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Effect of methanol, ethanol, DMSO and hexane on *Chlorella vulgaris* in the context of phytohormone delivery

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ABSTRACT

Phytohormones have the potential to improve microalgae culture productivity. Still, not every phytohormone is water-soluble, and most have to be dissolved in organic solvents before being delivered to the cells. Unfortunately, most studies neglect or do not report the effect of the solvent. This is a problem as the solvent itself can have an effect, and not accounting for it would lead to confounding and potentially erroneous interpretations. Consequently, this work evaluates the effects of classical solvents (methanol, ethanol, DMSO, and hexane) in the context of phytohormone delivery. Thus, *Chlorella vulgaris* was exposed to different dosages of those solvents. The cultures were monitored for three days using optical density to evaluate growth, the OJIP test to assess photosynthetic apparatus status, and flow cytometry to measure viability. Individual outputs and integrated biomarkers were used to analyze the results. Overall, ethanol triggers cell flocculation for the tested concentrations (as low as 0.1%vol) and lysis above 0.5%vol. Methanol also led to flocculation above 0.5%vol, but started to impair viability from 0.1%vol on. DMSO and hexane appear as viable solvents, with no deviation from control for 0.25 and 0.1%vol dosage. Their dosages can be increased to 1%vol while still claiming admissibly perturbed cultures, as absolute values remain in line with control, but dispersion increases. Finally, a validation run was carried out. DMSO-delivered salicylic acid was used to modify *Chlorella vulgaris* lutein production. Results confirmed the efficiency of salicylic acid and the safety of DMSO at the chosen dosage.

1. Introduction

Over the past decades, microalgae have emerged as a promising platform for biomolecule production. Their envisioned applications range from quality food/feed (Koyande et al., 2019; Madeira et al., 2017) to high-value compounds (Levasseur et al., 2020), with possible valorization as biofuel from extraction process leftovers (Enamala et al., 2018). Among the targeted high-value biomolecules lie pigments, which can be used as food dye or potent antioxidants. Their flagship is astaxanthin, with historical applications in aquaculture and potential in the nutraceutical industry (Higuera-Ciapara et al., 2006). Still, other carotenoids ought to be acknowledged. For example, lutein has demonstrated benefits on human health, but is present in our diet in too small amounts to leverage the full extent of its benefits. This state of fact leaves room for microalgae-sourced supplementation, as the conventional lutein supplements are not produced sustainably (Camarena-Bernard and Pozzobon, 2024; Buscemi et al., 2018).

Still, producing these molecules using microalgal biotechnological means is expensive. Consequently, scholars and engineers have explored different strategies to reduce production costs. For example, combining heterotrophy and phototrophy sequentially, through a process called trophic conversion (Levasseur and Pozzobon, 2025), allows improving overall process productivity for astaxanthin (Hata et al., 2001; Wan et al., 2015) and lutein (Flórez-Miranda et al., 2017; Camarena-Bernard et al., 2024). The underlying concept is quite simple: heterotrophy is used to produce a massive quantity of low quality microalgae (low pigment, low protein content), while phototrophy subsequently drives them to express the targeted metabolites. Still, this strategy requires both fermenters and large-scale photobioreactors as a dilution might be required to allow heterotrophic cells sufficient light access (Barros et al., 2019).

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Table 1
Literature survey of the molecules used as phytohormones and associated solvents in the context of lutein production.

Species	Phytohormone	Concentration	Solvent			Ref.
			Ethanol	DMSO	Water	
Acutodesmus sp.	Melatonin (MT)	2.5-10 µM	X			Raman and Ravi (2011)
Botryococcus braunii	6-benzylaminopurine (6-BA)	0.01-5 mg/L			X	Du et al. (2020)
	Abscisic acid (ABA)	0.1-50 mg/L			X	
	2,4-epibrassinolide (EBR)	0.001-1 mg/L			X	
	Ethephon (ETH)	0.005-0.2 mg/L			X	
	Gibberellic acid (GA)	0.1-20 mg/L			X	
	1-naphtaleneacetic acid (NAA)	1-50 mg/L			X	
	Salicylic acid (SA)	0.1-50 mg/L			X	
	Spermidine (SPD)	0.1-50 mg/L			X	
Chlorella vulgaris	3-indoleacetic acid (IAA)	0.1 µM	X			Piotrowska-Niczyporuk and Bajguz (2014)
	Indole-3-butyric acid (IBA)	0.1 µM	X			
	Phenylacetic acid (PAA)	1 µM	X			
	1-naphtaleneacetic acid (NAA)	1 µM	X			
Chromochloris zofingiensis	1-aminocyclopropane-1-carboxylic acid (ACC)	5 mg/L			X	Chen et al. (2020)
	2,4-Dichlorophenoxy acetic acid (24D)	5 mg/L		X		
	2-Chlorobenzoic acid (CA)	5 mg/L	X			
	Gibberellic Acid (GA)	6.9 mg/L	X			
	Indole-3-acetic acid (IAA)	7.8 mg/L		X		
	Indole-3-butyric acid (IBA)	9.9 mg/L		X		
	Indole-3-propionic acid (IPA)	10 mg/L	X			
	Abscisic acid (ABA)	10 mg/L	X			
	Gibberellic Acid (GA)	5-50 mg/L	X			
	3-indoleacetic acid (IAA)	15-250 mg/L	X			Chen et al. (2022)
	Indole-3-propionic acid (IPA)	10-240 mg/L	X			
	Indole-3-butyric acid (IBA)	10-130 mg/L	X			
	1-naphtaleneacetic acid (NAA)	10-60 mg/L	X			
	1-aminocyclopropane-1-carboxylic acid (ACC)	5-40 mg/L			X	
Desmodesmus F51	Salicylic acid (SA)	100-500 µM			X	Ahmed et al. (2019)
Dunaliella salina mut tyd4	Abscisic acid (ABA)	0.1-10 µM	X			Liu et al. (2025)
	Gibberellic Acid (GA)	0.1-10 µM	X			
	Melatonin (MT)	0.1-10 µM	X			
Haematococcus pluvialis	Salicylic acid (SA)	10-500 µM	X			Kozlova et al. (2017)
	Methyl jasmonate (MJ)	10-500 µM		X		Raman and Ravi (2011)
Scenedesmus quadricauda	Epibrassinolide (EBL)	0.5-100 nM		X		Kozlova et al. (2017)
	Brassinolide (BL)	0.5-300 nM		X		
	3-indoleacetic acid (IAA)	1 nM-1µM		X		
	Abscisic acid (ABA)	5-500 µM	X			
	24-epibrassinolide (EBL)	0.5-2 nM		X		Kozlova et al. (2018)
	3-indoleacetic acid (IAA)	5-1000 nM		X		

In this context, the use of phytohormones appears as a strategy to increase productivity while avoiding the trouble and the capital investment required to manage the two trophic modes. The term *Phytohormone* is a broad umbrella that encompasses various organic molecules used as metabolic signalers. Owing to their properties, a small quantity can greatly affect the cells' metabolism. Of course, such a strategy requires fine-tuning, mostly by screening the different compounds at different concentrations (Chen et al., 2022; Kozlova et al., 2017). Nevertheless, successes can be underlined. For example, methyl jasmonate has been shown to increase biomass and carotenoid production in *Haematococcus pluvialis* - synonym *H. lacustris* (Nakada and Ota, 2016) - (+4.6% biomass, +46.4% total carotenoids, +17.7% lutein, and +15.9% astaxanthin Raman and Ravi (2011)), as well as lutein in *Brachiomonas submarina* and *Kirchneriella aperta* (+115.9%, +37.6% (Mc Gee et al., 2020)). Similarly, abscisic acid led to a higher biomass and carotenoid production in *Scenedesmus quadricauda* (+67.4% biomass, +22.5%, respectively (Kozlova et al., 2017)), or +50.0% lutein content in *Chromochloris zofingiensis* (Chen et al., 2020). Many more phytohormones could be reviewed, but for the sake of concision they will not be exemplified further.

However, from a scientific perspective, the use of phytohormones raises questions, especially when efficient screening and mechanistic explanations are sought after. Indeed, most of those molecules are not water-soluble and have to be delivered using an organic solvent. Table 1 offers an oversight of the phytohormones used to improve carotenoid production. As one can see, 10 out of 19 molecules are not soluble in water. For example, 3-indoleacetic acid (IAA) is poorly soluble in water but is greatly soluble in ethanol. Epibrassinolide (EBL) is hydrophobic but classically delivered in DiMethyl SulfOxide (DMSO).

As these phytohormones have to be delivered using a solvent, one could wonder whether the experimenter is observing the effect of the phytohormone alone, the solvent alone, or a combination of both. Indeed, the ecotoxicology community has shown for a long time that organic solvents affect microalgal cells. This community focused on the impact of pollution on ecosystems, from low-level contamination to industrial spillages. Despite being fragmented (compound-wise, dosage-wise, and strain-wise), some general trends have been obtained by reviewing the field (Miazek et al., 2017). First, methanol and ethanol show a dual response: at low concentrations, they can promote growth, while they inhibit at higher concentrations. Aromatic solvents (*e.g.*, benzene) also exhibit mixed effects, from stimulatory to inhibitory (or simply no effect at all). Furthermore, glycol solvents (*e.g.*, propylene glycol), cyclic solvents (*e.g.*, cyclohexane, tetrahydrofuran), chlorinated solvents (*e.g.*, trichloromethane), and ionic liquids (*e.g.*, tetrabutylphosphonium) mostly have an inhibitory effect. While Miazek et al. survey shows that there are numerous possibilities regarding which molecules to study, this work will focus on the solvents most frequently encountered in microalgal biotechnology: methanol, ethanol, and DMSO. While not encountered in studies focusing on carotenoids, methanol was added to the list as it is used as a phytohormone carrier in other

Table 2
Tested solvents and volume fractions.

Fraction	Solvent fraction (%vol)			
	Ethanol	Methanol	DMSO	Hexane
Low	0.1	0.1	0.25	0.1
Medium	0.5	0.5	1	1
High	1	1	3	5

fields of microalgal biotechnology (Cruz et al., 2023). In the same sense, and to extend the applicability of this work, hexane will also be added to the list. Indeed, it is a non-polar solvent, contrary to the three former ones. Hence, its evaluation may open new doors for chemical compound delivery to the cells.

Having chosen the molecules to work with, previous work evaluating the effects of the picked solvents have to be acknowledged. For example, in pioneering work, ethanol was shown to be a possible substrate for *Chlorella* growth (Wardas et al., 1983). The authors cultivated the strain under illuminated and dark conditions with ethanol content from 0.058 to 0.18%vol. After 12 to 16 days, they observed a +31 to 167% increase in cell density in the presence of ethanol compared to control. In addition, no lag phase was evidenced. These observations underline the hormetic effect of ethanol: stimulatory at low dosage and inhibitory above. The inhibitory counterpart is species dependent, as demonstrated by Okumura et al. who exposed nine marine microalgae species to different solvents (Okumura et al., 2001). Ethanol EC_{50} (on growth) varies from 4.6 10^{-3} to 2.4%vol, a three order of magnitude span. In addition, the authors reported that methanol was less toxic to microalgae than ethanol, with EC_{50} ranging from 1.8 10^{-2} to 3.5%vol for the same strains. This higher bound was also evidenced for *Raphidocelis subcapitata* (f.k.a. *Selenastrum capricornutum* or *Pseudokirchneriella subcapitata* (Machado and Soares, 2024)) (Cho et al., 2008b,a). Moving from continuous to acute exposure tests, one can note the work of Cañavate and Lubian, who investigated methanol and DMSO as potential cryoprotectants (Cañavate and Lubian, 1994). They exposed six different species to concentrations ranging from 5 to 30%vol with exposure duration from 1 min to 4 h. The conclusions are, of course, dependent on species, concentration, and exposure time. Nevertheless, a general trend can be observed: a higher methanol concentration over a longer time is more harmful to the cells. However, the author evidences that methanol is also capable of hormesis up to 15%vol with the strain *Chaetoceros gracilis*.

Moving from alcohol to other solvents, DMSO comes next. Contrary to methanol and ethanol, DMSO EC_{50} range is narrower (0.35 to 2.1%vol) (Okumura et al., 2001). Still, even when no effect on growth nor chlorophyll content can be observed, DMSO can alter lipids (higher malondialdehyde - MDA, a product of lipid peroxidation - observed in *Euglena gracilis*, yet at an unspecified DMSO level) (Li et al., 2009), and damage cell membranes (Gallardo-Rodríguez et al., 2012). In the case of acute exposition to DMSO (5 to 30%vol), a trend similar to the methanol one can be observed: a higher dosage and a longer exposure induce more important damages (Cañavate and Lubian, 1994). Still, in this context, DMSO is much better handled by the cells than methanol, with about half higher dosage tolerance.

Works dealing with hexane are few. Yet, Wardas et al. tested its addition to a *Chlorella* culture (phototrophy, hexane at 2%vol) (Wardas et al., 1983). Compared to control, they observed a 1 to 2 days lag phase and a 30 to 40% lower final cell density.

While insightful, the aforementioned studies mainly focused on cell growth. While, of course, crucial for a biotechnological process, it only partially reflects a potential solvent's side effect. Indeed, microalgae status can be characterized on many more dimensions, such as pigment and lipid profiles, photosynthesis efficiency,... The later is of particular interest as microalgae photosynthetic apparatus is known to be quite sensitive to perturbation. In some cases, growth can appear unaltered while photosynthesis-related readings (fast chlorophyll fluorescence, a.k.a. OJIP, assays) are affected (Liu et al., 2020; Luo et al., 2019). This observation can even be exploited to create a fast, short-term photosynthesis-based pollution sensor (Cho et al., 2008b). Consequently, the present investigation focuses on three levels of analysis to quantify the effect of solvent addition to a culture in the context of phytohormone delivery. The first one is overall growth, the second is photosynthetic apparatus status (probed using an OJIP test), and the last one is cell viability. The methodology will be applied to *Chlorella vulgaris*, as it is a model strain widespread in both academic research and industrial biotechnology. Finally, the tested solvents will be methanol, ethanol, DMSO, and hexane.

2. Materials and methods

2.1. Strain, culture medium & maintenance

Chlorella vulgaris CV 211-11b, bought from SAG Culture Collection, Germany, was chosen for this study. Bold's Basal Medium (Andersen and Phycological Society of America, 2005) with tripled nitrogen content (classically referred to as B3N) was used throughout this study. Mother culture cells were cultivated in continuous mode in ultra-thin flat panel photobioreactors (6 mm thickness, 125 mL working volume, 1.8 vvm, 25 °C) under very diluted conditions (OD at 750 nm below 0.1), as detailed elsewhere (Pozzobon, 2022). Briefly, the objective was to expose the cell to constant and uniform illumination under nutrient-replete conditions. Inline optical density was used as the signal-controlling automated dilution, *i.e.*, turbidostat mode of operation. This way, it was possible to ensure consistent inoculum physiological status for all the runs. Finally, the incident illumination was set at 45 μmolPhotonPAR/m^2/s, the sparged gas was air, and the system was operated in triplicate to produce 360 mL of fresh culture upon withdrawal.

2.2. Test procedure & tested conditions

Chlorella vulgaris cultures were harvested from the photobioreactors at the beginning of the week. The cells and the medium were unaltered and dispatched into 250 mL flasks. Each flask was filled with 24 mL of culture and the tested solvent (absolute grade, source from Fisher Scientific) to the desired concentrations (see below). Then, the flasks were placed into an orbital shaker. The culture conditions were extremely close to the ones of the mother culture, *i.e.*, 45 μmolPhotonPAR/m^2/s under air, at room temperature. This procedure was chosen to minimize the source of possible stress between inoculum preparation and test runs. For example, harvesting cells by centrifugation could have induced damages (Pozzobon et al., 2024). Similarly, medium transfer, or even modification, could have led to an osmotic stress (Los and Murata, 2004), potentially altering membrane fluidity (Pozzobon et al., 2025). Furthermore, using a very dilute inoculum ensures that regular, nutrient-replete growth could unfold upon placement in the orbital shaker. The cultures were monitored twice a day over three days and a half.

The volume of the solvent addition depends upon the targeted concentrations. The remaining volume to reach 25 mL within the flask was added using milli-Q water. Table 2 presents the different tested solvent fractions for the investigated compounds. These concentrations were determined based on our laboratory practices, literature (Silva et al., 2009), and on the will to determine a *safe* (*i.e.*, not affecting the cell) range for each solvent. Furthermore, preliminary microplate cultures were led to assess the tentative validity of the selected range (data not shown). All the tests were carried out in biological triplicate, with triplicated control (1 mL milli-Q water addition), resulting in 12 flasks operating simultaneously, in the same orbital shaker to minimize potential sources of deviation.

2.3. Analytical procedures

Twice daily, a 3-mL sample was withdrawn from the flask. Upon removal from the orbital shaker, each flask was weighted to evaluate evaporation. The sample was then used for optical density measurement to acquire photosynthetic apparatus status and cell viability.

Optical density measurements were carried out at 680 and 750 nm (Shimadzu UV-1900). Reading obtained at 750 nm were used as a proxy of cell concentration (Griffiths et al., 2011). Samples featuring a value above 0.5 were diluted so that they fell within the linearity range (below 0.7 with this apparatus and this strain). At the end of the run, the optical density reading of each flask was treated individually (*i.e.*, 12 growth signals in total per run), corrected by evaporation contribution, and used to determine cell growth rate. To do so, the slope of the linear portion signal in a semi-log plot (about 3 days, *i.e.*, 6 points) was extracted using the ordinary least square method.

OJIP tests were led using FL 6000 Fluorometer (PSI). Prior to being exposed to saturating illumination, the samples were placed in a dark enclosure for 15 min (dark adaption). The test lasted 5 s in total, with a saturating pulse intensity of 3500 μmolPhotonPAR/m^2/s (far above the recommended 10 times the cultivation light intensity). The obtained signals were first checked for their overall quality. Indeed, in some cases where cells died, readings turned out to be noisy and trendless. Afterwards, signals were processed using Strasser et al. guidelines (Strasser et al., 2000). This procedure yields many indicators. Here, it was chosen to focus on the functional state of the PSII. Consequently, Φ_0 (*i.e.*, energy transfer from the antennae to the reaction center, also referred to as Fv/Fm) and ψ_0 (*i.e.*, the ability to use the channel energy to recover an electron) will be the flagships for the discussion.

Finally, once gone through optical densities measurements and the OJIP assay, the sample was divided in two for flow cytometry analysis (BD LSR Fortessa X-20 flow cytometer, mounted with blue - 488 nm - and yellow-green - 561 nm - lasers). The first part of the sample was used to assess cell viability. To do so, cells were dual-stained using Propidium Iodide (in short PI) and Fluoresceine DiAcetate (in short FDA) simultaneously. The staining protocol was adapted from a previously described method (Pozzobon et al., 2020). In a nutshell, FDA identifies metabolically active cells based on intracellular esterase activity, while PI selectively stains non-viable cells (via DNA binding) with compromised membrane integrity. From a practical standpoint, 200 μL aliquot of the culture was mixed with 800 μL of FDA (at 120 μM, in 1%vol DMSO) and 10 μL of PI (at 1.5 mM, in water). The stained cells and the second part of the sample (not stained cells) were incubated together in the dark for 15 min before being presented to the analyzer. Between each sample analysis, the flow cytometer was rinsed with a tube of Milli-Q water to prevent cross-contamination. For each sample, 30,000 events were recorded.

For both stained and pristine *Chlorella vulgaris* cells, five signals were recorded: Forward SCatter (FSC) as a proxy for cell size, Side SCatter (SSC) for cell complexity, and chlorophyll autofluorescence (blue laser, channel 695/40 nm detection), FDA fluorescence (blue laser, channel 530/30 nm), and PI fluorescence (yellow-green laser, channel 610/20 nm).

2.4. Validation run

Once a suitable solvent and dosage have been identified (DMSO at 0.25%vol - see below), a validation run was carried out. For this test, *Chlorella vulgaris* cells were grown in contact with salicylic acid (500 μM final concentration) for 10 days. Salicylic acid was chosen as its effect on *Chlorella vulgaris* is less documented than on other strains. This allows for the incidental use of the validation run to bring original data. The culture conditions were the same as those previously described. In addition to the test runs, two controls were carried out: cells in B3N medium alone and cells in B3N medium with DMSO at 0.25%vol but no phytohormone. The three conditions were triplicated, leading to nine flasks being monitored once a day by optical density measurement.

At the end of the culture, cells were harvested and washed twice by centrifugation (4 °C, 11000 rpm, 10 min). Biomass was then frozen and freeze-dried (1-day primary drying, 1-day secondary drying, Christ alpha 1-2 LD +). Biomass powder was stored in the dark at -20 °C before being used for pigment composition assay. For this assay, about 1 mg of freeze-dried microalgae powder was homogenized in 5 mL of pure methanol using the MP Biomedicals FastPrep42 bead beater. The suspension was cooked for 20 min at 60 °C (shaded from light) (Porra, 1990). The liquid was then filtered (0.22 μm) and immediately presented to a spectrophotometer (Shimadzu UV-1900, dual beam, quartz cuvettes). Spectra were obtained from 340 to 750 nm (1 nm resolution), preprocessed following Lichtenthaler's guidelines (Lichtenthaler and Buschmann, 2001), before being presented to Pozzobon and Camarena's machine learning algorithm (based on partial least squares) (Pozzobon and Camarena-Bernard, 2022), which determined chlorophyll a, chlorophyll b, lutein, violaxanthin, and zeaxanthin concentrations in the extract.

2.5. Data processing & statistical analysis

Unless stated otherwise, data are presented as mean ± standard deviation of the three replicates.

When processing flow cytometry reading, three cell populations emerged: alive (FDA-positive and PI-negative), dead (FDA-negative and PI-positive), and a third one referred to as *sluggish* (FDA-negative and PI-negative). This third population was not encountered to a sizable extent over the course of our previous investigations involving flow cytometry. We hypothesize that they correspond to cells having low to no esterase activity, but uncompromised DNA. When evaluating overall viability, they were not counted as viable cells.

As the tested solvents were not evaluated simultaneously, integrated biomarkers approach was used to compare them. This method was developed to compare multidimensional biological samples exposed to different conditions, produced at different places or different times, might be challenging (Beliaeff and Burgeot, 2002). In a nutshell, they allow for scaling the different dimensions characterizing a sample (S, Eq. (1)) and aggregating them into a score (IBM, Eq. (4)). The only requirement is that all the individual dimensions are oriented in the same manner (*e.g.*, the higher, the better). In this work, the integrated biomarkers comprised: growth rate, final OD 680/750 nm ratio, Φ_0, ψ_0, and viability.

$$S_i = \frac{s_i - \hat{s_i}}{\sigma_{s_i}} - (min(\frac{s_i - \hat{s_i}}{\sigma_{s_i}}) < 0)(min(\frac{s_i - \hat{s_i}}{\sigma_{s_i}})) \quad (1)$$

$$A_i = \frac{S_i}{2} sin\beta(S_i cos\beta + S_{i+1} s_i \beta) \quad (2)$$

$$\beta = Arctan(\frac{S_{i+1} sin\alpha}{S_i - S_{i+1} cos\alpha}) \quad (3)$$

$$IBM = \sum_{i=1}^{n} A_i \quad (4)$$

Whence s_i are the raw measurement on dimension i, $\hat{s_i}$ their average, σ_{s_i} their standard deviation, α is $\frac{2\pi}{n}$, n the number of dimensions (5 here).

Finally, statistical significance is assessed using ANOVA testing (two-tailed, post-hoc Honestly Significant Difference with Bonferroni correction, significance level $p < 0.05$) or Welch's (unpaired) t-test (for pairwise comparison, two-tailed, $p < 0.05$). The tests were performed using the *statsmodels* Python package (Seabold and Perktold, 2010).

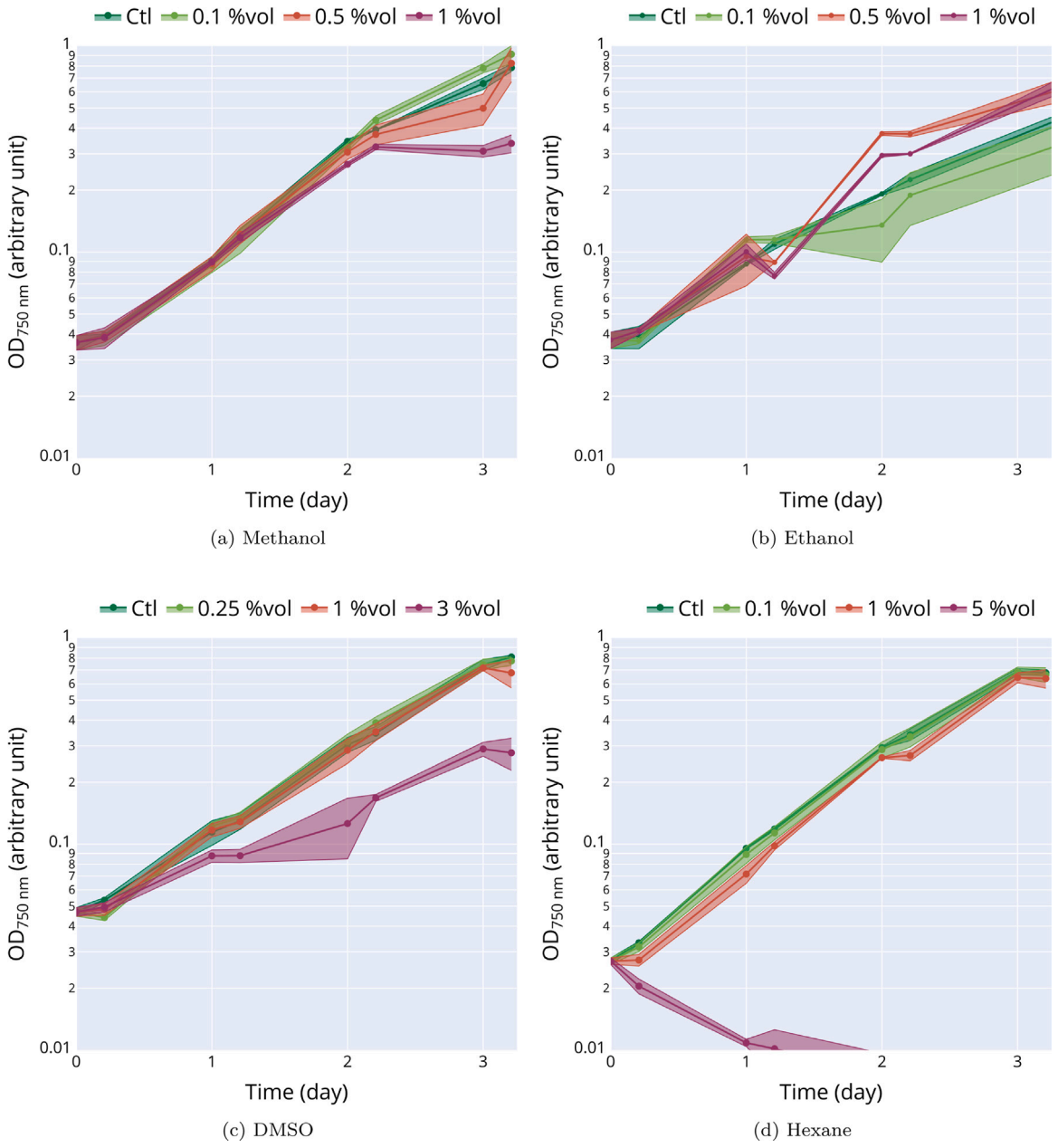


Fig. 1. Growth dynamics of *Chlorella vulgaris* exposed to different solvents. Solid line - mean of the three replicates. Shaded area - standard deviation (n=3). Points - measurements.

3. Results & discussion

3.1. General comment

Before diving into quantitative indicators analysis, some qualitative comments are to be made. First, methanol and ethanol additions led to flocculation for all the tested conditions (except for the lowest methanol dosage), while their respective control runs exhibited no such behavior. Then, the 5%vol hexane cultures crashed. Contamination was kept at a marginal level, except for ethanol runs, which saw a surge of bacteria. Those experiments were repeated and led to the same results. Based on these observations, one can rule out the medium and high dosages of methanol (flocculation), the high dosage of hexane (crash), and ethanol as a whole (flocculation).

3.2. Cell growth

Fig. 1 presents the growth dynamics of *Chlorella vulgaris* for the four tested solvents, with three dosages and control for each. As one can see, throughout the run, the control cultures (dark green line on the graph) were able to grow exponentially (graphs in logarithmic scale), after a 5-hour lag phase. Solvent addition induced behaviors that were solvent and dosage-dependent. Among these behaviors, it is likely that the flocculation observed for methanol and ethanol perturbed the optical density measurements. They will nevertheless be reported and discussed for the sake of completeness.

Methanol addition led to a deviation in terms of overall dynamics for the highest dosage (1%vol) after two days of culture. The time-dependency of solvent effect was previously encountered by Cho's team, who reported deviation from control being noticeable on growth

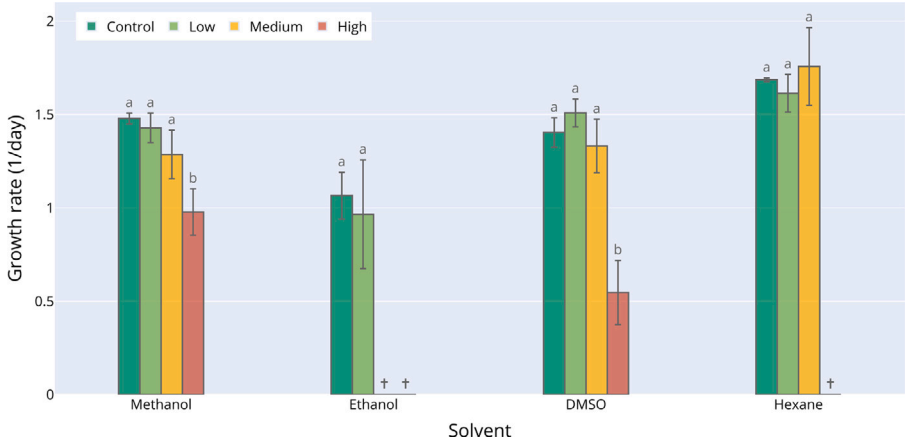


Fig. 2. Growth rate of *Chlorella vulgaris* exposed to different solvents. Cross - bacterial growth or negative growth rate. Bar - mean of the three replicates. Error bar - standard deviation (n=3)

after 72 to 96 h (Cho et al., 2008a). On the contrary, the three other solvents did not exhibit a temporal shift and deviated from the start when doing so. For example, DMSO 3%vol exhibited a markedly lower cell proliferation than the control from the beginning. The same goes for hexane 5%vol, which saw a consistently decreasing cell population. Overall, the highest dosages systematically showed a sizable deviation from control. Furthermore, this deviation was always negative, except for ethanol. Still, in the case of ethanol, microscope observations revealed the complete disappearance of microalgae in favor of alleged ethanol-metabolizing bacteria.

Moving from qualitative to quantitative observations, it is possible to compute the growth rate for each run. These results are presented in Fig. 2. On one side, increasing methanol concentration led to a progressive decrease in growth rate (from 1.48 ± 0.03 1/day for control to 0.98 ± 0.12 for 1%vol). Yet, it only becomes statistically significant from 1%vol on. On the other, DMSO and hexane exhibited a relatively stable growth rate for control, low, and medium dosages (min *p*-value of 0.364 for DMSO and 0.472 for hexane, respectively). Fig. 2 also reveals that controls are not strictly comparable between experiments. Indeed, their growth rates span from 1.06 ± 0.12 1/day for the ethanol runs to 1.68 ± 0.01 1/day for the hexane ones. This can be explained by the fact that this work was led over the course of fluctuating extreme weather conditions, including a heat wave. Consequently, despite a usually reasonable level of temperature control in our facility, the room temperature evolved to a point that impacted the experiments. Still, consideration being relative to control, the effect of this deviation can be deemed marginal. In addition, the integrated biomarkers approach will allow managing the discrepancies between the controls (see below).

Finally, growth dynamics and growth rates might be the only indicators that can be compared to the literature. First of all, methanol- and ethanol-induced flocculation were not reported previously. So it might be a variety-specific behavior. Then methanol tolerance up to 1%vol falls within the documented range (1.8 10^{-2} to 3.5 %vol (Okumura et al., 2001)). On the contrary, our results suggest a high sensitivity to ethanol of *Chlorella vulgaris* CV 211-11b, with only 0.1%vol dosage allowing for almost regular growth. This observation contrasts with the previous ones, which underlined a hormesis effect (tested range: 0.058 to 0.18 %vol (Wardas et al., 1983)). Furthermore, one should note that in the case of Wardas et al. work, manual cell counting was used. Hence, it can be stated with a high degree of confidence that flocculation or excessive bacterial presence would have been noticed and reported if any. Another element pointing towards a strain-specific effect is the report of Wu et al. who showed that marine *Chlorella* sp. MEM17 was able to tolerate high fractions of ethanol, with an EC_{50} of 6 %vol (Wu et al., 2024). Moving to DMSO, observing regular growth up to 1%vol is in line with other authors' reports (EC_{50} ranging from 0.35 to 2.1 %vol) (Okumura et al., 2001). Finally, 2%vol hexane was previously shown to alter cell growth dynamics (Wardas et al., 1983), which aligns with our finding here (no deviation at 1%vol, no growth at 5% vol).

Overall, based on growth monitoring, one can add the highest dosage of DMSO to the exclusion list.

3.3. Photosynthetic apparatus status

Numerous indicators can be extracted from an OJIP test. Here, Φ_0 (*i.e.*, energy transfer from the antennae to the reaction center, also referred to as Fv/Fm) and ψ_0 (*i.e.*, the ability to use the channel energy to recover an electron) were chosen for their ease of interpretation (Fig. Fig. 3). The first general comment is that both indicators follow the same trend, with ψ_0 being more sensitive than Φ_0. Then, it can also be noted that runs exhibiting lower growth feature altered signals. This is not surprising, as photosynthesis is the driver of cell proliferation in this work. Still, the keen observer may notice some subtle details. For example, while 0.5%vol methanol would appear as exhibiting a growth similar to the control (p = 0.118), its Φ_0 and ψ_0 signal markedly differ from the control ones on day 3. Also, high doses of DMSO induce somewhat lower quantum yields, which are reflected in the growth rate. Finally, high doses of hexane irreparably damage the photosystems, with extremely low OJIP readings after 5 h of exposure, and flat signals afterwards. On the contrary, low and medium dosages followed the same steady trend as the controls for both Φ_0 and ψ_0 indicators. From an operational point of view, one can note that resorting to OJIP assays allows reaching this conclusion after 5 h of exposure, while growth rate measurement required a few days.

Finally, the reading obtained from ethanol-exposed cultures aligns with the growth rate and qualitative observations. Indeed, for the two highest ethanol dosages, the OJIP signals rapidly flattened, which aligns with bacteria overwhelming the microalgae population. Here, the only relevant results is the recovery dynamics of the low ethanol dosage cultures, which required two days to come back to nominal performances.

Diving further into the observed signals, it can be noted that the spread of the replicates increases with the dosage. Consistently, the low dosage had a spread equal to that of the control (excluding ethanol), while the medium dosage induced a somewhat higher variation. This observation is of interest when designing an experiment. Indeed, while inducing variability is not as problematic as inducing a bias, it might still be worth considering, especially when statistical testing is intended (see Fig. 3).

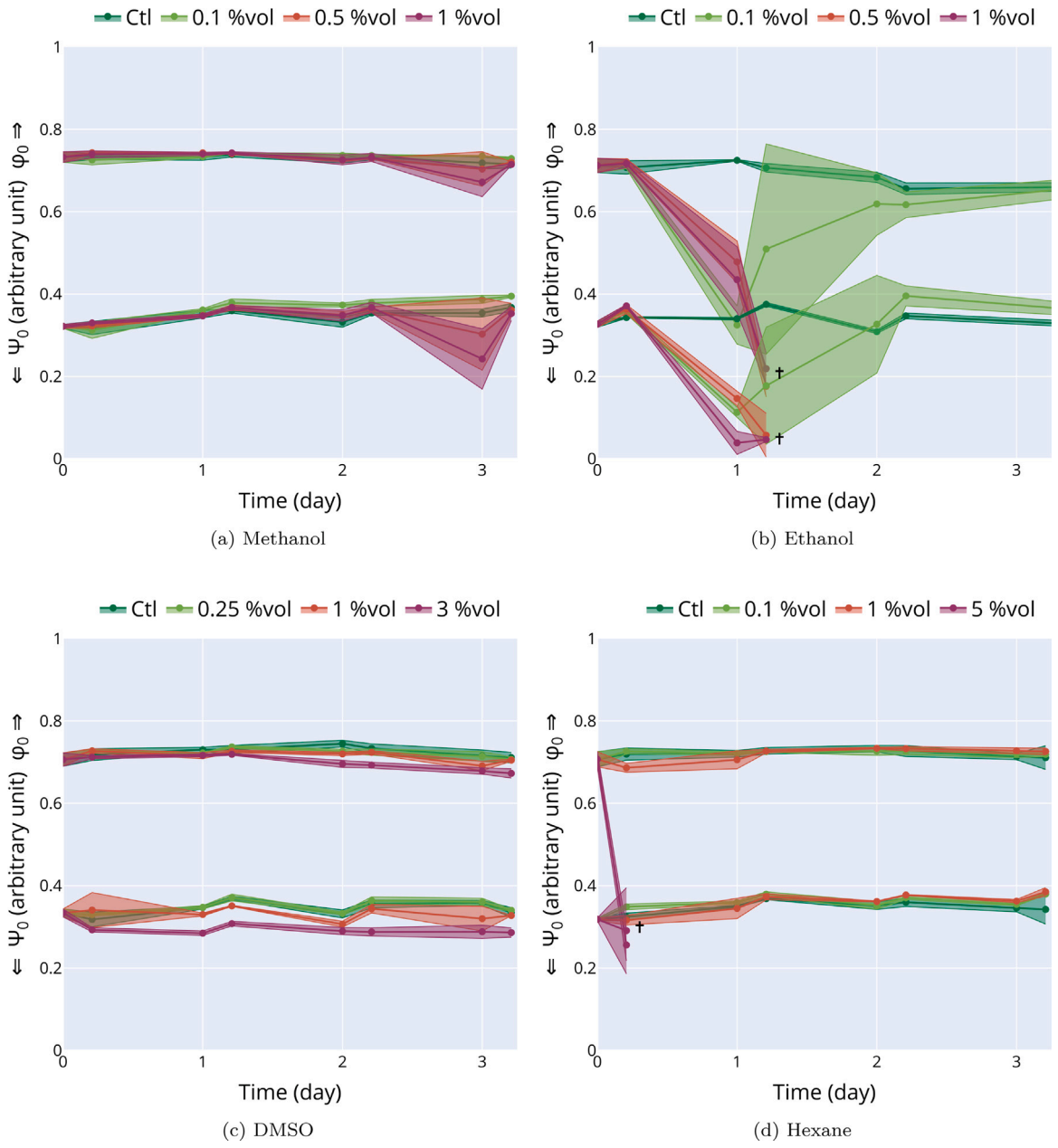


Fig. 3. Φ_0 and ψ_0 indicators of *Chlorella vulgaris* exposed to different solvents. Cross - measurements stopped because of unexploitable OJIP readings. Solid line - mean of the three replicates. Shaded area - standard deviation (n=3). Points - measurements.

3.4. Viability

Fig. 4 presents the results of the end-point viability assay. *Chlorella vulgaris* cells were identified by successive gating over chlorophyll autofluorescence signal, size (FSC signal), and cytoplasmic complexity (SSC signal). As one can see, three populations can be identified: alive (FDA-positive and PI-negative), dead (FDA-negative and PI-positive), and sluggish (FDA-negative and PI-negative). Whilst the live cells represented above 90 % of the overall population in the control runs, they dramatically dwindle for some solvent assays. Interestingly, the complementary population is not made of dead cells, but of sluggish cells. For the highest dosage, the sluggish cell fraction can be the main type of cells: 45.8% of the population on average for methanol, 59.5% for on average for DMSO, and 92.2% for hexane (the remaining cells being simply dead in this case). These observations yield insights into the consequences of solvent exposure. Indeed, methanol, DMSO, and hexane can show large populations of sluggish cells. These types of cells can be described as inactivated, as FDA negativity implies very low to no esterase activity. Still, their inactivity does not necessarily mean their death (in the sense of compromise of their DNA, as PI negativity implies that DNA is not accessible). Furthermore, no clear difference could be detected in the cell autofluorescence signal, suggesting roughly equivalent chlorophyll contents. All in all, this physiological state calls for further investigations, which fall outside of the scope of this work. In contrast to methanol, DMSO, and hexane, ethanol leads to cell rupture and overall cell disappearance of the suspension. Finally, it is worth noting that flow cytometry can only analyze cells in suspension. Hence, in the case of the flocculated cultures, this analysis only probes the cells not belonging to the flocs (ethanol, all concentrations, and methanol, medium and high dosage).

In terms of alignment with the other assays, the flow cytometry ones are congruent. The viable population decreases as the methanol fraction increases. Still, the viability is hindered even for the lowest dosage ($p = 0.0055$), incidentally ruling out methanol from the list of potential

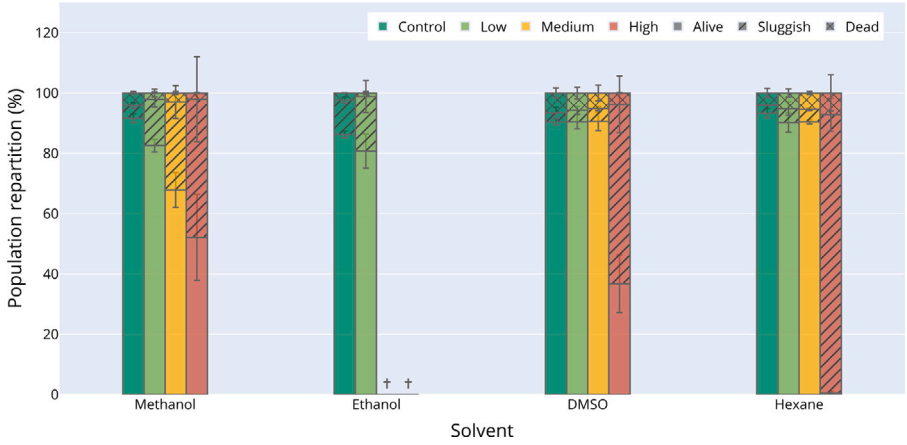


Fig. 4. End-point viability assay results. Cross - measurements stopped because no photosynthetic microorganisms could be detected. Legend: alive - FDA-positive and PI-negative, dead - FDA-negative and PI-positive, sluggish - FDA-negative and PI-negative. Bar - mean of the three replicates. Error bar - standard deviation (n=3).

Table 3
Integrated biomarkers values and variation coefficients for DMSO and hexane runs.

Solvent					
DMSO			Hexane		
Fraction (%vol)	IBM values	Var. Coef.	Fraction (%vol)	IBM values	Var. Coef.
0	1.7 ± 0.07	0.74%	0	1.69 ± 0.01	4.37%
0.25	1.77 ± 0.06	4.37%	0.1	1.63 ± 0.07	3.63%
1	1.6 ± 0.2	8.69%	1	1.73 ± 0.15	12.65%
3	0.74 ± 0.09	24.57%	5	0.5 ± 0.12	12.41%

solvents. This voids the possible use of methanol, even at the lowest dosage. The low and medium dosages of DMSO and hexane also yield values similar to their control ($p = 0.998$ and 0.203, respectively).

3.5. Integrated biomarkers

At this point, ethanol can be ruled out as it induces cell flocculation and lysis. The same goes for methanol, as it also triggers cell flocculation at medium and high dosages, but also increases cell mortality, at the lowest one. In contrast, DMSO and hexane seem viable options for phytohormone delivery at low and medium concentrations. Fig. 5 compares the two remaining solvents on the different dimensions studied in this work. Each dimension was plotted with respect to the average value of control to scale the values and ease comparison and manage trial-to-trial variation, such as the ambient temperature difference due to the heat wave. The first qualitative comment is that high dosages of each solvent diverge substantially from the control. Going further, one can notice that medium dosages seem to induce a higher amount of variability, especially for DMSO.

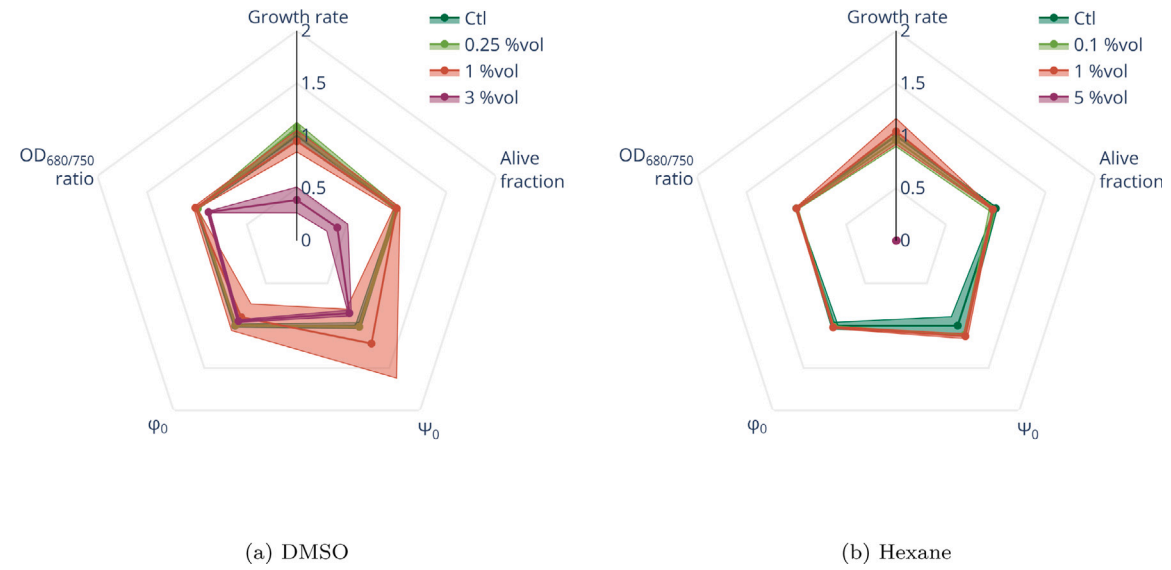
In terms of quantitative comparison, Table 3 displays the value of the integrated biomarkers for all the tests involving DMSO and hexane. As a token of the quality of the experiments and the efficiency of the integrated biomarker approach, one can notice how close the two controls are (1.7 ± 0.07 and 1.69 ± 0.01, for DMSO and hexane, respectively) and how low the variation coefficients are (below 5%). The integrated biomarkers also substantiate and confirm the intuitive perception that for both solvents, low dosages yield performance undifferentiated from control. This conclusion stands in terms of absolute values and dispersion. Increasing the solvent amount (from 0.25 to 1%vol for DMSO and from 0.1 to 1%vol for methanol) also allows for culturing in conditions similar to the control, yet at the cost of an increased dispersion. Finally, the highest dosages induce a dramatic shift from the reference, which is not a surprise, as it was observed for all the individual indicators.

3.6. Validation run

With adequate solvent concentration identified, it was possible to carry out the validation run. *Chlorella vulgaris* was exposed to salicylic acid (500 µM final concentration, in DMSO at 0.25%vol) in order to increase cell lutein content. Fig. 6 reports the growth dynamics of the two control runs (regular medium and regular medium with 0.25%vol DMSO) and phytohormone-boosted run. The two control runs showed extremely close growth profiles, while the phytohormone one deviated slightly before catching up. Moving pigment profile comparison, the two control runs yielded similar results (minimum p-value of 0.553 over the five pigments). This is a token of the quality of the procedure that allows the introduction of a phytohormone via a solvent that does not alter the intrinsic cell behavior. Regarding the finality of the test, salicylic acid indeed increased cell lutein content (+22%, p-value of 0.027). The runs with the regular medium and regular medium with 0.25%vol DMSO yielded cell containing 1.72 ± 0.09 and 1.67 ± 0.04 mg/g, respectively, while the salicylic runs reached 2.09 ± 0.18 mg/g. These results align with Czerpak et al. findings who reported a +23 to +40% total carotenoid content in *Chlorella vulgaris* exposed to 100 µM of salicylic acid (Czerpak et al., 2002).

4. Conclusion

Chlorella vulgaris was exposed to methanol, ethanol, DMSO, and hexane to examine solvent effects in the context of phytohormone delivery. Ethanol triggers cell flocculation for the tested concentrations (as low as 0.1%vol) and lysis above 0.5%vol. This behavior contrasts with literature, and suggests a possible strain-specific effect among the vast *Chlorella* genus. Methanol also led to flocculation above 0.5%vol, but started to impair viability from 0.1%vol on. DMSO and hexane appear as viable solvents, with no deviation (in terms of absolute value and dispersion, and over the course of a validation run) from control for 0.25 and 0.1%vol dosage. Their dosages can be increased to 1%vol while still claiming admissibly perturbed cultures, as absolute



(a) DMSO (b) Hexane

Fig. 5. Radar plot summarizing the different component of the integrated biomarkers. Each dimension is plotted with respect to the average value of control.

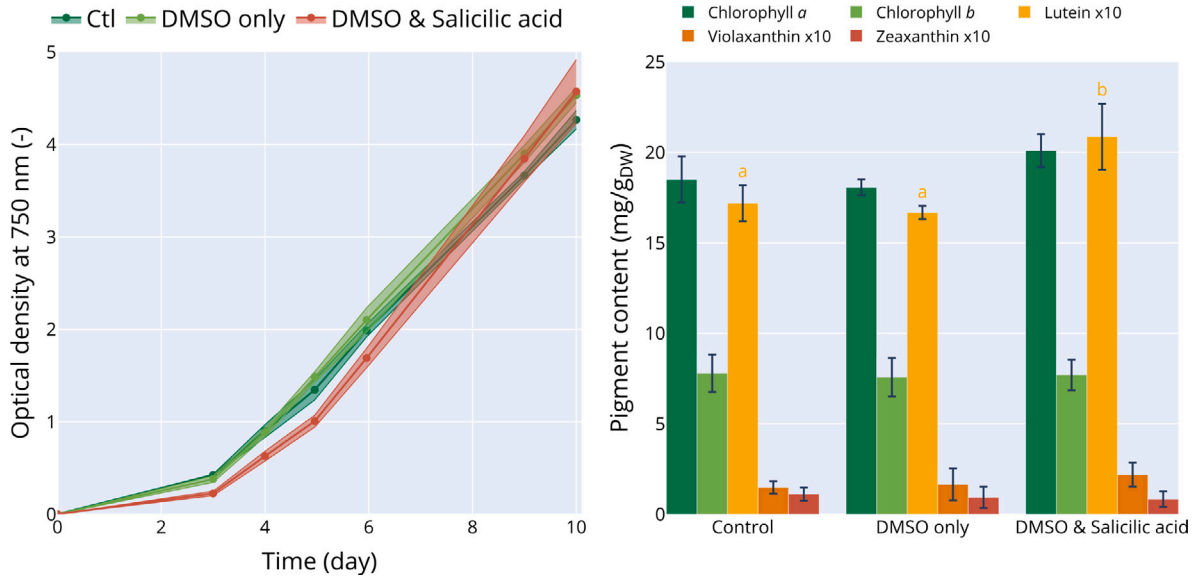


Fig. 6. Left - Culture growth dynamics. Solid line - mean of the three replicates. Shaded area - standard deviation (n=3). Points - measurements. Right - Cell pigment profiles. Bar - mean of the three replicates. Error bar - standard deviation (n=3, except for DMSO only runs for which a sample was lost during the extraction process). Letter - Statistical difference obtained by post-hoc Honestly Significant Difference test.

values remain in line with control, but dispersion increases. While the identified solvent fraction may appear low, one has to remember that phytohormones are signalers exhibiting effects at minimal dosage. All in all, these results constitute a valuable dataset that clearly shows which lines are not to be crossed to ensure reliable phytohormone delivery. Finally, a sluggish population, whose detailed investigation is a perspective for further work, was identified over the course of the viability assays.

CRediT authorship contribution statement

Victor Pozzobon: Writing – original draft, Visualization, Validation, Supervision, Software, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Diego Lopez Salas:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Camille Laury:** Writing – review & editing, Visualization, Validation, Investigation, Formal analysis, Conceptualization. **Cristobal Camarena-Bernard:** Writing – review & editing, Conceptualization. **Wendie Levasseur:** Writing – review & editing, Supervision, Methodology, Formal analysis, Conceptualization.

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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Data availability

Data will be made available on request.

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