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Antioxidant Bioprospecting in Microalgae: Characterisation of the Potential of Two Marine Heterokonts from Irish Waters

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Abstract
Microalgae constitute a heterogeneous and diverse range of organisms capable of accumulating bioactive metabolites, making them promising feedstock for applications in the nutraceutical, functional food, animal feed, biofertilisation or biofuel sectors. There has been renewed interest in recent times in natural sources of antioxidants, particularly as health products and preserving agents. Microalgae strains isolated from aquatic habitats in Ireland were successfully brought into culture. The 91 strains were grown phototrophically in nutrient-enriched media to generate biomass, which was harvested and assessed for antioxidant potential. Extracts were screened for antioxidant activity using a modified volumetric Trolox-ABTS assay and the Folin-Ciocalteu method. Two heterokont marine strains of interest were further studied to ascertain variations in antioxidant capacity across different stages of batch culture growth. The antioxidant activity of extracts of bacillariophyte cf. *Stauroneis* sp. LACW24 and ocrophyte cf. *Phaeothamnion* sp. LACW34 increased during growth with a maximum being observed during the late stationary or early death phase (2.5- to 8-fold increases between days 20

Research Highlights
- Microalgae strains (91) from Ireland were screened for antioxidant potential
- 2 selected marine heterokonts showed greatest antioxidant activity in late stationary phase
- Diatom strain LACW24 returned up to 65.0 μM Trolox eq per g (DW) under silicate deficiency
- Heterokont strains LACW24 and LACW34 contained 5.9 and 3.0 mg g^{-1} DW fucoxanthin, respectively
- Corresponding extracts caused no cytotoxicity on mouse cell lines after 48-h exposure

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and 27). Strains LACW24 and LACW34 contained 5.9 and 3.0 mg g^{-1} (DW) of the xanthophyll fucoxanthin, respectively. Extracts of strains also showed no cytotoxicity towards mouse cell lines. These results highlight the potential of these strains for biomass valorisation and cultivation upscaling and to be further considered as part of ongoing bioprospecting efforts towards identifying novel species to join the relatively narrow range of commercially exploited marine microalgae species.

Keywords Marine microalgae · Antioxidants · Fucoxanthin · Cytotoxicity

Introduction

Microalgae constitute promising natural resources from which high-value metabolites can be refined for applications in a variety of industries. Primary and secondary metabolites including polysaccharides, lipids, pigments, proteins, vitamins or antioxidants produced by microalgae have hence multiple applications in the food, cosmetic and natural medicine sectors [1–3]. The functional properties of compounds extracted from microalgae are numerous and include for example antioxidant, anti-inflammatory, antimicrobial or anti-carcinogenic activities [4–7]. The main microalgal species used to date for the production and extraction of high-value metabolites comprise the dinoflagellate *Crypthecodinium*, the chlorophytes *Haematococcus pluvialis*, *Chlorella* spp., *Dunaliella salina* as well as some cyanobacteria such as *Arthrospira platensis* and *Aphanizomenon flos-aquae* [8].

There has been increased interest worldwide in the biotechnology of microalgae, noticeably for the exploitation of their antioxidant properties [9–13]. Bioprospecting efforts to isolate new strains with promising bioactive attributes have hence been carried out [9, 11, 14–23]. The role of antioxidants in the prevention of fat spoilage is well established as they counteract the free radical-mediated chain reactions within lipids that can cause food deterioration. Oxidation of fatty acids can happen during food processing, storage and cooking and adversely affects food quality by reducing its nutritional value and shelf life [24–26]. With antioxidant activity in chicken meat, eggs and vegetable puree has recently been shown to have beneficial effects [2, 26–28]. There also has been much attention in the cosmetic applications of naturally sourced anti-ageing and UV protection biomolecules extracted from microalgae by several companies [29–31].

Oxidative stress arises within cells when the electron flow becomes uncoupled with the homeostasis of the metabolism [32]. This leads to the transfer of unpaired single electrons to other biomolecules and subsequent chain reactions generating free radicals, which can rapidly cause damage to membrane lipids, proteins or DNA [33]. Microalgae can produce a variety of natural antioxidant metabolites in response to their environment within the cell membrane, cytoplasm and organelles [34, 35]. The antioxidant activity in microalgae involves both non-enzymatic and enzymatic actors. Non-enzymatic antioxidants include tocopherols, carotenoids, phenolic compounds, coenzyme Q, fatty acids, glutathione, ascorbate, peptides, polyamines or mycosporine-like amino acids [36]. Typical enzymes with an antioxidant capacity include the superoxide dismutase, glutathione reductase, peroxidases and catalase.

Health concerns have been reported regarding the addition of synthesised antioxidants to food supplements and functional foods, providing a potential area of opportunity for naturally derived microalgal products. Recent studies have investigated possible adverse effects and health benefits of natural and synthetic antioxidants. In particular, synthetic antioxidants such as butylated hydroxytoluene (BHT) or butylated hydroxyanisole (BHA) have come under

increased scrutiny in recent years [37–39]. Naturally sourced alternatives may in this context be viewed as preferable options.

In the present study, the screening of antioxidant activity in extracts of newly established microalgal cultures isolated from the West of Ireland was carried out. Two marine heterokont strains, which previously showed promising content in the polyunsaturated fatty acid EPA [40], were then tested for potential variation in antioxidant activity at various stages of the typical growth of batch cultures. The potential cytotoxicity of extracts of these strains was then assessed using mouse cell lines.

Materials and Methods

Sample Procedure

Microalgal cells were isolated from samples taken in the West of Ireland from several freshwater, marine and terrestrial habitats throughout 2013–2014. Samples were collected using plankton hauls or directly with sterile bottles where surface cell aggregates were visible. Samples were briefly scanned by inverted light microscopy and where necessary were filtered through a 100-μm mesh filter to remove excess zooplankton and ciliates. All samples were supplemented with defined media and maintained in an illuminated incubator at 15 °C under a photon flux density of ~ 100 μmol m^{-2} s^{-1} and a 14:10 light dark photoperiod cycle.

Cell Isolations and Culturing

Single cell isolations were carried out by microcapillary using an inverted light microscope [41]. Isolated cells were initially placed in 96-well plates and progressively subcultured into 24-well plates, then test tubes as single monoalgal cultures. These were kept in a controlled environment in 100-ml glass tubes within an incubator at 15 °C and a photon flux density of ~ 100 μmol m^{-2} s^{-1} on a 14:10 light dark photoperiod cycle for 30 days. Nutrition media (f/2, Bold's Basal, BG11, IMR½) were made up using autoclaved and/or filtered water [42].

Estimation of Biovolumes

Cell biovolumes were estimated for the strains successfully brought into culture. A minimum of 30 individual cells in 96-well plates was measured using a calibrated graticule on an inverted microscope at × 400 magnification immediately after being preserved in formalin or acidic Lugol's iodine. Cells were gently turned when necessary with a needle to obtain the dimensions needed. The average cell biovolume was estimated using previously published equations [43].

Cell Enumeration and Biomass Harvest

Culture aliquots were homogenised and fixed with acidic Lugol's iodine. Large cells were enumerated in 96-well plates. Cells under 10 μm in size were counted using an upright light microscope with a haemocytometer. All counts were carried out in triplicates. Variable volumes of homogenised cultures were harvested by centrifugation (10,000 rpm for 5 min) during the stationary phase of growth to obtain 0.4 mm^3 of biomass pellets.

Wet Biomass Processing

Biomass pellets were re-suspended in 2 ml of 50% ethanol and placed in a bead beater for 45 s at full power. After incubation of 20 min in darkness at room temperature, the samples were centrifuged at 10,000 rpm for 4 min, and the supernatants were collected into new tubes. The pellets were re-suspended again in 2 ml of solvent and subjected to a further 20-min incubation. The two extracts were then combined and stored in darkness at −20 °C and analysed within 48 h.

Determination of Total Antioxidants by Trolox Equivalent Antioxidant Assay (TEAC)

A commonly used assay for the screening of antioxidants is the Trolox equivalent antioxidant assay (TEAC) [20, 44]. This assay highlights the chemical reactions of hydrogen-atom transfer and single-electron transfer of reactive oxygen species. Briefly, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) is converted to its radical blue coloured monocation $ABTS^+$ by reaction with potassium persulphate, which is then reduced in the presence of hydrogen-donating compounds to a colourless state with absorption maxima at wavelengths of 645 nm, 734 nm and 815 nm, the absorption values for microalgal extracts being typically measured at 734 nm.

For the assay, a stock of 7 mM $ABTS^+$/ 2.45 mM $K_2S_2O_8$ was diluted with 50% ethanol so that the addition of an aliquot of 1050 μl of solvent to 3000 μl of diluted ABTS+/ $K_2S_2O_8$ solution displays an absorbance value of 0.700 ± 0.05 at 734 nm in a 1-cm light path spectrophotometer cuvette. Then, sample extract aliquots of 1050 μl were mixed with 3000 μl of diluted ABTS+/ $K_2S_2O_8$ working stock solution. At 6 min, samples and 50% ethanol solvent blanks were transferred into 1-cm light path spectrophotometer cuvettes and the absorbances are measured at 734 nm.

Trolox (6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) is a water-soluble analogue of vitamin E, which exhibits radical scavenging and antioxidant activities. It is used as a standard in the assay using a calibration range of 0 to 30 μM [44]. These are then compared to the total antioxidant activity of the microalgae extracts to be screened. As per the microalgal extracts, 1050 μl of increasing concentrations of Trolox prepared in 50% ethanol were added to 3000 μl of diluted ABTS+/ $K_2S_2O_8$ working stock solution prior to the measurements. The antioxidant activity of the microalgal extracts was expressed in Trolox equivalents (μM).

Determination of Total Antioxidants with the Folin-Ciocalteu Assay

The determination of antioxidant activity by the Folin-Ciocalteu assay is well documented for the quantification of phenolic compounds [45, 46]. The reaction of the Folin-Ciocalteu reagent with phenols is based on the reduction potential of the compounds present in an extract with the phenolate ion, the uptake of oxygen being completed near or above ~pH 10.

Gallic acid is a natural phenol that was used as standard in the Folin-Ciocalteu assay to which the activity of the extracts to be screened is compared. The gallic acid calibration range used to quantify reactive oxygen species typically is 0 to 2 mg l^{-1}. The reaction takes place in a final volume of 4 ml in a 1-cm light path spectrophotometry cuvette. An aliquot of 400 μl of microalgae extract in 50% ethanol is mixed with 2000 μl of tenfold diluted Folin-Ciocalteu reagent and incubated for 4 min in darkness at room temperature. Sodium carbonate (75 g l^{-1}) is then added to bring the final volume to 4000 μl. This solution was then incubated for 4 h in

darkness at room temperature. The absorbance of the samples was measured by spectrophotometry at 760 nm. The antioxidant activity of the microalgal extracts was expressed in gallic acid equivalents (mg l^{-1}).

Variation in the Antioxidant Activity During Batch Culture Growth

This study further focused then on two marine species previously shown to contain promising levels of the high-value lipids EPA and DHA [40]: the bacillariophyte cf. *Stauroneis* sp. LACW24 and the ocrophyte cf. *Phaeothamnion* sp. LACW34. The strains were cultured in 300 ml of f/2 medium in 500 ml Erlenmeyer flasks to ascertain at which stage of their growth antioxidant activity was the highest. In addition, the diatom strain LACW24 was grown in f/2 medium without silicate to potentially stimulate its bioactivity [47]. The strains were inoculated at a starting biomass of 0.05 mm^3 ml^{-1}, and four replicate flasks were used for each. The growth of the cultures was monitored periodically, and sampling was performed on three different occasions prior to carrying out the antioxidant assays. To reduce potential interferences with growth dynamics caused by the excessive removal of culture volume and nutrients via sampling, a reduced sample biomass of 0.1 mm^3 was collected from all strains on each of the sampling occasions. This was based on preliminary trials which indicated that this amount of biomass was sufficient to detect antioxidant activity in the corresponding extracts. The specific growth rates of the cultures were determined according to the following formula:

$$r = (\ln \mathrm{Nt}\,)/\ln \mathrm{No}\,\Big)/\triangle t$$

where r is exponential growth rate, No is the cell concentration at time zero, Nt is cell concentration at time t and Δt is the length of the time interval in days between Nt and No.

Pigment Extraction and Fucoxanthin Analysis by HPLC-UV-DAD

Pigment extraction was carried out according to McGee et al., [48] whereby 1 mg of biomass was extracted with 500 μl ice cold 100% v/v ethanol with glass beads using a FastPrep FP120 for 40 s at a speed of 4.0 m s^{-1}. Deionized water and acetone were added to make the solution up to a 90% v/v acetone in a final volume of 1.5 ml and vortexed. Extracts were filtered through 0.2-μm PTFE HPLC syringe filters and transferred into amber vials and stored at − 80 °C and analysed within 24 h.

Pigment extracts were analysed at constant room temperature on a Varian ProStart HPLC binary system coupled with a ProStar 310 UV and 335 PDA detectors with a sample loop of 20 μl. The separation of pigments was carried out using a Phenomenex Onyx C18 100 × 4.6 mm ID monolithic column coupled with a Phenomenex Onyx C18 guard cartridge 10 × 4.6 mm ID. The gradient employed was that of McGee et al. [48] with a stepped gradient solvent programme which consisted of 10% B initial isocratic condition for 0.10 min, a linear gradient to 65% B from 0.10 to 2.00 min, isocratic hold at 65% B for 2 min, linear gradient to 90% B for 1 min followed by a hold at 90% B for 1 min and a final re-equilibration step to the initial conditions from 6.01 to 7.50 min. The composition of the mobile phases was as follows: mobile phase A consisted of an ammonium acetate (0.5 M): methanol (20:80 v/v) and mobile phase B comprised of acetonitrile: acetone (30:70 v/v). A calibration curve ranging was made using a fucoxanthin standard (Sigma-Aldrich). Pigments were identified by comparing retention times and UV-vis spectral fine structures to pigment standards (DHI) and an in-house pigment library [48].

Cell Lines and Cell Viability assays

Two normal mammalian cell lines (MC3T3-E1 mouse bone and C2C12 mouse muscle cell lines) were used in this study. MC3T3-E1 and C2C12 cells were maintained in DMEM medium (Sigma-Aldrich), containing 10% and 15% (v/v) Fetal Bovine Serum (FBS) (GIBCO, Life Technologies), respectively. The C2C12 cells were obtained from the American Type Culture Collection (ATCC); the MC3T3-E1 cells lines were kindly donated by Prof. G. Ghersi (University of Palermo, Palermo, Italy). Cells (3×10^3 per well) were distributed into each well of 96-well microtiter plates. After 24 h, at time 0, the medium was replaced with fresh complete medium complemented with ethanolic extracts of strains LACW24 and LACW34 at different doses. Cells were cultured for 24, 48 and 72 h. At the end of the treatment, cytotoxicity assays were performed using tetrazolium compound-based CellTiter 96® AQueous One Solution Cell Proliferation (MTS) assay (Promega) according to the manufacturer's instructions. Cell viability was expressed as a percentage of the absorbance measured in the treated cells versus the control cells (vehicle alone without microalgal extract). Values were expressed as means ± SD of three separate experiments, each performed in triplicate.

Data Treatment

ANOVAs followed by Tukey's post-hoc tests were carried out to identify significant differences between the average antioxidant activities of extracts, average antioxidant activities of the extracts collected at various stages of the batch culture growth phases and the viabilities of cell line exposed to microalgal extracts of varying strength.

Results

Determination of Total Antioxidant Activity with the TEAC Assay

The TEAC assay was carried out based on the construction of a Trolox calibration curve to report the antioxidant activity of the extracts. A threshold limit was set to highlight the 10% strains exhibiting the highest total antioxidant activity. These were made up of three freshwater and six marine strains. The average antioxidant activity obtained for the nine strains was 22.4 μM Trolox eq. (s.d. 5.9). The maximal activity was observed for the marine *Rhodomonas* strain APSW10 with 33.3 μM Trolox eq. (s.d. 1.5) and was significantly higher than those of the other strains (ANOVA, $n = 27$, $p < 0.05$). Overall, 9% of the extracts showed an activity over 16 μM Trolox eq., 33% between 16 and 10 μM Trolox eq. and 58% under 10 μM Trolox eq. (Fig. 1). There were also no significant differences in the average activities returned amongst the main taxonomic phytoplankton groups (ANOVA, $n = 91$, $p > 0.05$).

Determination of Total Antioxidant Activity by the Folin-Ciocalteu Method

The Folin-Ciocalteu assay was carried out based on the construction of a gallic acid calibration curve. A 10% threshold was also selected to identify the best performing strains. These were made up of three freshwater and six marine strains. The average antioxidant activity obtained for these strains was 5.5 mg l^{-1} gallic acid eq. (s.d. 0.8). The maximal activity was observed for

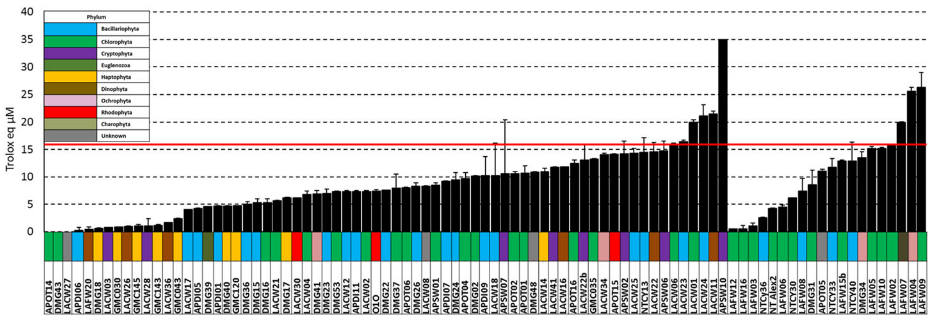

Fig. 1 Ranked antioxidant capacity of the 91 microalgae strains screened using the TEAC assay. The threshold was set to highlight strains in the upper 10 percentile. Error bars indicate standard deviation ($n = 3$). The colour coding indicates the main microalgal groups

the freshwater *Chloromonas* sp. strain LAFW02 with 7.1 mg l^{-1} gallic acid eq. (s.d. 0.1). Nevertheless, there was no significant difference between the average values obtained for the nine extracts that reached the 10% threshold (ANOVA, $n = 27$, $p > 0.05$). Overall, 7% of the extracts showed an activity over 5 mg l^{-1} gallic acid eq., 28% between 5 and 2 mg l^{-1} gallic acid eq. and 65% under mg l^{-1} gallic acid eq. (Fig. 2). Although positively correlated, the data of the TEAC and Folin-Ciocalteu assays showed a low coefficient of determination (Fig. 3). There were also no significant differences in the average activities returned amongst the main taxonomic phytoplankton groups (ANOVA, $n = 91$, $p > 0.05$).

Variation in the Antioxidant Potential of Selected Marine Strains During Growth

The marine strains cf. *Stauroneis* sp. LACW24 and cf. *Phaeothamnion* sp. LACW34 were selected for assessing antioxidant activity throughout their growth (Fig. 4). Sampling was carried out on days 15, 20 and 27 to capture their antioxidant capacity during distinct stages of their growth.

The strains were actively growing during days 10 and 20. The stationary phase thereafter was relatively short and quickly followed by the death phase. The highest biomass yield was similar for both strains, except for the silicate limited LACW24 set. The specific growth rates

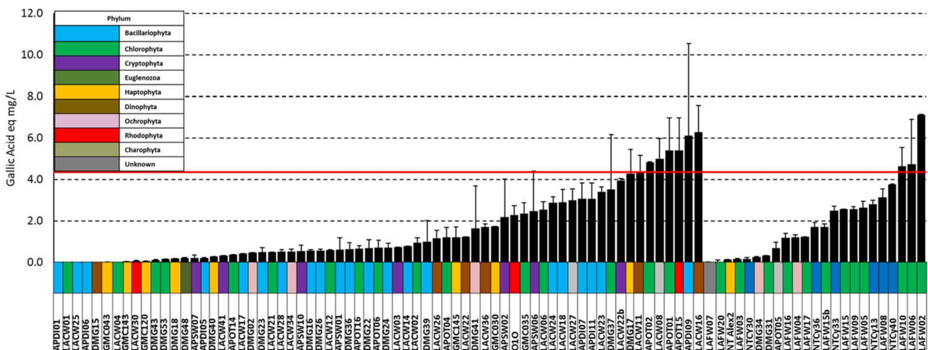

Fig. 2 Ranked antioxidant capacity of the 91 microalgae strains screened using the Folin-Ciocalteu assay. The threshold was set to highlight strains in the upper 10 percentile. Error bars indicate standard deviation ($n = 3$). The colour coding indicates the main microalgal groups

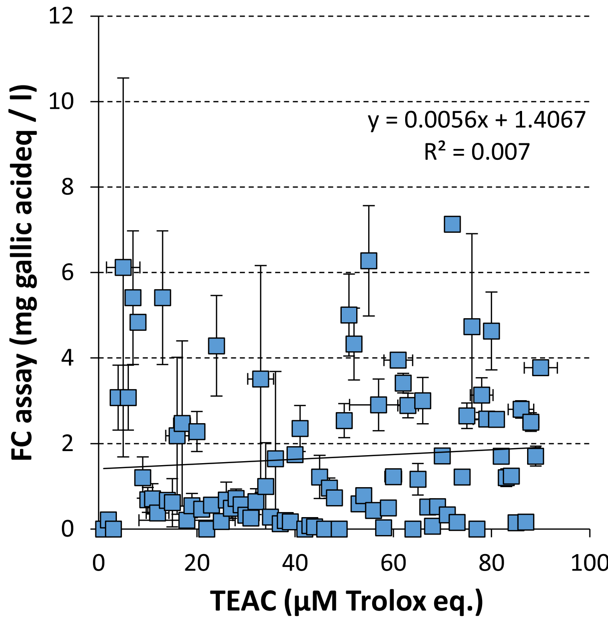

Fig. 3 Scatterplot of the data obtained with the TEAC and Folin-Ciocalteu assays for the antioxidant activity screening of the 91 strains. Error bars indicate standard deviation ($n = 3$)

were relatively low with those of LACW24Si + (0.27 ± 0.03) and LACW34 (0.27 ± 0.10) being significantly greater than that of LACW24 (0.08 ± 0.03) (ANOVA, $n = 12$, $p < 0.05$).

Total antioxidant capacity measured via the TEAC assay (Fig. 5) showed increases as the cultures aged. The antioxidant activity of strain LACW24 grown without silicates was

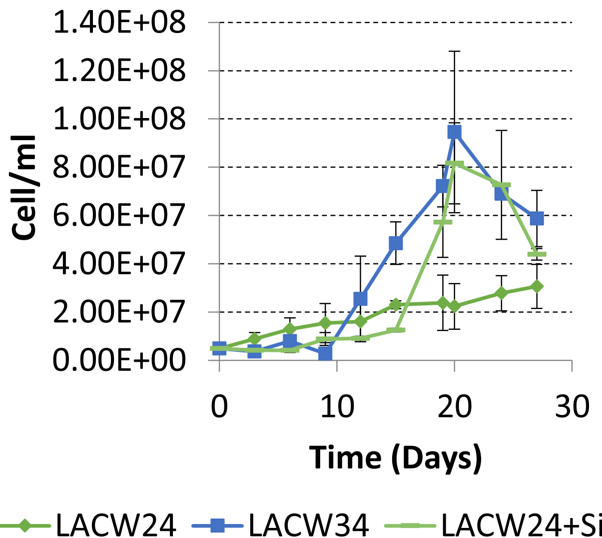

Fig. 4 Batch culture-based growth dynamics of selected marine microalgae strains. Values are the average of 4 replicates. For clarity, error bars were not placed on the figure. Strain LACW24 was grown in the presence (+Si) and absence (−Si) of silicate

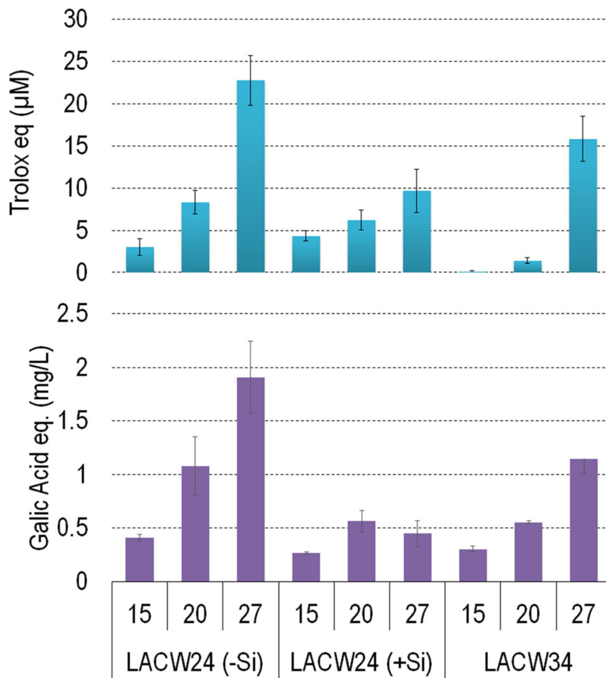

Fig. 5 TEAC (top) and Folin-Ciocalteu (bottom)-based antioxidant capacity of extracts of strains LACW24 and LACW34 at distinct stages of their batch culture growth. Values are the average of 4 replicates (± 1 s.d.)

significantly higher on day 27 (ANOVA, $n = 36$, $p < 0.05$). For comparison purposes with the literature, additional TEAC measurements were carried out using freeze-dried biomass, which

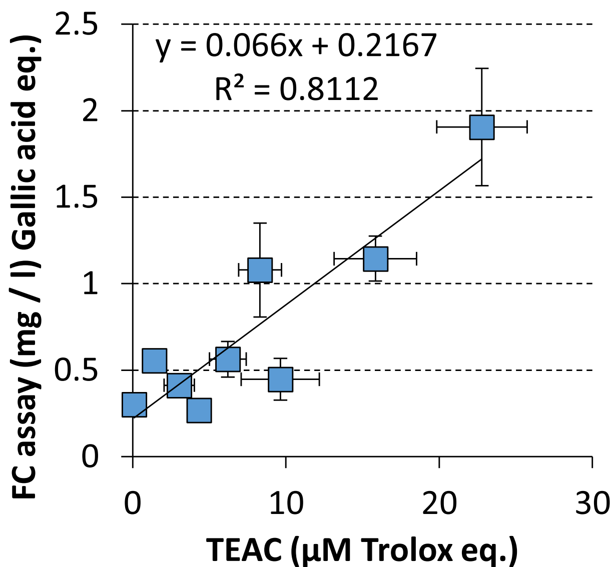

Fig. 6 Scatterplot of the data obtained with the TEAC and Folin-Ciocalteu assays for the antioxidant activity measurements carried out on LACW24 and LACW34. Error bars indicate standard deviation ($n = 4$)

returned 33.4 and 51.3 μmol Trolox eq. per g of biomass for strains LACW34 and LACW24, respectively. Interestingly, this increased to 65.0 μmol Trolox eq. per g of biomass for strain LACW24 when cultivated under silicate deficiency.

The results obtained with the Folin-Ciocalteu assay returned similar patterns to those obtained with the TEAC assay (Fig. 6). The antioxidant activity of the diatom strain LACW24 grown without silicate was also significantly higher than any of the other extracts on day 27 (ANOVA, $n = 36$, $p < 0.05$).

Fucoxanthin Content and Cytotoxicity of Extracts of LACW24 and LACW34

The ethanolic extracts of strains LACW24 and LACW34 were analysed by HPLC-UV-DAD, returning a pigment profile typical of heterokonts (Fig. 7). The high-value xanthophyll

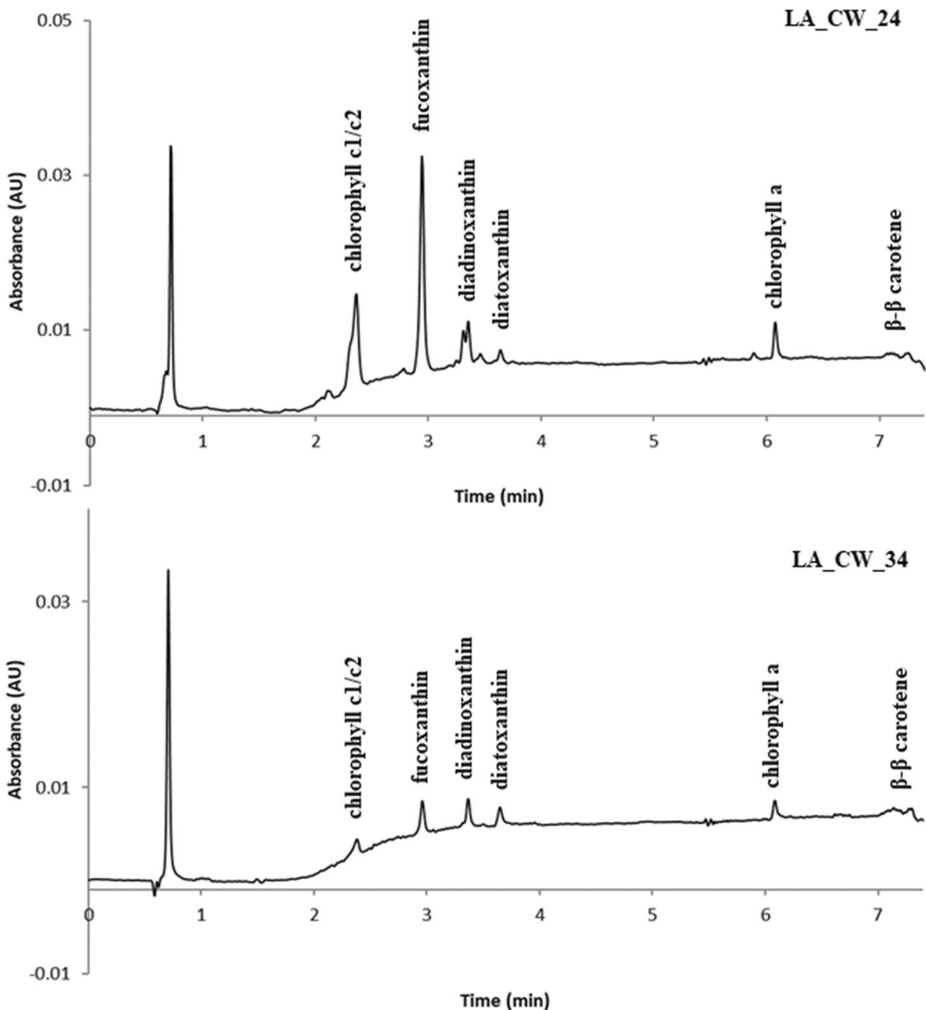

Fig. 7 HPLC-DAD chromatograms of extracts of strains LACW24 and LACW34 indicating the main pigments detected, including fucoxanthin

pigment fucoxanthin was quantified in the extracts, returning yields of 5.9 and 3.0 mg g^{-1} DW for LACW24 and LACW34, respectively. Cell viability assays were performed in C2C12 and MC3T3-E1 standard mammalian cell lines using different concentrations of LACW24 and LACW34 extracts (Fig. 8). Both extracts proved not toxic for the cells and did not affect cell viability after 72 h of treatment in the two cell lines used. In C2C12 cells, 48-h treatment with extracts of LACW24 and LACW34 slightly increased cell viability by approximately 20% at concentrations of 50 μg ml^{-1} and 75 μg ml^{-1}. This increase was lost after 72 h of treatment when cell viability was back to the values of the control.

Discussion

Microalgae have shown promise over the last two decades as sources of high-value metabolites, including antioxidants. In this study, volumetric assays were used to evaluate the antioxidant activity potential of 91 newly isolated microalgal strains from Irish waters. The TEAC assay, which acts as a measure of total activity by capturing both hydrophilic and lipophilic antioxidants, has been a cornerstone method for reporting antioxidant activity. Here, this assay was relatively fast compared to the Folin-Ciocalteu assay, which is typically viewed as more targeted towards polyphenol compounds. The antioxidant potential of microalgal cultures has been examined in other studies and showed that both carotenoids and phenolic compounds significantly contribute, albeit differentially, to the total antioxidant activity [16, 49]. Such studies also reported variable results using solvents of different polarities. The correlation between antioxidant activity results when screening many species using various assays is not always evident and is influenced by specificity and mechanisms of action [50]. This would explain why the TEAC and Folin-Ciocalteu data obtained in the present study showed extensive variation, probably as a result of differential contributions of various types

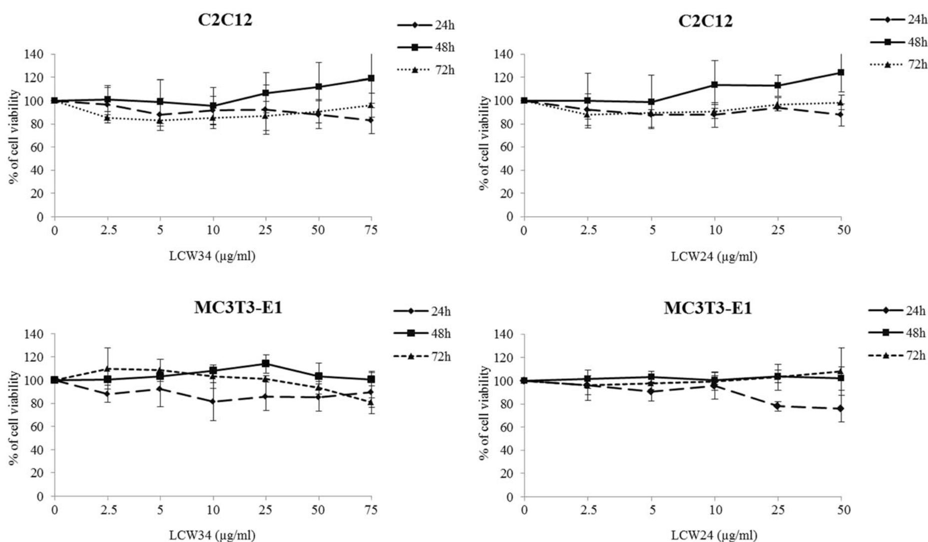

Fig. 8 Cell viability of standard mammalian cell lines (C2C12 and MC3T3-E1) exposed to ethanolic extracts of strains LACW24 and LACW34 at varying concentrations and for different timing of incubation (24, 48 and 72 h). Values were expressed as means ± SD of three separate experiments, each performed in triplicate

of antioxidant compounds. No particular taxonomic groups of algae also seemed to possess high antioxidant activity as a constitutive trait. Antioxidant activity was seen to be species-specific, in contrast to pigment or fatty acid signatures, which have been shown in previous studies to constitute biomarkers of particular algal groups [48, 51, 52].

The screening results obtained for some species echoed those obtained in other studies [15, 53] Extracts of some freshwater cyanobacteria such as *Lyngbya* sp. also showed high antioxidant activity, which may be attributable to phycobiliproteins [23, 54–57]) found that extracts of the dinoflagellate *Amphidinium carterae* returned substantial antioxidant activity. *Prorocentrum micans* LACW16 also yielded elevated antioxidant activity, indicating that dinoflagellates may constitute promising sources of antioxidants. The best performing strains were interspersed across the main microalgal lineages and included *Rhodomonas marina* APSW10, *Scenedesmus* sp. LAFW09, *Amphidinium carterae* LACW11 or cf. *Stauroneis* sp. LACW24. Previous screening studies have highlighted microalgal marine strains to have high antioxidant potential, including the haptophyte *Isochrysis galbana*, the ochrophyte *Nannochloropsis oleoabundans*, the diatoms *Phaeodactylum tricornutum* and *Chaetoceros calcitrans*, the cyanobacteria *Synechococcus* sp. and *Nostoc ellipsosporum* as well as the chlorophyte *Chlamydomonas nivalis* [14]. Likewise, studies focusing on freshwater microalgae have also identified promising species with antioxidant activity such as *Chlorella vulgaris*, *Tetraselmis* sp., *Haematococcus pluvialis* or *Scenedesmus obliquus* [14, 19, 20]. The generic low light and temperature parameters used in the present study, which reflected the conditions in Ireland at the time of the isolation of the strains (typically cloudy weather with a water temperature near 15 °C), were not those typically used for optimal growth [14, 58]. However, microalgae such as cryptophytes have been shown to be very tolerant of low temperature and light [59], the species *Rhodomonas marina* in particular, having previously shown appreciable antioxidant activity and polyunsaturated fatty acid content [17].

The heterokont strains LACW24 and LACW34 showed the highest antioxidant activities, more so under silicate deficiency for the latter. In general, the xantophyll fucoxanthin is abundantly present within diatoms and has been associated with antioxidant activity [60]. This pigment has been noted as a promising dietary and weight loss supplement for the treatment of obesity [61]. Fucoxanthin was detected in both strains at levels of 5.9 and 3.0 mg g^{-1} DW for LACW24 and LACW34, respectively. Interestingly, these strains also returned promising signatures of polyunsaturated fatty acids with elevated proportions of the high-value lipid EPA and or DHA [40]. Other studies have indicated that extracts of heterokonts, such as the diatom *Navicula clavata*, might display antioxidant activity associated with phenolic compounds [62, 63].

Markets for natural antioxidants have typically focused on the food additive, nutraceutical, cosmetic and animal feed sectors. Microalgal carotenoids have displayed potential to counteract excitotoxicity and hence reduce the ensuing reactive oxygen species generation [64]. However, it is essential when considering microalgae-derived metabolites and their suitability for incorporation to product formulation to evaluate their toxicological potential. Complex extracts exhibiting antioxidant activity might still exert some level of toxicity to humans and animals. For example, some studies have shown that extracts of the dinoflagellates *Amphidium carterae*, *Ostreopis ovata* and *Alexandrium minutum* could return high antioxidant activity [9]. Considering that some of these dinoflagellates are notorious for synthesising potent biotoxins, future downstream work focusing on selecting promising microalgal strains for the biorefinery of antioxidants and other metabolites should encompass further toxicological testing of

promising fractions. In this regard, it is encouraging that ethanolic extracts of strains LACW34 and LACW24 did not cause cytotoxicity in an in vitro assay using two different mammalian cell lines after exposure for 72 h.

Recent studies have assessed antioxidant potential, using the cellular lipid peroxidation activity assays, which considers the bioavailability of the compounds of interest [9]. Further investigations focusing on the quantification and cellular localisation of antioxidant compounds would help to clarify the biochemical functions associated with the observation of antioxidant activity while screening extracts [65]. The cellular localisation of antioxidants such as carotenoids and flavonoids may be realised using Nile Red and Naturstoff reagent [66, 67]. The miniaturisation of the assays in a well-plate format would also facilitate in the future the high throughput screening of multiple strains grown under a variety of culture conditions.

Conclusion

A selection of native microalgae strains from Ireland was assayed for antioxidant capacity. The marine strains cf. *Stauroneis* sp. LACW24 and cf. *Phaeothamnion* sp. LACW34 showed promising antioxidant activity and no cytotoxicity when cultivated in a batch culture mode, showing potential for further biorefinery studies. Future physiological enhancement of these strains by modulating culturing conditions should permit increasing cellular yields of antioxidants and other valuable metabolites.

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Author Contributions Lorraine Archer, Eoin Gillespie and Nicolas Touzet conceived and designed the experiments. Lorraine Archer conducted experiments and analyses. Lorraine Archer and Nicolas Touzet analysed the data. Lorraine Archer, Dónal McGee, Andrea Paskuliakova, Gary McCoy and Nicolas Touzet contributed towards the isolation, maintenance and characterisation of the strains. Rachael Parks conducted the HPLC DAD and quantification of fucoxanthin. Antonella Cusimano and Giorgia Adamo designed, conducted and analysed the cytotoxicity in vitro assays, and Antonella Bongiovanni supervised these experiments. Lorraine Archer and Nicolas Touzet drafted the core of the manuscript; all authors contributed to finalising and editing the manuscript.

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