In vitro Diving Simulation: A New Approach to Assess Biological Impact of Hydrocarbons at Depth

Matthieu Dussauze[a]*, Karine Pichavant-Rafini[a], Stéphane Le Floch[b], Alexandra Marziou[a]
Philippe Lemaire[c], Ludovic Fronteau[d], Marc Belhomme[a], Michaël Theron[a]
[a] Laboratoire ORPHY EA4324, Université de Bretagne Occidentale, Brest, France
[b] *Cedre*, Centre de Documentation, de Recherche et d'Expérimentations sur les Pollutions Accidentelles des Eaux, Brest, France
[c] Total Fluides S.A, Paris la Défense, France
[d] Département Génie Mécanique, Université de Bretagne Occidentale, Brest, France
* matthieudussauze@yahoo.fr

Abstract

There has been little research on the biological impact of oil in deep-sea environment. Hence, this study aimed to evaluate the potential interest of a new *in vitro* approach for the assessment of consequences of hydrocarbon exposure under high hydrostatic pressure. Two simple and new tools were used in this study: the high pressure microscopy system and the high pressure oxygen consumption system. They are based on cellular bioassay protocols and allow to study the concomitant effect of high hydrostatic pressure and oil contamination. This work was conducted on hepatocytes of an OSPAR model fish, turbot (*Scophthalmus maximus*). Cells were beforehand exposed to the water soluble fraction (WSF) of a crude oil and then they were exposed to hydrostatic pressures using one of the two experimental systems. Cell mortality (using propidium iodure and annexin V) and cell oxygen consumption were measured and four experimental conditions were tested (control condition at atmospheric pressure, control condition under pressure, WSF condition at atmospheric pressure and WSF condition under pressure).. Thanks to these new tools allowing *in vitro* diving simulation, we can study the effects of a contaminant on the capacity of marine species to face hydrostatic pressure. It opens new perspectives on the analysis of the biological impact of chemical dispersion of oil at depth.

1 Introduction

The oil demand associated with the depletion of easily exploitable oil deposits led the oil industry to intensify the search for new oil areas and to exploit deposits previously considered as economically unprofitable. These new oil research areas are mostly located in the Arctic and in "offshore" areas in deep waters. Thus, due to an increase of human activity in these areas, the probability of oil spills will undoubtedly increase. These chemical releases, whether chronic or accidental, could upset the balance of these fragile ecosystems which are still not fully understood from an ecotoxicological point of view. In case of an oil spill at sea, several factors must be considered to limit the impact of pollution in the context of costs and benefits for the environment (NEBA) (Koyama and Kakuno, 2004). In this context, the question of the effects of hydrocarbons in the new oil research areas should be carefully considered.

A rapid survey of the literature on the effects of hydrocarbons in deep-sea environments clearly shows that, except two papers (Vevers et al., 2009; Olsen et al., 2015), no experimental studies have been performed on this subject. Consequently, the need to obtain information from ecotoxicological trials in the deep-sea context and particularly under high hydrostatic pressure is obvious (Mestre et al., 2013; Broje et al., 2014). Indeed, the fact that hydrostatic pressure, as hydrocarbons, is known to have important effects on biological systems (Somero, 1991; Sébert et al., 1998; Theron et al., 2001) makes likely the existence of synergistic effect between these two parameters. Thus, in order to fully understand the impact

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of a pollutant at depth, it is fundamental to perform ecotoxicological tests under high hydrostatic pressure. Conventional experimental approaches of the effects of high pressure require the use of heavy and expensive pressure chambers making it difficult to produce data in this area. This manuscript describes two simple new methods based on cellular bioassays protocols allowing the study of the concomitant effect of high hydrostatic pressure and oil contamination. The first one is designed to use fluorescence microscopy techniques and the second one makes it possible to measure cellular oxygen consumption under pressure.

2 Materials and Methods

2.1 Chemicals and Materials

The petroleum oil used in the study was a crude oil provided from a deep-sea well. Propidium iodide (PI) and annex V was bought from Sigma-Aldrich (St. Louis, MO, USA). Sapphire windows NT43-631 (diameter, 12.75 ± 0.15 mm; thickness, 1.00 ± 0.10 mm) were ordered from Edmund Optics Ltd. (Barrington, NJ, USA). HPLC pump 422 was bought from Kontron AG (Eching, Germany). A pressure indicator model and pressure gauge CPH6400 was bought from WIKA Alexander Wiegand SE & Co. KG (Klingenberg am Main, Germany). An OXY-4 mini, 4-channel minisensor oxygen meter was bought from PreSens Precision Sensing GmbH (Regensburg, Germany).

2.2 Physical Descriptions of Systems Configuration

2.2.1 High Pressure Fluorescence Microscopy System

This experimental system (Figure 1) is adapted from the scuba diving simulation system described by Wang et al. (2013); it is composed of three functional parts: the hyperbaric chamber, the perfusion and pressure control system, and the temperature control.

The flow through hyperbaric chamber is identical to the one described by Wang et al. (2013) and fits on a microscope stage (Figure 2). Briefly, it is made from poly-methyl-methacrylate (PMMA) and is penetrated at its center by a small cylinder shaped hole and closed by two optical sapphire windows sealed with two rubber O-rings. Cells are introduced into the chamber on the bottom window where a microscopic observation can be performed with an inverted microscope. A HPLC pump is connected with HPLC polyetheretherketone (PEEK) tubing to the hyperbaric chamber. The cell culture media is kept at atmospheric pressure and pumped to the pressure chamber by the HPLC pump. The hydrostatic pressure is increased in the system using a HPLC variable counter-pressure generator. The hydrostatic pressure generated in the perfusion system is recorded continuously with a pressure gauge. The temperature control of the system is ensured with a water circulation in a co-axial tubing system and in the hydrostatic chamber.

Cell observations were performed in normobaric condition and under high hydrostatic pressure through the hyperbaric chamber on the stage of a fluorescence inverted microscope (Zeiss Axio observer D-1; Carl Zeiss, Jena, Germany) with an EC epiplan neofluar LD 50 objective (NA, 0.55; WD, 9.1 mm).

2.2.2 High Pressure Oxygen Consumption System

This second experimental system (Figure 3) consists in four hyperbaric chambers (Figure 4) made from PMMA and connected to an HPLC pump with PEEK tubing. These chambers have in their center a 1.5 mL hole: the chamber well where the cell suspension is introduced. A homogenization of the chamber well is performed by a magnetic stir bar. The sealing of each chamber is realized with a rubber O-rings and a screwed 4 mm thick PMMA cover. A PEEK tubing is connected to the chamber well. The temperature control of the system is ensured with a water circulation in the body of the chamber.

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The increase of hydrostatic pressure in the chamber is made possible connecting the chamber well PEEK tube to an HPLC pump and forcing a cell culture media through a HPLC variable counter-pressure generator. The hydrostatic pressure generated in the system is recorded continuously with a pressure gauge. During each experiment two chambers were kept at atmospheric pressure and two chambers were exposed to high hydrostatic pressure. The oxygen partial pressures in the chamber well were recorded during the experimentation using an oxymeter Oxy-4 (Presens, Regensburg, Germany). The oxygen sensor spots are placed on the top of the chamber well on the PMMA cover. The optical fiber connected to the oxymeter is placed on the external wall of the well cover, facing the optode sensor spot.

2.3 Testing Methods

2.3.1 Physical Performances

The maximal sustainable pressure in the two systems was determined up to 10.1 MPa and in speed ranges of compression and decompression from 1 to 10 MPa/min. The effect of the pressure increase on the oxygen sensor was evaluated at 3 different oxygen partial pressures (155, 115 and 0 Torr).

2.3.2 Biological Analyses

2.3.2.1 Animals

Juvenile turbot (*Scophthalmus maximus*) (from 150 to 200g) used in this experiment were hatched in the aquaculture facility of *France turbot* (Noirmoutier, France). In the laboratory, fish were acclimatized in a 300 L seawater tank for two weeks. Photoperiod was according to the season (12:12). Oxygen saturation (> 90 %), pH (8.10 ± 0.16) and temperature (maintained at 17°C) were measured daily. Turbot were fed daily with dried commercial pellets provided by the hatchery.

2.3.2.2 Cell Isolation and Contamination

Turbots were killed by a blow to the head and the liver was rapidly removed. Hepatocytes were then isolated by the collagenase digestion method as previously described by Ollivier et al. (2006) and finally collected in a 320 $mOsm.kg^{-1}$ isotonic solution (in $mmol.l^{-1}$: 152 NaCl, 3.4 KCl, 0.8 $MgSO_4$, 0.33 Na_2HPO_4, 0.44 KH_2PO_4, 5 $NaHCO_3$, 10 Hepes, 5 Glucose, 1.5 $CaCl_2$, pH 7.63). Cells were allowed to rest at 17°C under slight agitation during at least 1 hour before the experiments.

Once isolated, cells were exposed one hour to the soluble fraction of a crude oil. Firstly, a water accommodated fraction of oil was prepared using a standardized CROSSERF protocol (Chemical Response to Oil SpillS: Ecological Research Forum, Singer et al., 2000). However, in this experiment, instead of seawater, the WAF was prepared with the isoosmotic solution ensuring the hepatocyte viability. Four experimental conditions was evaluated: a control normobaric condition (C), a condition at normobaric pressure with WAF (WAF), a condition under pressure (P) and a condition exposed to pressure and WAF (P-WAF). Hydrocarbon concentrations were assessed with GC-MS using a Stir Bar Sorptive Extraction method (Lacroix et al. 2014).

2.3.2.3 Cell Exposure to Pressure

In the case of the microscopy chamber, a cell suspension was loaded in the hyperbaric chamber on a sapphire window coated with polylysin and sealed. Cells were then perfused 40 min at 1 ml/min with isosmotic solution containing PI (3 mmol/L) and annexin V. Two cell states have been verified with the addition of these two chemical substances. Apoptosis, process of programmed cell death, was checked using PI and necrosis, cell injury which results in the premature death of cells, was checked using annexin V. Under fluorescence

microscopy, survival of hepatic cells was monitored during diving simulation. PI colored the cell nuclei of cells dead by necrosis in red and Annexin V colored in green cells which began to enter in apoptosis. Cells which were colored both green and red were cells who dead by an apoptosis. Comparing picture at the beginning and at the two hours of experimentation, mortality during simulated dive with or without WAF was assessed.

In the case of the high pressure consumption system, between 3 and 4 million cells (suspended in isosmotic solution) were placed into each chamber well. The chambers were sealed, connected to the pressurizing system and maintained 40 min at atmospheric pressure. In both systems the pressure was increased at a speed of 1 MPa/min up to 7 MPa in the microscopy chamber and to 10 MPa in the respiration system; the pressure was then maintained 15 minutes and the chambers decompressed at 10 MPa/min. The temperature was maintained constant at 17°C.

Cell analyses were performed during the dive with fluorescence microscopy or through oxygen consumption. Control samples loaded in the same systems but kept at atmospheric pressure were analyzed at the same time.

3 Results and Discussion

The problem of ecotoxicological impact of oil on deep habitat is recent. Indeed, only one study (Vevers et al., 2009) was performed before the *Deepwater Horizon* accident. The need of ecotoxicological data is crucial to perform a relevant NEBA during an oil spill. To try to respond to this problem, two main approaches are possible. Firstly, the ecological approach is very informative but faces a major problem: the technical difficulty to access to these deep marine areas. The second option to assess the effects of oil pollution in depth is an experimental approach that offers several possibilities. Firstly, to experimentally contaminate animals from deep-sea ecosystems at atmospheric pressure. This type of study has already been carried out on a benthic amphipod (Olsen et al., 2015) and promises significant opportunities with several other species. The main limitations of this approach are the necessity to bring deep-sea species at atmospheric pressure in good physiological condition and the cost of catching, bringing and maintaining deep ecosystem animals in a laboratory.

The second possibility is to experimentally expose to pressure contaminated organisms or to perform contamination under pressure. Indeed, since the aim of ecotoxicological studies is to analyze the effect of a substance in different environmental contexts, it seems judicious to perform this kind of pressure studies. But the cost of the hyperbaric chambers needed to expose animals to high hydrostatic pressure is clearly the main limitation of this approach.

The goal of this paper is to propose easy and low cost approaches, complementary to the one described previously, that make it possible to study concomitantly the effects of oil contamination and hydrostatic pressure. They are based on cellular ecotoxicological assays and were performed on an OSPAR model species representative of benthic fish in Europe marine cost, the turbot *Scophthalmus maximus*.

3.1 Microscopy Coupled with Micro-Hyperbaric Chamber

Composed of a small flow-through hyperbaric chamber fitting on classical microscopy equipment, this experimental system was constructed to perform a real time monitoring of cells under pressure.

During the physical performance tests compression could easily be performed at speeds ranging from 0.1 to 1 MPa/min. whatever the compression speed, the sapphire window of the hyperbaric chamber appeared to break at 7 MPa preventing the simulation of depth greater than 700 m. In order to explore a wider range of pressures, the use of thicker sapphire

windows could be envisaged. There is nevertheless a risk of degradation of the microscope images since even high power objectives are designed to be used with 0.18 mm cover slides.

As shown in Figure 5, the microscopy observation of the cells during the simulated dive could be performed using fluorescent microscopy. Indeed, annexin V and propidium iodide (respectively staining apoptotic in green and dead cells in red) could be used under a pressure of 4 MPa to determine mortalities (see Table I) on cells exposed to a WAF at 95.8µg.L^{-1} of total hydrocarbons as previously described.

These results show that, at 4 MPa, the pressure does not lead to a higher mortality for cells exposed or not exposed to hydrocarbons. These preliminary results must be

3.2 Oxygen Consumption under Pressure

This second experimental system appeared to support pressures up to 10.1 MPa, equivalent to a depth of 1000 m. It also worth noticing that prototypes (with 3 cm thick walls) were able to cope with 40MPa of hydrostatic pressure; higher pressure could not be tested due to the limitation of the HPLC pump. As previously, compression speed that can be used range at least from 0.1 to 1 MPa/min.

In these chambers, optodes were used to measure the oxygen partial pressure. Since, as far as we know no studies were performed under pressure with this kind of material, the effect of the hydrostatic pressure on the optode response has been performed (Figure 6). The results show a moderate drift of the response of the oxygen sensor close to 2.5 % at a partial pressure of oxygen of 155 Torr (100% of air saturation).

Cellular oxygen consumption measurements were performed at atmospheric pressure and at 10.1 MPa of hydrostatic pressure as shown in Figure 7. This experimental system makes possible the assessment of the effects of Hydrostatic pressure up to 10.1 MPa on the aerobic metabolism of contaminated and uncontaminated cells. With its 4 chambers, it allows to perform measurements in duplicate in normobaric and hyperbaric conditions concomitantly.

This experimental system is used in this work on isolated cells but could also be used with small animals such zooplankton. It could be used for example with *Acartia tonsa*, one other OSPAR model species that measures about 1.5 mm and can be easily obtained.

4 Conclusion and Perspectives

With these new tools, it is possible to the performance of ecotoxicological cell tests but also to work on key species of the marine trophic chain. They open new experimental perspectives and allow to perform high pressure experiments easily on any lab bench. With these tools, it could be interesting to analyze the effects of oil contamination on Diel Vertical Migration (DVM) performed for example by *Acartia tonsa*. The DVM species are crucially important in marine ecosystems and are considered as the largest displacement of biomass on earth (Hays, 2003). Furthermore, with naturally barotolerant species of such zooplankton, the question of the acclimation of a deep species at atmospheric pressure or a surface species at high hydrostatic pressure can be solved.

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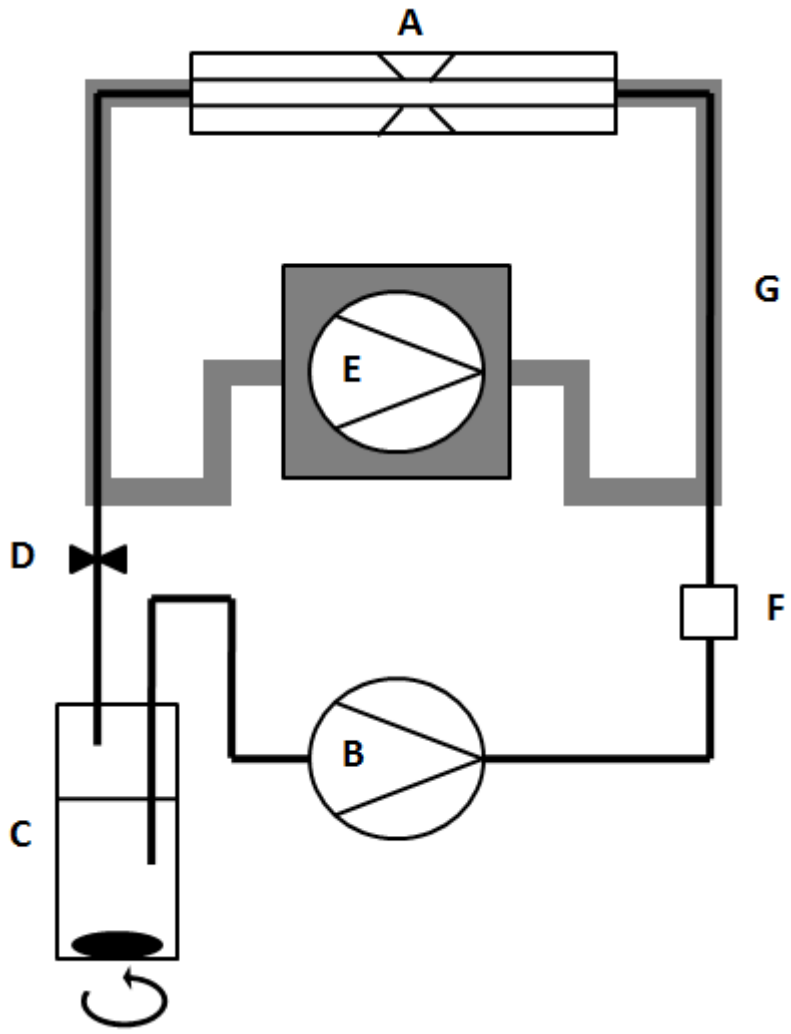

Figure 1 Details of systemic configuration, including hyperbaric chamber (A), HPLC pump (B), liquid supplier (C), filling valve (D), thermostatic water circulation system (E), pressure gauge (F) and coaxial tubing (G). Adapted from Wang et al (2013).

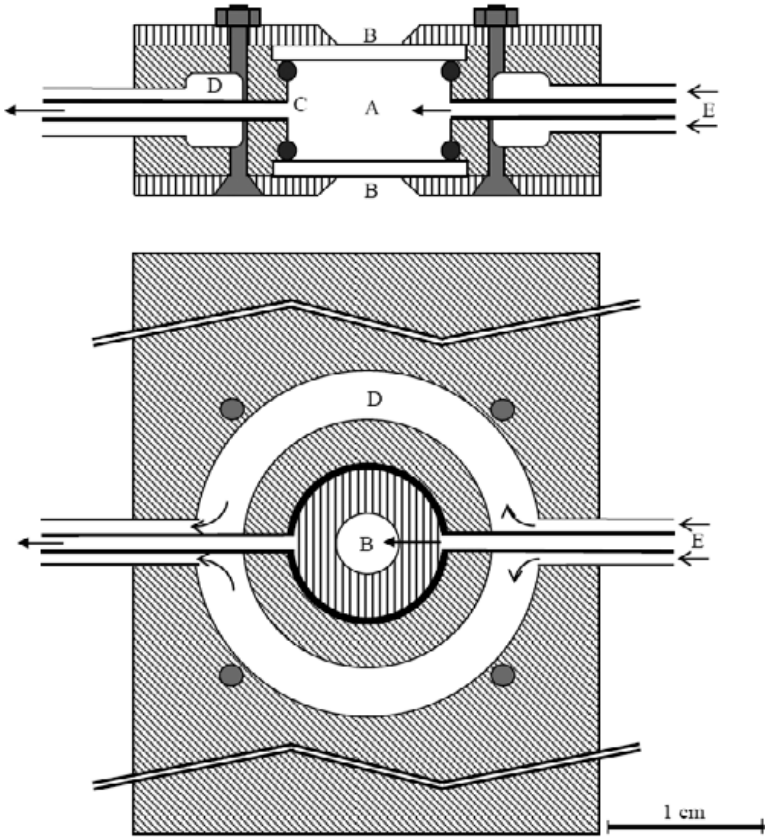

Figure 2 Schematic view of hyperbaric chamber from Wang et al (2013), including chamber well (A), sapphire windows (B), perfusion of the chamber (C), thermostatic water circulation system (D) and coaxial tubing (E).

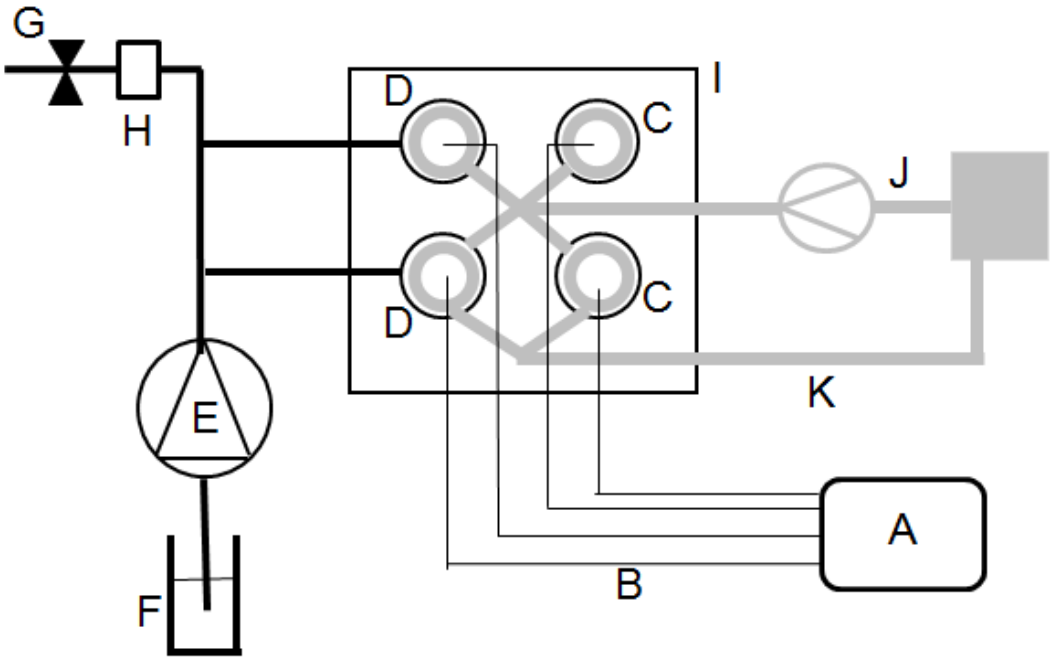

Figure 3 Details of systemic configuration, including oxymeter Oxy-4 (A), Optical fiber (B), hyperbaric chamber at atmospheric pressure (C), hyperbaric chamber under hydrostatic pressure (D), HPLC pump (E), liquid supplier for HPLC pump (F), filling valve (G), pressure gauge (H), magnetic stirrer (I), thermostatic water circulation system (J) and coaxial tubing (K).

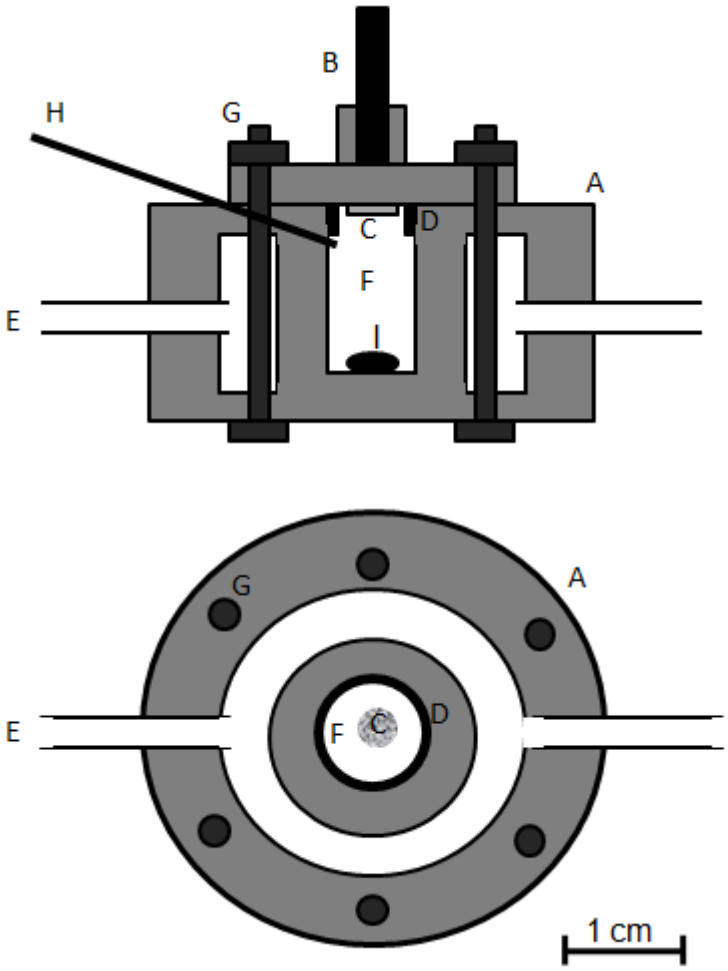

Figure 4 View of one hyperbaric chamber, including respiration chamber (A), optical fiber of the oxymeter (B),oxygen captor (C), thermostatic water circulation system (E), Chamber well (F), O-ring (D) and sealing system (G) allowing an increase of pressure, HPLC polyetheretherketone tubing link to HPLC pump (H) and stir bar allowing agitation in the chamber well (I).

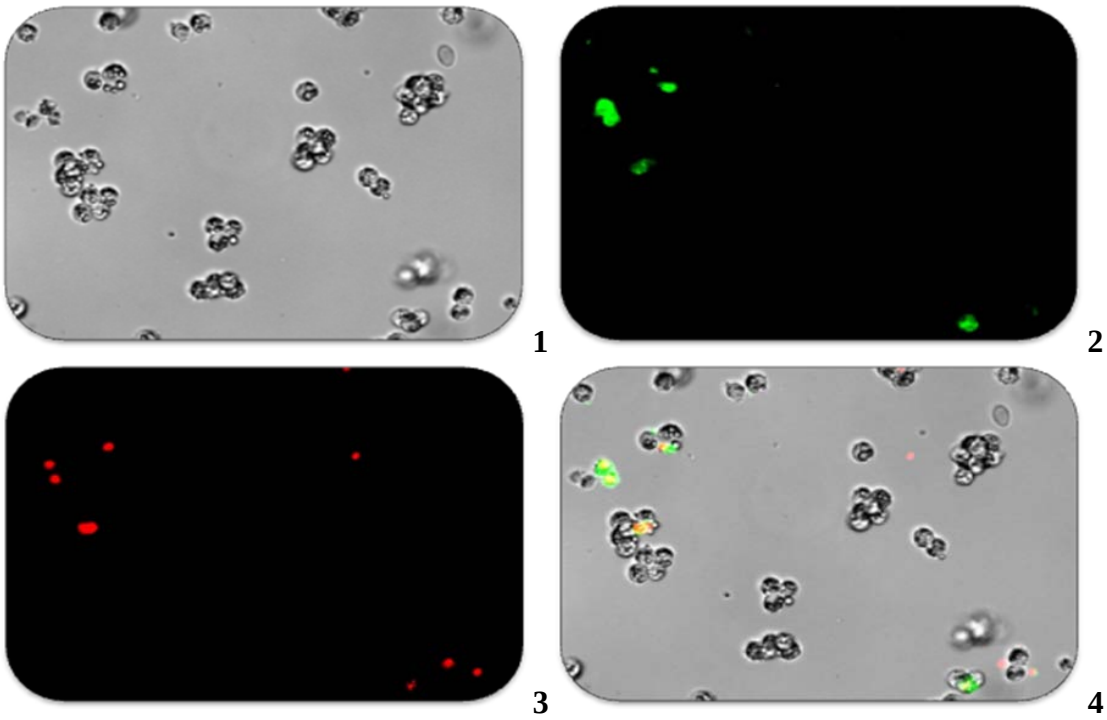

Figure 5 Real-time observation of cell survival under microscope (1) and fluorescence microscope (2 and 3) and with marking superposition under microscope (4) during *in vitro* diving simulation of hepatic cells.

Table 1 Results of cellular mortality during the two hours of experiment at different pressure and for cells exposed or not exposed to hydrocarbons. C : control normobaric condition; WAF : condition at normobaric pressure with WAF; P : condition under pressure; P-WAF: condition exposed to pressure and WAF.

Experimental condition	Hydrostatic pressure (MPa)	Number of tested cells	Number of dead cells at the beginning	Mortality at the beginning (%)	Number of dead cells at the end	Mortality at the end (%)	Increase of mortality during experimentation (%)
C	0.1	416	31	7	33	8	0,5
P	1.1	449	43	10	44	10	0,2
P	2.1	35	5	14	6	17	2,9
P	3.1	107	11	10	11	10	0,0
P	4.1	173	30	17	40	23	5,8
WAF	0.1	433	46	11	60	14	3,2
P-WAF	1.1	139	15	11	18	13	2,2
P-WAF	3.1	111	13	12	14	13	0,9
P-WAF	4.1	88	6	7	7	8	1,1

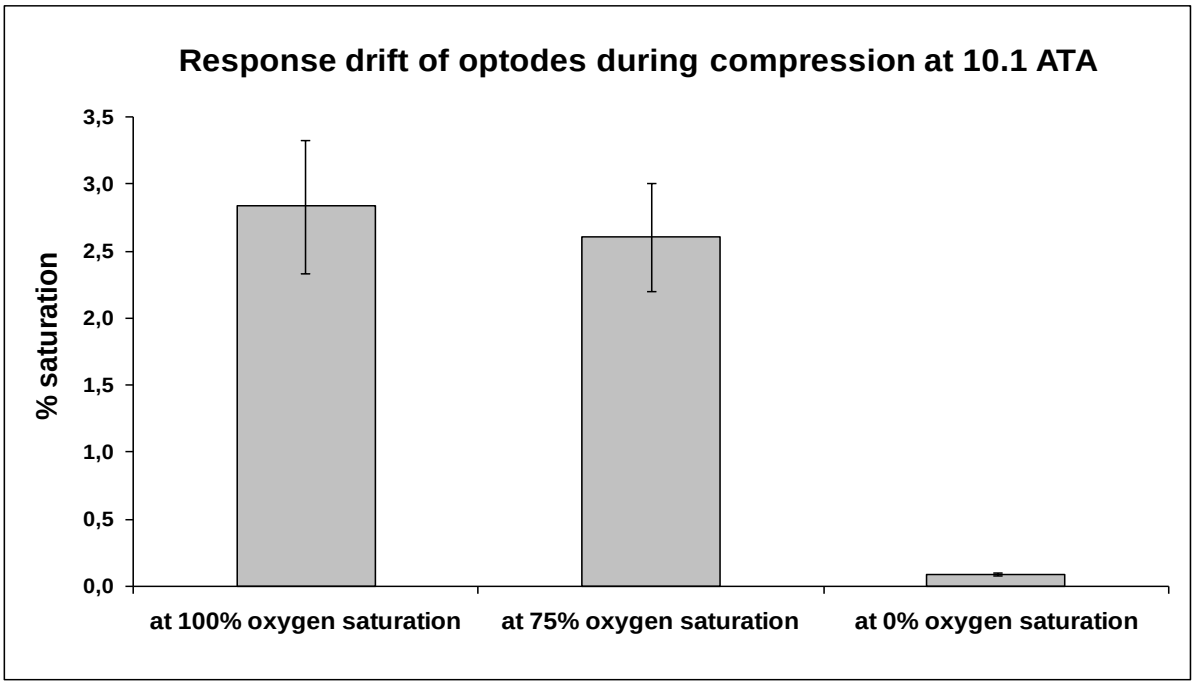

Figure 6 Response drift of optodes at 10.1 MPa for different level of oxygen saturation in the well of the respiration chamber.

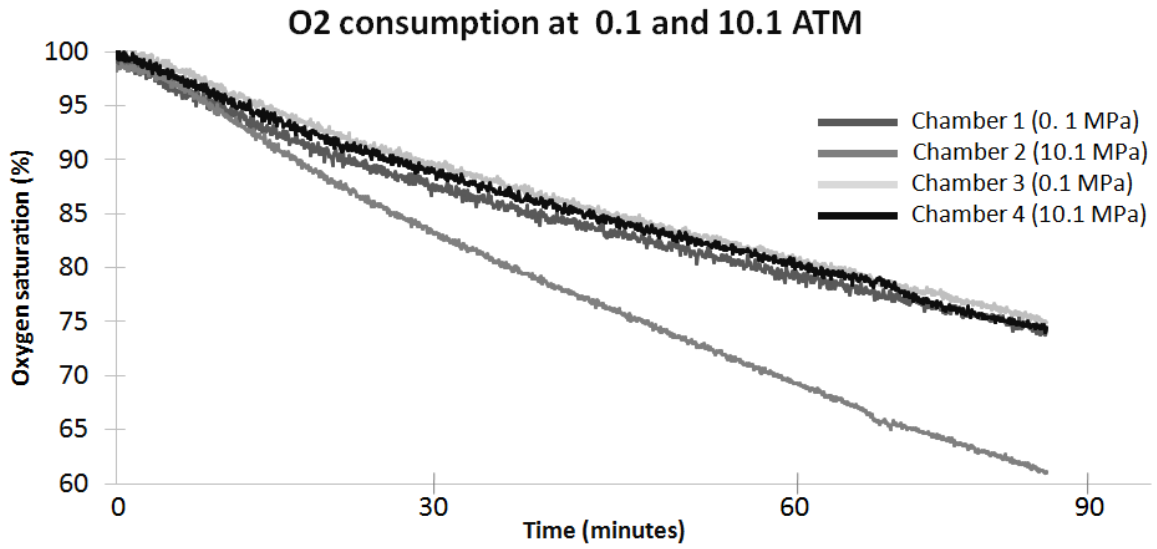

Figure 7 Oxygen consumption (% of O_2 saturation) during 90 minutes of hepatic cells placed in respiration chamber with two pressures, 0.1 (respiration chambers 1 and 3) and 10.1 MPa (respiration chambers 2 and 4) and measured using an oxymeter.