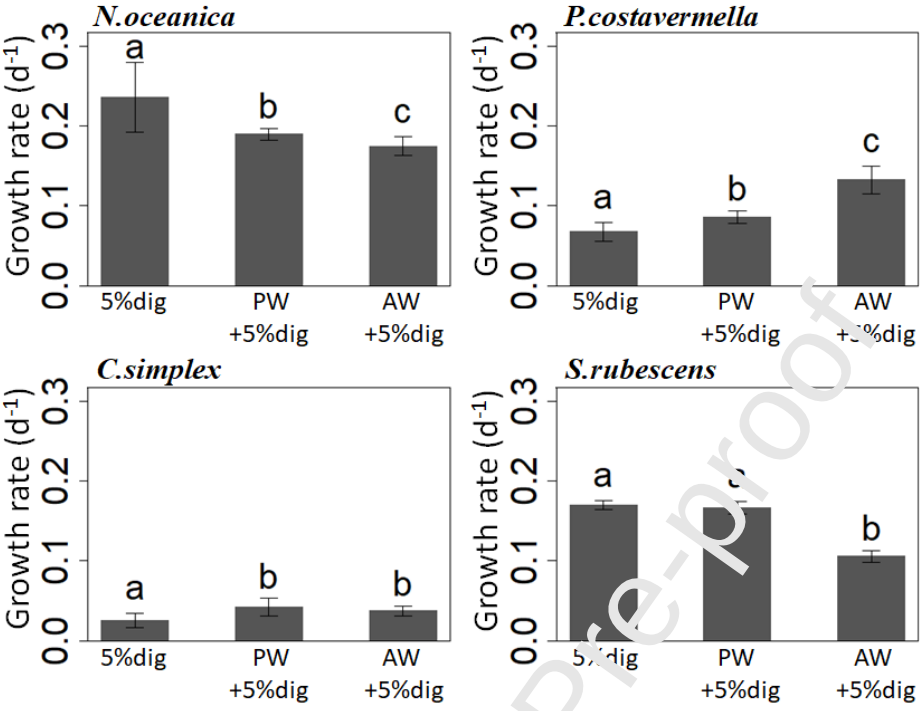
consistent with this previous study as optimum salinity of *N.oceanica*, *P.costavermella*, *C.simplex* and *S.rubescens* were determined between 14-41 $g \cdot L^{-1}$ for these strains, with a decrease of growth at higher salt concentrations. For *D.salina* and *T.suecica*, optimal salinities were determined between 81-95 $g \cdot L^{-1}$ and 41-68 $g \cdot L^{-1}$, respectively.

Concerning growth in *f* medium with 5% v/v digestate and 5% v/v digestate alone, it appeared that digestate limited growth for *D.salina*, *T.suecica* and *P.costavermella*, as growth rates were lower than in *f* medium alone. *N.oceanica* and *C.simplex* were not impacted by the digestate, as growth rates were similar for all conditions. At 40 $g \cdot L^{-1}$ of salinity, *S.rubescens* was the only microorganism with better growth in digestate, with growth rate approximately 37% higher than in *f* medium. Previous work [16] investigated the growth performances of *Nannochloropsis oculata* in seawater supplemented with 5% of liquid digestate at 30 $g \cdot L^{-1}$ of salinity. With this strain, growth rates monitored were higher using digestate (0.4 d^{-1}) than $f_{/2}$ medium (0.3 d^{-1}). The use of $f_{/2}$ instead of *f* medium probably explain the lower growth rate compared to digestate, this trend not being observed in this study with *N.oceanica* strain. In general, higher growth rates were monitored, potentially due to the use of bottle photobioreactors instead of microplate, not optimal for mixing, lighting and pH control.

2. Growth in artificial produced water and artificial aquifer water supplemented with digestate

In order to valorise saline industrial effluents as part of culture medium for microalgae and cyanobacteria, culture tests were performed to determine the impact of such media on growth. As discussed earlier, only the microalgae *N.oceanica*, *P.costavermella*, *C.simplex* and the cyanobacterium *S.rubscens* showed interesting growth rates at 40 $g \cdot L^{-1}$ in medium with 5% v/v digestate. Growth rates measured for these microorganisms at 40 $g \cdot L^{-1}$ salinity are shown in Figure 2.

Figure 2: Growth rates of *N.oceanica*, *P.costavermella*, *C.simplex* and *S.rubscens* after 9 days of growth in artificial produced water or artificial aquifer water supplemented with 5% v/v digestate and seawater. Values correspond to mean ± standard deviation, n=12. Same letter above bars in microalgae histogram indicates no significant difference between the tests (p-value > 0.05).



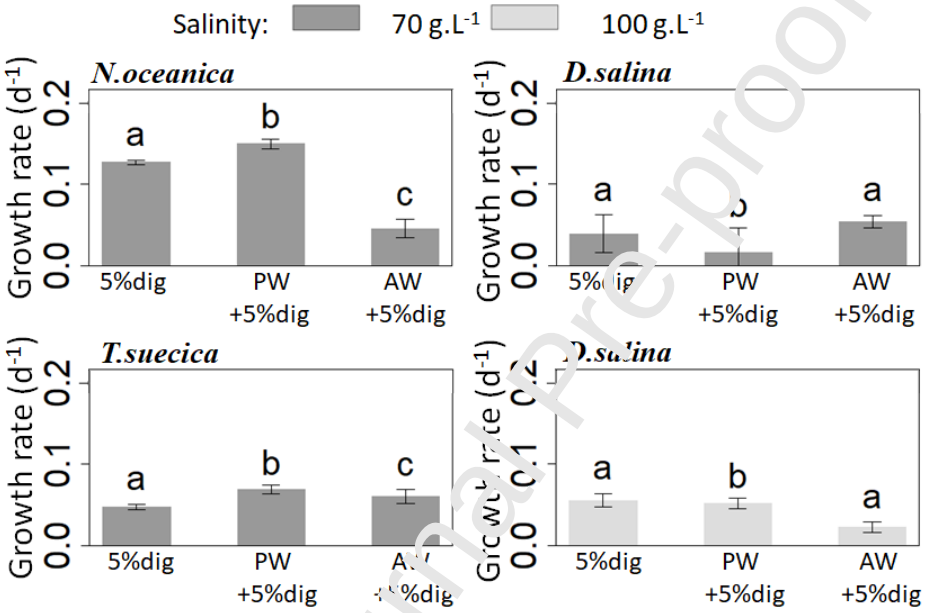
N.oceanica exhibited the highest growth rates (approximately 0.2 d^{-1}) among the tested strains, followed by *S.rubescens*, *P.costavermella* and *C.simplex*. Growth in aPW or aAW had different impacts on growth rates depending on the strain. More precisely, aPW had a little inhibition effect on growth rates of *N.oceanica* (-20%) whereas it had a significant stimulative effect on *P.costavermella* (+28%) and *C.simplex* (+63%). Effects of aAW were greater, with inhibition effect of -26% and -38% on *N.oceanica* and *S.rubescens*, respectively, and +96% stimulative effect on *P.costavermella*. Considering the growth rates measured, *N.oceanica* is the most promising strain at 40 $g \cdot L^{-1}$ of salinity.

Working at similar salinity (40 $g \cdot L^{-1}$), Racharaks *et al*. [15], and Parsy *et al*. [16] monitored growth rates of 0.3 d^{-1} cultivating *Nannochloropsis* sp. in underground water supplemented with liquid digestate, being similar to growth rates measured for *N.oceanica* in this study (approximately 0.2 d^{-1}). The higher values could be due to the photobioreactor used, as both

authors used 250-500 mL bottle reactors with mixing instead of static microplates in this study.

Only *D.salina*, *T.suecica* and *N.oceanica* were considered at higher salinities as other strains were not really adapted to these salinities. Results are shown in Figure 3.

Figure 3: Growth rates of *N.oceanica*, *D.salina* and *T.suecica* after 9 days of growth in artificial produced water or artificial aquifer water supplemented with 5% v/v digestate and seawater. Values correspond to mean ± standard deviation, n=12. Same letter above bars in microalgae histogram indicates no significant difference between the tests (p-value > 0.05).



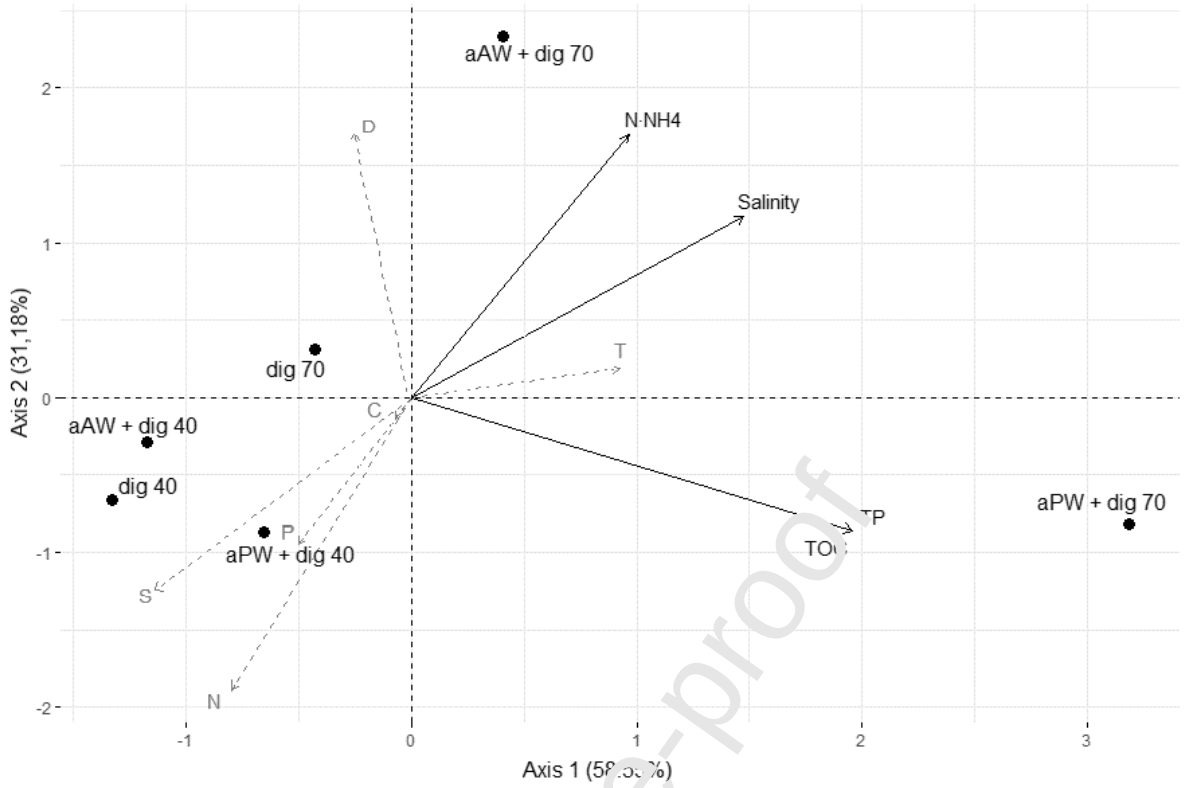
At 70 g·L^{-1}, aPW had a stimulation effect on growth rates of *N.oceanica* (+18%) and *T.suecica* (+46%). aAW had inhibition effects on growth rates of 64% for *N.oceanica*, but also a stimulation effect of 27% on *T.suecica*. *D.salina* was not impacted by the presence of aAW, however, growth was decreased by 59% with aPW. Considering the growth rates measured, *N.oceanica* is also the most promising strain at 70 g·L^{-1} of salinity, followed by *T.suecica*. *D.salina* showed lower growth rates than the two other strains. At 100 g·L^{-1} of salinity, only *D.salina* was able to grow. At this salinity, aPW had no toxic or stimulative impact, while aAW had a 60% inhibition effect.

Regarding the results for all salinities, growth with aPW was globally higher than in aAW. It can be hypothetically explained by the presence of phosphates in aPW (2 $mgP \cdot L^{-1}$) whereas there was no additional phosphorus in media with aAW. In addition, aPW add a small amount of organic carbon potentially used by microorganisms for mixotrophic growth. Previous work studied the effects of copper, iron, barium, chromium, zinc and arsenic with these six strains of microalgae and cyanobacteria [17]. However, no toxic effects were observed at aPW or aAW concentrations for these metals individually, so lower growth in aAW is not due to its higher concentration of metals than aPW (Table 1) except if cocktail effects occurred.

Working at 70 $g \cdot L^{-1}$ of salinity, Parsy *et al*. [16] did not observed growth cultivating *Nannochloropsis* sp. in PW and liquid digestate. The low growth rate monitored in this study could be explain by the different Nannochloropsis strain, as well as the use of aPW instead of real PW used by Parsy et al. [16] which had a more complex and potentially more toxic composition.

To visualize the influence of culture media parameters on growth rates measured, a PCA was performed and is presented in Figure 4. For the analysis, parameters such as salinity, TOC, NH_4^+ and PO_4^{3-} concentrations were considered. The growth rates of the different strains are also shown to represent how they correlate.

Figure 4: Principal component analysis summarising the information carried by the parameters of the culture media and the impact on the growth rate of the different selected strains. The solid arrows represent the variables, the dotted arrows represent the growth rates for each strain. Strains: D: *D.salina*; N: *N.oceanica*; T: *T.suecica*; P: *P.costavermella*; C: *C.simplex*; S: *S.rubescens*. Media parameters: TOC: Total organic concentration; N-NH4: ammonium concentration; TP: phosphate concentration; Salinity: TDS concentration. Each point represents a culture medium, in which growth rates were measured with 12 replicates for each strain.



Axes 1 and 2 carry 89.73% of the information in the initial dataset, with 58.55% and 31.18% for axis 1 and 2, respectively. Regarding the variables, phosphate and TOC concentrations are highly correlated. This result was expected since among the 6 media considered, all are composed with 5% v/v digestate and only those with aPW have an excess of phosphorus and TOC, since aAW did not contained any. NH_4^+ concentration and salinity are also highly correlated, as loads of aPW and aAW were increased to work at higher salinity, and both contained similar NH_4^+ concentrations. Groups of variables (salinity-NH_4^+ and TOC-PO_4^{3-}) were less correlated. As media at 40 g·L^{-1} contained less aPW or aAW, these media are grouped in the PCA. Media at 70 g·L^{-1} are split in two groups as they presented higher difference looking considered variables. Arrows reprensenting growth rates of strains *N.oceanica*, *P.costavermella* and *S.rubescens* are attracted to the group of media at 40 g·L^{-1} salinity, being coherent with results observed, while *T.suecica* and *D.salina* are more attracted to media at higher salinity. Growth of *C.coccomyxa* being very low, no information is given regarding the variables considered to perform the PCA. To obtain a performent microalgae

culture in all the different medium, it would be wise to select several species that point to the different PCA groups, such as *D.salina, T.suecica* and *N.oceanica* or *S.rubescens*.

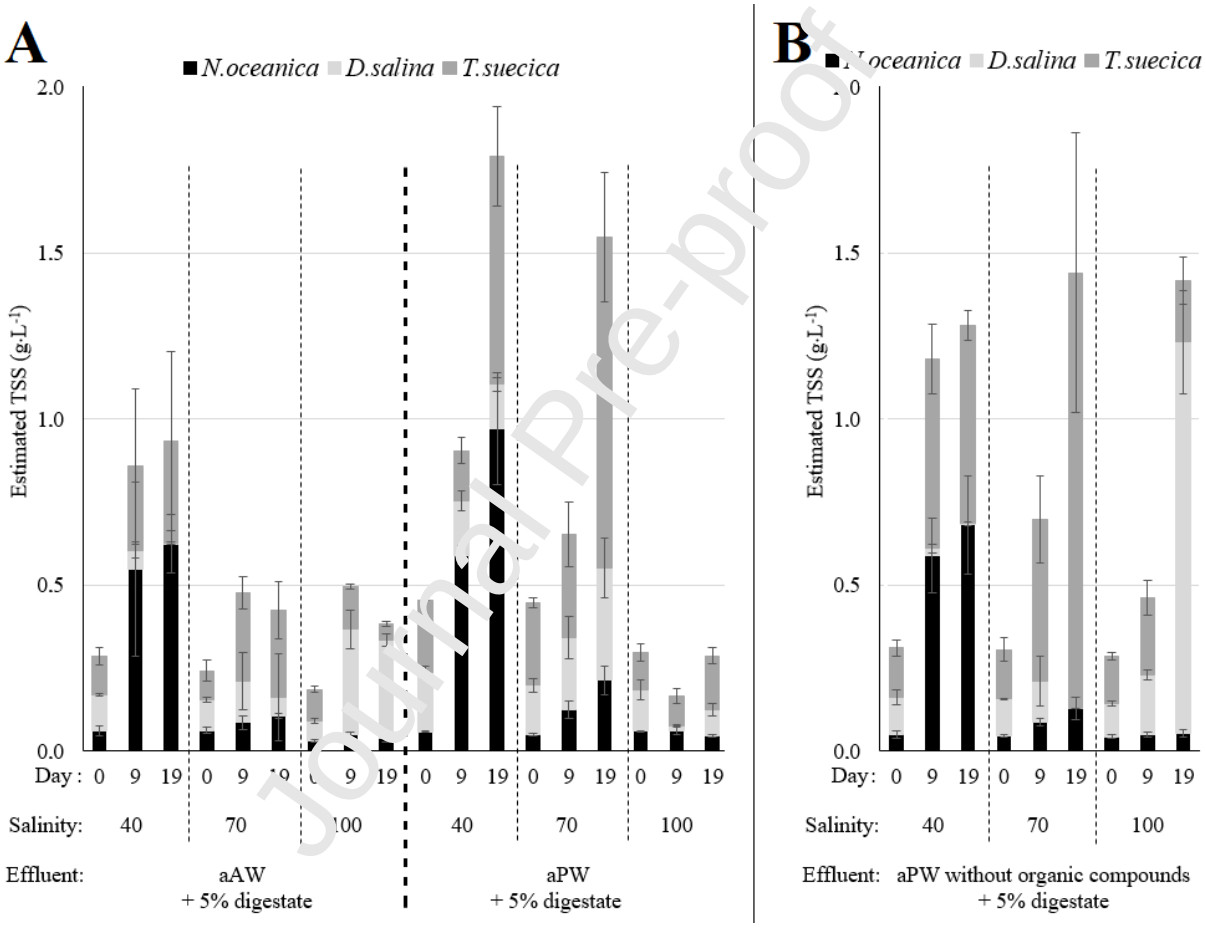
In this study, PCA was performed considering only 4 parameters. However, there is no limit to the number of parameters that can be included in the analysis, but too many can make the interpretation more difficult. PCA is a good visual representation to show the preferences of microalgae and cyanobacteria towards the culture media and to see the correlations that may exist between the different parameters. Nethertheless, it requires a large amount of data to be able to observe trends and derive information from them.

Considering all results obtained in each medium for the six strains, it appears that *N.oceanica* is the best strain for all the media tested at salinities of 40 and 70 $g \cdot L^{-1}$. *S.rubescens* showed equivalent growth rates at 40 $g \cdot L^{-1}$ of salinity, but *N.oculata* was more performant at 70 $g \cdot L^{-1}$ in terms of growth rates and productivity (data not shown), becoming more interesting than *S.rubescens*. As demonstrated in a previous study, growth rate of *N.oceanica* drop rapidly when salinity increases, making it poorly performing at salinities above 70 $g \cdot L^{-1}$ [17]. *T.suecica* is the second-best performing microorganism after *N.oceanica* at 70 $g \cdot L^{-1}$. *D.salina* is the only one able to grow at salinities of 100 $g \cdot L^{-1}$ and higher. Considering a process that would have an increasing salinity if an increasing quantity of PW or AW is used, working with a consortium of these 3 microalgae would ensure the growth of at least one microalga when conditions vary. *D.salina*, *N.oceanica* and *T.suecica* strains were therefore selected to perform mixed cultures at a larger scale and to follow the evolution of the population of the consortia at various salinities. The use of this consortium

3. Growth in supplemented saline effluents with microalgae consortium

Three microalgae *N.oceanica*, *T.suecica* and *D.salina* were grown in both aPW and aAW in higher scale photobioreactors. Estimated concentration of each strain in the consortia in various saline effluents are shown in Figure 5-A.

Figure 5: A: Concentration of *N.oceanica*, *D.salina* and *T.suecica* cultivated simultaneously in aPW and aAW supplemented with 5% v/v digestate. B: Concentration of *N.oceanica*, *D.salina* and *T.suecica* cultivated simultaneously in aPW without organic compounds supplemented with 5% v/v digestate. Values correspond to mean ± standard deviation, n=3.



Highest TSS_{theo} were obtained in aPW at 40 and 70 $g \cdot L^{-1}$ of salinity, with 1.8 and 1.5 $g \cdot L^{-1}$, respectively, after 19 days. Growth in aAW was lower with 0.9 $g \cdot L^{-1}$ reached at 40 $g \cdot L^{-1}$ of salinity. In the other conditions, final TSS_{theo} did not reached 0.5 $g \cdot L^{-1}$. Regarding results at 40 $g \cdot L^{-1}$ of salinity, *N.oceanica* became predominant (> 50 %) after 9 day and still was after 19 days. At the end of the batch, *T.suecica* represented between 33 and 47 % of the TSS_{theo}, showing it is an interesting strain also at this salinity when it is cultivated in microalgae

consortia. *D.salina* concentration did not increase after 19 days, demonstrating the impossibility to grow the strain in digestate at this salinity as shown in previous results. At 70 $g \cdot L^{-1}$ of salinity, *D.salina* was not able to grow and growth of *N.oceanica* was limited as concentration never exceed 25% of the total microalgae concentration. At this salinity, *T.suecica* became predominant in all the saline effluents, with more than 62% of TSS_{theo}. Finally, at 100 $g \cdot L^{-1}$ of salinity, *N.oceanica* and *T.suecica* were not able to grow. *D.salina* was the only strain that showed a significant but low growth in aAW.

As monitored with individual tests in microplates, growth with aPW was higher than in aAW, potentially explained by the presence of phosphates and organic carbon in aPW, and/or due to toxic cocktail effects due to aAW composition.

To determine if *D.salina* growth was limited in aPW because of the organic compounds, aPW without organic compounds was also investigated. Estimated concentration of each strain in the consortia in various saline effluents are shown in Figure 5-B. Similar results were obtained at 40 and 70 $g \cdot L^{-1}$ of salinity, with no growth of *D.salina*, similar TSS_{theo} of *N.oceanica* and *T.suecica* at 40 $g \cdot L^{-1}$, and a large predominance of *T.suecica* at 70 $g \cdot L^{-1}$ of salinity. In contrast to aPW, growth in aPW without organic compounds at 100 $g \cdot L^{-1}$ was high, with a final TSS_{theo} concentration of 1.4 $g \cdot L^{-1}$ and a predominance of *D.salina* (83%) after 19 days.

In the literature, only a few papers concerning the study of consortia cultivated in industrial wastewater are available [26]. However, no study was found concerning microalgae consortium growth using saline industrial wastewater. Moreover, the growth of consortia is often studied from a wastewater remediation point of view, mainly focusing on carbon, nitrogen and phosphorus removal. Cinq-Mars *et al*. [27], worked on the characterization of two microalgae consortia grown in industrial wastewater for biomass valorization. Wastewater was composed of a mix of various wastewaters: 45% v/v from a lactulose

producer, 41% v/v from a dairy product industry, 10% v/v from a cleaning product industry and 4% v/v from landfill leachate. Wastewater salinity was not mentioned. Authors identified the genus present in their two consortia after 50 days culture, acclimating the cells to their wastewater, and showed that both consortia had the same majority of eukaryotic species, a green alga of the Trebouxiophyceae class (phylum Chlorophyta), with 100 and 86% proportion in the green algae and cyanobacteria consortia, respectively. Concerning prokaryote species, the cyanobacteria consortia was dominated (60%) of a cyanobaetria of the Gomphosphariaceae family. Babatsouli *et al*. [28], studied a bacterial-microalgae consortia in a fixed-bed photobioreactor during a wastewater treatment. Authors used a consortium cultivated in seawater and bioaugmented with *Picochlorum* sp. cells. After 3 months of wastewater treatment (wastewater salinity of 0.2 – 1 $g \cdot L^{-1}$), a biofilm mainly composed of *Picochlorum* sp. and *Stichococcus* sp. was developed on the fixed-bed. Authors performed taxonomic analyses via pyrotag sequencing, to see bacteria, photosynthetic eukaryotes and fungi diversities. Both articles had a taxonomic approach to quantify diversity and understand the consortia composition. The taxonomic approach was not used in this study, the objective being to follow and quantify the growth of each microalga to better understand this cultivation process.

Regarding the results, a large range of salinities can be used to grow the consortia of microalgae *N.oceanica, T.suecica* and *D.salina*, as one strain always become predominant at a particular salinity (*N.oceanica* at 40 $g \cdot L^{-1}$, *T.suecica* at 70 $g \cdot L^{-1}$ and *D.salina* at 100 $g \cdot L^{-1}$), ensuring the production of biomass. It appears that mixed culture is a good solution to have a biomass production during a culture process where the culture media will evolve in terms of salinity (evaporation/raining/input variations) and composition (input variations). In addition to biomass production, the water treatment efficiency of the microalgae consortium has been investigated to determine the possibility to treat saline industrial effluents. It was shown that

these microorganisms were able to eliminate up to with 100%, 77%, and 99% of ammonium, chemical oxygen demand, and aromatic compounds, respectively, after 23 days (lab-scale, batch tests) [29].

Conclusion

This study investigated the growth of six halotolerant photosynthetic microorganisms in saline industrial effluents supplemented with liquid digestate. Among the tested strains, *N.oceanica*, *T.suecica* and *D.salina* showed the best growth performances, with growth rates up to 0.2 d^{-1} in the best conditions. These three strains were selected to make consortium cultures to study the evolution of their populations depending on the effluent and salinity used. Each strain became the majority depending on the salinity, whereas the type of industrial effluent had smaller impacts. *N.oceanica* was predominant at 20 $g \cdot L^{-1}$, *T.suecica* at 70 $g \cdot L^{-1}$ and *D.salina* at 100 $g \cdot L^{-1}$, ensuring the production of biomass in a cultivation process with varying salinity, due to evaporation or change of salinity in the effluents used. In the perspective of using these microalgae consortia in industrial saline effluents, additional tests should be carried, to verify the feasibility of such a process at higher scale in continuous or semi-continuous culture.

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Formal analysis (Application of statistical, mathematical, computational, or other formal techniques to analyze or synthesize study data)
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Declaration of interests

☒ 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.

Highlights:

- Liquid digestate was used as a source of nutrient to support microalgae growth in saline produced water and aquifer water.
- Six candidate strains were investigated at various saline effluent loads to identify the best performing ones.
- Depending on the salinity, *Nannochloropsis oceanica*, *Tetraselmis suecica* and *Dunaliella salina* had the best performances at 40, 70 or 100 g.L-1, respectively.
- *N.oceanica*, *T.suecica* and *D.salina* became predominant over the two others at salinity of 40, 70 or 100 g.L-1, respectively, during consortium culture.