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# A comparison of quantitative and qualitative superoxide dismutase assays for application to low temperature microalgae

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## Abstract

Antioxidant enzymes such as superoxide dismutase (SOD) play a key role in the removal of reactive oxygen species produced during visible and ultraviolet irradiance stress in microalgae and plants. However, little is known about the enzymatic antioxidative stress responses in ecologically important Antarctic marine microalgae. SOD in particular is difficult to analyze, possibly due to problems in obtaining sufficient quantities necessary for reliable and reproducible enzymatic assays. The aim of the present work was to create a sensitive, easy-to-use and reliable method for SOD determination in Antarctic microalgal material by comparing and optimizing existing protein extraction procedures and SOD assays in the marine Antarctic diatom *Chaetoceros brevis*. Optimization was achieved in cell disruption (sonication) and protein extraction procedures, extraction buffers, SOD assay methods (xanthine/xanthine oxidase and NBT/riboflavin photometric quantitative methods and native gel electrophoresis qualitative method) and the assay temperature. Protein extraction was optimal at low sonication amplitudes after a few pulses, irrespective of the type of buffer used. Extraction efficiency varied highly between the tested buffers; most protein was extracted in the presence of 1% of Triton X-100. SOD activity was best quantified using the NBT/riboflavin method in combination with a buffer containing potassium phosphate and Triton X-100. Moreover, the NBT/riboflavin method was demonstrated to be the most reliable and sensitive method at low temperatures (5 °C).
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*Keywords:* Superoxide dismutase; Xanthine/xanthine oxidase assay; NBT/riboflavin assay; Native gel electrophoresis; Marine Antarctic diatom

## 1. Introduction

Antarctic marine microalgae are known to experience high irradiance stress in situ. The extent to which microalgae are subjected to excess irradiance depends on factors like the intensity and spectral composition of incoming irradiance, position in the water column and the associated attenuation of irradiance [1–3]. Natural solar ultraviolet radiation (UVR: 280–400 nm) can reduce water column productivity in Antarctic marine waters by more than 50% [1,4]. Springtime Antarctic stratospheric ozone depletion causes an additional integrated productivity loss between 4% and 12% [1,5,6].

The detrimental effects of UVR-driven photoreactions to essential biomolecules can interrupt metabolic pathways and cause an overreduction of electron transport chains (ETC). When the consumption of reduced equivalents cannot match the production, electrons from ETCs can leak to molecular oxygen ($O_2$) and initiate formation of reactive oxygen species (ROS) [7–9]. ROS may damage photosystem II reaction center proteins by which photosynthesis could decrease and cause viability loss [10].

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Microalgae have developed mechanisms that offer protection against high levels of solar radiation to prevent overreduction of ETCs. Yet, when these protection systems are not adequate, ETCs become overreduced and ROS will accumulate rapidly. As a response, cells can counteract ROS by using a sophisticated network of antioxidant enzymes which actively scavenge the various ROS intermediates [12].

The first reactive oxygen species (ROS) formed is the superoxide anion ($O_2^{\cdot-}$). Through a series of uncontrolled univalent reductions, $O_2^{\cdot-}$ can be converted into the hydroxyl radical ($HO^\cdot$) which is among the most reactive oxygen species known to chemistry [13]. To prevent production of $HO^\cdot$ and oxidation of biomolecules, $O_2^{\cdot-}$ is converted into hydrogen peroxide ($H_2O_2$) by the enzyme superoxide dismutase (SOD; [14,15]). SOD is not only the first line of defence but also the only enzyme capable of catalyzing this reaction. Therefore SOD holds a key position within the antioxidant network.

SOD has been shown to be present in all aerobic cells [16]. In plants, SOD varies in response to environmental stressors such as chilling [17–19], metal exposure [20], hypersalinity [21,22] and UVR [23]. In contrast, relatively little information is available on SOD activity in marine microalgae. Most of the studies are conducted with temperate marine microalgae, demonstrating alterations in SOD activity in response to metal exposure [24–26], salinity [27,28], change in visible irradiance [29–31] and UVR [32–35]. Studies describing SOD responses in marine Antarctic microalgae are very scarce [36,11], which is surprising because of the ecological significance of the mentioned excess irradiance responses.

The limited information on SOD activity in marine Antarctic microalgae is possibly related with the difficulties in obtaining sufficient biomass for reliable and reproducible measurements. Methods so far used to measure SOD activity in microalgae are the xanthine/xanthine oxidase (X–XOD) [37] and the NitroBlue Tetrozolium/Riboflavin (NBT/RF). The first one is used for photometric measurements only while the second is used also for Native-PAGE [38]. The commonly used X–XOD method [37] requires such high quantities of biomass that obtaining reliable SOD assessments is virtually impossible for microalgal field material. In contrast, this method works fine for low temperature macroalgae as was demonstrated by Aguilera et al. [39,40] and Dummermuth et al. [41]. Own preliminary experiments with Antarctic microalgae showed, first of all, that standard cell disruption and protein extraction techniques were not satisfactory in terms of extraction efficiency. Secondly, the X–XOD assay did not seem suitable for application at the low temperatures required for analyzing Antarctic microalgal material. Therefore, it was the aim of the present work to find the optimal combination of cell disruption, protein extraction and SOD activity procedures at low temperature. To this end we tested a combination of various sonication procedures, protein extraction buffers and SOD assays. The material we used was derived from experiments with the common Antarctic diatom *Chaetoceros brevis*.

## 2. Materials and methods

### 2.1. Experimental procedures

Experiments were done using extracts of the marine Antarctic diatom *C. brevis* (isolate CCMP 163). *C. brevis* was grown at 4 °C and 25 $\mu$mol m$^{-2}$ s$^{-1}$ PAR (14L:10D cycle) in modified *f*/2 medium [42]. Samples were harvested by filtration or centrifugation (see below for procedures) when the cultures contained approximately $1 \times 10^5$ cells/ml. Samples were stored at $-80$ °C and analyzed within two months. A comparison between filtration and centrifugation for harvesting biomass had shown that filtration onto 47 mm polycarbonate filters (2.0 $\mu$m pore size; Osmonics, USA) yielded 25% more material than centrifugation (1500*g*) for 20 min at 4 °C (Falcan 6/300) (own unpublished results). Therefore, unless indicated otherwise (experimental series no. 1), cells were harvested by filtration.

The following type of experiments were done: (1) effects of disruption procedures on protein extraction efficiency; (2) effects of four extraction buffers on protein extraction efficiency; (3) effects of four extraction buffers on SOD activity and (4) temperature dependence of SOD activity assays.

### 2.2. Cell disruption and protein extraction efficiency

To study disruption procedures, 48 tubes containing 50 ml of *C. brevis* culture were centrifuged after which supernatants were discarded. Twenty four cellular pellets were resuspended in 1.5 ml buffer A containing 50 mM KH$_2$PO$_4$ (Merck, Darmstadt, Germany), pH 7 and 0.1 mM EDTA (Merck, Darmstadt, Germany) [43] and transferred to a 60 ml glass beaker. The other 24 cellular pellets were resuspended in buffer X containing 50 mM KH$_2$PO$_4$, pH 7.8, 0.1 mM EDTA, 1% Triton X-100 (Aldrich, Steinheim, Germany) and 2% PVP (Acros, NJ, USA), modified from Gechev et al. [44] and also transferred to a 60 ml glass beaker. To determine the effect of pulse frequency on protein extraction, duplicates of 1 ml from both cell suspensions were sonicated applying a range between 1 and 5 pulses with 30 s intervals at a fixed amplitude of 25 $\mu$m (100 W High Intensity Ultrasonic processor, Sonics and Materials, Inc., Newtown, USA). The optimal disruption was determined for 1 ml duplicates by sonication at amplitudes between 30 nm and 90 nm with 2 pulses and 30 s intervals. Samples were kept on ice throughout the disruption procedures, yet immediately after sonication the temperature of the extract was registered (Thermotime, Cresta, The Netherlands) and the protein content of the extracts were measured according to Bradford using BSA as protein standard [45].

## 2.3. Comparison of extraction buffers

To compare protein extraction efficiency, four extraction buffers were tested: buffer A (50 mM $KH_2PO_4$, pH 7.8 and 0.1 mM EDTA [43]; buffer B (50 mM $KH_2PO_4$, pH 7.8, 0.1 mM EDTA, 1% Triton X-100); buffer X (50 mM $KH_2PO_4$, pH 7.8 and 0.1 mM EDTA, 1% Triton X-100, 2% PVP and complete protease inhibitor cocktail (Roche, Mannheim, Germany)); and buffer Y (50 mM $KH_2PO_4$, pH 7.8, 0.1 mM EDTA, 1% Triton X-100, 2% PVPP (Sigma, Steinheim, Germany) and complete protease inhibitor cocktail [44]). For this experiment as well as experiments 3 and 4, approximately $2 \times 10^6$ cells were harvested by filtration (polycarbonate filters, 2.0 μm pore size; Osmonics, USA). The cells were disrupted by sonication ($2 \times 30$ s, at $A = 30$) at 4 °C in 1.5 ml of one of the four buffers. All crude extracts were centrifuged for 20 min ($20,000g$) at 4 °C after which the supernatants were collected ($n = 3$). One hundred and fifty microliter of the supernatant was used for quantifying the protein concentration in triplicate according to Bradford [45].

## 2.4. Comparison of four different protein extraction buffers on SOD activity

Aliquots of supernatants obtained as described in Section 2.3 were used for determining SOD activity applying both the xanthine/xanthine oxidase (X–XOD) assay [37] and the Riboflavin/NitroBlue Tetrazolium (RF/NBT) assay [38,46]. Both indirect methods involve the inhibition of, respectively, Cyt-*c* and NBT reduction. In the X–XOD method, SOD competes with Cyt-*c* for $O_2^{\cdot-}$ generated by the xanthine/xanthine oxidase system. *SOD activity* is defined as the amount of sample required for 50% inhibition. Cyt-*c* reduction was measured for 3 min at 4 °C in a 1.5 ml assay mix containing SOD buffer 1 (50 mM $KH_2PO_4$ and 0.1 mM EDTA at pH 7.8) 10 μM Cyt-*c* (Sigma), 50 mM xanthine (Sigma, Steinheim, Germany)) and XOD (Sigma, Steinheim, Germany) at 550 nm on a Cary 3E UV/vis double beam spectrophotometer (Varian, Middelburg, The Netherlands) equipped with a temperature controlled cell attached to a water bath. The blanc coloration slope was 0.025 AU/min. For activity determination in extracts, extract volumes of sample ($V_{sample}$) were added such that an inhibition ($I$) between 45% and 65% was achieved. The volume of sample needed to obtain 50% inhibition ($V_{50}$) was then calculated according to $V_{50} = V_{sample} * 50\%/I$. Specific activity (SA) was calculated as $1/(V_{50} * [protein])$. In the NBT/RF method, SOD competes with NBT for $O_2^{\cdot-}$ generated by the RF under illumination. NBT reduction was measured (in duplicate) in five reaction mixtures containing 0, 12.5 μl, 25 μl, 50 μl and 100 μl extract. The 1.5 ml reaction mixtures contained SOD buffer 2 (50 mM $KH_2PO_4$, pH 7.8, 0.1 mM EDTA, and 0.25% Triton X-100), 2 mM riboflavin (Sigma, Steinheim, Germany) and 57 μM NBT (Sigma, Steinheim,

Germany). The reaction mixtures were incubated in the dark for 30 min at 4 °C. Since generation of superoxide radicals in the NBT/RF assay is driven by light, samples were subsequently illuminated from above for 15 min by 4 fluorescence tubes (Philips TLD/18 W, 30 cm distance) giving 199 μmol photons $m^2 s^{-1}$. Afterwards, absorbance was measured at 560 nm on a Cary 3E UV/vis double beam spectrophotometer. Fifty percent inhibition was calculated by regression using the linear part of a natural semi-log curve after which the SA was calculated as described for the X–XOD method.

SOD activity was visualized using native 12% polyacrylamide gel electrophoresis (Native-PAGE). Samples containing 15 μg protein were loaded onto a gel using 5 μl protein loading buffer. After electrophoresis (80 V), activity of SOD in the gel was visualized by the staining procedure described by Beauchamps and Fridovich [38] and Parida et al. [21]. The gels were soaked in 35 ml staining buffer containing 0.25 mM RF, 5 mM NBT, and 0.5 mM TEMED (Amresco, Solon USA) for 30 min. in the dark at 4 °C and subsequently illuminated from above for 15 min by 4 fluorescent tubes (18 W each, 30 cm distance) giving 199 μmol photons $m^2 s^{-1}$. SOD bands appeared as light bands on a blue/purple background. The reaction was stopped by rinsing with Milli Q.

## 2.5. SOD activity assays at different temperatures

Both quantitative assays were performed as described in Section 2.4 at temperatures of 6 °C, 12 °C, 18 °C, 24 °C and 30 °C. Regarding the X–XOD method, temperature dependence was determined by the activity of XOD required to obtain a blanc slope of 0.025 AU/min. The measurements were done in triplicate. Generation of superoxide radicals in the NBT/RF assay is driven by light. To determine the effect of light intensity besides the effect of temperature, SOD measurements were performed under three different light intensities for each of the five different temperatures. Therefore, the reaction mixtures were incubated at the designated temperature in a water bath for 10 min prior to irradiation and kept within the water bath during illumination from above with 133, 166 and 199 μmol photons $m^{-2} s^{-1}$. The three irradiance intensities were applied by positioning the reaction mixtures at three different distances from the 4 fluorescent tubes. Irradiance was measured with a spherical light meter (QSL-100, Biospherical Instruments) in air. Measurements were done on four replicates. Statistical analyses were done on all results using standard One way ANOVA significance tests. Error bars represent standard deviations.

## 3. Results

### 3.1. Disruption of cells

Applying an increasing range of sonication amplitudes resulted in enhanced extract temperatures regardless of

the buffer used. Despite the fact that extracts were sonicated on ice, temperatures increased dramatically with increasing pulses, frequency and amplitude up to 25 and 50 °C at the highest pulse frequency and amplitude levels, respectively (Fig. 1). At the same time, the concentration of protein did not increase with increasing amplitude irrespective of the buffer used. Moreover, the protein concentration decreased significantly when applying sonication amplitudes of 60 μm or higher, probably due to the destructive effects of heat on protein integrity. Applying 2 sonication pulses of 30 s at 30 μm amplitude already resulted in obtaining a maximum amount of extracted protein.

## 3.2. Extraction buffer

Because of its membrane dissolving capacity, the presence of Triton X-100 in buffers B, X and Y contributed significantly ($p < 0.05$) to the protein extraction efficiency (Figs. 1 and 2c). On average, Triton X-100 (buffer X) yielded between 2 and 2.5 times more protein than the phosphate buffer (buffer A) alone and was efficient without cell disruption by sonication (Fig. 1a). Addition of the matrix forming molecules PVP and PVPP decreased the extraction efficiency of proteins significantly ($p < 0.05$) by approximately 20% (Fig. 2c).



Fig. 2. Effect of extraction buffers A (sole phosphate buffer), B (phosphate and Triton X-100), X (phosphate, Triton X-100 and PVP) and Y (phosphate, Triton X-100 and PVPP) on the amount of protein required for 50% inhibition ($V_{50}$) and protein extraction efficiency. $V_{50}$ values using the xanthine–xanthine oxidase (X–XOD) method (a). $V_{50}$ values using the Riboflavine/NitroBlue Tetrazolium (NBT/RF) method (b). Effect of extraction buffer on protein extraction efficiency (c). a: differs significantly from buffers B and Y ($p > 0.0005$); b: differs significantly from buffers A and X ($p > 0.0005$); c: differs significantly from buffers B, X and Y ($p > 0.0005$); d: differs significantly from buffers A, X and Y ($p > 0.0005$); and e: differs significantly from buffers A and B ($p > 0.0005$).
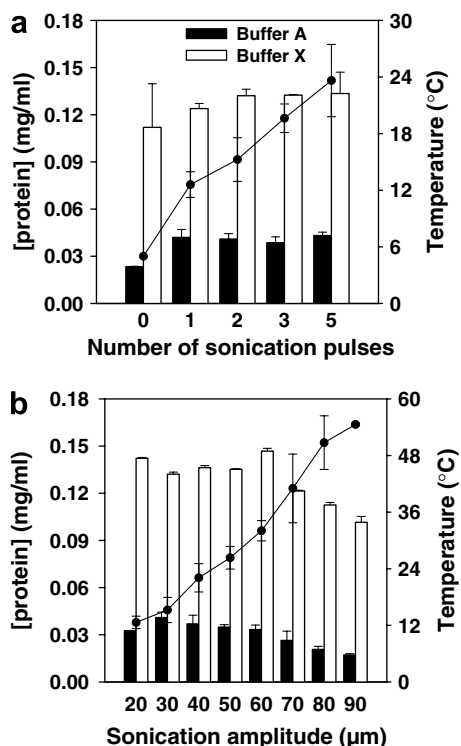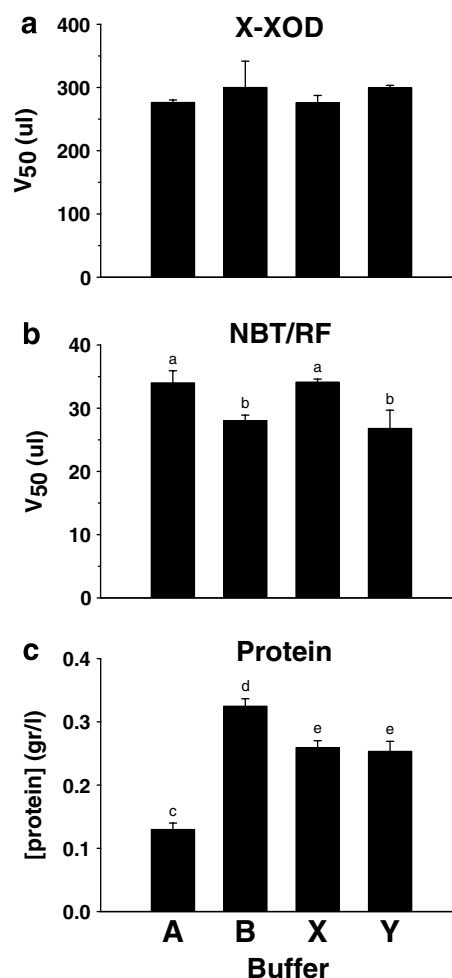


Fig. 1. Effects of sonication on cell disruption efficiency and extract temperature: (a) effect of sonication pulses and (b) effect of sonication amplitude. Cell disruption procedures were tested in extraction buffers A (dark bars) and X (white bars) and expressed in amount of protein extracted. Extract temperature is indicated by the black dots.

## 3.3. Protein extraction buffer and SOD activity

$V_{50}$ values of the X–XOD method were nearly 10 times higher than the $V_{50}$ values of the NBT/RF method, regardless of the buffer used (Fig. 2a and b). The differences between the $V_{50}$ values within each assay were small when the four protein extraction buffers were compared, but were only significant ($p < 0.05$) in combination with the NBT/RF method. Despite these differences, they were minor compared to the differences between the protein extraction efficiency of the four buffers. As a result, the large variation in specific SOD activities (SA) could rather be explained by differences in protein extraction efficiency between the four buffers (Fig. 2c), than by differences in
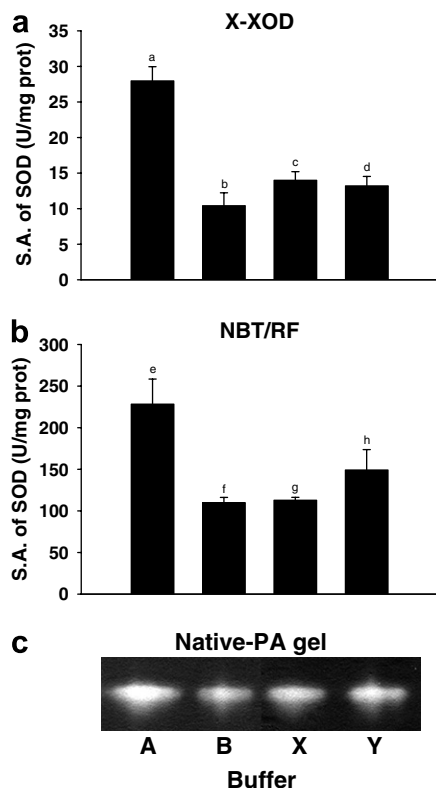
Fig. 3. Effect of protein extraction buffers A (phosphate buffer), B (phosphate and Triton X-100), X (phosphate, Triton X-100 and PVP) and Y (phosphate, Triton X-100 and PVPP) on specific activity of SOD measured by the xanthine–xanthine oxidase (X–XOD) method (a), the Riboflavine/NitroBlue Tetrazolium (RF/NBT) method (b) and visualized on native polyacrylamide (Native PA) gel (c). Twenty microgram of protein was applied on gel. a: differs significantly from buffers B, X and Y ($p > 0.005$); b: differs significantly from buffers A and X ($p > 0.05$); c: differs significantly from buffers A and B ($p > 0.05$); d: differs significantly from buffer A ($p > 0.0005$); e: differs significantly from buffers B, X and Y ($p > 0.001$); f: differs significantly from buffers A and Y ($p > 0.05$); g: differs significantly from buffer A ($p > 0.0005$); and h: differs significantly from buffers A and B ($p > 0.05$).

$V_{50}$ values (Fig. 3a and b). This was also confirmed by Native-PAGE (Fig. 3c).

### 3.4. Temperature dependence

Because the X–XOD method uses the enzyme xanthine oxidase (XOD) to generate superoxide radicals, this assay depends on temperature and requires a higher concentration of XOD at lower temperatures to obtain a similar activity slope as compared to the same assay at 25 °C (Fig. 4a). In contrast, temperature marginally affected the NBT/RF method (Fig. 4b). Because superoxide radical formation is based on a photochemical reaction, this method was indeed found to be more dependent on irradiance intensity than on temperature (Fig. 4b). The SOD activity of *C. brevis* showed no significant dependency on temperature at all, regardless of the method used (X–XOD: slope $= -0.3234$, $r^2 = 0.3628$; NBT/RF: slope $= -0.1622$, $r^2 = 0.0041$), although significant ($p < 0.05$) variability
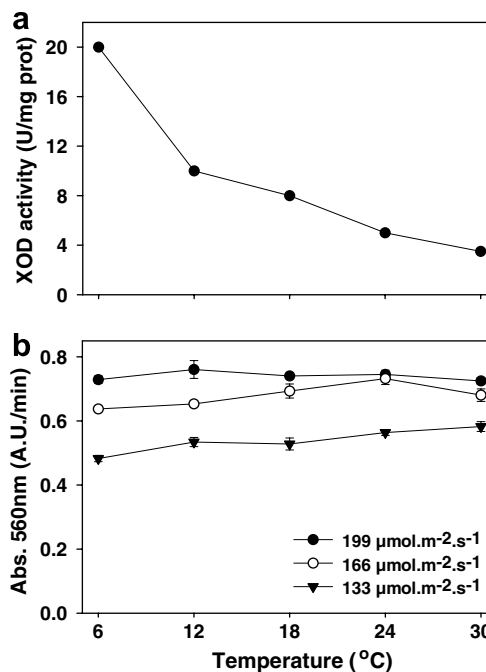


Fig. 4. Temperature dependence of the SOD activity assay. Temperature effect on the xanthine–xanthine oxidase (X–XOD) method indicated as the amount of xanthine oxidase (XOD) activity required to obtain a slope of 0.025 AU/min (a). Temperature effect on coloration of blancs using the Riboflavine/NitroBlue Tetrazolium (RF/NBT) method at three different irradiance levels (b).

was observed. This was confirmed by Native-PAGE (data not shown).

### 4. Discussion

Studies on microalgal SOD responses, especially those on polar microalgal species are virtually non-existent. An important probable cause for this could be the difficulty in obtaining sufficient biomass required for the SOD activity assays. There are several ways to assess microalgal biomass, such as chlorophyll concentration, carbon concentration or cell number, but most of them are subject to large variations caused by species specific differences and environmental circumstances. Therefore, in our study, we used protein concentration as a measure of biomass because it exhibits less variation when compared with other biomass parameters.

Specific activity of SOD is typically normalized to the amount of biomass like protein or cell concentration. Earlier studies showed that high protein concentrations or high cell numbers derived from large culture volumes (up to several hundred ml) were needed for a reproducible SOD assessment, regardless of the assay used [32,33,47,48]. Therefore, the low cell densities typically found in the field pose detection and accuracy problems to either protein harvest and/or SOD activity measurements. Thus, it is essential to optimize cell collecting and disrupting procedures. Because filtered cultures of *C. brevis* yielded 25%

more protein than centrifuged cultures (data not shown) cells were standardly harvested by means of filtration, except in the case of the cell disruption experiments. Here, cells were harvested by centrifugation after which the pellets were resuspended and joint together in the designated extraction buffer. In this way identical cell concentrations, as required for the cell disruption tests were obtained. This was not possible using filter derived material that showed a slight variability in cell concentration. For the cell disruption experiment, only buffers A (a simple phosphate buffer) and X (protease inhibitor, Triton X-100 and PVP), were compared which differed most in composition. Protein extraction was shown to be most effective using buffer X applying 2 sonication pulses at an amplitude of 25 μm (Fig. 1). Although buffer X yielded higher protein concentrations applying 2 pulses at an amplitude of 60 μm or 5 pulses at an amplitude of 25 μm, the temperature of the extract raised as well (up to 35 and 25 °C, respectively). In general, an increased number of pulses did not result in increased extraction efficiency. Moreover, an increase in sonication amplitude even reduced protein concentrations: in buffer A this was initiated by amplitudes over 40 μm whereas for buffer X this was initiated by amplitudes over 70 μm. Likely, increased extract temperatures at elevated amplitudes cause denaturation, followed by aggregation and precipitation, especially of low temperature proteins. In addition, because buffer X contains Triton X-100, denatured proteins remain in solution, even at higher temperatures that would otherwise cause aggregation and precipitation.

Concerning the efficiency of the tested protein extraction buffers, buffer B was most efficient yielding 60% more protein than buffer A and 20% more than buffers X and Y. This can be explained by the presence of Triton X-100 which enables buffers B, X and Y to dissolve membraneous structures to release membrane associated or enclosed proteins. It must be emphasized that the Triton X-100 strength needs to be well below 0.5% (in this study 0.1%) in order to avoid interference with Coomassie dye. In contrast to Triton X-100, the presence of PVP and PVPP had a negative effect on the amount of extracted protein. Their purpose is to indiscriminately abstract phenolic and polyphenolic impurities from protein extracts which could otherwise affect activity assays. However, PVP/PVPP not only precipitates interfering phenols but also proteins containing amino acids with phenolic residues (i.e. tyrosine and phenylalanine). On the other hand, although buffers B, X and Y yielded more protein, the presence of more chemical constituents (compared with buffer A) could also affect SOD functioning and activity. Therefore, SOD assays were tested for all four buffers. These tests showed that the amount of extract required to obtain 50% inhibition ($V_{50}$) were rather similar (discussed below) when considering the four buffers (Fig. 2a and b). Evidently, SOD is (almost) entirely present in the cytosolic fraction of the cell. As a result, calculated specific SOD activities, typically normalized to protein, revealed lower values when applying more

efficient protein extraction buffers, such as buffers B, X and Y, simply because of higher protein concentrations obtained in these buffers (Fig. 2c). So, it is important to realize that Triton X-100 does not stimulate SOD extraction but it enhances overall protein extraction efficiency and therewith the accuracy of SOD activity calculation of low biomass samples.

Both qualitative SOD assays measure activity in an indirect way, using a reducible coloration agent as an activity reporter. This makes both methods highly susceptible for interference with non-SOD substances which are able to react with $O_2^{\cdot-}$ radicals or reduced as well as oxidized coloration agents. Because SOD activity is measured differently in both assays, the interference of non-SOD substances is also expressed in a different way. Since most non-enzymatic superoxide scavengers interfere with the SOD activity assay immediately after superoxides are generated, the cytochrome-$c$ inhibition slope started very irregular and was determined after 30 s following xanthine oxidase addition. Therefore, despite the presence of reactive substances, these SOD activity values were found to have very low standard deviations. The RF/NBT method on the other hand measures SOD activity via the inhibition of NBT reduction against the sample concentration. Here, accumulated reduced NBT is measured by which these values not only represent SOD activity but include non-enzymatic scavenging of superoxide radicals as well. Uptil now it seems unfeasible to distinguish between enzymatic and non-enzymatic inhibition of NBT reduction. Therefore, to minimize the contribution of non-enzymatic substances (i.e. metal ions and reducing equivalents), the amount of sample used is kept relatively low (compared to the X–XOD method). Also, because non-enzymatic superoxide scavengers react with the generated $O_2^{\cdot-}$ radicals immediately after its production, the reaction time is set on 15 min by which the enzymatic proportion is increased. As a result of both conditions, the contribution of the enzymatic superoxide scavenging activity is optimized while the inhibition of NBT reduction stays within its detection limits. Finally, (poly)phenolic compounds are able to produce $O_2^{\cdot-}$ or reduce NBT and thereby could strongly interfere with SOD activity. This would underline the significance of adding PVP or PVPP to the extraction buffer. However, this was not supported by our experiments, because the application of buffers X and Y (containing PVP or PVPP) in both SOD assays, did not significantly reduce $V_{50}$ values. (Fig. 2a and b). Therefore, buffer B, containing Triton X-100 only, is most suitable for SOD activity measurements in *C. brevis*. Yet, it is advisable to test the need for PVP/PVPP when using other (microalgal) species.

The $V_{50}$ values did not significantly differ between the four buffers, when using the X–XOD method. The $V_{50}$ values for the NBT/RF method on the other hand varied slightly but significantly, with highest values for buffers A and X. Generally, the X–XOD method required higher extraction volumes (275–300 μl) for a $V_{50}$ inhibition than the NBT/RF method (27–34 μl). In terms of *C. brevis*

protein this means that the X–XOD method required at least 36 μg protein to obtain 50% inhibition while the RF/NBT method was accurate when applying 3.5 μg protein or less. Thus, reliable and sensitive SOD activity measurements were more dependent on the assay used than on the applied protein extraction buffer. Because of the above mentioned difficulties in obtaining sufficiently high microalgal biomass, their SOD activity is best measured using the NBT/RF method. This method was also found to be less sensitive to temperature than the X–XOD method, although the observed lower XOD activity at lower temperatures could simply be counteracted by increasing the amount of XOD (Fig. 4). Surprisingly, regardless of the method used, the enzyme activity of *C. brevis* SOD did not reveal an optimum at lower temperatures pursuant to its growth optimum (data not shown).

Besides these two quantitative methods, SOD activity can also be detected by Native-PAGE, which is a qualitative method showing species specific SOD isozyme composition and their relative activity. This method is an excellent tool to visualize relative changes in SOD activity as well as pinpointing which isozyme species causes that change. Yet, the variety in isozyme composition makes it difficult for Native-PAGE to compare overall SOD (isozyme) activities between species. Also different species with similar values of SOD activity can show different intensities of SOD activity on a Native-PAGE without obvious reasons. Because of both properties, Native-PAGE is primarily restricted to determine intraspecific, qualitative differences. Native-PAGE separating extracts of *C. brevis* showed that applying an amount between 5 μg and 20 μg protein gave an optimal resolution to detect differences in activity (Fig. 5). The presence of one visible band indicates that one particular isozyme is predominant but that does not exclude the presence of more SOD isozymes. In conclusion, quantifying the band density cannot be used adequately as a measure for SOD activity.

Comparing existing data on SOD activity in microalgae is difficult because of the broad range of microalgal species used, the use of different biomass extraction procedures and the application of different SOD assays. In addition, various studies normalize SOD activity to cell number or Chl *a*, although most studies normalize to protein as initially proposed by McCord and Fridovich [37]. Virtually all studies involve temperate to tropical microalgal species

[28,29,33,35] whereas only a few report about SOD activity in polar microalgae [18,45]. Values of SOD activities range between 2 U/mg prot and 2300 U/mg prot for studies that applied the X–XOD method [30,47], and between 10 U/mg prot and 620 U/mg prot for studies that applied the NBT/RF method [24,48]. So far, there does not seem to be an obvious trend with higher SOD activity values for the NBT/RF assay as compared with the X–XOD assay, as found in our study.

We also measured SOD activity in numerous other Antarctic (*Phaeocystis antarctica*, *Thalassiosira antarctica*, *Chaetoceros dichaeta*) and temperate (*Thalassiosira weissflogii* and *Emiliania huxleyii*) microalgal species, which were cultured under similar irradiance conditions as *C. brevis*. Moreover, a natural community of benthic diatoms from the Dutch Wadden Sea was tested as well (data not shown). In all these samples, the amount of biomass was insufficient to measure SOD accurately using the X–XOD method but instead could be measured using the NBT/RF method (values varying between 83 U/mg prot and 95 U/mg prot). So, besides *C. brevis*, the NBT/RF method can also be used for other polar as well as temperate microalgal species.

In conclusion, our study shows that the NBT/RF assay is the most reliable assay for measuring SOD activity in marine (Antarctic) microalgae within a large temperature range. The addition of Triton X-100 (<0.5%) to standard phosphate buffers increases protein extraction efficiency, thereby increasing the accuracy of the SOD activity calculation. Studies using protein extraction buffer without Triton X-100 overestimate the SA of SOD at least 2 times compared to the actual value, according to our study. A constant systematic discrepancy between total and cytosolic protein could adjust existing SOD activity values but since there is no fixed ratio between total/cytosolic protein (because of difference in size, amount of metabolic enzymes, quantity of photosynthetic associated proteins) this seems unfeasible. Finally, with respect to future comparisons, the large differences in calculated SOD activities when using different protein extraction or SOD assays, corroborate the suggestion that SOD activity measurements in marine microalgae should be standardized.

## 5. Abbreviations

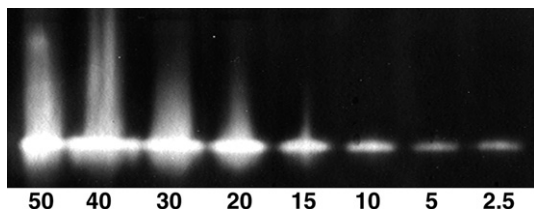| | |
|---|---|
| ETC | electron transport chains |
| ROS | reactive oxygen species |
| $O_2^{\cdot-}$ | superoxide anion |
| $HO^{\cdot}$ | hydroxyl radical |
| SOD | superoxide dismutase |
| X–XOD | xanthine/xanthine oxidase |
| NBT | NitroBlue Tetrozolium |
| APX | ascorbate peroxidase |
| GPX | glutathione peroxidase |
| PVP | polyvinylpyrrolidone |
| PVPP | polyvinylpolypyrrolidone |



Fig. 5. Enzymatic SOD activity applying on a 12.5% native polyacrylamide gel. Numbers indicate the amount of protein applied (μg).

## Acknowledgements

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