Flow cytometric assessment of morphology, viability, and production of reactive oxygen species of Crassostrea gigas oocytes. Application to Toxic dinoflagellate (Alexandrium minutum) exposure

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Abstract :

The Pacific oyster Crassostrea gigas accounts for a large part of shellfish aquaculture production worldwide. Aspects of morphological and functional characteristics of oyster oocytes remain poorly documented, and traditional techniques, such as microscopic observations of shape or fertilization rate, are time and space consuming. The purpose of this study was to assess for the first time viability and reactive oxygen species (ROS) production of Pacific oyster oocytes using flow cytometry (FCM) and to apply this method to determine oocyte responses to in vitro exposure to the toxic dinoflagellate Alexandrium minutum. A culture of A. minutum caused a significant increase in oocyte ROS production, which gradually increased with the age of the culture, but viability was not affected. Effect of the supernatant of the same A. minutum culture did not cause any significant modifications of oocyte morphology, viability, or ROS level. This study confirmed that some oocyte cellular characteristics can be assessed using FCM techniques. © 2014 International Society for Advancement of Cytometry

Keywords : Pacific oyster, gamete quality, ROS production, viability, flow cytometry, ecotoxicology

The Pacific oyster *Crassostrea gigas* has considerable commercial value worldwide, including in France. Oyster seed production relies upon gamete quality, fertilization success, and larval development. As gamete quality is the first step in the spat-production sequence, it is necessary to assess the quality of gametes in broodstock oysters first and foremost.

To date, bioassays to assess oocyte quality are limited, and those available are tedious and time-consuming. Oocyte quality can be described using morphological characteristics by microscopy (1). Although microscope observations are unavoidable and useful to validate flow cytometry (FCM), assays based on microscope observations often have little predictive value and can be subjective according to each observer. Determinations of biochemical content (proteins, carbohydrates and lipids) have been used as criteria of oocyte quality (2–4). More recently, proteomic profiles of oocytes were proposed as quality estimators (5,6). All of these biochemical assays, however, are fairly tedious to perform and provide only retrospective information. Fertilization rate, hatching rate and larval development are used as complementary end points to evaluate oocyte quality (7–10); however, as these processes depend also upon spermatozoa quality, variability and inconsistency from one experiment to another often are encountered.

One measure of the quality of oocytes is viability. Viability of oocytes was determined previously using light microscopy with trypan blue (1) or epifluorescence microscopy with fluorescein diacetate (FDA) and propidium iodide (PI) (11,12). However, Valdez-Raminez et al. suggest that membrane damage in oocytes is not the sole cause of larval rearing failure, but is an additional metric to estimate gamete quality (1). Furthermore, Kadomura et al. (13) suggest that reactive oxygen species (ROS) production of fish oocytes may be involved in survival rate of larvae. ROS production is a key cellular mechanism involved in immune defense or stress response, but excessive ROS can lead to oxidative damage of DNA (14).

ROS production of oocytes was measured previously using a spectrophotometer for oocytes of mouse (15) and *Xenopus* (16) or using epifluorescence microscope for porcine oocytes (17). To our knowledge, however, no literature is available to assess ROS production by bivalve oocytes.

Flow cytometry (FCM), coupled with fluorescent probes, has been used for years to assess mammal spermatozoa quality (18), and more recently has been adapted to spermatozoa of invertebrates (19–21). Although FCM tools are now well-developed to study oyster spermatozoa biology and physiology, such an approach has not been tested on oyster oocytes as these cells are close to the maximum cell size recommended by flow cytometer manufacturers. In the literature, mean diameter of oocytes of *C. gigas* ranges from 40 to 65 µm depending upon techniques used (22–24). Considering that mammalian cell diameters range from 10 to 30 µm, analysing oyster oocytes using FCM represents a real technical challenge.

Compared to microscopic or spectrophotometric methods, FCM combines multiple benefits, such as statistical reliability and time-savings. FCM can analyse several variables (morphology, cell activity…) simultaneously on each of more than hundred cells per second. Moreover, compared to spectrofluorimetric assays that yield a mean fluorescence value for all cells, FCM measures various parameters on individual cells, enabling differentiation of cell populations, thereby allowing more accurate and discriminating measurements. Thus, the rapidity and accuracy of FCM would make it an excellent method to assess oocyte physiological status in support of larval production in experimental and commercial hatcheries. Such a technological development also may allow in the near future physical sorting of oocytes with specific functional features.

To test the ability of FCM to detect oocyte responses to external stimuli, we sought an environmental stressor to which oyster gametes can be exposed in nature or in the hatchery. *In*

vivo, oysters exposed to toxic algae revealed alteration of spermatozoa quality (21,25). Gametes of *C. gigas* are released into the surrounding seawater where they are exposed to various environmental stressors (26,27) and possibly toxic algae (10,28–30). Blooms of *A. minutum* occur during summer, which is concomitant with the oyster spawning period. It is therefore expected that harmful algal blooms (HABs) may affect morpho-functional characteristics of oocytes from *C. gigas*. To date, studies testing HAB impacts upon bivalve oocytes used fertilization and hatching percentages as end points (10,31,32) but these processes also are dependent upon spermatozoa quality. Also, based upon microscopic observations of oocyte shape, the harmful dinoflagellate *Heterocapsa circularisquama* was shown to significantly reduce Japanese pearl oyster oocyte viability *in vitro* after 30 and 60 minute incubations (10). Using FCM to assess effects of toxic *A. minutum* upon oocytes of oysters could represent technical breakthrough allowing rapid analyses of morphology, viability, and ROS production of these cells.

The dinoflagellate *A. minutum* is distributed worldwide and produces paralytic shellfish toxins (PSTs). Blooms of toxic dinoflagellates have been responsible for massive mortalities of bivalves and also are toxic to human consumers (33). In addition to PSTs, *Alexandrium* species produce extracellular toxic compounds (haemolytic, ichtyotoxic, and allelopathic compounds) which are excreted and known to be noxious to other phytoplankton species (34,35). As growth phase of culture is known to impact *A. minutum* toxicity (34), oyster oocytes were exposed to culture or supernatants at three different times of exponential growth phase in the present study.

The aim of the present study was to: i) design and test two FCM assays (viability and ROS production) for use as biomarkers of oocyte quality and, ii) apply these measurements to oocytes of *C. gigas* to an *in vitro* exposure to the toxic dinoflagellate *A. minutum*.

MATERIAL AND METHODS

BIOLOGICAL MATERIAL AND TECHNIQUES OF ANALYSES

Oyster oocytes

Three-year-old Pacific oysters were collected from Pointe du Château, Bay of Brest, France. To collect oyster oocytes, gonads were stripped (36). Oocytes were suspended in 0.2 µm filtered sterile sea water (FSSW), filtered at 100 µm to remove pieces of gonad tissue and concentrated on a 20-µm sieve. For FCM analysis, oocyte concentration was adjusted to 50,000 oocytes.ml^{-1} of FSSW.

Flow cytometric analysis

Analyses of morphology, viability, and production of reactive oxygen species (ROS) were performed using an EasyCyte Plus cytometer (Guava Technologies, Millipore, Billerica, Massachusetts) equipped with a 488-nm argon laser and three fluorescence detectors; green (525/30 nm), yellow (583/26 nm), and red (680/30 nm). On Guava cytometers, cells are pumped into an exchangeable flow cell (100 µm diameter). Samples were acquired during 30 seconds at flow rate of 0.59 µl.sec^{-1}. Most of small debris were eliminated of the analysis with a forward scatter threshold at 90. Relative fluorescence values were expressed as arbitrary units.

Microscopy

To confirm FCM results, oocyte observations were performed using an inverted epifluorescence microscope (Axio observer.Z1, Zeiss, Germany) equipped with a Vivatome

module with filters for green (excitation 494/20 nm, emission 536/40 nm) and red (excitation 575/25 nm, emission 628/40 nm) fluorescences. Images were captured using a Zeiss Axiocam and analysed with Axiovision software.

To assess morphological features of oocytes, a 10 µl sample of oocytes was transferred to a Thomas cell, and morphological characteristics were observed under a phase-contrast microscope (Olympus BX51, X 10 objective) connected to a video camera (Qicam Fast 1394). Oocyte photomicrographs were taken and oocyte area (µm²), perimeter (µm), circularity (ranging from 0 to 1, a value of 1 indicating a perfect circle) and diameter (µm) were determined using Image J software (n=30 oocytes).

OPTIMIZATION EXPERIMENTS

Assessment of oocyte morphology and viability

Values from forward scatter (FSC: relative to cell size) and side scatter (SSC: relative to cell complexity) detectors were used as descriptors of oocyte morphological characteristics. Viability was measured using both SYBR-Green I (Molecular probes, Eugene, Oregon), which penetrates live and dead oocytes (green fluorescence, 500-530 nm) and propidium iodide (PI, Sigma, St Quentin Fallavier, France), which penetrates only cells with compromised membrane and then emits in the red fluorescence range (550-600 nm). Results were expressed as percentages of live cells. Aliquots of 200 µl oocyte suspensions (50,000 cells.ml^{-1}) were stained with SYBR-Green I (1/10,000 of the commercial solution) and PI (final concentration 10 µg.ml^{-1}) for 10 minutes in the dark at 18°C. The positive control for PI staining was obtained by heating oocytes at 60°C for 60 min. To confirm the FCM observations, stained oocytes were also observed under the epifluorescence microscope.

Assessment of oocyte ROS production

ROS production was measured using 2',7'-dichlorofluorescin diacetate DCFH-DA (Sigma, St Quentin Fallavier, France), a cell-permeable, non-fluorescent compound. DCFH-DA is hydrolysed intra-cellularly to form DCFH which turns to highly green fluorescent 2′,7′-dichlorofluorescein (DCF) upon oxidation by ROS in a quantitative manner. To adjust the settings for ROS measurement using DCFH-DA fluorescence, samples were analysed respectively with and without DCFH-DA staining. To determine optimal duration of incubation, the kinetics of green fluorescence development were analysed on oocytes from three individual females after 15, 30, 60, 90 and 120 minutes of incubation with DCFH-DA at 18°C. To control that measured DCF fluorescence corresponds to ROS production, oocyte samples were incubated with chemicals (Sigma, St Quentin Fallavier, France) known to induce oxidative stress; *tert*-Butyl hydroperoxide (TBHP) and cumene hydroperoxide (CHP) at 0.1, 1 and 10 mM and H_2O_2 at 0.01, 0.1 and 1 mM. Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), a water-soluble analog of vitamin E, and L-Ascorbic acid were tested as antioxidant substances at 0.1 mM. All tested concentrations of oxidants and antioxidants had no effect on oocyte viability, and did not change the pH of the assay. Upon addition of 2 µl of chemicals on 200 µl oocyte suspensions, samples were stained with DCFH-DA (final concentration 10 µM) at 18°C. To confirm the FCM observations, stained oocytes were also observed under the epifluorescence microscope.

APPLICATION TO OYSTER OOCYTE EXPOSURE TO *Alexandrium minutum*

Algal cultures

The toxic dinoflagellate, *Alexandrium minutum* (AM89BM) and the non-toxic dinoflagellate *Heterocapsa triquetra* (HT99PZ) were grown in filtered and autoclaved L1 medium (37), at

16°C with a dark:light period of 12:12h (100 µmol photons.$m^{-2}.s^{-1}$). The culture of *A. minutum* was harvested at three ages: (i) beginning of the exponential phase: day 2 after inoculation (5,600 cells.ml^{-1}), (ii) middle of the exponential phase: day 7 after inoculation (16,500 cells.ml^{-1}) and (iii) end of exponential phase: day 11 after inoculation (53,000 cells.ml^{-1}). Culture of *H. triquetra* was harvested at the end of the exponential phase (61,700 cell.ml^{-1}).

Exposure protocol

Oocytes were stripped from three individual females and prepared as described above. To conduct *in vitro* exposures, 100 µl of each oocyte suspensions (50,000 cells.ml^{-1}) were exposed for 2 hours at 18°C to 100 µl of culture or supernatants of *A. minutum* at the three different growth phases described above (day 2, day 7 and day 11 after inoculation), as well as to the supernatant of *H. triquetra* and FSSW as controls. Supernatants of *A. minutum* and *H. triquetra* cultures were prepared just before incubation with oocytes by filtration on cellulose acetate filters (0.22 µm Minisart, Sartorius, Göttingen, Germany) (35).

Assessment of morphology, viability and ROS production

Morphological features of oocytes were analysed by phase contrast microscopy and FCM. Viability and ROS production assays were performed separately by FCM as described above. In viability experiment, SYBR-Green I and PI were added 10 minutes before the end of the exposures and for ROS production assay DCFH-DA was added 1 hour before the end of the exposures.

STATISTICAL ANALYSIS

Results of oxidant and antioxidant effects on oocyte ROS production are presented as percentage of control (mean ± confidence interval, $\alpha=0.05$) but one-way analysis of variance (ANOVA) was performed on DCF fluorescence values of test conditions and controls. To compare the effects of treatments (culture and supernatants) on the different cellular measurements, an ANOVA was also performed. The assumption of homogeneity of variance was confirmed using Leven's Test for equality of error variances, and normality of residuals was verified. LSD tests were used to specify significant differences between individual treatments. Differences were considered significant when p-value was <0.05. Statistical analyses were performed using Statgraphics Plus 5.1 software (Manugistics, Inc, Rockville, MD, USA).

RESULTS AND DISCUSSION

OPTIMIZATION EXPERIMENTS

Morphology and viability

In this study, oocytes of oysters were analysed using microscope and for the first time using FCM. Using microscopy, area, perimeter, circularity, and diameter of oocytes appeared very homogenous (data not shown). Oyster oocyte diameter measured with microscopy (from 63 to 70 µm) was consistent with literature (22–24). Using FCM, morphology of oocytes was analysed with forward scatter FSC (relative cell size) and side scatter SSC (relative cell complexity) (Fig 1A). As Pacific oyster oocyte diameter is approximately 65 µm, amplifiers of forward scatter and side scatter were set to minima on the Guava flow cytometer. Relative cell complexity (SSC) mean of each female ranged from 1,964 to 2,040 a.u. and was less variable (CV ranged from 5.6 to 5.9%) than relative cell size (FSC) mean that ranged from 318 to 360 a.u. with a CV ranging from 19 to 24% (data not shown). Forward scatter variability may be explained by the fact that oyster oocytes were obtained by stripping of the gonads. This technique bypasses natural maturation processes of oocytes and provides oocytes with pear shape, with cells subsequently becoming round after incubation in seawater (23). The larger distribution of oocyte relative size observed by FCM can be attributed to the mix of oocytes with round and pear shapes (Fig 1B) which likely diffract light (FSC) differently. This difference is not observed when cells are measured directly by microscope, in which diameter seems to be the more variable dimension. Forward scatter measures diffracted light, and detector values are difficult to compare directly to biometric characteristics measured using the microscope. Nevertheless, relative cell complexity (SSC) of oocytes is an additional oocyte measurement by FCM not possible using a microscope.

Viability was assessed using SYBR-Green I and propidium iodide double staining. Live oocytes acquired bright green fluorescence, while dead oocytes contained both green and red fluorescence (Fig 2). Observation of SYBR-Green I/PI stained oocytes by epifluorescence microscopy confirmed flow cytometer measurements (Fig 2). Moreover, oocyte chromosomes appeared bright green, which confirms the staining of DNA with SYBR-Green I. This result is in accordance with previous assessment of oyster oocyte viability using fluorescent probes and epifluorescent microscopy (11,12), but the FCM technique developed here analyses 200 to 1,000 oocytes within 30 sec per sample, compared to longer microscopic observation of 200 oocytes per sample described by Paniagua-Chávez et al. (11).

ROS production

To date, no literature is available concerning analyses of ROS production by oyster oocytes. DCF fluorescence accumulate in oocytes with increasing time, as previously observed for various cellular models in marine organisms (38). To determine optimal incubation time, kinetics of ROS production were monitored after 15, 30, 60, 90 and 120 minutes of incubation with DCFH-DA probe (Fig 3). Green fluorescence increased linearly with time from 15 to 60 min. Thereafter, fluorescence increased faster between 60 and 120 min without reaching a plateau. This distinguished oocytes from oyster hemocytes, which generally reach a plateau or slowed around 120 minutes (38). This ROS increase may reflect a stress from the abnormally high oocyte concentration used, as oocytes are known to be impaired when maintained for too long at high concentration (> 5,000 cells.ml^{-1}) as shown by Song et al. (39). Furthermore, in standard hatchery procedures, oocyte fertilization generally is conducted within 1-4 hours of stripping or spawning of ripe females and at concentration of 100-1,000 cells.ml^{-1}. Considering the above statements and our higher oocyte concentration, an incubation of 60 min with DCFH-DA was chosen to assess ROS production.

Green fluorescence of DCF stained oocytes was close to 100 times higher than unstained oocytes (n=15) (Fig 4A). This confirmed an insignificant autofluorescence of oocytes. As voltage of the green detector was set close to the minimum on the Guava flow cytometer, this revealed that oocytes can produce a fairly large amount of ROS as measured by the DCFH-DA assay. Similar to results obtained by Kadomura et al. (13) on oocytes of the devil stinger fish using chemiluminescence, oyster oocytes produced large amounts of ROS without stimulants. DCF accumulation was also observed under the microscope (Fig 4B). Different levels of the green fluorescence revealed that all oocytes from the same sample do not produce the same amount of ROS, which demonstrates one advantage of FCM compared to a spectrofluorimeter that measures overall fluorescence in an oocyte sample.

Oxidant and antioxidant substances were used to confirm that DCF fluorescence measured in these conditions correspond to ROS production. As shown in figure 5, *tert*-Butyl hydroperoxide (TBHP), known to induce ROS production significantly increased DCF fluorescence of oocytes at 1 and 10 mM in a dose dependent manner (Fig 5A). Similar response upon exposure to cumene hydroperoxide (CHP) at 1 mM was observed, even though high cyto-toxicity was observed at 10 mM of CHP. Such induction of ROS production is consistent with results previously reported for *Xenopus* oocytes (40). However hydrogen peroxide (H_2O_2) did not increase DCF fluorescence in our cellular model (data not shown) unlike the ROS induction by this chemical observed in *Xenopus* oocytes (40). Addition of Trolox, a water-soluble analog of vitamin E, at 1 mM resulted in a drastic significant decrease of ROS production induced by 10 mM of TBHP (Fig 5B). It was to be noted that Trolox alone tends to decrease ROS production of oocytes as compared to control condition. L-Ascorbic acid, another antioxidant (41), was inefficient to reduce ROS production induced by 10 mM of TBHP but on contrary resulted in a further increase of ROS production (data not shown). Similarly, L-Ascorbic acid used in combination with $FeSO_4$ increased production of ROS in

Xenopus oocytes (40). Overall, observed effects of tested oxidants (TBHP and CHP) and antioxidant Trolox demonstrated DCFH-DA assay allowed measuring unambiguously ROS production by oyster oocytes. Unexpected increase of ROS production by oyster oocyte upon L-Ascorbic addition may reflect some species specific responses and deserve further in depth cellular studies.

Mean diameter of oyster oocytes is approximately 65 µm. Diameters of oocytes from other bivalves, are in this range or only slightly larger: *Mytilus edulis* is 60 to 75 µm (42), *Pecten maximus* is 65 to 70 µm (43), *Cerastoderma edule* is 55µm (44), so are likely to be amenable to FCM analyses using our protocols. However, oocyte diameter of the sea urchin *Strongylocentrotus droebachiensi* is approximately 155 µm and 200 µm for *Haliotis tuberculata coccinea* (45,46). Considering these oocyte diameter ranges, it appears necessary to develop larger FCM flow cells or capillaries to study cell physiology of oocytes from other biological models, and even larger to analyse embryos as fertilization increases cell size. Morphological characteristics, viability and ROS production were developed for oyster oocytes using FCM and then applied to assess susceptibility to *Alexandrium minutum* exposure.

APPLICATION TO OOCYTE CHANGES UPON EXPOSURE TO TOXIC *Alexandrium minutum*

Morphology and viability

Forward scatter (proxy of cell size) and side scatter (proxy of cell complexity) were not affected by exposures to *A. minutum* cultures or supernatants, compared to controls (FSSW and supernatant of *Heterocapsa triquetra*), regardless of the age of the culture. This result is consistent with microscopic observations of oocytes as no significant differences were

observed in area, perimeter, circularity, or diameter between treatments. Mean percentage of live oocytes did not change upon *A. minutum* exposure.

ROS Production

ROS production of oocytes measured with DCFH-DA assay was significantly higher upon exposure to 7-day-old (middle of exponential phase) and 11-day-old (end of exponential phase) culture when compared to the seawater control (Fig 6). We speculate that ROS production is associated with higher amounts of toxic compounds produced by aging, toxic *A. minutum* cells (35). Upon exposure to 2-day-old (beginning of exponential phase), culture of *A. minutum*, ROS production increased only slightly compared to the seawater control. Indeed, a clear dose dependant response of ROS production upon exposure to *A. minutum* culture was observed. A non-significant increase in ROS production was observed upon exposure to *A. minutum* supernatants. This result may be attributable to rapid decay of excreted, extracellular, toxic compounds (allelopathic, haemolytic, and ichtyotoxic) in supernatants (47), even though supernatants of cultures were prepared and filtered just before contact with oocytes. As opposed to supernatants, cultures (with live algae) continuously produced and excreted these hypothetical extracellular compounds during incubation with DCFH-DA. These results suggest that the contact between algal cells and oocytes was required to induce ROS production responses. Surprisingly, ROS production of oyster oocytes upon *H. triquetra* supernatant exposure was significantly lower than the seawater control and all *A. minutum* treatments. This result could be explained by the capacity of *H. triquetra* to produce anti-oxidant compounds (48,49). Another hypothesis could be that *A. minutum*, which is known to produce more pericellular superoxide than *Heterocapsa* sp*.,* has been shown to reduce protist survival via ROS production (47,50). This release of ROS by *A.*

minutum into the medium during exposure could participate to the increase of ROS observed in oocytes exposed to this culture.

Overproduction of ROS in marine organisms can be induced by many stressful conditions, such as heat shock, UV exposure, pollution, oxygen availability changes, or pathogens (51). Overall, these results reveal a modification in ROS regulation in oocytes upon *A. minutum* exposure. The observed increase of ROS production can thus reflect a stimulation of oocyte metabolism, which at high level can lead to DNA damage in the oocytes. To better understand how such increases in ROS affect oocytes, it would be interesting to search for relationships between ROS production, fertilization rate, hatching rate, and larval mortality upon *A. minutum* exposure. Further investigations are required to understand ROS production mechanisms in bivalve oocytes, and FCM seems to be an effective, rapid, and appropriate technology to address such questions.

RECOMMENDATIONS FOR OOCYTE STUDY USING FLOW CYTOMETERS

FCM has real promise to advance analysis of shellfish oocytes, but as oyster oocytes tend to sediment very rapidly, sample mixing is a crucial issue for such assays. As the Guava system is mixing the "n+2" sample while the "n" sample is being acquired in the microplate, this sequence leaves too much time for oocytes to sediment (mixed 1 minute before acquisition for n+2 samples). To avoid this sedimentation problem, we recommend transferring oocyte samples into microplate every four wells (1, 4, 7, 10 line sequence for instance). Although serial dilution gave a very good correlation coefficient (r^2=0.99; data not shown), caution must be taken in assessing oocyte concentration, as samples are not mixed continuously during acquisition by the flow cytometer. We recommend comparing counts obtained by FCM and microscopy when this variable is crucial for experiment design. To analyse oocytes

using FCM, caution regarding analyze chamber size and clogging is also recommended with other flow cytometers. However, first trials using another flow cytometer (FacsCalibur, Becton Dickinson) having an analyze chamber size of 100 µm did not result in any troubleshooting allowing application of our protocols to a large park of available flow cytometers.

CONCLUSION

To the best of our knowledge, this study demonstrated, for the first time the use of FCM to analyse cellular physiology of oyster oocytes. Measurements of viability and ROS production were established and applied to an environmental question concerning responses of oocytes to *A. minutum*. Such an approach could, indeed, be extended to chemical contaminants in a marine ecotoxicological context. Furthermore, assessment of oocyte quality is a crucial key to development of cryopreservation protocols. FCM was revealed to be a good tool allowing measurement of viability and ROS production on numerous oocytes in a short time (200-1,000 oocytes within 30 seconds). FCM is able to identify abnormal oocytes through morphological changes, increase in dead cells, or ROS production variations. This technique can be applied on a routine basis in both experimental and commercial hatcheries as prices of portable flow cytometers have decreased over the last ten years (about $20,000 for a "basic" instrument). Moreover, other fluorescent probes could be tested in the future to assess other functional physiologies in oyster oocytes.

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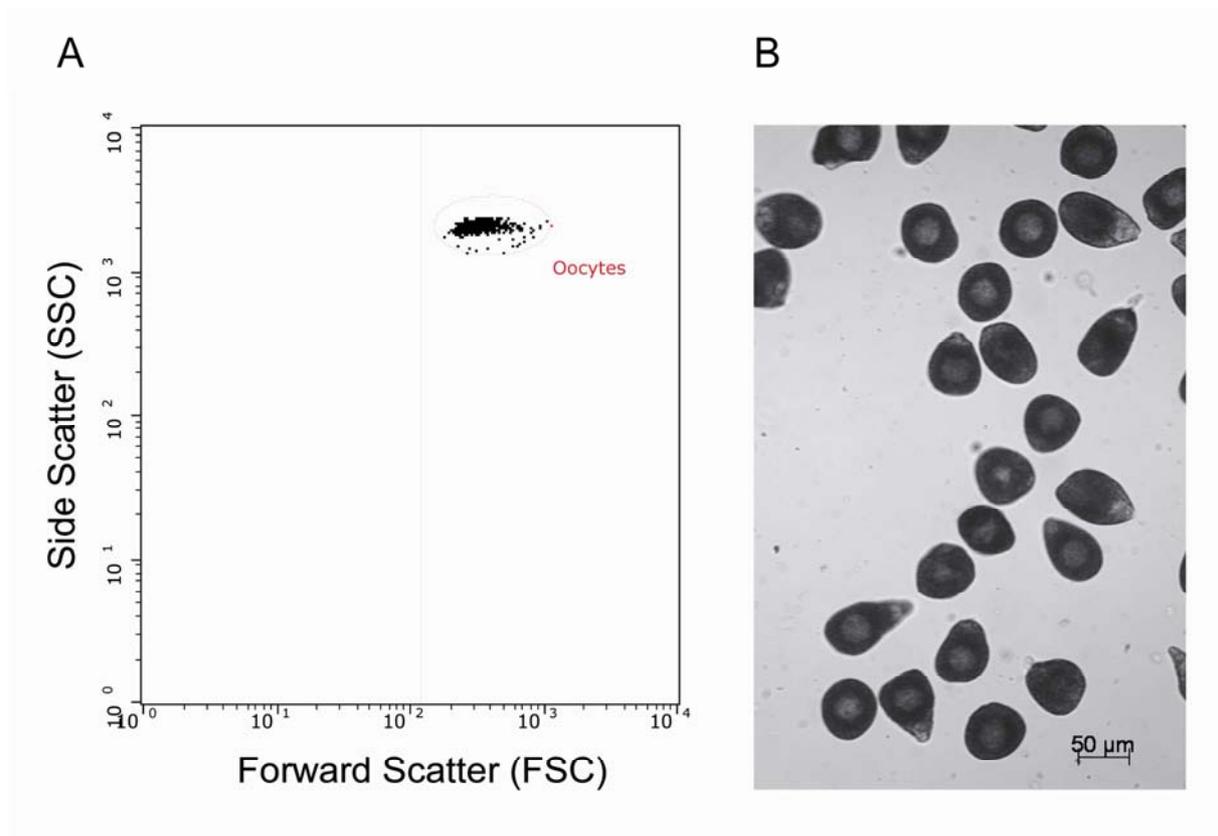
Figures

Figure 1. (A) Forward scatter (relative cell size) vs side scatter (relative cell complexity) cytogram of *Crassostrea gigas* oocytes. **(B)** Phase-contrast photomicrograph of *Crassostrea gigas* oocytes.
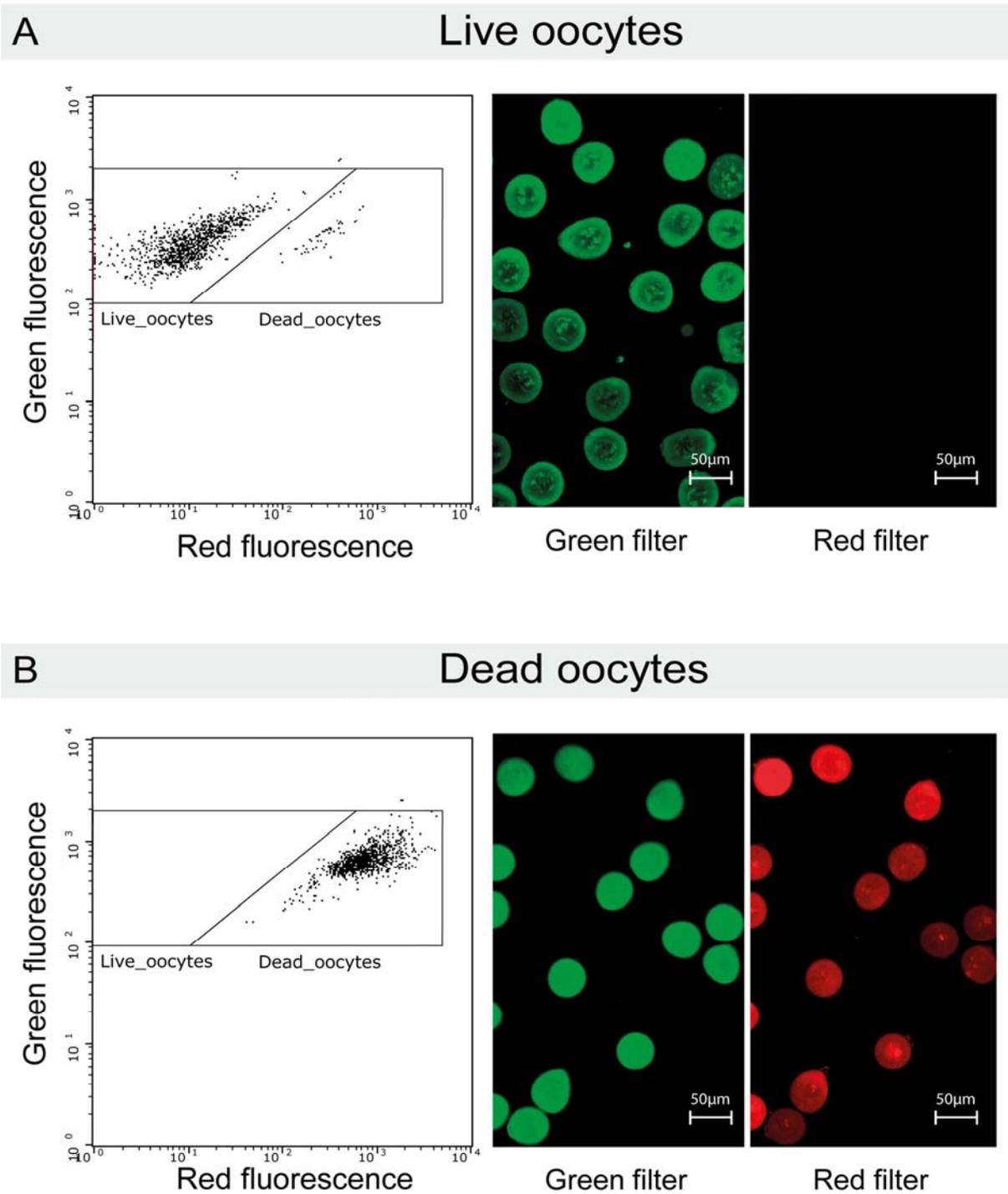
Figure 2. SYBR-Green and PI stained cytogram and epifluorescence microscopic observations of **(A)** live oocytes or **(B)** oocytes killed by 60 minutes at 60°C. Green fluorescence: excitation 494/20 nm and emission 536/40 nm, red fluorescence: excitation 575/25 nm and emission 628/40 nm.
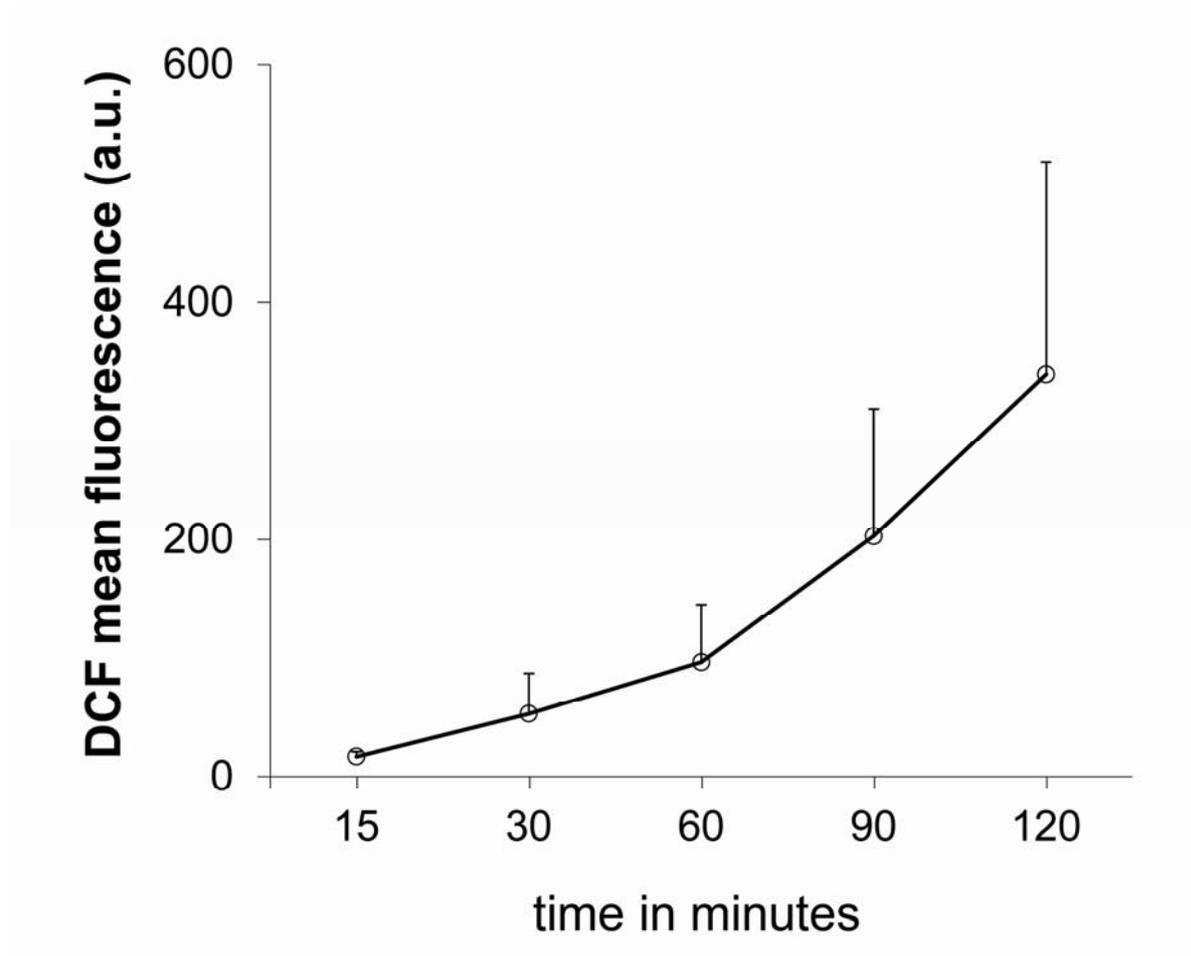
Figure 3. Kinetics of DCF fluorescence of oocytes incubated from 15 to 120 minutes with
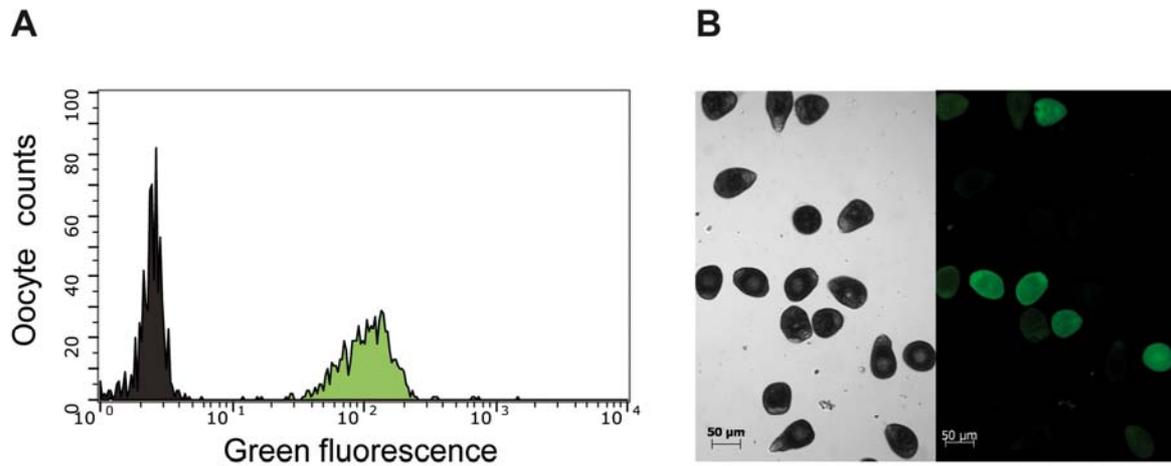
DCFH-DA. Results are expressed as mean ± CI (α=0.05), n=3.

Figure 4. (A) Distribution histogram of unstained oocytes (dark grey peak) and oocytes incubated 1 hour with DCFH-DA (green peak). **(B)** Phase-contrast and epifluorescence photomicrographs of oocytes incubated 1 hour with DCFH-DA (Bright field and green fluorescence: excitation 494/20 nm and emission 536/40 nm)
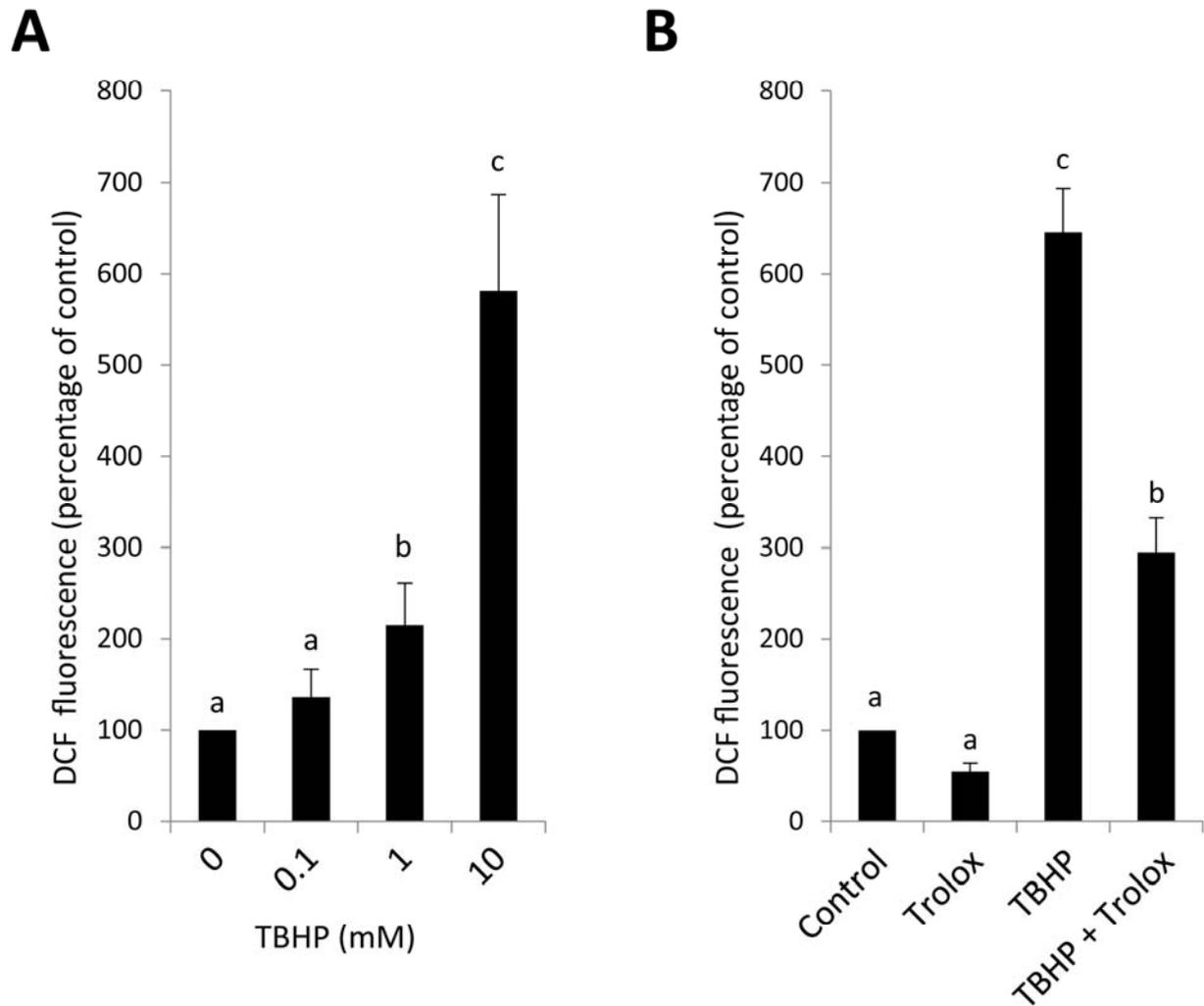
Figure 5. (A). Effect of *tert*-Butyl hydroperoxide (TBHP) from 0.1 mM to 10 mM on oocyte

ROS production using DCFH-DA assay. (B). Effect of the antioxidant Trolox at 0.1 mM,

TBHP at 10 mM, and TBHP at 10 mM + Trolox at 0.1mM on oocyte ROS production.

Results were expressed as percentage of control (mean ± CI (α=0.05), n=3) but an ANOVA

was performed on DCF fluorescence values of test conditions and control. Significant

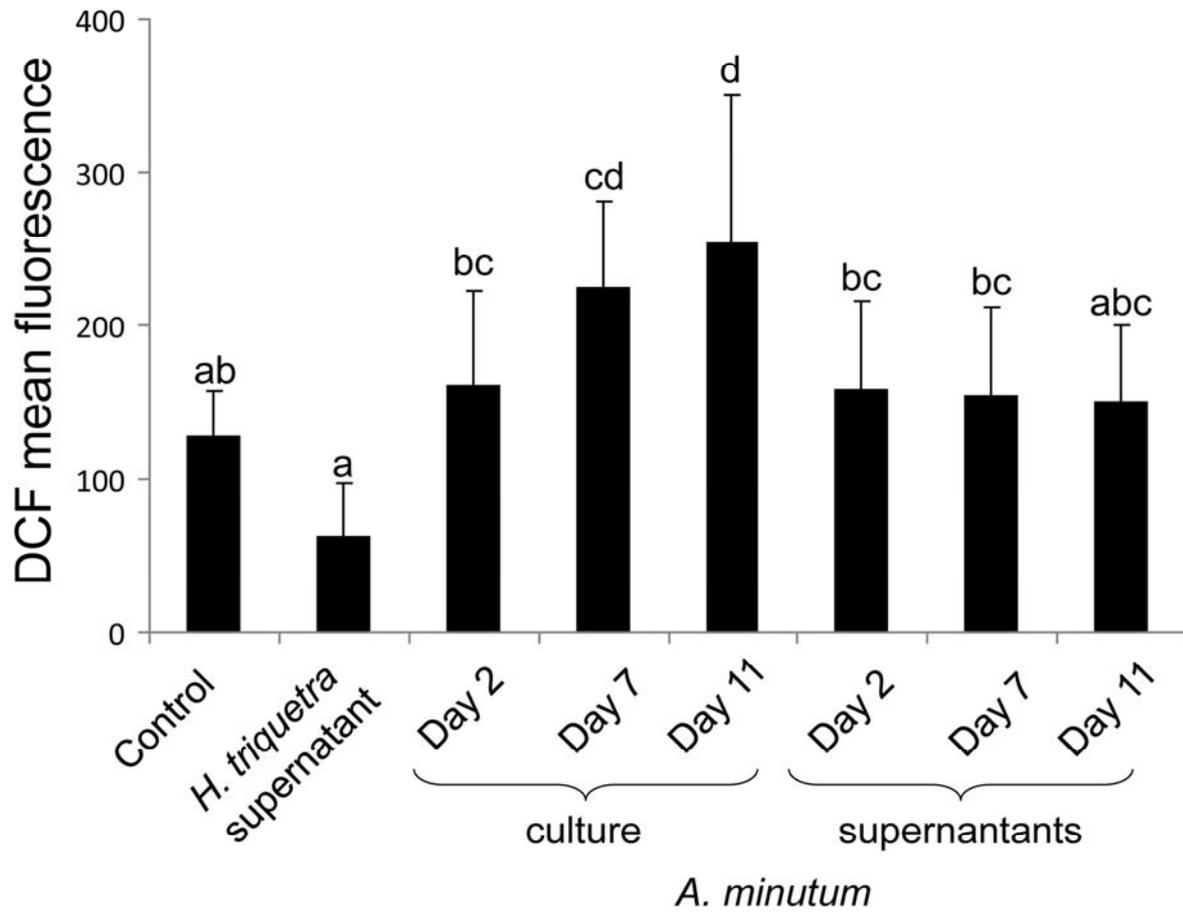differences between treatments are denoted by lowercase letters.

Figure 6. DCF green fluorescence (a.u.) of oocytes after 60 minutes exposure to 2-, 7- and 11-day-old *A. minutum* cultures or supernatants compared to FSSW or supernatant of *H. triquetra*. Results are expressed as mean ± CI (α=0.05), n=3. Significant differences between treatments are denoted by lowercase letters.