Lipophilic compounds, but not fucoxanthin, mediate the genotoxic effect of photoautotrophic grown *Phaeodactylum tricornutum* in Caco-2 and HT-29 cells

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ABSTRACT

Phaeodactylum tricornutum (*P. triconutum*) gained high interest for use in human nutrition. We aimed to investigate the effect of photoautotrophically cultivated *Phaeodactylum tricornutum* on genotoxicity, cytotoxicity, formation of reactive oxygen species and apoptosis in Caco-2 and HT-29 cells. Furthermore, the effect of processing (sonication, ball-milling) on these parameters was assessed.

P. triconutum dose-dependently induced DNA strand breaks (2 µg dry matter/mL – 200 µg dry matter/mL) in both cell lines. These breaks can mostly be repaired after a recovery time of 2 h. Using processing methods did not affect the genotoxicity. We found that a lipophilic fraction, but not fucoxanthin, might be responsible for the DNA damage. Neither the *P. triconutum* preparations nor fucoxanthin affected cell proliferation or revealed an apoptotic or necrotic activity.

In conclusion, *P. triconutum* showed a genotoxic potential in intestinal cells, mainly mediated by lipophilic constituents. Sonication and ball-milling might be considered as safe methods for processing of *P. triconutum*.

1. Introduction

Phaeodactylum tricornutum (*P. tricornutum*) is a pleomorphic diatom that can grow under varying conditions, which facilitate cultivations in salt as well as fresh water (Ryckebosch, Muylaert, Eeckhout, Ruyssen, & Foubert, 2011). The use of so-called "brown microalgae" in feedstuff (e.g. in aquacultures) and food products increasingly gained interest due to various valuable constituents such as poly-unsaturated fatty acids (PUFAs, e.g. ω-3 fatty acids), and phytochemicals (e.g. polyphenols and carotenoids) (Ryckebosch et al., 2011; Sorensen, Berge, Reitan, & Ruyter, 2016). Consequently, lipophilic sub-fractions in particular might be of interest for human nutrition.

When cultivated under optimal conditions, *P. tricornutum* biomass can achieve a high content of eicosapentaenoic acid (EPA) (Derwenskus et al., 2018; Meiser, Schmid-Staiger, & Trosch, 2004) as well as the carotenoid fucoxanthin (Derwenskus et al., 2018; Gille, Hollenbach, Trautmann, Posten, & Briviba, 2019). A number of health promoting effects such as anti-inflammatory (Heo et al., 2010; Maeda, Kanno,

Kodate, Hosokawa, & Miyashita, 2015; Serini et al., 2012; Vedin et al., 2008), antioxidant (Heo et al., 2012; Liu et al., 2000; Peng, Yuan, Wu, & Wang, 2011) and anti-obesity activity (Flachs et al., 2005; Flachs, Rossmeisl, & Kopecky, 2014; Jeon et al., 2010; Maeda, Hosokawa, Sashima, Funayama, & Miyashita, 2005) have been attributed to either EPA or fucoxanthin. However, these lipophilic constituents also possess the potential to promote concentration dependent adverse cellular effects. Several authors observed growth-inhibiting effects for EPA (Fasano et al., 2012; Kuan, Walker, Luo, & Chen, 2011; Zhang et al., 2015) as well as for fucoxanthin (Hosokawa et al., 2004; Kotake-Nara et al., 2001; Liu, Huang, Hosokawa, Miyashita, & Hu, 2009; Zhang et al., 2008) in different cell lines. Thus, in particular a lipophilic *P. tricornutum* fraction could be addressed in terms of potential toxic effects in future studies.

Cells of the luminal surface of the intestine are an important contact point after consumption of *P. tricornutum*-supplemented food products. In this light, primary human intestinal cells as well as differentiated Caco-2 cells would represent an optimal model. However,

undifferentiated Caco-2 and HT-29 cells are frequently applied to assess toxic effects of food products (Duh et al., 2018; Gille, Trautmann, et al., 2019; Hartwig et al., 2007; Roser, Pool-Zobel, & Rechkemmer, 2001). Numerous *in vitro* methods were established in order to evaluate toxicity in cells. In this study we focused on the cytotoxic, genotoxic and pro-apoptotic/necrotic activity of different *P. tricornutum* preparations in Caco-2 and HT-29 cells. In addition, we investigated the impact of sonication and ball-milling as well as the use of different sub-fractions including fucoxanthin on these cellular effects.

2. Materials and methods

2.1. Materials

Chemicals for microalgae cultivation (boric acid, calcium chloride dihydrate, cobalt(II) nitrate hexahydrate, copper(II) sulfate pentahydrate, dipotassium phosphate, EDTA, iron(II) sulfate heptahydrate, magnesium sulfate heptahydrate, manganese(II) chloride tetrahydrate, TRIS, potassium chloride, potassium dihydrogen phosphate, sodium chloride, sodium silicate, and zinc sulfate hexahydrate), magnesium sulfate heptahydrate and sodium ammonium hydrogen phosphate tetrahydrate as well as Kobe I agar-agar, D-glucose, sodium chloride, monosodium phosphate, potassium carbonate and sodium hydrogen phosphate were obtained from Carl Roth (Karlsruhe, Germany) or Merck (Darmstadt, Germany). Eaglés minimal essential medium (EMEM), Dulbecco s minimal essential medium (DMEM), and phosphate- buffered saline (PBS, pH: 7.4) were supplied by Lonza (Verviers, Belgium), non-essential amino acids (NEA) land trypsin-EDTA (0,25%) by Fischer Scientific (Schwerte, Germany), penicillin/streptomycin (P/S) and glutamine by Biozym (Hessisch Oldendorf, Germany), and fetal calf serum (FCS) by Biowest (Nuaillé, France). EDTA disodium salt (Na$_2$EDTA), laurosyl sarcosine sodium salt, malondialdehyde, xylenol orange, trypan blue, staurosporin, brefeldin A, acetyl chloride, sodium sulfate and butylated hydroxytoluene were purchased from Sigma Aldrich (Taufkirchen, Germany). Normal melting agarose was obtained from FMS (Rockland, USA) and low melting agarose from Cambrex (Rockland, USA). Solvents (*n*-hexane, methanol LC-MS grade), hydrogen peroxide (H$_2$O$_2$) and sodium hydroxide (NaOH) were acquired from Merck (Darmstadt, Germany).

2.2. Microalgae cultivation and biomass harvesting

P. tricornutum (strain UTEX 640) was supplied by the culture collection of Algae (SAG, University of Goettingen, Germany) and cultivated under photoautotrophic conditions as described earlier (Gille, Hollenbach, et al., 2019). The biomass was obtained by centrifugation of the liquid cultures, the supernatant discarded, and the remaining pellet (biomass) stored at –20 °C (Gille, Hollenbach, et al., 2019).

2.3. P. tricornutum preparations

Fresh *P. tricornutum* biomass of 200 mg (corresponding to 20 mg dry matter (dm)) were dissolved in 10 mL HBSS and stored immediately at –20 °C (unprocessed) or sonication was applied. The sonication step was conducted as described earlier (Gille, Trautmann, Posten, & Briviba, 2015) under the following conditions: 15 min, 5 cycles/min, frequency of 20 kHz, ultrasonic intensity of 9.8 W cm^{-2} with a SONOPULS HD 2070 homogenizer from Bandelin (Berlin, Germany). The samples were stored at -20 °C.

Prior to processing with the ball-mill, *P. tricornutum* biomass was lyophilized protected from light by using a Christ Alpha 1–2 LD freeze drier (Osterode a. Harz, Germany). The lyophilized samples were resuspended in deionized water and treated at a shaking frequency of 30 Hz for 10 min using a ball mill MM 300 from Retsch (Haan, Germany) after addition of three stainless steel beads (3 mm diameter). Subsequently, the samples were stored at –20 °C.

3. Sub-cellular fractionation by Folch extraction

To achieve a separation of *P. tricornutum* constituents due to their solubility in different solvents, the extraction method in accordance to Folch et al. was conducted (Folch, Lees, & Sloane-Stanley, 1957). Therefore, 5 mL HBSS were added to a sample of 20 mg (dm) *P. tricornutum* and mixed thoroughly before adding by 3 mL methanol and mixing for 2 min. Subsequently, 6 mL chloroform were added and mixed again thoroughly for 2 min. The mixture was centrifuged for 10 min at 4000*g* and the respective upper (methanol and HBSS; hydrophilic), lower (chloroform; lipophilic), and solid phase were transferred to separate tubes. Subsequently, the solvents were then evaporated under a stream of nitrogen and stored at -20 °C until cell treatment.

3.1. Characterization of P. tricornutum sub-fractions

The hydrophilic and lipophilic sub-fraction were subjected to analysis of carotenoids, α-tocopherol, fatty acids, and hydrophilic and lipophilic peroxides.

3.1.1. Carotenoids

The carotenoids were analyzed in accordance to a method that was published previously (Derwenskus et al., 2019; Gille, Hollenbach, et al., 2019).

3.1.2. α-Tocopherol

α-Tocopherol was measured in accordance to a statutory predicted method (CEN/Technical Commitee 275, 2015). In brief, 5 mL of each extract were mixed with 1 mL ascorbic acid (20 mg/mL) and 5 mL potassium hydroxide (50 g/100 mL). The mixture was incubated at 80 °C for 15 min. After the samples were cooled to room temperature, lipophilic compounds extracted two times with hexane, and the solvent evaporated under a stream of nitrogen. For HPLC analysis, the dried residue was resolved in 100 µL mobile phase (methanol/acetonitrile (50/50; v/v)). The HPLC system was equipped with a pump, auto sampler, column oven, and fluorescence detector from VWR-Hitachi as well as a Luna® C18 LC column (5 µM, 250 × 4.6 mm) from Phenomenex (Aschaffenburg, Germany). The separation was conducted using with an isocratic program and the resulting peak was detected with a fluorescence detector (excitation and emission wavelengths, 288 and 329 nm, respectively).

3.1.3. Fatty acids

Fatty acid composition was analyzed by GC–MS. Dichloromethane lipid extracts were dried under nitrogen, dissolved in n-hexane and derivatized with methanolic acetyl chloride (Ecker, Scherer, Schmitz, & Liebisch, 2012). Fatty acid methyl esters (FAME) were analyzed by GC–MS on a Trace GC 1310 equipped with a split/splitless injector and coupled to a TSQ Duo (all from Thermo Scientific, Germany). 1 µL sample was injected using a split ratio of 1:8 at 260 °C and a purge flow of 3 mL/min helium. FAME were separated on a 70% cyanopropyl polysilphenylene-siloxane column (TR-FAME) with the dimensions 60 m × 0.25 mm × 0.25 µm (Thermo Scientific, Germany). Helium was used as carrier gas with a flow rate of 1.2 mL/min. The following temperature program was used: 50 °C (3 min)/50 > 140 °C (15 °C/min)/140 > 220 °C (3 °C/min)/220 > 250 °C (15 °C/min). Detection of individual FAME was performed on a triple quadrupole mass spectrometer using a combined SIM/Scan mode with time segments and electron ionization. Transfer line and ion source were held at 250 °C and 200 °C, respectively. Compounds were identified by NIST library search and quantified by external calibration using one specific quantifier ion and up to two qualifier ions. A fatty acid methyl esters (FAME) standard mix plus additional single FAME compounds (all from Supelco/Sigma-Aldrich) were used for calibration.

3.1.4. Ferrous oxydation xylenol orange (FOX)-assay

The FOX-assay was applied to assess hydrophilic and lipophilic peroxides in the methanol or chloroform fractions obtained by Folch extraction. The method was performed as described previously with some modifications (Gay, Collins, & Gebicki, 1999). The sub-fractions obtained by Folch extraction were applied to the test as methanolic solutions. Catalase and tris(2-carboxyethyl)phosphine (TCEP) were added to both sub-fractions to examine the potential decompensation or reduction of existing peroxides by pre-incubating with 40 μL of a 200 U/mL catalase solution or 40 μL of a 80 mM TCEP solution. To 40 μL of each test sample, 40 μL iron (II) sulfate (1.5 mM, in 25 mM sulfuric acid) and 280 μL xylenol orange solution (150 μM, in 25 mM sulfuric acid) were added and incubated for 30 min at room temperature. Following incubation, 100 μL of the mixture was transferred to one well of a 96 well plate and the absorbance measured at 560 nm (Tecan microplate reader, Tecan GmbH, Crailsheim, Germany).

3.2. Caco-2 cell culture and cell treatment

Caco-2 and HT-29 colon carcinoma cells were supplied by the German collection of Microorganisms and Cell Cultures (DMSZ, Braunschweig, Germany). Caco-2 cells were cultivated in EMEM (supplemented with 10% (v/v) FCS, 1% (v/v) glutamine, 1% (v/v) NEA, and 1% (v/v) P/S), whereas HT-29 cells were grown in DMEM (supplemented with 10% (v/v) FCS, 1% (v/v) NEA, and 1% (v/v) P/S) at 37 °C in humidified air with 5% (v/v) CO_2. Cells were cultured in 6 well-plates (Caco-2 cells: $0.02 * 10^6$/well; HT-29: $0.1 * 10^6$/well) in order to conduct the comet assay, WST-1 assay, and apoptosis measurement or in 96 well-plates (Caco-2 cells: $1 * 10^3$/well; HT-29: $5 * 10^3$/well) for the assessment of ROS formation. In all tests, cells were grown for 5–6 d, changing the growth medium every 2–3 d, and utilized at a confluence of 70–85%. To achieve differentiation, Caco-2 cells were grown for at least 18 d in 6 well-plates, changing the growth medium every 2–3 d. The differentiated cells were used for experiments between day 18 and 21.

For cell incubation, the suspensions of unprocessed, sonicated and ball-milled *P. tricornutum* were diluted to a concentration of 200 μg, 20 μg and 2 μg dm/mL, respectively. The hydrophilic, lipophilic and solid sub-fraction were resolved in HBSS containing 0.05% tetrahydrofuran (THF) and applied to the cells according to a concentration of 100 μg dm/mL. To assess genotoxic effects, Caco–2 and HT-29 cells were incubated with the *P. tricornutum* preparations, fucoxanthin, or 100 μM H_2O_2 diluted in HBSS (containing $CaCl_2$). After 2 h, the incubation solutions were aspirated, the cells washed twice with PBS, and used for subsequent tests.

To test the time dependent repair of *P. tricornutum*-induced DNA damages and the effect on cell proliferation, the Caco-2 cells were incubated for 2 h with *P. tricornutum* (20 μg dm/mL) and for an additional 2 h or 24 h with EMEM (complete medium). Followed by aspiration of incubation solutions or EMEM and the cells were applied for comet assay or WST-1 assay.

To observe an apoptotic effect (Annexin staining and caspase-3 activation), the optimal incubation time and positive substances were established in pre-studies. Non-differentiated, differentiated Caco-2 cells, as well as HT-29 cells were treated with the appropriate positive controls, *P. tricornutum* preparations (20 μg dm/mL) or fucoxanthin for 48 h (non-differentiated Caco-2 cells), 4 h (differentiated Caco-2 cells), or 24 h (HT-29 cells). Afterwards, the cells were trypsinized and applied apoptosis and necrosis were measured.

To determine the uptake of fucoxanthin as well as the extracellular and intracellular formation of fucoxanthinol, Caco-2 cells (undifferentiated and differentiated) and HT-29 cells were treated with 3 μM fucoxanthin for up to 24 h. 500 μL of the supernatant of each well were collected after 2 h, 12 h or 24 h of incubation, 400 μL THF containing 0.25 mg BHT/mL were added and the samples stored at −20 °C until fucoxanthin/fucoxanthinol extraction. At the aforementioned time

points, cells were washed twice with PBS, trypsinized and counted in a hemocytometer (following trypan blue staining). 250 μL of HBSS as well as 400 μL THF containing 0.25 mg BHT/mL were added to each cell lysate and stored at −20 °C until fucoxanthin/fucoxanthinol extraction.

Throughout all experiments, the cell vitality was assessed by trypan blue staining (blue staining of DNA in necrotic cells). Therefore, the cells were trypsinized with 0.5 mL trypsin/EDTA, stained with trypan blue, and counted in a hemocytometer.

3.3. Single cell microgel electrophoresis assay (comet assay)

The extent of DNA damage was determined by the single cell gel electrophoresis assay (comet assay) in accordance with the method described by Briviba, Graf, Walz, Guamis, and Butz (2016). In brief, a number of 2×10^5 cells were mixed with 85 μL of 0.7% low melting point agarose and added to a microscope slide covered with 0.5% normal melting agarose. The slides were placed in lysis buffer (100 mM Na_2EDTA, 1% Triton X 100, 2.5 mM NaCl, 1% lauroyl sarcosine sodium salt, 10% dimethyl sulfoxide, 10 mM Tris, pH 10) for 1 h with a subsequent conduction of DNA unwinding in an alkaline electrophoresis buffer (1 mM Na_2EDTA, 300 mM NaOH; pH 13) for 20 min. Afterwards, electrophoresis was performed at 25 V, 300 mA for 40 min. For quantification, the DNA was stained with the fluorescence dye DAPI (4′,6-Diamidin-2-phenylindol; 0.5% (w/v)) and fifty randomly selected cells were analyzed with fluorescent microscopy (DM 400B, Leica Microsystems; Mannheim, Germany) using the imaging software of Perceptive Instruments (Halstead, UK). The extent of DNA damage is expressed as the percentage of DNA in the comet tail (fluorescent intensity).

3.4. Cell viability and proliferation (WST-1 assay)

The viability and proliferation of Caco-2 cells was assessed by the WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium-sodium salt) assay in accordance with the manufacturer's protocol. Following the treatment, the cell layer was washed twice with PBS and incubated with the WST-1 solution for 1 h. Afterwards, the absorbance of the formed formazan was determined at 450 nm (reference wavelength 690 nm) using a Tecan microplate reader (Tecan Deutschland GmbH, Crailsheim, Germany).

3.5. ROS measurement (DCFH-DA assay)

The pro-oxidative activity of the *P. tricornutum* preparations in Caco-2 and HT-29 cells was measured by applying a dichlorofluorescein diacetate (DCFH-DA) probe. In brief, the growth medium was discarded, the cell layer washed once with 150 μL PBS, and incubated for 30 min with 100 μL of a 10 μM DCFH-DA solution (in HBSS). Afterwards, the cell layer was washed with 150 μL PBS and treated for 2 h with positive controls or *P. tricornutum* preparations. Finally, the fluorescence was measured at 485 nm (excitation) and 530 nm (emission) using a Tecan microplate reader (Tecan Deutschland GmbH, Crailsheim, Germany). In order to exclude disturbances in measurement, the fluorescence of each test preparation was determined using the aforementioned conditions in a cell-free environment. For blank measurements, cells were exposed to HBSS instead of the DCFH-DA probe followed by the incubation with the test preparations. Tert-butylhydroperoxide (TBHP) and H_2O_2 at a concentration of 100 μM were used as positive controls.

3.6. Determination of malondialdehyde

Malondialdehyde (MDA) were measured in accordance to the method described previously with slight modifications (Briviba et al., 2004). In brief, a 500 μL sample (*P. tricornutum* suspension) was mixed

with 10 µL butylated hydroxytoluene solution (BHT, 10 mM, dissolved in ethanol), 350 µL trifluoroacetic acid (20%, v/v), and 500 µL thiobarbituric acid (1.4%, w/v) and incubated at 95 °C for 45 min. Followed by centrifugation for 10 min (16,000g) and analysis of the MDA content in the supernatant by HPLC (same system as used for tocopherol determination). Potassium phosphate (0.02 mM; pH 6.5) and methanol (65/35, v/v) were used as mobile phase and the flow rate was set to 0.75 mL/min. Standard solutions of MDA in a range b 0.1–2 µM were used for calibration. The resulting peak was detected with a fluorescence detector (excitation and emission wavelengths, 515 and 550 nm, respectively).

3.7. Fucoxanthin/fucoxanthinol extraction and analysis via HPLC

The extraction procedure and analysis with HPLC were conducted in accordance with previous studies (Gille, Hollenbach, et al., 2019). In brief, the samples were thawed in the dark and mixed briefly. To each sample 700 µL heptane (containing 0.25 mg BHT/mL) were added, followed by mixing for 1 min and centrifuging at 12,000g for 2 min. This procedure was repeated three times for each sample, the extracts were combined, and the solvents evaporated under a steam of nitrogen and stored at −20 °C until analysis. The amount of fucoxanthin or fucoxanthinol was determined by reversed-phase HPLC accordingly to a method published earlier (Gille, Hollenbach, et al., 2019).

3.8. Measurement of apoptosis and necrosis

In order to assess the induction of apoptotic signals, the activation of caspase-3 as well as the translocation of phosphatidylserine to the outer leaflet was determined. Therefore, the FITC Active Caspase-3 Apoptosis Kit from BD Pharmingen™ (Heidelberg, Germany) and the PE Annexin V Kit from BioLegend (Koblenz, Germany) were utilized in accordance to the manufacturer's protocol. Moreover, the Zombie Violet™ Fixable Viability Kit from BioLegend (Koblenz, Germany) was applied in combination with the latter to determine necrosis. In brief, cells of one well (HT-29, differentiated Caco-2 cells) or 2 wells (Caco-2) of a 6-well plate were trypsinized, washed once in PBS, and resuspended in 2 mL ice-cold PBS. At first, 100 µL of the Zombie Violet™ dye working solution (dilution: 1/1000 in DMSO) was added. The mixture was incubated for 15 min at room temperature protected from light, centrifuged (250g, room temperature, 5 min), and the pellet washed twice in ice-cold PBS. Second, the Annexin V-staining was performed by resuspending the cell pellet in 100 µL Annexin V Binding Buffer, adding 5 µL of PE Annexin V, and incubating the mixture for 5 min at room temperature protected from light. The cell pellet was washed twice with ice-cold PBS, resuspended in 500 µL BD Cytofix/ Cytoperm solution, and incubated for 20 min on ice in the dark. The cell suspension was centrifuged (250g, room temperature, 5 min), followed by two washing steps with 500 µL BD Perm/Wash buffer (diluted 1/10). This was followed by resuspending the cell pellet in 100 µL BD Perm/ Wash buffer, adding 20 µL of FITC rabbit Anti-Active Caspase-3, and incubating for 30 min at room temperature protected from light. Afterwards, the cells were washed once with 1 mL BD Perm/Wash buffer, resuspended and 500 µL BD Perm/Wash buffer, and stored on ice until measurement on a FACSVerse™ flow cytometer (Becton Dickinson, Heidelberg, Germany). The data analysis was conducted with the software FlowJo 10.4.2.

3.9. Statistic

Data are presented as mean ± standard deviation. The statistical analysis was determined by one-way ANOVA followed by Tukey-Kramer test or Dunn's test. For this, SigmaPlot software was used (version 13.0, Systat Software GmbH; Erkrath, Germany). Values were considered as significantly different if p value < 0.05.

Table 1
Analytical parameters of lipophilic *P. tricornutum* sub-fraction.

Constituent	Concentration (µg/mg dm)
Fatty acids:	
C14:0	5.06
C15:0	0.26
C16:0	13.71
C16:1_9 cis	30.99
C16:2_9,12 cis*	0.06
C16:3_6,9,12 cis*	0.24
C17:0	0.10
C18:0	1.76
C18:1_9 cis	0.73
C18:2_9,12 trans	0.39
C18:2_9,12 cis	1.16
C18:3_6,9,12 cis	0.25
C18:3_9,12,15 cis	0.59
C20:4_5,8,11,14 cis	0.63
C20:5_5,8,11,14,17, cis	21.62
C22:0	0.17
C22:6_4,7,10,13,16,19 cis	2.59
C24:0	1.70
C24:1_15 cis	0.33
Carotenoids:	
Fucoxanthin	3.83
Zeaxanthin	0.55
β-Carotene	traces
others	
α-Tocopherol	4.07

dm - dry matter (*P. tricornutum* biomass).
 * Tentative identification, semi-quantitative.

4. Results

4.1. Characterization of P. tricornutum preparations

The *P. tricornutum* biomass consists of 10% dry matter. In order to characterize the investigated biomass, several analytical parameters were assessed in the *P. tricornutum* test preparations with a focus on the lipophilic sub-fraction.

MDA is a byproduct of lipid peroxidation, especially formed from polyunsaturated fatty acids. It is highly reactive to macromolecules such as proteins, RNA, and DNA to form crosslinks and adducts (Marnett, 1999; Uchida, 2000; Voitkun & Zhitkovich, 1999). The content of TBARS (MDA) in the *P. tricornutum* test preparations did not show any differences due to the use of processing methods. The preparation of unprocessed *P. tricornutum* biomass revealed an amount of 4.34 ± 0.48 nmol/mg dm, that of the sonicated biomass 4.92 ± 2.64 nmol/mg dm, and that of the ball-milled biomass 3.12 ± 0.48 nmol/mg dm.

Table 1 shows the content of several constituents in the lipophilic sub-fractions. None or merely traces of these constituents were found in the hydrophilic sub-fraction. As already reported previously, palmitic acid (13.71 µg/mg dm), palmitoleic acid (30.99 µg/mg dm) and EPA (21.62 µg/mg dm) represented the main fatty acids in *P. tricornutum* (Derwenskus et al., 2019). Moreover, it contained 4 µg/mg dm α-tocopherol. The main carotenoid was fucoxanthin with 3.83 µg/mg dm, followed by zeaxanthin (0.55 µg/mg dm) and merely traces of β-carotene. In addition, we lacked to detect hydrophilic and lipophilic hydroxides as measured with the FOX-assay.

4.2. Genotoxic effects of P. tricornutum preparations and fucoxanthin in Caco-2 and HT-29 cells

In all conducted experiments, neither the treatment with *P. tricornutum* preparations nor controls resulted in cell viability below 95% in both cell lines (data not shown). H_2O_2 (positive control) significantly led to an induction of DNA strand breaks in Caco-2 as well as HT-29 cells (Table 2). All *P. tricornutum* preparations showed a dose-dependent

Table 2

Induction of DNA strand breaks by *P. tricornutum* preparations in Caco-2 and HT-29 cells.

Treatment		Caco-2 cells Tail intensity (%)	HT-29 cells Tail intensity (%)
Control (HBSS)		5.24 ± 2.50[a]	3.78 ± 0.59[a]
H_2O_2 (100 µM)		30.27 ± 11.55[b]	19.04 ± 8.84[b]
Unprocessed	200 µg dm/mL	39.58 ± 9.17[b]	24.50 ± 6.73[b]
	20 µg dm/mL	16.55 ± 1.86[ab]	12.18 ± 1.58[ab]
	2 µg dm/mL	4.89 ± 1.51[a]	5.42 ± 2.04[a]
Sonicated	200 µg dm/mL	28.33 ± 2.29[b]	21.05 ± 2.48[b]
	20 µg dm/mL	16.25 ± 4.18[ab]	7.82 ± 1.85[ab]
	2 µg dm/mL	5.96 ± 2.62[ab]	4.73 ± 0.78[ab]
Ball-milled	200 µg dm/mL	37.57 ± 8.51[b]	25.56 ± 4.49[b]
	20 µg dm/mL	14.51 ± 3.20[ab]	9.90 ± 2.27[ab]
	2 µg dm/mL	7.93 ± 0.76[a]	4.99 ± 0.32[a]

Data are means of at least four independent experiments ± standard deviation. Different letters indicate statistical significant differences as determined separately for each processing method by one-way ANOVA on ranks followed by the Dunn's test (p < 0.05).

genotoxic effect in both cell lines. The DNA damage induced by the preparations of the highest concentrations (200 µg dm/mL) was comparable with that observed for H_2O_2 (100 µM). The treatment with a concentration of 2 µg dm/mL showed no significant induction of DNA strand breaks. Moreover, the extent of genotoxicity did not differ following the use of sonication or ball-milling (Table 2).

To narrow a potential sub-fraction that triggered the DNA strand breaks, *P. tricornutum* was fractionated by Folch extraction. Thereby, a lipophilic, hydrophilic, and solid sub-fraction were obtained and applied to Caco-2 and HT-29 cells. In both cell lines, the use of the lipophilic fraction resulted in a significant genotoxic effect comparable to H_2O_2. The hydrophilic and solid sub-fractions revealed no induction of DNA damage (Fig. 1). Since fucoxanthin is an abundant carotenoid in *P. tricornutum* and present in the lipophilic fraction, we further tested its genotoxicity (Table 3). There was no induction of DNA strand breaks in HT-29 cells. In Caco-2 cells, there might be a moderate genotoxic effect. However, it was not dose dependent and not significantly higher than the solvent control (ethanol).

4.3. ROS formation in Caco-2 and HT-29 cells

The treatment of Caco-2 cells with unprocessed and sonicated *P. tricornutum* at a concentration of 200 µg dm/mL revealed a significant intracellular formation of ROS, which could be diminished using 20 µg dm/mL and 2 µg dm/mL. In contrast, the application of the preparation containing 200 µg dm/mL of ball milled biomass resulted in a faint amount of intracellular ROS. The other preparations of ball milled *P. tricornutum* (20 µg dm/mL and 2 µg dm/mL) showed no ROS formation (Fig. 2A).

Likewise, the application of unprocessed and sonicated *P. tricornutum* at the highest concentration resulted in a significant increase of intracellular ROS in HT-29 cells. The treatment with ball milled biomass (200 µg dm/mL) led to a moderate ROS formation; however, not significant due to high variation of the values. The incubation with preparations containing 20 µg dm/mL and 2 µg dm/mL of unprocessed, sonicated, or ball milled biomass showed faint or no intracellular ROS formation (Fig. 2B).

4.4. Repair of P. tricornutum-induced DNA damage in Caco-2 cells

Fig. 3 reveals the time dependent repair of DNA damage and the effect on cell proliferation after treatment with either H_2O_2 or unprocessed *P. tricornutum* biomass (20 µg dm/mL). The H_2O_2-induced DNA damage was not fully repaired after a recovery time of 2 h, whereas there was no difference in genotoxicity and proliferation
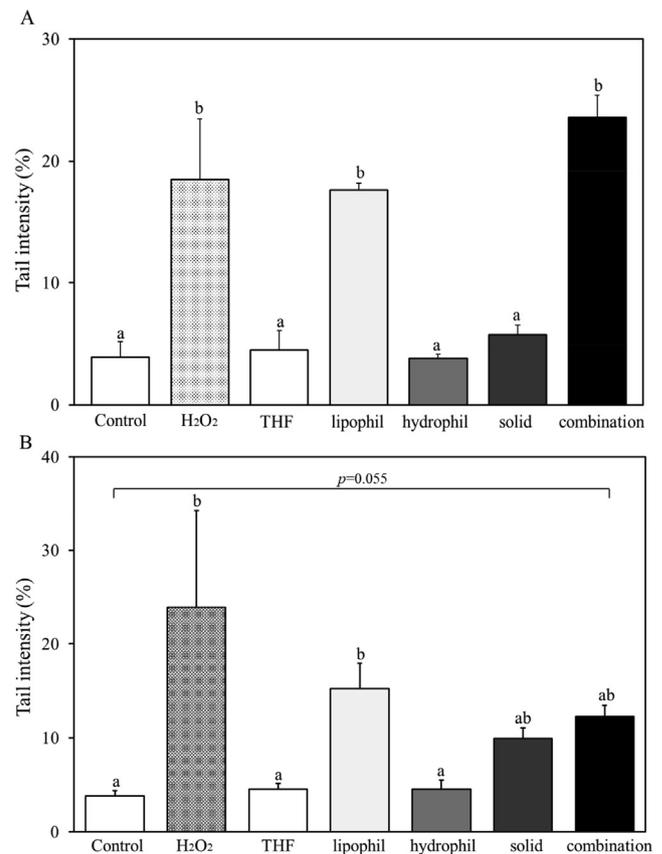
Fig. 1. Induction of DNA strand breaks by several *P. tricornutum* sub-fractions obtained by Folch extraction in (A) Caco-2 and (B) HT-29 cells. C: control treatment; THF: tetrahydrofuran; data are means of at least three independent experiments ± standard deviation. Different letters indicate statistically significant differences as determined by one-way ANOVA followed by Dunn's test (p < 0.05).

Table 3

Induction of DNA strand breaks by fucoxanthin in Caco-2 and HT-29 cells.

Treatment		Caco-2 cells Tail intensity (%)	HT-29 cells Tail intensity (%)
Control (HBSS)		3.84 ± 0.89[a]	4.05 ± 0.10[a]
Ethanol		5.10 ± 1.05[ac]	4.01 ± 0.84[a]
H_2O_2 (100 µM)		19.75 ± 2.74[b]	15.11 ± 1.49[b]
Fucoxanthin	3 µM	7.35 ± 2.38[ab]	4.88 ± 1.02[a]
	1.5 µM	8.24 ± 1.93[bc]	4.48 ± 0.67[a]
	0.15 µM	7.46 ± 1.56[bc]	4.24 ± 0.59[a]
	0.015 µM	6.45 ± 1.70[ac]	3.86 ± 0.30[a]

Data are means of three independent experiments ± standard deviation. Different letters indicate statistical significant differences as determined by one-way ANOVA on ranks followed by the Dunn's test for Caco-2 cells (p < 0.05) or Tukey Kramer Test for HT-29 cells (p < 0.05).

compared to control treatment 24 h post treatment. Contrary, the *P. tricornutum*-induced DNA damage was already lowered after 2 h of recovery. However, even after a 24 h-recovery period the genotoxic effect remained significantly higher in comparison to cells that received control treatment. The incubation with *P. tricornutum* did not impair the cell proliferation at any point in time.

4.5. Metabolization of fucoxanthin in Caco-2 and HT-29 cells

Previously, we observed the intracellular formation of fucoxanthinol in differentiated Caco-2 cells after an incubation with *P. tricornutum* preparations (Gille, Hollenbach, et al., 2019). Differentiated
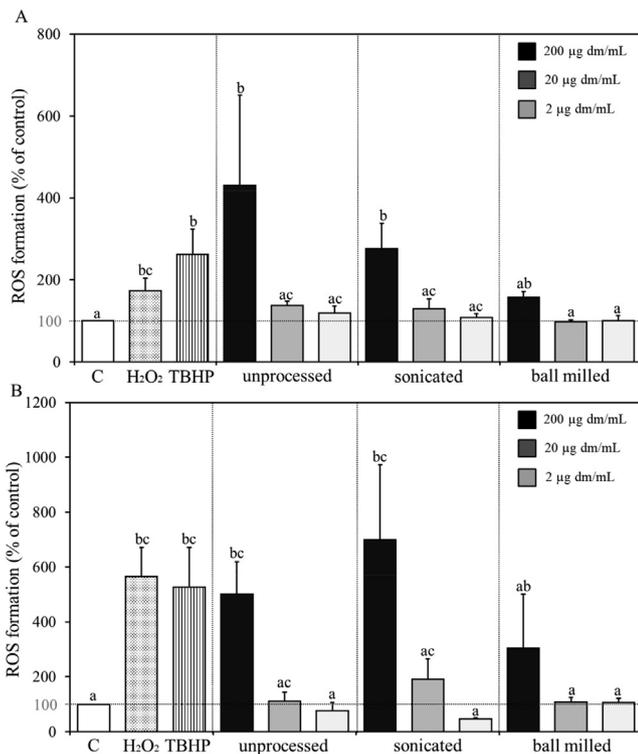
Fig. 2. Formation of cellular reactive oxygen species (ROS) by *P. tricornutum* preparations in (A) Caco-2 and (B) HT-29 cells. C: control treatment; TBHP: *tert*-butylhydroperoxide; data are means of at least three independent experiments ± standard deviation. Different letters indicate statistically significant differences as determined separately for each processing method by one-way ANOVA on ranks followed by the Dunn's test (p < 0.05).

Caco-2 cells show more similarities with intestinal cells (Annaert, Kinget, Naesens, de Clercq, & Augustijns, 1997; Hidalgo, Raub, & Borchardt, 1989; Pinto et al., 1981; Spalinger, Seidman, Menard, & Levy, 1998). Hence, fucoxanthin and its metabolites might lead to different cellular effects (Asai, Sugawara, Ono, & Nagao, 2004). For this reason, we investigated the time-dependent uptake of fucoxanthinol and extracellular/intracellular formation of fucoxanthinol in the applied cell lines (Fig. 4). The differentiated Caco-2 cells accumulated fucoxanthin after 2 h and the amount significantly increased up to 165.15 nmol/1 * 10^6 cells throughout the incubation for 24 h. After an incubation of 12 h, fucoxanthinol was extracellularly found and significantly accumulated in the cells. However, there was no considerable difference after 24 h incubation for both, the intracellular and cellular fucoxanthinol content. In general, the undifferentiated Caco-2 and HT-29 cells showed a similar metabolization of fucoxanthin. Both cell lines showed a higher intracellular fucoxanthin content (undifferentiated Caco-2 cells: 319.74 nmol/1 * 10^6 cells; HT-29 cells: 204.54 nmol/1 * 10^6 cells) than observed for the differentiated Caco-2 cells after 24 h of incubation. Contrary to the differentiated Caco-2 cells, fucoxanthinol was formed intracellularly since there is no fucoxanthinol measureable in the supernatant. However, the intracellular fucoxanthinol content was similar between the tested cell lines and showed the highest amount after the incubation of 24 h (undifferentiated Caco-2 cells: 425.06 nmol/1 * 10^6 cells; differentiated Caco-2 cells: 345.86 nmol/1 * 10^6 cells; HT-29 cells: 345.93 nmol/1 * 10^6 cells).

4.6. Apoptotic and necrotic response in Caco-2 and HT-29 cells

The apoptotic effect was assessed by measuring the activation of intracellular caspase-3 and the translocation of phosphatidylserine (Annexin V- staining) in Caco-2 cells (undifferentiated and
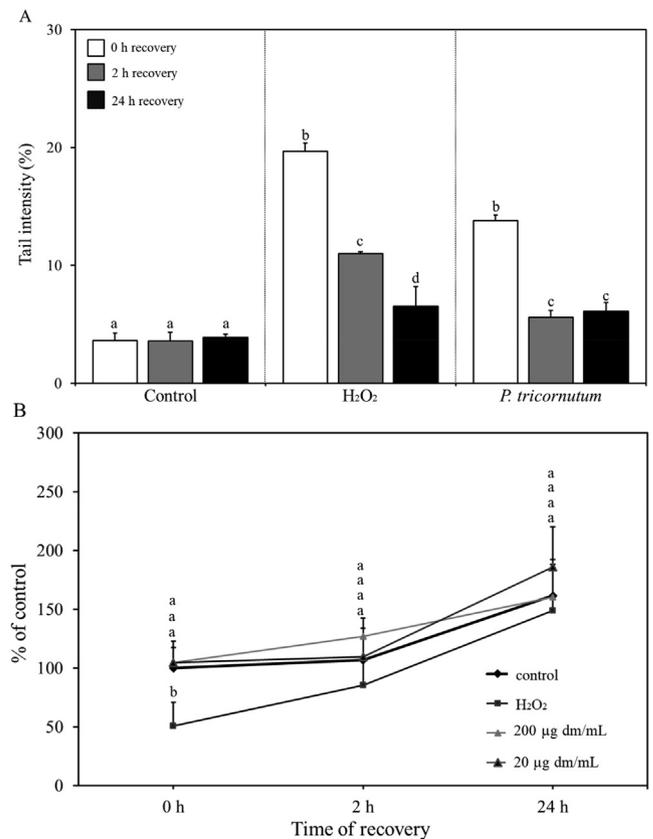

Fig. 3. Effect of recovery on (A) repair of DNA strand breaks and (B) cell proliferation after treatment with *P. tricornutum* in Caco-2 cells. C: control treatment; data are means of three independent experiments ± standard deviation. Different letters indicate statistically significant differences as determined separately for H_2O_2 and *P. tricornutum* by one-way ANOVA in (A) on ranks followed by the Dunn's test (p < 0.05) for H_2O_2 or by Tukey-Kramer test (p < 0.05) for *P. tricornutum*, and in (B) by Tukey-Kramer test (p < 0.05).

differentiated) and HT-29 cells. In addition, necrosis was determined by staining with Zombie Violet™ dye. The applied cell lines differ in their sensitivity for several positive controls and incubation times. The optimal incubation time to achieve an apoptotic effect was 48 h for undifferentiated Caco-2 cells, 4 h for differentiated Caco-2 cells, and 24 h for HT-29 cells.

In undifferentiated Caco-2 cells, the treatment with 50 mM butyrate showed an increase of Annexin V-staining and activation of caspase-3, which is significantly higher in comparison to the incubation with EMEM (solvent control) as well as unprocessed and ball-milled *P. tricornutum*. None of the *P. tricornutum* or fucoxanthin preparations showed an increase of these parameters or necrosis compared to the treatment with EMEM. Moreover, we observed a huge loss of cells when using 10 μM fucoxanthin. Therefore, 3 μM was used as the highest concentration (Table 4).

None of the applied treatments showed an apoptotic effect in differentiated Caco-2 cells when determining Annexin-V positive stained cells. However, the incubation with staurosporin (0.5 μM)/brefeldin A (0.5 μM) resulted in a significant increase of activated caspase-3 compared to the treatment with EMEM as well as the *P. tricornutum* preparations. Again, there was no significant increase of intracellular activated caspase-3 or necrotic effect when using the *P. tricornutum* and fucoxanthin preparations in comparison to the control treatment (Table 5).

In HT-29 cells, the staurosporin (0.5 μM)/brefeldin A (0.5 μM) treatment showed a significantly higher amount of Annexin V-positive stained cells compared to the other treatments, except for the fucoxanthin preparations. A similar result could be observed for intracellular
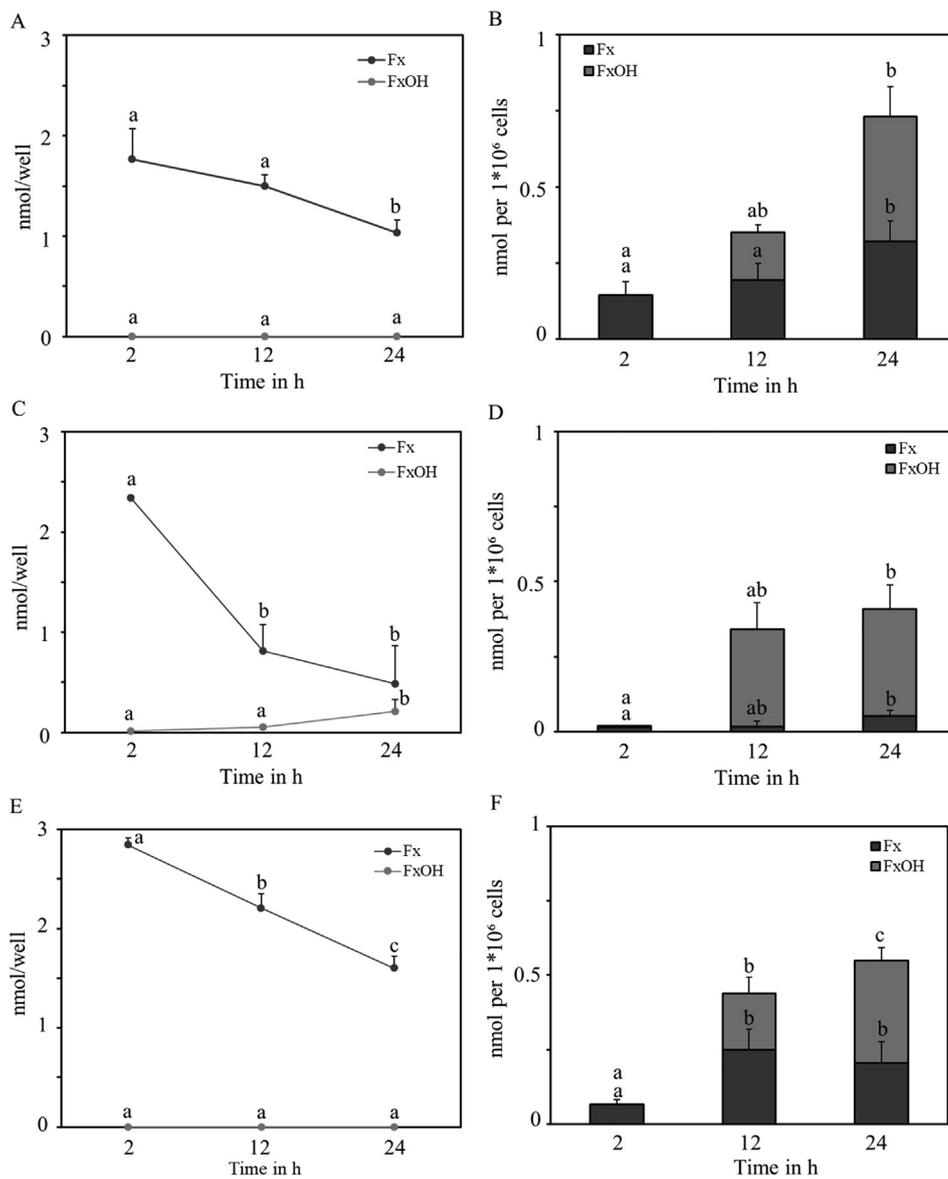
Fig. 4. Extracellular formation of fucoxanthinol (left) and intracellular uptake of fucoxanthin/fucoxanthinol (right) in undifferentiated Caco-2 cells (A,B), differentiated Caco-2 cells (C,D), and HT-29 cells (E,F). The cells were incubated with 3 μM fucoxanthin. At different time points, the supernatant and cells were harvested and applied for fucoxanthin/fucoxanthinol (Fx/FxOH) determination by HPLC. Data are means of three independent experiments (numerous wells) ± standard deviation. Different letters indicate statistically significant differences as determined separately for fucoxanthin and fucoxanthinol by one-way ANOVA on ranks followed by the Tukey-Kramer test ($p < 0.05$).

Table 4

Apoptosis rate (Annexin V), caspas-3 activation and necrosis rate of Caco-2 cells after treatment with controls or *P. tricornutum* preparations (20 μg dm/mL) and fucoxanthin.

Treatment	Annexin V (%)	Caspase-3 activation (%)	Necrosis (%)
Control (EMEM)	2.79 ± 1.28[ac]	1.99 ± 0.66[a]	3.77 ± 1.85[a]
DMSO	3.41 ± 0.91[ab]	2.21 ± 1.00[a]	5.32 ± 1.47[a]
Butyrate	4.50 ± 0.87[b]	12.76 ± 2.83[b]	7.17 ± 2.64[a]
Unprocessed	2.23 ± 1.07[ac]	1.54 ± 0.95[a]	6.61 ± 5.10[a]
Sonicated	3.14 ± 1.09[abc]	2.46 ± 1.09[ab]	7.72 ± 7.01[a]
Ball-milled	1.51 ± 0.52[c]	0.78 ± 0.15[a]	4.49 ± 1.65[a]
Fucoxanthin (1.5 μM)	3.83 ± 0.44[ab]	2.49 ± 0.94[ab]	3.95 ± 1.02[a]
Fucoxanthin (3 μM)	4.07 ± 1.12[ab]	3.61 ± 0.55[ab]	5.01 ± 0.92[a]

Data are means of four independent experiments ± standard deviation. Different letters indicate statistically significant differences as determined by one-way ANOVA followed by Tukey test for Annexin staining or on ranks followed by the Dunn's test for others ($p < 0.05$).

Table 5

Apoptosis rate (Annexin V), caspase-3 activation and necrosis rate of differentiated Caco-2 cells after treatment with controls or *P. tricornutum* preparations (20 μg dm/mL) and fucoxathin.

Treatment	Annexin V (%)	Caspase-3 activation (%)	Necrosis (%)
Control (EMEM)	2.46 ± 1.47[a]	3.31 ± 1.36[a]	15.52 ± 9.19[ab]
DMSO	3.10 ± 1.91[a]	3.57 ± 0.66[ab]	17.40 ± 12.03[ab]
Staurosporin/ Brefeldin	2.01 ± 0.55[a]	13.02 ± 5.60[b]	12.60 ± 5.13[ab]
Unprocessed	2.74 ± 1.38[a]	2.41 ± 0.75[a]	15.65 ± 9.32[ab]
Sonicated	4.83 ± 1.89[a]	2.17 ± 1.07[a]	5.91 ± 2.10[a]
Ball-milled	2.28 ± 1.02[a]	2.08 ± 0.96[a]	21.22 ± 3.03[b]
Fucoxanthin (1.5 μM)	3.04 ± 1.28[a]	3.58 ± 0.86[ab]	8.46 ± 3.41[ab]
Fucoxanthin (3 μM)	3.98 ± 2.39[a]	4.29 ± 3.37[ab]	10.10 ± 4.26[ab]
Fucoxanthin (10 μM)	3.31 ± 0.24[a]	2.59 ± 0.38[a]	8.99 ± 3.81[ab]

Data are means of four independent experiments ± standard deviation. Different letters indicate statistically significant differences as determined by one-way ANOVA on ranks followed by the Dunn's test ($p < 0.05$).

Table 6
Apoptosis rate (Annexin V), caspase-3 activation and necrosis rate of HT-29 cells after treatment with controls or *C. vulgaris* preparations (20 µg dm/mL) and fucoxanthin.

Treatment	Annexin V (%)	Caspase-3 activation (%)	Necrosis (%)
Control (DMEM)	3.17 ± 0.88[a]	0.45 ± 0.16[a]	2.29 ± 1.27[ab]
DMSO	2.52 ± 0.70[a]	0.49 ± 0.18[a]	2.76 ± 1.70[ab]
Staurosporin/ Brefeldin	12.67 ± 6.38[b]	16.57 ± 7.49[b]	6.86 ± 4.13[b]
Unprocessed	2.99 ± 0.78[a]	0.32 ± 0.12[a]	1.13 ± 0.55[a]
Sonicated	2.31 ± 0.26[a]	0.38 ± 0.02[ab]	1.25 ± 0.33[a]
Ball-milled	2.71 ± 0.24[a]	0.22 ± 0.05[a]	1.21 ± 0.59[a]
Fucoxanthin (1.5 µM)	3.63 ± 1.04[ab]	0.84 ± 0.38[ab]	2.24 ± 2.34[ab]
Fucoxanthin (3 µM)	3.99 ± 2.57[ab]	1.18 ± 0.51[ab]	2.31 ± 2.24[ab]
Fucoxanthin (10 µM)	6.57 ± 4.69[ab]	1.30 ± 1.17[ab]	1.56 ± 1.56[a]

Data are means of four independent experiments ± standard deviation. Different letters indicate statistically significant differences as determined by one-way ANOVA on ranks followed by the Dunn's test ($p < 0.05$).

activated caspase-3. Moreover, there was a small necrotic effect following the incubation with staurosporin (0.5 µM)/brefeldin A (0.5 µM), which was significantly increased compared to the incubation with unprocessed and ball-milled biomass (Table 6).

5. Discussion

The present study aimed to investigate cytotoxic, genotoxic, and pro-apoptotic/necrotic effects of several *P. tricornutum* preparations in Caco-2 (undifferentiated and differentiated) as well as HT-29 cells. The use of intestinal cells would be ideal, since they are an early contact point when consuming *P. tricornutum*-supplemented food products. Differentiated Caco-2 cells show morphological and biochemical similarities with intestinal cells (Hidalgo et al., 1989; Pinto et al., 1981). However, the measurement of DNA damage in these cells is technically difficult and the use of undifferentiated cells is more convenient. Moreover, undifferentiated Caco-2 and HT-29 cells enter haven proven to be a reliable model for testing genotoxicity and apoptosis of food products (Duh et al., 2018; Gille, Trautmann, et al., 2019; Hartwig et al., 2007; Roser et al., 2001). Processing methods have proven to be necessary to enable a proper release and bioaccessibility of *P. tricornutum* nutrients (Gille, Hollenbach, et al., 2019). However, techniques such as sonication and ball-milling might bear a risk of forming reactive by-products such as radicals and oxidants (Carail, Fabiano-Tixier, Meullemiestre, Chemat, & Caris-Veyrat, 2015). Therefore, biomass that is processed with these methods needs to be tested for further adverse effects.

The treatment with unprocessed and processed *P. tricornutum* preparations resulted in a dose-dependent genotoxic effect in Caco-2 as well as HT-29 cells. The extent of DNA strand breaks did not differ between the preparations of unprocessed, sonicated, or ball-milled biomass. An effect due to contaminations, e. g. with pathogenic microorganisms, can be neglected, since the cultivation was conducted under controlled, axenic laboratory conditions. However, the induction of oxidative stress might be an explanation since all preparations also led to a dose-dependent formation of intracellular ROS. Since *P. tricornutum* contains a high amount of PUFAs, we assumed that lipid peroxidation products would form, especially due to the use of processing methods. However, there was no difference of TBARS content in the preparations. Interestingly, the cellular ROS formation in undifferentiated Caco-2 cells and HT-29 cells varied between the applied processing methods. The preparation of the unprocessed and sonicated biomass indicated a similar outcome, whereas ball-milled biomass showed a diminished potential of forming intracellular ROS. However, the underlying mechanisms for the observed effects are presently

unclear and should be considered in further studies.

Data reveal that the genotoxic effect was triggered by the lipophilic sub-fraction (Folch extraction). In contrast, we previously observed that the DNA damage induced by the microalgae *Chlorella vulgaris* (*C. vulgaris*) was mainly mediated by the solid sub-fraction containing whole algal cells, the cell wall, and fragments (Gille, Trautmann, et al., 2019). The occurrence of lipid peroxides in the lipophilic fraction might be excluded due to the results obtained by the FOX (data not shown) and TBARS-assay. Fucoxanthin is an abundant carotenoid in *P. tricornutum* and present in this sub-fraction. The maximal fucoxanthin concentration applied via a *P. tricornutum* preparation was approximately 1.5 µM (preparation of 200 µg dm/mL) in the depicted experiments. Fucoxanthin is hydrolyzed to fucoxanthinol through enzymatic activity, e.g. in the intestine (Asai et al., 2004; Sugawara, Baskaran, Tsuzuki, & Nagao, 2002). Asai et al. found that fucoxanthinol exhibits a stronger anti-proliferative effect in HepG2 cells than fucoxanthin (Asai et al., 2004). We previously showed the formation of fucoxanthinol when incubating differentiated Caco-2 cells with *P. tricornutum* preparations (Gille, Hollenbach, et al., 2019). These cells show several morphological and biochemical similarities with enterocytes, such as the occurrence of brush-border membrane enzymes (Hidalgo et al., 1989; Pinto et al., 1981). Likewise, the activity of an endogenous lipase and carboxylesterase were described on the apical membrane of these cells (Annaert et al., 1997; Spalinger et al., 1998). Though the applied cell lines differed in the cellular amount of fucoxanthin and fucoxanthinol, we could not observe a deviating effect on genotoxicity and apoptosis/necrosis. Consequently, none of the detected adverse cellular effects could be related to fucoxanthin accumulation or the formation of fucoxanthinol. Thus, both compounds might be assessed as harmless. In contrast to the depicted results, several authors observed cytotoxic, pro-oxidative, and pro-apoptotic effects for fucoxanthin in different cell lines (Kotake-Nara et al., 2001; C. L. Liu et al., 2009; Z. Zhang et al., 2008). Moreover, Hosokawa et al. proposed the induction of apoptosis in colon cell lines due to the fragmentation of DNA and decreased protein level of Bcl-2 (Hosokawa et al., 2004). The deviating results might stem from a different experimental setup (e.g. incubation at a different cell status), use of higher concentrations of up to 25 µM fucoxanthin, and the focus on other cellular effects/different outcome. Complementing the depicted data, Beppu et al. did not observe a mutagenic activity of fucoxanthin or fucoxanthinol when applying Ames test and micronucleus assay (Beppu, Niwano, Sato, et al., 2009). Moreover, there were no physiological anomalies or toxic effects following fucoxanthin supplementation in mice (Beppu, Niwano, Sato, et al., 2009; Beppu, Niwano, Tsukui, Hosokawa, & Miyashita, 2009).

Besides fucoxanthin, the lipophilic fraction comprises other constituents, that themselves or their related metabolites might have a genotoxic potential. *P. tricornutum* contains high amounts of PUFAs such as EPA, which have shown growth-inhibitory and pro-apoptotic effects in several colon cancer cell lines including Caco-2 and HT-29 cells (Fasano et al., 2012; Kuan et al., 2011; Xia & Li, 2012; Zhang et al., 2015). In addition, compounds such as 4-hydroxy-2-hexanal (HHE) are derived from ω-3 PUFAs (Guichardant, Bacot, Moliere, & Lagarde, 2006; Van Kuijk, Holte, & Dratz, 1990) and are highly reactive towards nucleophiles (Esterbauer, Schaur, & Zollner, 1991; LoPachin & Gavin, 2014).

An extended induction of DNA damages and diminished capacity of DNA repair potentially lead to apoptosis. The DNA strand breaks caused by 20 µg *P. tricornutum* dm/mL were already diminished after a recovery period of about 2 h. Neither the *P. tricornutum* preparations nor H_2O_2 indicated an adverse effect on cell proliferation. In line with that, none of the applied preparations led to an increase of apoptosis in undifferentiated Caco-2 (after 48 h), differentiated Caco-2 (after 4 h), or HT-29 cells (after 24 h). So far, *P. tricornutum* was investigated less in terms of use in food products, whereas *C. vulgaris* represents one microalgae that has been used traditionally for human nutrition and other applications (Mata, Martins, & Caetano, 2010; Safi, Zebib, Merah,

Pontalier, & Vaca-Garcia, 2014). Compared with the depicted results, we previously observed a similar outcome of the investigated cellular effects including the extent of genotoxicity with *C. vulgaris* preparations. In addition, ball-milled *P. tricornutum* as well as *C. vulgaris* biomass was fed to C57Bl/6J mice in different concentrations for two weeks. The diets were well tolerated by the mice and neither histological nor serum analysis showed any toxicity (Neumann et al., 2018). Moreover, it should be considered that fucoxanthin-rich seaweeds have traditionally been used in Asian countries such as Japan, Korea and China for several purposes and no adverse health effects have been reported (Brown et al., 2014; Kumar & Brown, 2013).

6. Conclusions

To our knowledge, this is one of the first studies investigating the genotoxic effect of *P. tricornutum* in intestinal/colon cell lines. Our data indicate a dose-dependent induction of DNA damage; however, we were unable to identify specific constituents that caused the *P. tricornutum*-induced genotoxicity. Potentially, a multitude of lipophilic compounds or related metabolites could be responsible for the observed effects. Moreover, the induced DNA strand breaks could already be diminished after 2 h and there was no hint of an impairment of cell proliferation or apoptotic/necrotic effects. However, more research on the trigger and underlying mechanisms is required. In addition, the main carotenoid fucoxanthin merely showed mild genotoxic and no pro-apoptotic or necrotic activity. In general, neither the induction of DNA strand breaks nor further adverse effects were related to processing with sonication or ball-milling.

7. Ethics statements

This work did not include any human subjects or animal experiments.

Declarations of interest

The authors declare that there are no conflicts of interest.

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