The physiological response of the marine platyhelminth *Macrostomum lignano* to different environmental oxygen concentrations.

Short title: Response of a flatworm to oxygen variation

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ABSTRACT

Respiration rate of meiofauna is difficult to measure, and the response to variations in the environmental oxygen concentrations has so far been mainly addressed through behavioral investigation. We investigated the effect of different oxygen concentrations on the physiology of the marine platyhelminth *Macrostomum lignano*. Respiration was measured using batches of 20 animals in a glass microtiter plate equipped with optical oxygen sensor spots. At higher oxygen saturations (>12kPa), animals showed a clear oxyconforming behavior. However, below this values, the flatworms kept respiration rates constant at 0.064 ± 0.001 nmol $O_2 \cdot l^{-1} \cdot h^{-1} \cdot ind^{-1}$ down to 3 kPa po_2, and this rate was increased in 30% in animals that were reoxygenated after enduring a period of 1.5h in anoxia. Physiological changes related to tissue oxygenation were assessed using live imaging techniques with different fluorophores in animals maintained in normoxic (21 kPa), hyperoxic (40 kPa), near anoxic (≈0 kPa) conditions and subjected to anoxia-reoxygenation. Ageladine-A and BCECF both indicated that pH_i under near anoxia increases by about 0.07 to 0.10 units. Mitochondrial membrane potential, $\Delta\psi_m$, was higher in anoxic and hyperoxic compared to normoxic conditions (JC1). Staining with ROS sensitive dyes, DHE for detection of superoxide anion ($O_2\bullet^-$) formation and C-H_2DFFDA for other ROS species aside from $O_2\bullet^-$ (H_2O_2, HOO• and $ONOO^-$), both showed increased ROS formation following anoxia reoxygenation treatment. Animals exposed to hyperoxic, normoxic and anoxic treatments displayed no significant differences in $O_2\bullet^-$ formation, whereas mitochondrial ROS formation as detected by C-H_2DFFDA was higher after hyperoxic exposure and lowest under near anoxia compared to the normoxic control group. *M. lignano* seems to be a species tolerant to a wide range of oxygen concentrations (being able to maintain aerobic metabolism from extremely low po_2 and up to hyperoxic conditions) which is an essential prerequisite for successfully dealing with the drastic environmental oxygen variations that occur within intertidal sediments.

Keywords: flatworm, live-imaging, meiofauna, respiration.

Abbreviations used in the text:
$\Delta\psi_m$=mitochondrial membrane potential
C-H2DFFDA= 5-(and-6)-carboxy-2',7'-difluorodihydrofluorescein diacetate
DHE=dihydroethidium
NH-FSW=Filtered sea water supplemented with 15mM Na-HEPES

50 pH_i=intracellular pH
51 Po_2=oxygen partial pressure
52 ROS=reactive oxygen species
53 SDT=sodium diethyl-dithiocarbamate trihydrate
54 SOD=superoxide dismutase
55

INTRODUCTION

Marine meiofauna colonize the upper sediment layer of the ocean which, especially in coastal and intertidal sediments, can be highly variable with respect to oxygenation. On one hand, wave action and coastal currents often lead to mechanical mixing of oxygen rich surface water into the upper loosely packed sediment layers. Relatively compacted sediment surfaces in shallow waters are often densely colonized by benthic microalgae that cause daily peaks of photosynthetic oxygenation in the upper 1-2 cm of sediment surface. On the other hand, coastal sediments contain high amounts of organic matter from decaying macroalgae or terrestrial and riverine coastal runoff. The chemical and microbial oxygen demand in these sediments is high so that oxygen that diffuses downwards is rapidly consumed. This produces steep sedimentary redox gradients between the upper, sometimes even hyperoxic and the lower suboxic to anoxic layers, which often span no more than 10 to 50 cm below which the sediment becomes chronically anoxic and sulfidic (rich in hydrogen sulfide, H_2S) (Fenchel and Finlay, 2008). These sediments are colonized by highly diverse macro- and meiofauna communities, the composition of which varies depending on sediment stability, grain size composition, organic matter content and oxygenation. Whereas macrofauna organisms often build sedimentary burrows that they oxygenate through irrigation with oxygen rich surface waters, meiofauna organisms move between the sand grains, seeking optimal positioning between too high and too low environmental oxygenation according to their metabolic and nutritional requirements. In very few cases, marine meiofauna were shown to actively establish suitable environmental oxygen levels in their closest environment between the sediment grains (Corbari et al., 2005). Much more often it was shown that the meiofauna stratify in response to the sedimentary oxygen gradient (Corbari et al., 2004) and different groups of meiofauna have been described as oxophilic, microoxophilic and thiobiotic according to their preferred positioning in the small scale chemical and redox gradients within the sediment column and around macrofauna burrows (Fenchel and Finlay, 1995; Morrill et al., 1988). This behavior precludes not only that meiofauna species have different metabolic strategies to cope with low and variable environmental oxygenation, but also that they would have means to sense oxygen (and hydrogen sulfide) and react flexibly to changing environmental oxygenation. These reactions include vertical migrations within the sediment column, often away from too high surface oxygenation (Corbari et al., 2005), and/or adjustment of metabolic rate to cope with reduced oxygen availability. However, soft bodied meiofauna worms, such as nematodes and platyhelminths, generally deal with fluctuating tissue oxygenation and changes in environmental redox state because of their small size and fast equilibration time. Whereas several studies have assessed the behavioral (Rosenberg et al., 1991; Tinson and Laybourn-Parry, 1985) and ecological responses to fluctuating oxygenation (Wetzel et al., 2001), studies of the physiological response to fluctuating chemical and oxygenation gradients are scarce.

A seminal paper by Morrill et al. (1988) reported antioxidant activities to be better established in thiobiotic than in oxybiotic meiofauna, giving rise to the idea that i) normoxic oxygen levels could already be “hyper-oxic” for these low oxygen adapted species, ii) that the oxidation of toxic H_2S could give rise to the formation of reactive oxygen species (ROS) in thiobiotic, low oxygen adapted meiofauna.

In the present study, we investigated the physiological effects of hyperoxic and anoxic exposure conditions in the interstitial marine flatworm *Macrostomum lignano* (Rhabditophora, Macrostomorpha) Ladurner et al, 2005. *M. lignano* has been established as a new model in sexual selection (Schärer et al., 2005) and evolutionary

and developmental studies (Egger et al., 2006; Morris et al., 2006), but especially in stem-cell research (Bode et al., 2006; Pfister et al., 2007) and the role of these stem-cells for the process of ageing (Mouton et al., 2009).

Platyhelminths are transparent and, thus, elegant model organisms to study cellular and subcellular processes *in vivo*. Using "live imaging" techniques we provide a first set of data regarding the physiological responses of individual flatworms to extreme states of environmental oxygenation and, further, on the effect of anoxia and subsequent reoxygenation on ROS forming processes, mitochondrial membrane potential and tissue pH. We developed a technique to record oxygen partial pressure (po_2) dependent oxygen consumption rates using batches of 20 flatworms in order to describe their metabolic response to variable environmental oxygenation.

In this study we used non-invasive optical techniques to understand how an anoxia tolerant, animal responds to high, low and fluctuating oxygenation, especially with respect to ROS formation, mitochondrial functioning and maintenance pH homeostasis during anoxia-reoxygenation, a detrimental situation in many human pathologies.

MATERIALS AND METHODS

Animal culturing

Cultures of *M. lignano* (DV-1 line) were reared at the Alfred Wegener Institute (Bremerhaven, Germany) in Petri dishes with Guillard's F/2 medium at room temperature (RT) (20ºC) and fed weekly the diatom *Nitzschia* sp. For experimentation, however, a sterile medium composed of seawater filtered over a 0.2µm Whatman filter and supplemented with 15mM Na-HEPES (NH-FSW) was used. Buffering was necessary to avoid pH changes in the medium when setting anoxic or hyperoxic conditions.

Respiration measurements

Two different treatments were considered for the respiration measurements: normoxic (21kPa) and anoxic (0kPa) followed by reoxygenation. For the latter, animals were kept for 1.5h under anoxic conditions (kept in a gas tight glovebox which was equilibrated with 100% N_2 and immersed in an anoxic medium which was prepared by flushing with 100% N_2) and later reoxygenated by bubbling with air for an additional 1.5h. Prior to conducting respiration measurements, experimental animals were isolated from the cultures and maintained without food for ca. 20h in NH-FSW. After the acclimation time, 3 groups of 20 animals were transferred with a micropipette to 3 individual wells of a glass microtiter plate (Mikroglas Chemtech GmbH, Mainz, Germany) previously equipped with oxygen sensor spots (SP-PSt3-NAU-D5-YOP, Precision Sensing GmBH, Regensburg, Germany) glued onto the bottom of each well. An additional well was maintained as control, and contained only NH-FSW and the sensor spot. Each of the wells was filled completely with NH-FSW to its maximum capacity which was 100 ± 0.5 µl, to avoid the formation of air bubbles when sealing the wells. Each of the four wells was sealed with a coverslip and the complete surface of the microtiter plate was additionally covered with a layer of auto-adhesive Armaflex® (Armacell Enterprise GmBH, Münster, Germany). Additional pressure was maintained on the complete surface of the plate in order to ensure air-tight sealing. Measurements were carried out with a 4-channel fiber-optical oxygen meter (Oxy-4) and non-invasive oxygen sensors (Precision Sensing GmBH, Regensburg, Germany) which were daily calibrated following the manufacturer's description. Data were recorded at 15sec intervals and the experiments were stopped when the oxygen was completely consumed from each of the animal chambers, which usually took around 16h. All experiments were conducted at

RT. Data corresponding to the first 30min following the start of the experiment were discarded in order to avoid interference from stress related to the manipulation of the worms. Respiration rates were expressed as nmol $O_2 \cdot L^{-1} \cdot h^{-1} \cdot ind^{-1}$. The critical oxygen pressure (p_c) (Tang, 1933) was calculated using the equation of Duggleby (1984).

Optical measurements

In this study intracellular pH, mitochondrial membrane potential, mitochondrial density and reactive oxygen species (ROS) concentrations were measured by "live imaging" techniques, which consist in applying specific dyes and *in-vivo* visualization using a Leica TCS SP5II confocal microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany) and a CCD camera system (Visitron Systems GmbH, Puchheim, Germany).

Four treatments were considered in our study: control (21kPa), anoxia (0kPa), hyperoxia (40kPa) and anoxia followed by reoxygenation. Experiments were carried out in a gas tight glovebox which was equilibrated with either 100% N_2 (anoxia) or 40% O_2/60% N_2 (hyperoxia) using a Wösthoff gas mixing pump (Wösthoff GmbH, Bochum, Germany). Anoxic medium was prepared by flushing with 100% N_2, while hyperoxic NH-FSW medium was obtained by flushing with a 40% O_2-60% N_2 mixture. Thirty minutes flushing were enough for equilibration in all cases as confirmed by measurements of the oxygen content using optical oxygen sensors and the Presens Oximeter (see above).

Treatments were conducted with batches of 10 animals which were manipulated at the same time in 2ml Utermöhl chambers for 1.5h. The microscopic chamber in which the animals were anoxically incubated was sealed while still inside of the glovebox and under the gas stream. The chamber was then transferred to the confocal microscope and for the anoxic treatment, it should be noted that we cannot exclude some minor reoxygenation. Thus we decided to use the term of "near anoxia" for the treatment that was aimed to be strict anoxia. After finishing the incubation, a fluorophore was added to the animals in normoxic, anoxic (severely hypoxic) and hyperoxic treatments.

For the anoxia-reoxygenation treatment we can assure that the worms were incubated in absolute anoxia, whereupon the chamber was opened and medium and animals were allowed to reoxygenate for an additional 1.5 h. After this time, the dye was added to the reoxygenation treatment. All dyes were used individually for separate batches of animals in order to avoid any possible interference among dyes. The list of the different dyes used in the present study along with their mechanisms of function is given in table 1, while incubation and analysis conditions for each of these dyes are detailed in table 2.

For both of the pH-sensitive dyes used in the present study (ageladine-A and BCECF), further calibration was needed in order to assign numerical pH values to the experimentally recorded intensities/ratios. For ageladine-A (Fujita et al., 2003) this was done following the method described by Bickmeyer et al. (2008) and Bickmeyer (2012) in which the average values obtained for each of the treatments are expressed as a proportion of the control values, and by assuming that non-specialized cells under these control conditions would present a putative pH_i of 7.3-7.4. On the other hand, and since BCECF fluorescence intensity at 490 nm linearly increases between 6.4 and 7.8 pH (Silver, 2003), BCECF results were also converted to pH_i by constructing a calibration curve using the Nigericin technique (Nigericin sodium salt, N7143-5mg, Sigma Aldrich, Germany) described by Thomas et al. (1979).

Fluorimetric analysis of the response of the animals to the treatment conditions were carried out in sealed microscope slides using a Leica TCS SP5II confocal microscope.

For validation of the pH_i results, further analyses were carried out using a wide-field fluorescence microscope Zeiss Axiovert-10 (Zeiss GmBH, Germany) (equipped a CCD camera system, Visitron, Puchheim, Germany) and applying BCECF staining. For analysis, animals were anesthetized in a 2:1 mixture of 7.14% $MgCl_2 \cdot 6H_2O$ and NH-FSW (Pfannkuche and Thiel, 1988). One picture per individual and per PMT, when ratio calculation was required, was taken for each of the animals using a 10x objective lens. In order to avoid photobleaching, a short period (<5 sec) of low resolution (256 x 256 pixel) live scanning was applied for focus adjustments and afterwards only one single scan (512 x 512 pixel) was run of each individual. Autofluorescence was suppressed by adjusting the threshold, and phototoxicity was minimized by the multiphoton laser for low wavelengths scans.

Image analysis was carried out using Leica LAS Lite software (Leica Microsystems CMS GmbH 2011). Quantification was carried out by plotting 5 transects per animal (always perpendicular to the longitudinal axis of the animal and in all cases selecting the areas of highest intensity, Fig. 1). The maximum value for each transect was obtained and the values of the 5 transects were averaged for each individual.

Experiments were repeated for a minimum of two times (if results were identical) and up to 12 times, in order to confirm the differences between treatments. Data from the different replicas were pooled. However, since dye uptake by the animals varied among days, the confocal settings needed to be adjusted in order to optimize the quality of the images taken. Thus, data normalization was required prior pooling. Data were normalized by expressing all values within one experimental replica (40 worms) as percentage of the maximum recorded value in that experiment. The maximum value was set to equal 100%. Statistical analyses were performed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Resulting data were compared using ANOVA (followed by Student-Newman-Keuls *a-posteriori* multiple comparison test) when data complied with the assumptions for parametric analyses. When this was not the case, Kruskal-Wallis tests were conducted.

RESULTS

Respiration measurements

For the normoxic treatment, Po_2 dependent respiration rates were analyzed in a total of 10 pools, each consisting in 20 animals. Figure 2 shows that respiration rates decreased rapidly (by 70%) between 19 to 12.5kPa in the chamber. Below this po_2, *M. lignano* starts to regulate respiration down to a low critical po_2. Thus, for *M. lignano*, a first critical oxygen partial pressure (p_c) was established at 13 kPa (p_{c1}), which marks the point at which the organisms are switching between conformity to regulation. Between 12.5 and approximately 3kPa the respiration rates of the worms were constant around 0.064 ± 0.001nmol $O_2 \cdot l^{-1} \cdot h^{-1} \cdot ind^{-1}$. A second p_c appears at 1.36 ± 0.32 kPa (p_{c2}), where respiration rates started to decrease again with declining po_2. Thus, the analysis revealed a high and a low *p*c above which (p_{c1}) and below which (p_{c2}) the worms switch from regulating to conforming, po_2 dependent respiration. The same pattern was observed for the animals that were previously maintained under anoxic conditions and later reoxygenated. However, the interval at which animals maintained constant respiration rates, values were significantly higher (0.083 ± 0.001 nmol $O_2 \cdot l^{-1} \cdot h^{-1} \cdot ind^{-1}$) ($F=458.23$; $p<0.001$).

Activity observations

Normoxic and hyperoxic animals moved rapidly in the Petri dishes with behavioural characteristic best described as unidirectional burst swimming. For microscopic analysis at these high oxygenation levels, the worms had to be anesthetized with $MgCl_2$. Contrary, movement were drastically reduced in anoxia and often the worms started to swim in circles. Many worms were completely immobilized and displayed only epithelial cilia movements. Movements were completely reactivated by reoxygenation, with animals often reaching normoxic levels of activity after 1.5 h under reoxygenated conditions.

pH measurements

Staining with the pH sensitive dye ageladine-A (Fig. 3a and supplementary material fig. 1a) indicated that the tissue pH levels in anoxic worms or the ones exposed to anoxia-reoxygenation were significantly more alkaline compared to normoxic and hyperoxic animals; ANOVA ($F=10.38$; $p<0.001$). Further estimation of the pH values (using the values of normoxic animals as a reference and assuming that non-specialized cells have a pH of 7.4) indicated a pH-increase by approximately 0.10 units under near anoxia and anoxia-reoxygenation.

Staining with BCECF (supplementary material fig. 1b) corroborated the general pattern obtained with ageladine in which anoxic and reoxygenated individuals were significantly more basic than the normoxic and hyperoxic treatment groups ($F=3.70$; $p<0.05$) (Fig. 3b). As these results contradicted all our assumptions that animals exposed to near anoxia should become, if anything, more acidic, we applied yet another approach with the wide-field fluorescence imaging microscope and BCECF. Again, animals under near anoxia and anoxia-reoxygenation had the most alkaline values (supplementary material fig. 2) (showing an average increase of 0.07 units) whereas the normoxic and hyperoxic treated worms had a tissue pH of 7.46.

Mitochondrial density and membrane potential specific staining

MitoTracker staining indicated most mitochondria to be located in the animals body wall in epithelial and muscle cells, as well as around the mouth area (Fig. 4). MitoTracker Green FM (supplementary material fig. 3a) stained different treatment groups with different intensities ($K=54.09$; $p<0.000$). Animals kept under hyperoxic conditions (40 kPa) were stained with highest intensity, theoretically suggesting maximum mitochondrial density in hyperoxic worms, followed by individuals under normoxic conditions which displayed less intensive staining (Fig. 5a). Animals kept in anoxia had the lowest staining values with MitoTracker green, and individuals that were subsequently reoxygenated returned to staining intensity recorded in normoxia.

To determine whether the significant differences between differently oxygenated groups were associated with oxygenation dependent capacities for dye uptake, a second experiment was performed in which the animals were stained prior to the exposure to the different oxygenation treatments. With this approach we obtained exactly the same group specific pattern as in the first experiment in which the dye was applied following incubation at different oxygen levels. Therefore we used MitoTracker Deep Red 633 in a third approach (supplementary material fig. 3b), which did not produce a clear group-specific pattern. Pooled data from the 4 replicas conducted with MitoTracker Deep Red 633, yielded no significant differences among treatments ($K=6.98$; $p=0.072$) (Fig. 5b). High resolution confocal imagery confirmed staining of individual mitochondria in *M. lignano* cells (Fig. 6).

Staining with the dye JC-1 (supplementary material fig. 3c) indicated significant differences in the chemiosmotic H^+ potential ($\Delta\psi_m$) across the mitochondrial inner membrane among treatments (F=15.14; p<0.001) (Fig. 5c). The smallest potentials were measured in animals exposed to normoxia and in the ones that were reoxygenated after 1.5 h of anoxic incubation. Animals exposed in hyperoxic and anoxic media showed significantly more red/green staining, indicating an elevated membrane potential, i.e. a stronger H^+ gradient.

Staining for ROS detection

Superoxide anion ($O_2^{\bullet-}$) concentrations were assessed with dihydroethidium (DHE) (supplementary material fig. 4a). DHE staining was similar in animals exposed to anoxic, normoxic and hyperoxic conditions. Animals that were reoxygenated after a period of 1.5h in anoxia had significantly increased DHE staining compared to all other groups (K=15.77; p<0.001) (Fig. 7a). Formation of other ROS species aside from $O_2^{\bullet-}$ (H_2O_2, HOO• and $ONOO^-$) in *M. lignano* cells were assessed using C-H_2DFFDA (supplementary material fig. 4b), which again showed important differences between treatments (K=80.82; p<0.000). The staining intensity decreased in the order anoxia-reoxygenation = hyperoxia> normoxia > near anoxia (Fig. 7b) indicating ROS formation to decrease between hyperoxia and anoxia as expected.

Effects of superoxide dismutase (SOD) and sodium diethyl-dithiocarbamate trihydrate (SDT) on ROS staining

To scrutinize for the causes of the high DHE signal in anoxically incubated worms that should theoretically not be able to form $O_2^{\bullet-}$, we applied a SOD solution (Sigma S-2515) and the SOD inhibitor SDT (Sigma D-3506) to anoxic animals stained with DHE (Fig. 8a). The first results with neither SOD nor SDT addition again lead to significant DHE staining (above the background signal) in anoxically incubated specimens, although the values were significantly lower than those obtained with normoxic animals. SOD addition to anoxically incubated animals significantly reduced DHE staining, as $O_2^{\bullet-}$ radicals were converted to H_2O_2 at higher rates. Contrary, addition of the SOD-inhibitor SDT caused 2-fold higher DHE fluorescence in anoxic compared to normoxically exposed animals, because less $O_2^{\bullet-}$ radicals were converted to H_2O_2. Around 90% of the animals died under anoxic exposure with addition of SDT. After performing these tests that indirectly confirm the specificity of DHE for $O_2^{\bullet-}$ detection, we assessed the effect of SOD addition on DHE and C-H_2DFFDA staining in an experiment with both dyes (Fig. 8b). Addition of SOD to the medium of anoxically incubated worms once again significantly reduced $O_2^{\bullet-}$ staining in the DHE-stained worms compared to the normoxic control group. Contrary, SOD addition significantly increased C-H_2DFFDA staining, suggesting higher H_2O_2 concentrations in anoxically incubated SOD supplemented animals than in normoxic controls.

DISCUSSION

M. lignano as marine model organism for live imaging of physiological stress

In the present study we used living *M. lignano* individuals for the application of live imaging techniques. The transparency of this organism allowed an adequate and satisfactory measurement of the physiological parameters taken into account. Moreover, its small size also favoured a fast and complete dye uptake making live imaging with the whole animal possible.

Po_2 dependent respiration in M. lignano

Respiration measurements in *M. lignano* cannot be conducted with single animals, and instead require batches of 20 specimens maintained in small volumes (90-100 µl) of liquid. As the respiration rates are extremely low, measurements need to be conducted in small glass or plexiglass chambers without linings made of Teflon, known to be an oxygen binder.

Exposing *M. lignano* to decreasing oxygen concentrations during the respiration measurements indicated that the worms maintain constant consumption rates between 3 and 12.5kPa. This could be indicating that this is the species' optimum range of respiration and the animals are actually regulating oxygen consumption, most likely by adjusting ciliary beat frequency. Velocity of ciliary pumping movements may determine the water flow over the body surface which, in this species, also represents the respiratory epithelium. We could not quantify ciliary beat frequency in the present study but, however, we did not observe remarkable differences in cilia movements under different oxygen concentrations. Without being able to rule out the hypothesis that animals are actively regulating ventilation through cilia beating, it should also be considered the possibility that such regulation does not exists and that, in fact, results are driven by mitochondria saturation above 3kPa and that the activity of an alternative oxidase could be responsible for the increase in O_2 consumption above 12.5kPa. Regardless the reason, the range of constant respiration characterizes *M. lignano* as aerobic hypoxia tolerant species. Similar po_2 dependent respiration patterns have been reported for other marine meiofauna such as oligochaetes (Giere et al., 1999) and the authors suggested this to be an important prerequisite for successfully inhabiting intertidal sediments with microscale spatial variations of the oxygen concentration.

Animals that were reoxygenated after enduring a 1.5h period under anoxia, showed a 30% increase in their respiration rates, probably due to the stress of the procedure.

Responses to high oxygen concentrations

$\Delta\psi_m$, and the way the mitochondrial membrane potential is affected by different oxygen concentrations, has mainly been addressed in mammalian cell cultures. For *M. lignano* the JC1 measurements under normoxia and hyperoxia suggest the increase in oxygen consumption not to be due to mitochondrial uncoupling (opening of the mitochondrial membrane transmission pore, MTP), but to a more intensive respiration, respectively electron flow, which causes increased mitochondrial membrane potential ($\Delta\psi_m$) in hyperoxically exposed worms. Contrary mitochondrial uncoupling and reduction of $\Delta\psi_m$ under hyperoxic conditions has been observed in mammalian microvascular cells (Sastre et al., 2000) and pneumocytes (Guthmann et al., 2005) which impressively highlights the difference between oxyconforming flatworms and oxyregulating mammalian cells. We did not observe aggregation of worms under normoxic/hyperoxic conditions indicating collective re-breathing as observed in ostracods when facing too high oxygen levels (Corbari et al., 2005). Indeed, *M. lignano* would warrant more thorough investigations of its migration or aggregation behaviour within oxygenation structured micro-environments (Fenchel and Finlay, 2008). However, we did observe that the locomotory activity of the worms was conspicuously higher in normoxic and hyperoxically manipulated treatments, with no observable difference between both oxygenation states. Thus, as in many other infaunal species (Abele et al 1998), normoxic oxygen levels are already high for these plathyhelminths and their behaviour indicates an attempt to reduce oxygenation in their sedimentary environment. This increased activity is fuelled by the enhanced respiration above 12.5kPa.

The C-H_2DFFDA measurements further indicate an increase in ROS (H_2O_2, HOO• and $ONOO^-$) production compared to normoxic worms, whereas no effect of hyperoxia on $O_2•^-$ concentrations was observed in the DHE measurements. A cross-experiment in which we added either SOD or SOD inhibitor STD to the medium confirmed that the DHE signal depends primarily on the amount of $O_2•^-$ in the cells. The measurements with ROS sensitive fluorophores therefore indicate that under hyperoxia the worms SOD activity converts excess $O_2•^-$ to H_2O_2. This conversion is abolished by the SOD inhibitor STD which killed the animals when they were exposed under hyperoxic conditions and STD. Mitochondrial production of ROS increases linearly with increasing oxygen concentration (Turrens et al., 1982), and hyperoxia has been shown to elicit oxidative stress and antioxidant responses in marine model organisms (Abele et al., 1998; Lushchak and Bagnyukova, 2006). Interestingly, 12.5kPa is also the limit at which mitochondria of the mammalian lung start to release H_2O_2 at a faster rate compared to lower oxygen levels, indicating antioxidant defenses starting to be overwhelmed. It seems a bit far-fetched to suggest a common upper *p*c for such distinct systems as meiofauna species and human lung epithelia, but the comparison illustrates the wide applicability of the hypothesis that in very diverse systems p_{O_2} is kept at very low levels to prevent hyperoxic ROS production.

pH of the tissue in hyperoxia was shown to be slightly more acidic than in animals kept under normoxic conditions. This can result from various mechanisms such as intensified hydrolysis of ATP and oxidative inhibition of the Na/H^+ antiporter under enhanced oxidative stress, as reported for brain cells (Mulkey et al., 2004) and for crustaceans (Abele-Oeschger et al., 1997). Furthermore, in fish and some crustaceans hyperoxic shocks can result in a reduction of ventilation rates, which cause extracellular acidosis (Gilmour and Perry, 1994; Gilmour, 2001; Wheatly and Toop, 1989).

Responses to anoxia and subsequent reoxygenation

Under anoxic conditions *M. lignano* showed drastically reduced movements, presumably an energy saving behaviour in response to the lack of oxygen. A similar response to anoxia has been reported for a wide variety of invertebrate species, like bivalves (e.g. de Zwaan and Wijsman, 1976) or crustaceans (e.g. Hervant et al., 1995).

Even though we did not obtain numerical $\Delta\psi_m$ results, our data indicate that *M. lignano* mitochondria mount a stronger H^+ gradient under low oxygen concentrations than at normoxic conditions (Fig. 5). Compared at normoxia, hypoxia tolerant invertebrates have lower $\Delta\psi_m$ values than cold-blooded vertebrate and mammalian mitochondria (Abele et al., 2007; Brookes et al., 1998; Keller et al., 2004). Anoxic *M. lignano* also maintained high $\Delta\psi_m$ values as hyperoxic individuals, reinforcing the view that the species not only tolerates low oxygen concentration but keeps up the mitochondrial proton gradient down to very low p_{O_2}. Under severely hypoxic to anoxic conditions < 1.5 kPa oxygen consumption rates started to decline as oxygen became limiting. Maintenance of the high proton gradient would then be due to reduced energy expenditures including complete absence of swimming mobility so that energetic requirements can be estimated to be minimal. This leads to the conservation of a high H^+ gradient because of a reduced phosphorylation activity and proton backflow at the ATPase.

On the other hand, anoxic animals also featured the most alkaline (least acidic) intracellular pH. This is paradoxical in the sense that for many organisms, cells and

tissues, severe hypoxia has been demonstrated to lead to a decrease in cellular ATP, increase of the free intracellular calcium concentrations (e.g. Kristián and Siesjö, 1996) and acidification of extra- and intracellular pH (e.g. Bickler, 1992). Only few studies have observed hypoxic alkalization in cancer cells (see review by Webb et al., 2011) and certain mammalian cells and are in keeping with our results (Mitsufuji et al., 1995). Such an alkalization of the intracellular pH during anoxia could well be an adaptive strategy to avoid apoptosis, known to be associated with intracellular acidification (Lagadic-Gossman et al., 2004; Matsuyama et al., 2000). But the question remains how such an increase of pH_i can be achieved. In cancer cells this has been associated with changes in the expression and/or activity of membrane transporters and ion pumps (see Webb et al., 2011). Other authors like Mitsufuji et al. (1995) suggested that the increasing pH values might be attributed to the activation of the Na/H^+-antiporter during hypoxia. Since the Na^+ gradient across the cellular membrane is maintained by the Na,K-dependent ATP-ase, the driving force for the proton export by the Na/H^+-antiporter is an energy (ATP) dependent process. The Na/H^+-antiporter exports intracellular protons, thus increasing pH_i and preventing acidosis until the cellular stores of ATP are exhausted. We have, however, demonstrated that mitochondrial membrane potential and potentially also the electron flow are maintained down to very low oxygen concentrations. Since ATP requirements are reduced through the reduction of locomotion, we assume ATP to be available for the upkeep of the Na^+ gradient and so to stabilize secondary active proton export during severe hypoxia, which would explain less acidic values in our near anoxia exposed worms. Since ATP concentration was not measured in the present study, this hypothesis cannot be corroborated. In future studies ATP concentrations and involvement of membrane transporters in stabilizing intracellular pH can be tested by luminescence assays (ATP+APD) and using specific inhibitors for membrane transporters. Another factor that could theoretically contribute to the more alkaline pH values during periods of low oxygen concentrations would be the decrease in ATP hydrolysis, which also involves H^+ release, due to the reduction of the worms' muscular activity. Although we consider hypoxic alkalinisation to be an important new finding based on live imaging techniques and confirmed by usage of two different pH-sensitive dyes, further investigations of this phenomenon are recommended. While no exact calibration in vivo has been yet developed for the use of Ageladine-A, BCECF has a reported accuracy of 0.07 pH units (Franck et al., 1996), which is within the range shift that we have detected in the present study. Further experiments are planned to see whether pH_i alkalinisation during hypoxia in the worms is accompanied by an extracellular acidification as observed in cancer cells (Webb et al., 2011).

The tenet that mitochondrial ROS formation increases linearly with oxygen concentration in cells (Turrens et al., 1982) is also disputable for the worms. Whereas the H_2O_2 formation was in agreement with this theory, $O_2\bullet^-$ formation follows a different pattern: *M. lignano* individuals under near anoxia showed similar $O_2\bullet^-$ concentrations as normoxic and hyperoxic animals. Under conditions of strict anoxia and thus in the absence of oxygen, ROS can definitely not be produced. In consequence, under so-called anoxia, minimal traces of oxygen must still have been present, enough to cause some $O_2\bullet^-$ formation in the worms. It is still under debate if ROS can be produced under hypoxia (Hermes-Lima et al., 1998; Hermes-Lima and Zenteno-Savin, 2002) and some evidence of this happening first came from direct ROS measurements (Vanden Hoek et al., 1997), followed by detection through resulting DNA damage (Englander et al., 1999). These results can, however, also be explained by the low K_m

for O_2 of mitochondrial nitric oxide synthase, which would lead to production of NO, estimated to be around 5-10% of the normal steady rate of NO production (Alvarez et al., 2003). This NO may bind to and inhibit cytochrome oxidase, causing an increase of its K_m for oxygen and, consequently, increased reduction of the upstream electrons transporters such as complex I and III and in consequence, enhance formation of $O_2^{\bullet -}$ under hypoxic conditions (reviewed by Turrens, 2003). Further, NO can react with superoxide to form toxic peroxynitrite which can lead to all sorts of macromolecular damage, including DNA damage, and presumably also induce antioxidant systems. Based on the results obtained through the use of SOD and STD and their effect on anoxic individuals, we suggest that SOD might be inhibited during anoxia, which would additionally contribute to the high $O_2^{\bullet -}$ and the low H_2O_2 concentrations recorded under these conditions. For other invertebrate species such as clams, a decrease in SOD expression and activity has also been observed (Monari et al., 2005).

Effect of oxygenation on MitoTracker fluorescence

Mitochondria in *M. lignano* are mainly concentrated in muscular and epithelial cells, providing energy to the numerous cilia and for body contraction. Although both MitoTracker dyes stained the same location, MitoTracker Green FM and MitoTracker Deep Red 633 gave significantly different results (Fig. 5 and supplementary material fig. 3) with respect to the comparison across treatments. Only with MitoTracker Green we observed a decrease in staining under anoxia, which suggests fluorescence of this dye to be negatively affected by anoxic exposure, even though it has been reported to be independent of membrane potential (Métivier et al., 1998). Some studies have demonstrated that this may not be true (Keij et al., 2000), and similar oxygenation dependent patterns of JC-1 and MitoTracker Green FM in *M. lignano* strongly suggest that MitoTracker Green is indeed sensitive to $\Delta\psi_m$. In that case, MitoTracker Green FM cannot be considered as a useful probe for mitochondrial density in experiments involving variable tissue oxygenation. This is further supported by the fact that MitoTracker Deep Red 633 staining was independent of the experimental oxygenation state and can thus be considered a better tool for determining mitochondrial mass (Haugland, 2002) in this kind of experiment.

CONCLUSIONS

In an attempt to establish the platyhelminth *M. lignano* as an applicable whole animal model for physiological investigations, we determined its peculiar physiological response to anoxic and hyperoxic exposure at different levels of cellular functioning. The worms are hypoxia tolerant and maintain the mitochondrial proton gradient and presumably also ATP levels during at least two hours of anoxic exposure. Oxyconforming respiration below 3 kPa apparently satisfies the worm's maintenance metabolism and prevents onset of cellular acidosis, while mobility ceases altogether and only ciliary movements are observable in anoxia and severe hypoxia. Elevated DHE fluorescence indicated superoxide formation in near anoxia which might be attributable to diminishment of SOD activity at low tissue oxygenation. However, we suggest that *M. lignano* could also be an interesting model to study hypothetical respiratory chain superoxide formation at low tissue oxygenation under the possible influence of hypoxic NO formation. The response to hyperoxia also differed from mammalian systems, but aligned with other marine invertebrate infauna that increase respiration rates in a p_{O_2} dependent manner above an upper critical p_{C_2}. This is often interpreted as an attempt to reduce excess oxygen in the animals environment and could be characteristic of

556 species that have successfully adapted to deal with the vagaries of environmental
557 oxygenation in intertidal sediments.
558
559
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754 FIGURE CAPTIONS
755
756 Fig. 1: Quantification process of the resulting confocal images: a total of 5 transects
757 were used for each of the animals under study. These were always perpendicular to the
758 longitudinal axis of the animal and were located on the areas with the highest
759 fluorescence intensities. A. Animal stained with MitoTracker Deep Red 633. B.
760 Transmission image of the same individual.
761
762 Fig. 2: Oxygen consumption rates of *Macrostomum lignano* at different environmental
763 oxygen partial pressures (kPa). Values are pooled in 1% intervals. Black dots represent
764 mean oxygen consumption rates for the 10 replicas conducted, while grey diamonds
765 represent the values obtained for animals that were, after reaching anoxia, further
766 reoxygenated. Whiskers represent the s.e.m. p_{c1}= upper critical oxygen partial pressure;
767 p_{c2}= low critical oxygen partial pressure
768
769 Fig. 3: Bar diagrams showing the quantitative results obtained for the pH measurements
770 through A) Ageladine-A staining and B) BCECF staining (obtained with the confocal
771 microscope). Subfigures 1 (A1 and B1) show the results corresponding to one
772 individual replica of the experiment. Since for each case the pattern was consistent
773 throughout the replicas, data was normalized and pooled (refer to materials and methods
774 section for more details). The corresponding results are shown in subfigures 2 (A2 and
775 B2). Whiskers represent the s.e.m. N=number of replicas conducted.
776
777
778 Fig. 4: Staining of *M. lignano* with MitoTracker Deep Red 633. A) Transmission image
779 of *M. lignano*. B) MitoTracker Deep Red 633 fluorescence of the same individual and
780 showing how the highest mitochondrial densities can be found in the animals' body
781 wall, in epithelial and muscle cells. C) View of the head showing how high
782 mitochondrial density is also registered around the mouth (white arrow).
783
784
785 Fig. 5: Bar diagrams showing the quantitative results obtained for the mitochondrial
786 measurements through A) MitoTracker Green FM and B) MitoTracker Deep Red 633
787 staining for the assessment of mitochondrial density and C) JC-1 staining for the
788 determination of mitochondrial membrane potential ($\Delta_{\psi m}$). Subfigures 1 (A1, B1, C1)
789 show the results corresponding to one individual replica of the experiment. Since for
790 each case the pattern was consistent throughout the replicas (except for MitoTracker
791 Deep Red 633), data was normalized and pooled (refer to materials and methods section
792 for more details). The corresponding results are shown in subfigures 2 (A2, B2, C2).
793 Whiskers represent the s.e.m. N=number of replicas conducted.
794
795 Fig. 6: High resolution fluorescence images of individual cells of *M. lignano* stained
796 with MitoTracker Green FM under anoxic (A) and normoxic (B) conditions. Confocal
797 imagery. Scale bar=15µm.
798
799
800 Fig. 7: Bar diagrams showing the quantitative results obtained in the ROS quantification
801 through A) DHE and B) C-H_2DFFDA staining. Subfigures 1 (A1, B1) show the results
802 corresponding to one individual replica of the experiment. Since for each case the
803 pattern was consistent throughout the replicas, data was normalized and pooled (refer to

materials and methods section for more details). The corresponding results are shown in subfigures 2 (A2, B2). Whiskers represent the s.e.m. N=number of replicas conducted.

Fig. 8: Results on the effect of the inhibition of SOD under anoxic conditions: (a) effect of SOD and SDT on anoxic animals. Groups associated with the same letter (a-b-c-d) belong to the same subset based on a *a-posteriori* multiple comparison test Student-Newman-Keuls (S.N.K.) (K=27.41; $p<0.001$); (b) effect of SOD on the concentrations of O_2^- (DHE values) (b1) and H_2O_2 (C-H_2DFFDA values) (b2) in anoxic animals. Whiskers represent the s.e.m. *** $p<0.001$.

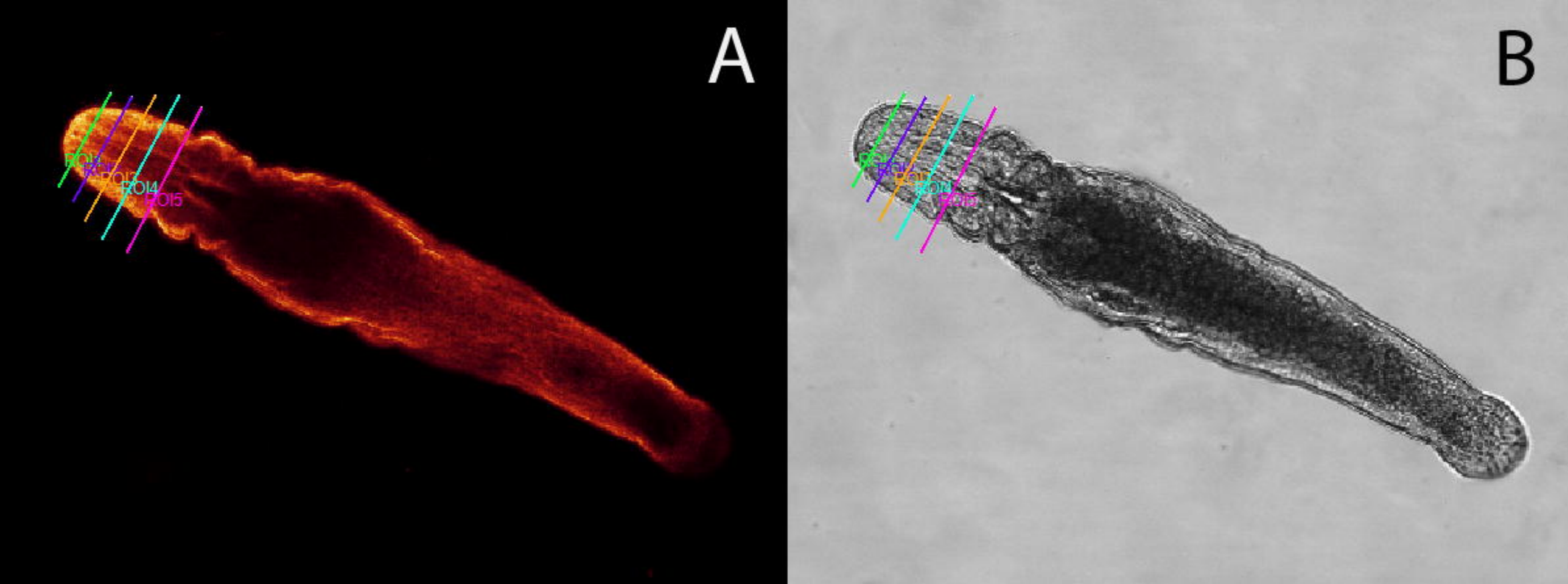
SUPPLEMENTARY MATERIAL CAPTIONS

Fig. 1: Representative fluorescence images obtained during the pH measurements under the confocal microscope for each of the treatments considered. A) Ageladine-A staining. B) BCECF staining.

Fig. 2: Wide-field fluorescing imaging of *M. lignano* individuals stained with BCECF under the four treatments considered in the study: a) anoxia followed by reoxygenation, b) Near anoxia, c) Normoxia and d) Hyperoxia.

Fig. 3: Representative fluorescence images obtained during the mitochondrial measurements under the confocal microscope for each of the treatments considered. Use of A) MitoTracker Green Fm and (B) MitoTracker Deep Red 633 for mitochondrial mass estimation and (C) JC-1 for $\Delta_{\psi m}$ calculation.

Fig. 4: Representative fluorescence images obtained during ROS quantification under the confocal microscope for each of the treatments considered. Use of A) DHE and B) C-H_2DFFDA for O_2• and H_2O_2 quantification, respectively.


A
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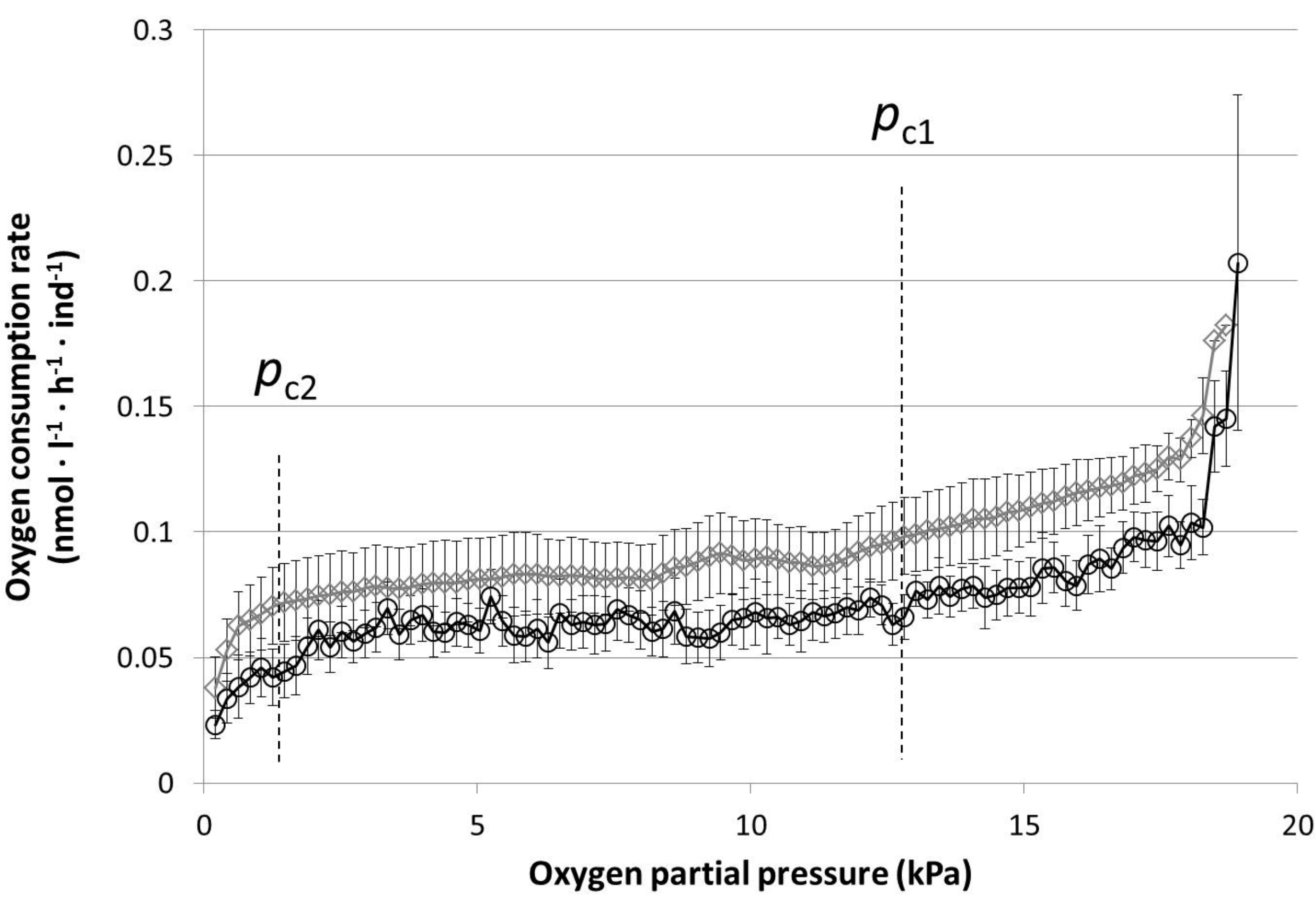

0.3
0.25
0.2
0.15
0.1
0.05
0
p_{c1}
p_{c2}
Oxygen consumption rate
(nmol · l-1 · h-1 · ind-1)
0
5
10
15
20
Oxygen partial pressure (kPa)

A1

Ageladine-A

Intensity (a.u.)

40
30
20
10
0.0

b
b
a
a

+pH

A2

Ageladine-A

N=4

Percentage of maximum intensity (%)

80
60
40
20
0.0

a
a
b
b

+pH

7.50

7.40

Anoxia + Reoxyg.

Anoxia

Normoxia

Hyperoxia

B1

BCECF

Ratio (exitac. 490nm / exitac. 440nm) (a.u.)

12.5
10.0
7.5
5.0
2.5
0.0

b
a
b
b

+pH

B2

BCECF

N=2

Percentage of ratio (ex. 488nm/ex. 440nm)(%)

80
60
40
20
0.0

a
a
b
b

+pH

7.53

7.46

Anoxia + Reoxyg.

Anoxia

Normoxia

Hyperoxia


A
B
C

A1 MitoTracker Green FM

Intensity (a.u.)

300
200
100
0.0

b c b a

+Mitoc. density

B1 MitoTracker Deep Red

Intensity (a.u.)

300
200
100
0.0

b b b a

+Mitoc. density

A2 MitoTracker Green FM N=2

Percentage of max. intensity (%)

100
80
60
40
20
0.0

b c b a

+Mitoc. density

Anoxia + Reoxyg.
Anoxia
Normoxia
Hyperoxia

B2 MitoTracker Deep Red N=4

Percentage of max. intensity (%)

100
80
60
40
20
0.0

+Mitoc. density

Anoxia + Reoxyg.
Anoxia
Normoxia
Hyperoxia

C1 JC-1

Ratio (monomeric / aggregate form) (a.u.)

2.0
1.5
1.0
0.5
0.0

a b a b

+ Δψm

Anoxia + Reoxyg.
Anoxia
Normoxia
Hyperoxia

C2 JC-1 N=2

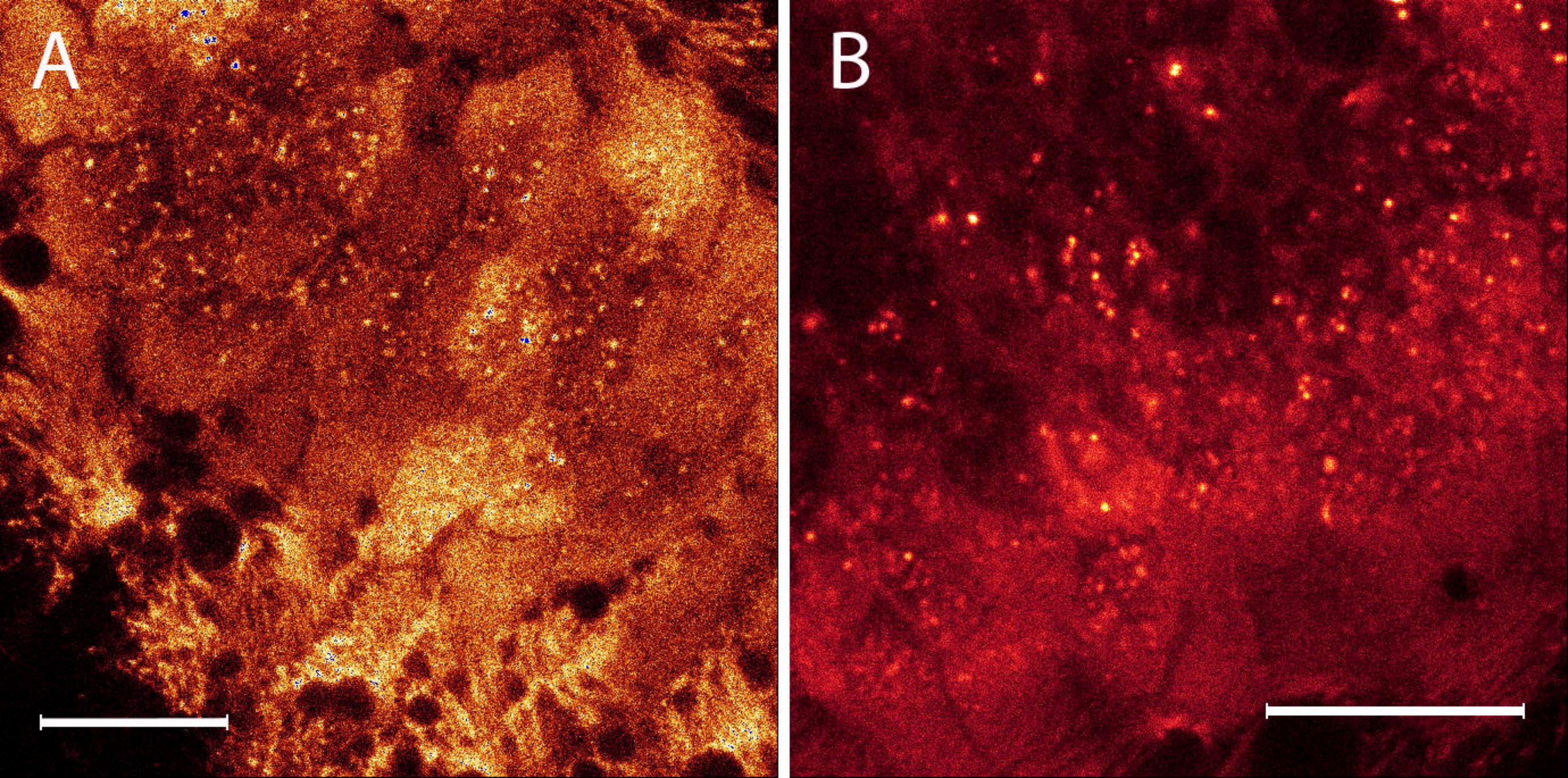
Percentage of ratio (monomeric / aggregate form) (%)

100
80
60
40
20
0.0

a c b c

+ Δψm

Anoxia + Reoxyg.
Anoxia
Normoxia
Hyperoxia


A
B

A1

Dihydroethidium

Ratio (ethidium / dihydroethidium) (a.u.)

4.0
3.0
2.0
1.0
0.0

a
b
b
b

+O_2^-

A2

Dihydroethidium

N=3

Percentage of ratio (ethidium/dihydroethidium)(%)

80
60
40
20
0.0

a
b
b
b

+O_2^-

Anoxia + Reoxyg.
Anoxia
Normoxia
Hyperoxia

B1$_a$

C-H_2DFFDA

Intensity (a.u.)

300
200
100
0.0

a
d
c
b

+H_2O_2

B2

C-H_2DFFDA

N=2

Percentage of maximum intensity (%)

100
80
60
40
20
0.0

a
c
b
a

+H_2O_2

Anoxia + Reoxyg.
Anoxia
Normoxia
Hyperoxia

A

Dihydroethidium

Ratio (ethidium / dihydroethidium) (a.u.)

5.0
4.0
3.0
2.0
1.0
0.0

a
d
c
b

$+O_2^-$

Anoxia + SDT
Anoxia + SOD
Anoxia
Normoxia

B_1 DHE

B_2 C-H_2DFFDA

6.0
4.0
2.0
0.0

300
200
100
0.0

Intensity (a.u.)

$+O_2^-$

$+H_2O_2$

***
***

× SOD
Control
Control
× SOD

Table 1: Description of the dyes used during the present study:

Dye	**Parameter measured**	**Company/code**	**Mechanism of function**
Ageladine-A (in DMSO)	pH	**	Probe which exists as a nearly uncharged monomer under pH of 8.1-8.6. After crossing cellular membranes, becomes charged in the cytosol and acidic compartments of cells.
BCECF AM (in DMSO)	pH	Molecular Probes (B-3051)	Probe which absorption shifts whether it occurs as the basic (phenolate anion) or acidic (protonated) form.
MitoTracker Green FM (in DMSO)	Mitochondrial density	Molecular Probes (M-7514)	Non-fluorescent molecule which becomes fluorescent once accumulates in the lipid environment of the mitochondria.
MitoTracker Deep Red 633 (in DMSO)	Mitochondrial density	Molecular Probes (M-22426)	Non-fluorescent molecule which becomes fluorescent once accumulates in the lipid environment of the mitochondria.
JC-1 (in DMSO)	Mitochondrial membrane potential	Molecular Probes (T-3168)	This green fluorescent probe exists as a monomer at low $\Delta_{\psi m}$. With high $\Delta_{\psi m}$ values, JC-1 aggregates and shows a red fluorescence.
Dihydroethidium (DHE) (in DMSO)	$O_2\bullet^-$	Molecular Probes (D-23107)	Regularly shows a blue emission when excited with a 355nm laser. When oxidized by the presence of $\mathbf{O_2^-}$, it intercalated with the DNA and shows a red emission when excited with an argon laser.
C-H_2DFFDA (in Ethanol)	H_2O_2, HOO• and $ONOO^-$	Molecular Probes (C-13293)	Non-fluorescent molecule which is converted to a green-fluorescent form when the acetate groups are removed by intracellular esterases and oxidation occurs in the cell.

** Synthesized by Peter Karuso, Macquarie University, Sydney.

Table 2: Analysis conditions for each of the dyes used during the study.

					Excitation		Emission		
Dye	**Final conc. used (µM)**	**Incubation time (min)**	**N ind per batch**	**N repetitions (total N individuals)**	**λ_1 (nm)**	**λ_2 (nm)**	**PMT1 (nm)**	**PMT2 (nm)**	**Calculation**
Ageladine-A (in DMSO)	15	90	10	4 (50)	MP(370)	-	420-500	-	Maximum Intensity
BCECF AM (in DMSO)	5.0	90	10	2 (20)	MP(439)	488	520-550	-	Maximum values ratio λ2/λ1
MitoTracker Green FM (in DMSO)	0.33	30	10	2 (20)	488	-	500-550	-	Maximum intensity
MitoTracker Deep Red 633 (in DMSO)	0.33	60	10	4 (40)	633	-	640-680	-	Maximum intensity
JC-1 (in DMSO)	5	60	10	2 (20)	488	488	500-550	560-600	Ratio PMT1/PMT2
Dihydroethidium (DHE) (in DMSO)	3.3	30	10	3 (30)	MP (355)	488	400-440	620-660	Ratio PMT2/PMT1
C-H_2DFFDA (in Ethanol)	10.6	30	10	2 (20)	488	-	510-550	-	Maximum Intensity

MP=Multiphoton laser. Values in parenthesis indicate the effective excitation wavelength.