Influence of nonylphenol from multilayer plastic films on artificial insemination of shows

Cristina Nerín[1] & Qi-Zhi Su[1] & Paula Vera[1] & Noelia Mendoza[2] & Raquel Ausejo[2]

1 Department of Analytical Chemistry, GUIA Group, I3A, EINA, University of Zaragoza, María de Luna 3, 50018 Zaragoza, Spain
2 Department of Research and Development,Magapor SL, 50600 Ejea de los Caballeros, Zaragoza, Spain
* Cristina Nerín cnerin@unizar.es

Abstract
Artificial insemination is common practice in mass livestock farming. Recently, it was shown that chemicals leaching from multilayer plastic bags affect the fertility of boars, although common quality tests did not show any impact on the sperm. It is not clear whether this incidence was a single case or whether it could be a systematic problem. Therefore, we studied six multilayer plastic bags. A total of 49 compounds were found, but most of them were at very low intensity. Nonylphenols in the range of 19- 99 μg/g plastic were found. Migration tests using water and 10% ethanol as simulants, to mimic the behavior of semen with the extender, were performed. The most interesting migrants in terms of potential reprotoxicity were identified as nonylphenols. The identification in depth demonstrated the presence of 10 isomers of nonylphenol with a total concentration range between 16 to 58 μg/Kg simulant, among other migrants at lower concentration. The influence of these nonylphenols and their maximum tolerable concentration in direct contact with semen from boars was studied. Motility, viability, mitochondrial activity and acrosomes reacted were significantly affected at 10 mg/Kg of nonylphenols in contact with the sperm, but in vitro penetration rate was significantly decreased with only 2 mg/Kg. Insight into the mode of action is also provided.

Keywords Non-target analysis . Reprotoxicity . Migration fromplastics . Artificial insemination . Semen plastic bags

Introduction

Petroleum plastics have been widely used in our daily life for almost 100 years, because of their substantial benefits regarding their low weight, durability and lower cost compared to other materials [1]. Together with the long chains of atoms that constitute the polymeric matrix, various additives are needed to improve the performance, functionality and aging properties of the polymer [2]. However, in the last 20 years the reputation of plastics has suffered thanks to a growing concern about the potential threat they pose to human health. These concerns focus on the additives because of the evidence that these chemicals leach out of plastics into our food, water [2], and bodies [3]. Besides the additives, other non-intentionally added substances (NIAS), such as impurities from starting materials or by-products from the manufacturing process, are also raising safety concerns [4, 5]. Leachables from plastics, via a process called chemical migration, may also cause other problems, either altering the organoleptic properties of the packaged food [6, 7] or affecting the integrity of the packaged product, as happens in artificial insemination. In mass livestock farming, artificial insemination is widely used. In the porcine sector, semen collected from boars is diluted with a suitable solution, called extender, and packaged in a high barrier plastic bag until final use. In 2014, bisphenol A

diglycidyl ether (BADGE) together with a cyclic ester (lactone), leached out from plastic bags, were reported to cause reproductive failure in artificial inseminations [8]. A more recent study [9] demonstrated, as well, that a surfactant commonly used in a water-based adhesive formula also affected the fertility of boars when being in direct contact with the semen, after the migration from the package. The study suggests that chemical migration from plastic containers can also lead to unsuccessful reproduction in artificial insemination, and thus cause economic loss to the companies involved. However, little is known on this topic, and further investigation is warranted. Among the common additives used in the plastics sector, surfactants and antioxidants can be mentioned. A breakdown product and starting material of themost widely used nonionic surfactants, alkylphenol ethoxylates (APEOs) and/or a hydrolysis product of a commonly used antioxidant , tris(nonylphenyl)phosphite (TNPP), is a nonylphenol that has been detected in the environment as well as in many plastics [10–12]. Having a similar structure with an endogenous hormone, nonylphenol has the potential to mimic or even block the effects of the endogenous hormones [13, 14]. Moreover, nonylphenol has been reported to be neurotoxic [13], toxic to the coral reef fish species Pseudochromis fridmani [10], spermicidal [15], and problematic to plants' defense systems, leading to growth disorders [16]. For these reasons, the European Chemicals Agency (ECHA) has classified nonylphenols as substances of very high concern (SVHC) [17]. However, as far as we know, the effect of nonylphenol coming from plastic bags on artificial insemination has not yet been reported. The objective of the present study is to evaluate potential harms of chemicals coming from plastics used in artificial insemination on pig reproduction. Non-targeted screening was first done to identify the compounds present in the films by ultrasonic-assisted extraction and subsequent GC-MS analysis. The concentrations of confirmed migrants—up to ten different nonylphenol isomers in two simulants (10% ethanol and water)—were then quantified by HS-SPME-GC-MS. The influence of these nonylphenols and their maximum tolerable concentrations in direct contact with semen were studied in depth to elucidate the reprotoxic effect and the mode of action of these chemicals on spermatozoa.

Materials and methods

Reagents and plastic films

Dichloromethane, methanol, ethanol, diethyl phthalate, 1-octadecene, nonylphenol, 1-hexadecanol, methyl palmitate, 1-octadecanol, bis(2-ethylhexyl) phthalate, and squalene of analytical grade were purchased from Sigma-Aldrich (Madrid, Spain). Six different multilayer films (A, B, C, D, E, and F) used as boar semen containers in artificial insemination were obtained from Magapor S.L. They were all composed of 12 μmPET and 60 μmPE (inner layer) glued with an adhesive to build the multilayer structure. Ultrasonic assisted extraction of plastic films First, 0.40 g finely cut plastic films was weighed into an 18 mL glass vial. The samples were then extracted with 5 mL dichloromethane in an ultrasonic bath for 2 h (Brasonic 3510-MTH; Connecticut, USA). After removing all plastic films, the extracts were gently evaporated to dryness by a nitrogen concentrator at 40 °C (Techne DB-3; Staffordshire, UK). Subsequently, 1 mL of methanol was added to re-dissolve the extracts in the ultrasonic bath for 5 min. The final extracts were then analyzed by GC-MS. Two replicates were prepared for each sample and also for blanks.

Migration from plastic films

Each film sample was first transformed into bag form (10 cm × 10 cm) using a thermal sealer (300 SCT-2; Audion Elektro, Holland), and filled in with 10 mL of simulants (10%

ethanol and water) accordingly. Thereafter, the simulant-containing bags were incubated at 40 °C for 3 days in an oven (UF110; Memmert, Germany) for the migration test. Then, the simulant was transferred to a 18 mL vial for injection by head-space solid-phase micro-extraction coupled to a gas chromatography mass spectrometer (HS-SPME-GC-MS). Migration tests were conducted in triplicate and blank samples were simultaneously analyzed.

GC-MS and HS-SPME-GC-MS analysis

For non-targeted screening analysis, a gas chromatograph (7820 A) coupled to a mass spectrometer (5977B) was equipped with an HP-5 MS column (30 m × 0.25 mm id, 0.25 µm film thicknesses) from Agilent (California, USA). All samples were injected in liquid mode using 2 µL injection volume and an Agilent 7693 autosampler. The oven temperature was as follows: kept at 50 °C for 3 min, then increased by the rate of 10 °C/min to 300 °C, where it was held for 2 min. Scan mode with a mass range from 40 to 700 Da was applied to acquire data. An open-source software package, MS-DIAL (version 4.00) [18], was employed for automatic peak detection, alignment, and structure annotation of analytes using NIST 14 and Wiley libraries. The identification of compounds was then manually checked by retention index and reference standards, when available. For the quantification of nonylphenol in food simulants, G1530A gas chromatography coupled to a 5973 mass spectrometer from Agilent was applied. The separation was done in a polar column (DB-Wax, Agilent) to achieve better separation in shorter time with the following temperature ramp: held at 60 °C for 2 min, and then increased at a rate of 30 °C/min to 220 °C, and kept there for 10 min. Both 10% ethanol and water simulant samples were analyzed by HSNerín SPME-GC-MS. Each sample was first pre-heated at 90 °C for 2 min in the agitator, followed by 15 min extraction at the same temperature using the DVB/CAR/PDMS fiber. The fiber was then desorbed in the injection port of the GC for 2 min. The whole HS-SPME procedure was managed by a 6500 CTC autosampler. Both GC-MS systems used helium (99.999%) as the carrier gas at a constant flow rate of 1.0 mL/min. The inlet temperatures were all set at 250 °C and spitless modes were employed.

Sperm collection and treatments

Twenty ejaculates were collected from 5 different animals. Semen was manually collected by the double gloved hand technique. All ejaculateswere diluted 1:10 in commercial boar semen extender Vitasem® with antibiotics and then immediately sent to Magapor SL quality control laboratories. The ejaculates were used individually. Sperm concentration was determined using a Bürker counting chamber (Equimed-Medical Instruments, Cracow, Poland). Samples were collected in a specific preheated flask, with 100 ml of preheated extender (37 °C) to avoid thermal shock and sperm agglutination [19]. Only the rich fraction was collected because it contains 80-90% of sperm in the ejaculation. The semen was transported to a temperature-controlled laboratory at 17 ±2 °C. Different seminal replicates of the same ejaculate with a total volume of 90 ml were incubated with different concentrations of nonylphenol for 10 days in a storage chamber (Magapor SL, Zaragoza, Spain) at 16 °C. A control sample without nonylphenol was maintained under the same conditions.

Evaluation of sperm quality

To evaluate the quality of sperm, several parameters were analyzed: viability, mitochondrial potential, acrosome integrity and early apoptosis by flow cytometry, and

motility by a CASA system at 1, 3, and 7 days. In vitro penetration tests were performed to evaluate the effect on fertilization capacity in treated and control samples.

sHOST and sORT

Two tests, the short hypoosmotic swelling test (sHOST) and the short osmotic resistance test (sORT), were assessed for the evaluation of functional integrity of the sperm membrane and acrosome, which reflects the viability of spermatozoa [20]. The sHOST test was performed according to the original test [21] but using a lower osmotic pressure (75 vs 150 mOsm/kg) and a shorter period of time (15 vs 120 min), as previously described [22]. The percentage of viable spermatozoa (with curled tails) and the percentage of spermatozoa with intact acrosome were evaluated on a smear with eosin-nigrosin stain under light field microscope at ×1000 magnification.

Sperm motility analysis

Motility kinematic parameters underwent computer-assisted measurement using a CASA system (ISAS 1.0.4; Proiser SL, Valencia, Spain). Aliquots of 1 ml from each sample and control samples were warmed at 37 °C in a water bath. Then, a 3.5 µl drop was placed between prewarmed known volume chambers (Magapor SL, Zaragoza, Spain) and maintained at 37 °C during analysis by a heated slide holder. From a single field, 25 consecutive digitalized images were analyzed. The following parameters of quality movement were studied: the percentage of progressive motility (PM) and total sperm motility (TM); curvilinear velocity (VCL), i.e., the mean path velocity of the sperm head along its actual trajectory (µm/s); linear velocity (VSL), i.e., the mean path velocity of the sperm head along a straight line from its first position to its last position (µm/s); mean velocity (VAP), i.e., the mean velocity of the sperm head along its average trajectory (µm/s); linearity coefficient (LIN), i.e., (VSL/VCL) × 100 (%); straightness coefficient (STR), i.e., (VSL/ VAP) × 100 (%); wobble coefficient (WOB), i.e., (VAP/VCL) × 100 (%); mean amplitude of lateral head displacement (ALH), i.e., the mean value of the extreme side-to-side movement of the sperm head in each beat cycle (µm); and beat cross frequency (BCF), i.e., the frequency with which the actual sperm trajectory crosses the average path trajectory (Hz).

Flow cytometry analysis

A BD Accuri™ C6 (Becton Dickinson, Madrid, Spain) with BD software was used for all the measurements, and at least 40,000 events were counted in every experiment. The sperm population was gated for further analysis on the basis of its specific forward (FS) and side scatter (SS) properties; other non-sperm events were excluded. Viability/acrosome integrity The double staining technique with propidium iodide (to assess plasma membrane damage) and FITC-PNA (to assess acrosome status) was used. Five microliters of each staining ([FITC; 1 µg / mL; Sigma] and propidium iodide [PI; 0.75 mM; Sigma]) were added to 300 µL of sperm samples (4 × 107 cells per milliliter). The samples were incubated at 37 °C in the dark for 15 min. Argon laser and 525 and 675 nm filters were used to avoid overlaps.

Mitochondrial activity and early apoptosis

A double stain technique with YoPro (1 mM in dimethyl sulfoxide; Invitrogen), a DNA dye used as an apoptotic marker because it only permeates cells that are beginning to undergo apoptosis, and MitoT (10 µM in dimethyl sulfoxide; Invitrogen) was used to evaluate mitochondrial membrane potential (ΔΨm). Two microliters of each dye were added to 300 µl (4×107) spermatozoa, and the samples were incubated at RT in darkness for 15 min. YoPro fluorescent (FL) emissions were collected with a 525-nm filter and

MitoT with a 755-nm filter to avoid spectral overlap. Monitored parameters were FS log, SS log, FL1 (YoPro), and FL5 (MitoT), and calibration of overlapping fluorescence was carried out.

Sperm penetration assay

Sow ovaries were collected at the slaughterhouse and transported to the laboratory in 0.9% sodium chloride at 37 °C. The oocytes were obtained by the technique of cutting the follicles and those of intact pellucid zone were selected. They were randomly distributed in different wells of a Petri dish with 400 μL of fertilization medium (TCM 199, SIGMA). The sperm was diluted in the fertilization medium and added to the different wells with the oocytes, at a final concentration of 5 × 105 cells per milliliter in each well. The wells remained in a humidified atmosphere with 5% CO2 at 39 °C for 24 h. After incubation, the oocytes were fixed in 1.5% glutaraldehyde for 15 min and stained with Hoechst 33342 (1 μg/mL; Sigma) for an additional 15min at 37 °C. Groups of five to six oocytes were placed on a slide under a cover slip and examined with a fluorescence microscope at 400 ×. The number of oocytes penetrated the total mature oocytes was counted.

Results and discussion

Identification of volatile compounds present in the plastic films

Table 1 shows the list of 49 compounds detected in the extracts from plastic samples. Reference standards were injected to confirm the identity of the compounds when available. Nineteen of them were alkanes or alkenes, which are very common in both virgin and recycled polymers [23]. There were also trace amounts of diethyl phthalate, di-sec-butyl phthalate, and bis(2-ethylhexyl) phthalate with signal to noise ratio (S/N) 5, 7, and 8, respectively. The observed low levels of these compounds could be explained either by their use as catalysts or by cross contamination in the polymer production rather than plasticizers. Methyl palmitate, which is used as intermediate for detergents, emulsifiers, stabilizers, resins, lubricants, plasticizers, and defoamer in food contact coatings [24], was detected in all samples. Among all detected compounds, nonylphenol isomers have attracted our attention, since they have been reported as endocrine disrupters and have the potential to mimic or even block the effects of the endogenous hormones [16]. With that in mind, their presence in the plastic materials used for artificial insemination processes could be harmful and deserve further investigation in terms of potential adverse effect on pig reproduction. Peaks of nonylphenol isomers in the sample extracts were not visually found (Fig. 1) and could be easily overlooked by analysts when manually selecting significant peaks. However, using MS-DIAL, this situation was avoided, as the software utilizes extracted ions instead of total ions for peak detection. The use of a chromatographic deconvolution algorithm improved the quality of spectrum as well, especially for the analysis of complex chromatograms with severely overlapped peaks. With the help of MS-DIAL, 2 peaks were tentatively identified as nonylphenol isomers, while the rest of peaks remained unknown because the unidentified peaks had low intensities and/or unclear spectra. Their identification was finally confirmed by reference standards and concentrations were estimated between 10 ~ 50 μg/g extract corresponding to 19-99 μg/g in the plastic (Fig. 1). Quantification of nonylphenol in both 10% ethanol and water simulants by HS-SPME-GC-MS The screening process confirmed the presence of 10 nonylphenols at the ppm level in the samples. Thus, it was important to quantify their migration into simulants. As water and 10% ethanol used as simulants were not suitable for direct injection into GC-MS, HS-SPME-GC-MS was used for this

purpose. A polar column (DB-Wax) resulted in a better separation of compounds in shorter time (chromatograms shown in Electronic Supplementary Material (ESM) Fig. S1). As can be seen, in these conditions, the 10 nonylphenol isomers were detected before 14 min retention time in the DB-Wax column. Unlike liquid injection GC-MS, HS-SPME-GC-MS could detect nonylphenols at the ppb level. As shown in Table 2, the limit of detection (LOD) and limit of quantification (LOQ) of all nonylphenol isomers by HS-SPME-GC-MS were several times higher in 10% ethanol than those in water, suggesting much lower sensitivity in 10% ethanol. This fact could be attributed to the affinity of nonylphenol by ethanol, which would decrease its concentration in the vapor phase. Therefore, quantification of nonylphenols in these two simulants must be done separately, each one with its specific calibration plot under the same experimental conditions. There were 10 nonylphenol isomers detected in both simulants, and their migrations to 10% ethanol were all higher than those to water, which is in agreement with a previous study [25]. However, their exact structure annotations are still challenging. Among the 10 isomers, isomers 2, 3, and 9 had much higher migrations than the others. It can be pointed out that although the concentration of nonylphenols in the plastic was quite high, up to 50 μg/g, their concentration in migration simulants was much lower, with a total amount of nonylphenol (20.5–57.7 μg/kg). These values were several times higher than 10 μg/kg simulants, which is the limit established in the regulation 10/2011/EU for functional barriers, this being the value currently applied to unlisted/unauthorized substances in food contact materials domains. As can be seen, low intensity of the chromatographic peaks in plastic extracts does not necessarily mean safe levels in terms of migration. First, low intensity could be the result of por sensitivity of the analytical method. As was demonstrated in this case, the use of SPME and a polar GC column considerably increased the sensitivity of the analytical determination. Second, chemical migration may vary a lot depending on the chemical structure, diffusivity of the materials, and so on. Thus, apparently low intensity of GC peaks in plastic extracts could constitute a safety concern level of migration, as happened in this case with nonylphenol. For this reason, a comprehensive evaluation of food contact materials through non-targeted screening of migration samples using highly sensitive analytical methods, e.g. SPME, can be more reasonable and protective regarding human health. One key point in the application of these plastic bags as packaging of seminal doses is the maximum concentration of nonylphenols that sperm can tolerate without suffering any damage. Thus, a study of exposure of sperm to increasing concentrations of nonylphenol isomers was carried out.

In vitro seminal analysis

To study whether nonylphenol may interact with spermatozoa, several parameters were analyzed (total and progressive motility, viability, acrosome status and mitochondrial activity and functional integrity of the sperm membrane and acrosome). The samples were prepared with Vitasem® (Magapor SL), a specific extender for long-term preservation for boar semen, in optimal conditions of viability. Spermatozoa in direct contact with the potential toxic compounds at different concentrations nonylphenol (1 ppm, 1.5 ppm, 2 ppm, 2.3 ppm, 2.6 ppm, 10 ppm and 20 ppm) were tested after 1, 3, 5 and 7 days. At low concentrations (1 ppm and 1.5 ppm) there was no effect on seminal quality parameters or in vitro penetration testing (data not shown). At high concentrations (10 ppm and 20 ppm) a significant effect was seen in all seminal quality parameters (Fig. 2) and in vitro penetration testing (Fig. 3). Intermediate concentrations (2 ppm, 2.3 ppm and 2.6 ppm) showed no significant differences with respect to controls in sperm quality parameters up to 7 days of exposure. But when in vitro penetration testing was evaluated, a significant decrease in the penetration rate was seen after only 2 days of storage. The addition of

nonylphenol at high concentrations (10 ppm and 20 ppm) produces a very significant decrease in motility, viability and mitochondrial activity, and an increase in early apoptosis, and acrosomes reacted at only 1 day of exposure (Fig. 4). The decrease in the in vitro penetration test is up to 80% (data not shown). Based on these variables, it cannot be said that nonylphenol has a direct effect on reproduction, since it is masked by the effect on the rest of sperm quality parameters. With a motility of only 10% and a viability of 30% at day 1, it could not be concluded that the effect of nonylphenol is the cause of the decrease in the penetration test, since sperm samples with this damage in the membrane and acrosome and such low motility are not able to bind the oocyte. However, the study of the addition of intermediate concentrations of nonylphenol (2 ppm, 2.3 ppm and 2.6 ppm) allows us to better understand the effect of nonylphenol as a reproductive toxic, because, without affecting the sperm quality parameters, it significantly affects the binding to the oocyte. The nonylphenol toxicity is related to oxidative stress [26]. It disrupts the prooxidant and antioxidant balance. It was reported that nonylphenols can interfere with the reproductive system in fish, rodents, and mammals, causing cell death in the gonads and changes in reproductive parameters. Nonylphenols can cause oxidative stress by the production of reactive oxygen species (ROS), such as hydrogen peroxide (H2O2) and superoxide anion (O2−). ROS may damage cellular components and causes cell death. Its apoptotic effect on Sertoli cells in mammals was studied [27–29], but not its direct effect on sperm cells. Therefore, these results could help us better understand the mechanism of action of nonylphenols and support the mode of action previously studied on Sertoli cells. This compound causes an oxidative imbalance inside the sperm, in such a way that it goes unnoticed for the most common analysis of sperm quality at different cell levels, which suggests that it should produce an alteration of the regulatory genes. Subsequently, its effect is seen later in the penetration test. The mechanism of action of nonylphenols in sperm could be related to the binding to DNA and modification of any of the specific genes, as was recently described in germ cells [29].

Conclusions

Specific migration analysis of plastic bags used for artificial insemination of sows and non-targeted screening showed the presence of 10 nonylphenol isomers in water and 10% ethanol used as simulants of semen. They were all identified and quantified in the migration tests from many plastic films used for pig insemination processes. The direct exposure of sperm to 2 ppm of these nonylphenols showed a significant reduction in the in vitro penetration rate, although visual changes in other parameters were not initially detected.However, 10 ppm or higher concentrations of nonylphenols produced a very significant decrease in motility, viability and mitochondrial activity, and an increase in early apoptosis, and acrosomes reacted to only 1 day of exposure. Nonylphenols cause an oxidative imbalance inside the sperm and probably produce an alteration of the regulatory genes, which explains why the effect is observed later in the penetration rate. This fact could be attributed to the modification of any specific genes if nonylphenols bind DNA, as was reported in other cases. Relatively low concentration of nonylphenols was detected in our sample set, much lower than the tolerable concentration in the sperm, but concentration higher than 1 μg/Kg in migration should be avoided because it may cause negative effects on artificial insemination of sows, as was demonstrated in this research.

Acknowledgements

Qi-Zhi Su has received a grant from the China Scholarship Council (201706780010). The authors thank Gobierno de Aragón and FEDER Funds for the financial help given to GUIA group (University of Zaragoza) T53_17R.

References

1. Hopewell J, Dvorak R, Kosior E. Plastics recycling: challenges and opportunities. Philos Trans R Soc B Biol Sci. 2009;364(1526): 2115–26.
2. Hahladakis JN, Velis CA, Weber R, Iacovidou E, Purnell P. Anoverview of chemical additives present in plastics: migration, release,fate and environmental impact during their use, disposal and recycling. J Hazard Mater. 2018;344:179–99.
3. Muncke J, Backhaus T, Geueke B, MaffiniMV,Martin OV,MyersJP, et al. Scientific challenges in the risk assessment of food contact materials. Environ Health Perspect. 2017;125(9):1–9.
4. Nerin C, Alfaro P, Aznar M, Domeño C. The challenge of identifying non-intentionally added substances from food packaging materials: a review. Anal Chim Acta. 2013;775:14–24.
5. Geueke B. Non-intentionally added substances (NIAS). 2013;1–7.
6. Graíño SG, Sendón R, Hernández JL, de Quirós ARB. GC-MS screening analysis for the identification of potential migrants in plastic and paper-based candy wrappers. Polymers. 2018;10(7): 802.
7. Karolina W. Legal requirements for plastic materials and articles that come into direct contact with food. 2019;3(60):11–19.
8. Nerin C, Ubeda JL, Alfaro P, Dahmani Y, Aznar M, Canellas E, et al. Compounds from multilayer plastic bags cause reproductive failures in artificial insemination. Sci Rep. 2014;4:1–10.
9. Nerin C, Canellas E, Vera P, Garcia-Calvo E, Luque-Garcia JL, Cámara C, et al. A common surfactant used in food packaging found to be toxic for reproduction in mammals. Food Chem Toxicol. 2018;113:115–24.
10. Hamlin HJ, Marciano K, Downs CA. Migration of nonylphenol from food-grade plastic is toxic to the coral reef fish species Pseudochromis fridmani. Chemosphere. 2015;139:223–8.
11. Mottier P, Frank N, Dubois M, Tarres A, Bessaire T, Romero R, et a l . LC-MS/MS analytical procedure to quantify tris(nonylphenyl)phosphite, as a source of the endocrine disruptors
4-nonylphenols, in food packaging materials. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2014;31(5):962–72.
12. Fernandes AR, Rose M, Charlton C. 4-Nonylphenol (NP) in foodcontact materials: analytical methodology and occurrence. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2008;25(3):364–72.
13. Jie X, Li JM, Zheng F, Lei G, Biao Z, Jie Y. Neurotoxic effects of nonylphenol: a review. Wien Klin Wochenschr. 2013;125:61–70.
14. Sharma M, Chadha P, Madhu Sharma C. Toxicity of non-ionic surfactant 4-nonylphenol an endocrine disruptor: a review. Int J Fish Aquat Stud. 2018;6(2):190–7.
15. ECHA. Substances restricted under REACH. https://echa.europa. eu/de/substances-restricted-under-reach/-/dislist/details/0b0236e1807e2b6e. Accessed 12 Apr 2020.
16. de Bruin W, Kritzinger Q, Bornman R, Korsten L. Occurrence, fate and toxic effects of the industrial endocrine disrupter, nonylphenol, on plants - a review. Ecotoxicol Environ Saf. 2019;181:419–27.
17. ECHA. 4-Nonylphenol, branched and linear, as substance of very high concern becuase due to their endocrine disrupting properties they cause probable serious effects to the environment which give rise to an equivalent level of concern to those of CMRs and PBTs/v.2012.

18. Tsugawa H, Cajka T, Kind T,Ma Y, Higgins B, Ikeda K, et al.MSDIAL:data-independentMS/MS deconvolution for comprehensive metabolome analysis. Nat Methods. 2015;12(6):523–6.
19. Rodríguez-Martínez H, Saravia F, Wallgren M, Tienthai P, Johannisson A, Vázquez JM, et al. Boar spermatozoa in the oviduct. Theriogenology. 2005;63:514–35.
20. Rota A, Penzo N, Vincenti L, Mantovani R. Hypoosmotic swelling (HOS) as a screening assay for testing in vitro fertility of bovine spermatozoa. Theriogenology. 2000;53:1415–20.
21. Van Der Ven HH, Crabo BG, Zaneveld LJD. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. J Reprod Fertil. 1984;70(1):219–28.
22. Pérez-Llano B, Lorenzo JL, Yenes P, Trejo A, García-Casado P. A short hypoosmotic swelling test for the prediction of boar sperm fertility. Theriogenology. 2001;56(3):387–98.
23. Camacho W, Karlsson S. Quality-determination of recycled plastic packaging waste by identification of contaminants by GC-MS after microwave assisted extraction (MAE). Polym Degrad Stab.2000;71(1):123–34.
24. García Ibarra V, Rodríguez Bernaldo de Quirós A, Paseiro Losada P, Sendón R, Ibarra VG, Rodríguez A, et al. Identification of intentionally and non-intentionally added substances in plastic packaging materials and their migration into food products. Anal Bioanal Chem. 2018;410(16):3789–803.
25. Loyo-Rosales JE, Rosales-Rivera GC, Lynch AM, Rice CP, Torrents A. Migration of nonylphenol from plastic containers to water and a milk surrogate. J Agric Food Chem. 2004;52(7): 2016–20.
26. Aly HAA, Domènech Ò, Banjar ZM. Effect of nonylphenol on male reproduction: analysis of rat epididymal biochemical markers and antioxidant defense enzymes. Toxicol Appl Pharmacol. 2012;261(2):134–41.
27. Duan P, Hu C, Quan C, Yu T, Zhou W, Yuan M, et al. 4- Nonylphenol induces apoptosis, autophagy and necrosis in Sertoli cells: involvement of ROS-mediated AMPK/AKT-mTOR and JNK pathways. Toxicology. 2016;341–343:28–40.
28. Duan P, Hu C, Quan C, Yu T, Huang W, Chen W, et al. 4-Nonylphenol induces autophagy and attenuates mTOR-p70S6K/4EBP1 signaling by modulating AMPK activation in Sertoli cells.Toxicol Lett. 2017;267:21–31.
29. Tanaka T, Ono Y, Hikihara N, Yoshida A, Yamada H, Higaki S,et al. Transcriptome analysis revealed bisphenol a and nonylphenol affect reproduction. Reprod Toxicol. 2019;88:39–47.

Table 1 Compounds detected in plastic films by GC-MS

N°	Rt (min)	Name	CAS	Real RI	Ref. RI	Total Score	Ave. S/N	Samples
1	15.89	1-Dodecanamine, N,N-dimethyl-	112-18-5	1504	1509	82.8	565	D
2	16.99	Hexadecane	544-76-3	1599	1600	c.f.	29	A, B, C, D, E, F
3	17.09	Diethyl Phthalate	84-66-2	1607	1594	c.f.	5	A, B, C, D, E, F
4	17.18	7-Hexadecene, (Z)-	35,507-09-6	1616	1620*	89.7	3	A, B, C, D
5	17.37	Decane, 2-cyclohexyl-	13,151-73-0	1633	1611*	90.3	6	A, B, C, D
6	18.30	Pentadecanal-	2765-11-9	1718	1701	91.6	6	B, E
7	18.35	Nonylphenol isomer	–	1723	1720*	c.f.	11	A, B, C, D, E, F
8	18.43	Nonylphenol isomer	–	1731	1720*	c.f.	50	A, B, C, D, E, F
9	18.47	Nonylphenol isomer	–	1735	1720*	c.f.	23	A, B, C, D, E, F
10	18.68	Nonylphenol isomer	–	1755	1720*	c.f.	16	A, B, C, D, E, F
11	18.76	Nonylphenol isomer	–	1762	1720*	c.f.	97	A, B, C, D, E, F
12	18.86	Nonylphenol isomer	–	1771	1720*	c.f.	6	A, B, C, D, E, F
13	19.14	Octadecane	593-45-3	1798	1800	c.f.	41	A, B, C, D, E, F
14	19.19	1-Octadecene like	–	1803	–	–	4	A, B, C, D, E, F
15	19.21	5,5-Diethylpentadecane	360,410	1805	1805	87.8	10	A, B, C, D, E, F
16	19.37	Diphenyl Glycol	104-66-5	1822	1811	94.8	181	A, D, E, F
17	19.58	Dodecane, 2-cyclohexyl-	13,151-82-1	1841	1810 *	79.4	13	A, B, C, D, E, F
18	19.81	1-Hexadecanol like	–	1865	–	–	41	A, B, C, D, E, F
19	19.93	Di-sec-butyl phthalate	4489-61-6	1877	1908 *	77.2	7	A, B, C, D, E, F
20	20.41	Methyl palmitate	112-39-0	1926	1916	c.f.	6	A, B, C, D, E, F
21	20.73	Fichtelite	2221-95-6	1959	1942	87.3	28	A, B, C, D, E, F
22	21.00	Unknown (243, 70)	–	1987	–	–	75	A, B, C, D, E, F
23	21.10	Eicosane	112-95-8	1998	2000	c.f.	43	A, B, C, D, E, F
24	21.14	1-Dodecanol, 2-hexyl-	110,225-00-8	2003	1989	92.2	5	A, B, C, D, E, F
25	21.18	5,5-Diethylheptadecane	360,417	2007	2006	87.5	5	A, B, C, D, E, F
26	21.42	Unknown (97, 55)	–	2033	–	–	8	A, B, C, D, E, F
27	21.57	Cyclohexane, tetradecyl-	1795-18-2	2049	2071	85.8	26	A, B, C, D, F
28	21.71	5-Ethylnonadecane	360,431	2064	2034	84.8	6	A, B, C, D, E, F
29	21.77	10-Heneicosene (c,t)	95,008-11-0	2071	2060	82.8	68	A, B, C, D, E, F
30	22.07	Isooctyl laurate	84,713-06-4	2104	2112*	90.7	9	A, B, C, D, E, F
31	22.24	Unknown (134, 91)	–	2123	–	–	139	D
32	22.47	n-Nonadecanol-1	1454-84-8	2150	2156	91.9	14	A, B, C, D
33	22.90	Docosane	629-97-0	2198	2200	c.f.	26	A, B, C, D, E, F
34	22.93	1-Docosene	1599-67-3	2202	2195	92.3	4	A, B, C, D, E, F
35	23.49	Docosane, 2,21-dimethyl-	77,536-31-3	2268	2279*	88	8	A, B, C, D, E, F
36	23.57	1-Acetoxynonadecane	1577-43-1	2277	2308	92.9	79	A, B, C, D, E, F
37	24.17	1-Heneicosanol	15,594-90-8	2350	2365	91.4	16	A, B, C, D, E, F
38	24.58	Cycloeicosane	296-56-0	2401	2398 *	86.8	8	D
39	25.08	Cyclohexane, octadecyl-	4445-6-1	2465	2486	87.4	30	A, B, C, D, E
40	25.22	1-Heneicosyl formate	77,899-03-7	2483	2472*	92.9	68	A, B, C, D, E, F
41	25.73	1-Tricosanol	3133-01-5	2550	2528	85.4	12	A, B, C, D, F
42	25.78	Bis(2-ethylhexyl) phthalate like	–	2556	–	–	8	A, B, C, D, E, F
43	26.21	Tetracosane, 5-ethyl-5-methyl-	55,282-30-9	2614	2621*	85.3	7	A, B, C, D, E, F
44	26.64	Cyclohexane, eicosyl-	4443-55-4	2673	2669*	88.2	21	A, B, C, D
45	26.75	1-Tetracosanol	506-51-4	2689	2650*	91.1	28	A, B, C, D, E, F
46	27.45	Unknown (59, 72)	–	2788	–	–	362	A, B, C, D, E, F
47	27.77	Squalene	111-02-4	2835	2832	c.f.	55	A, B, C, D, E, F
48	28.70	Unknown (385)	–	2964	–	–	114	A, B, C, D, F
49	29.16	Unknown (296, 341, 324)	–	3024	–	–	104	A, B, C, D, E, F

Note: c.f. means confirmed by standard; * stands for predicted retention indices

Table 2 LOD, LOQ, and concentrations (mean ± sd) of each nonylphenol isomer migrating into water and 10% ethanol (μg/kg simulant)

Samples	Simulants	Isomer 1 L O D 0.06 L O Q 0.18	Isomer 2 L O D 0.14 L O Q 0.43	Isomer 3 L O D 0.08 L O Q 0.23	Isomer 4 L O D 0.05 L O Q 0.14	Isomer 5 L O D 0.09 L O Q 0.28	Isomer 6 L O D 0.06 L O Q 0.17	Isomer 7 L O D 0.05 L O Q 0.14	Isomer 8 L O D 0.07 L O Q 0.20	Isomer 9 L O D 0.10 L O Q 0.3	Isomer 10 L O D 0.28 L O Q 0.83	Total
LOD, LOQ	H2O	0.03, 0.10	0.08, 0.25	0.04, 0.15	0.03, 0.10	0.04, 0.13	0.05, 0.17	0.02, 0.05	0.02, 0.06	0.05, 0.21	0.04, 0.15	
	10% EtOH	0.15, 0.49	0.19, 0.63	0.26, 0.88	0.11, 0.38	0.21, 0.69	0.15, 0.49	0.14, 0.47	0.06, 0.20	0.30, 1.00	0.14, 0.48	
A	H_2O	0.9 ± 0.1	6.2 ± 0.6	4.5 ± 0.5	0.7 ± 0.06	0.9 ± 0.08	1.0 ± 0.2	0.6 ± 0.05	0.7 ± 0.1	4.3 ± 0.5	0.7 ± 0.01	20.5 ± 0.3
	10% EtOH	1.5 ± 0.03	9.6 ± 0.02	6.7 ± 0.3	1.2 ± 0.05	1.8 ± 0.02	1.4 ± 0.06	0.8 ± 0.07	1.0 ± 0.05	6.8 ± 0.5	1.1 ± 0.01	41.0 ± 0.2
B	H_2O	0.6 ± 0.1	4.8 ± 0.01	3.5 ± 0.1	0.5 ± 0.01	0.7 ± 0.02	0.7 ± 0.03	0.3 ± 0.04	0.6 ± 0.04	3.4 ± 0.1	0.7 ± 0.01	15.9 ± 0.1
	10% EtOH	2.0 ± 0.04	13.2 ± 1.0	9.3 ± 0.09	1.8 ± 0.1	2.0 ± 0.02	2.0 ± 0.09	1.0 ± 0.07	1.7 ± 0.1	9.6 ± 0.3	1.8 ± 0.01	57.7 ± 0.2
C	H_2O	0.9 ± 0.04	6.6 ± 0.2	4.8 ± 0.3	0.8 ± 0.1	0.9 ± 0.02	1.0 ± 0.1	0.6 ± 0.01	0.7 ± 0.02	4.4 ± 0.2	0.9 ± 0.1	21.7 ± 0.1
	10% EtOH	1.6 ± 0.2	9.8 ± 0.6	6.5 ± 0.3	1.3 ± 0.1	1.5 ± 0.02	1.5 ± 0.05	0.8 ± 0.04	1.2 ± 0.02	6.8 ± 0.3	1.5 ± 0.2	41.3 ± 0.2
D	H_2O	0.9 ± 0.1	6.6 ± 0.6	4.8 ± 0.4	0.7 ± 0.1	0.9 ± 0.1	1.1 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	4.4 ± 0.4	1.0 ± 0.01	21.6 ± 0.3
	10% EtOH	1.2 ± 0.03	9.8 ± 0.02	6.5 ± 0.12	1.3 ± 0.05	1.6 ± 0.1	1.6 ± 0.05	0.8 ± 0.04	1.3 ± 0.3	6.5 ± 0.2	1.4 ± 0.01	40.7 ± 0.1
E	H_2O	0.9 ± 0.1	7.0 ± 0.2	4.8 ± 0.2	0.6 ± 0.02	1.0 ± 0.1	1.0 ± 0.02	0.6 ± 0.1	0.8 ± 0.2	4.6 ± 0.4	0.9 ± 0.01	22.3 ± 0.2
	10% EtOH	1.2 ± 0.04	8.9 ± 0.05	5.8 ± 0.1	1.2 ± 0.06	1.7 ± 0.07	1.4 ± 0.07	0.9 ± 0.04	1.1 ± 0.05	6.5 ± 0.3	1.6 ± 0.2	38.2 ± 0.1
F	H_2O	1.0 ± 0.1	6.9 ± 0.6	4.9 ± 0.4	0.8 ± 0.2	1.0 ± 0.1	1.0 ± 0.1	0.7 ± 0.1	0.7 ± 0.2	4.8 ± 0.6	1.1 ± 0.2	22.9 ± 0.3
	10% EtOH	1.1 ± 0.2	10.5 ± 1.2	7.0 ± 0.9	1.2 ± 0.2	1.8 ± 0.3	1.5 ± 0.1	0.8 ± 0.1	1.2 ± 0.04	7.2 ± 0.2	1.6 ± 0.6	43.4 ± 0.5

Note: EtOH stands for ethanol

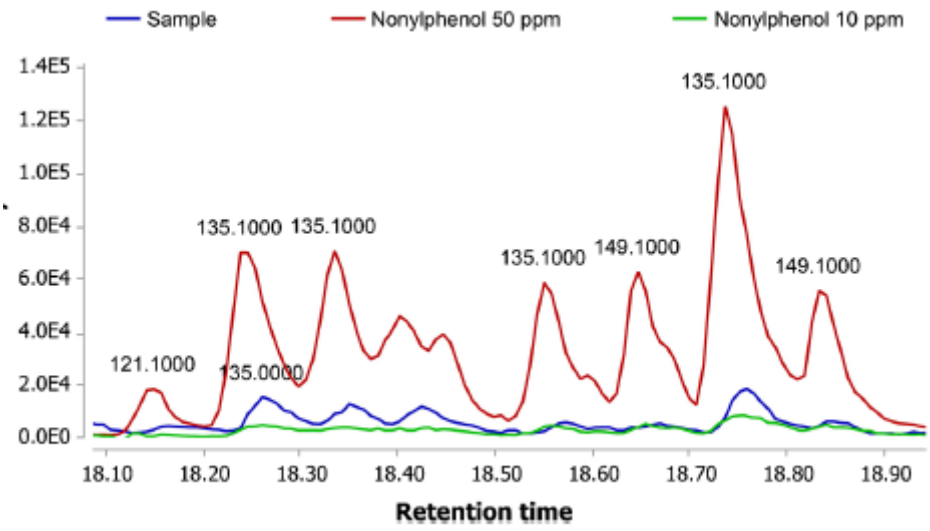

Fig. 1 Chromatograms of nonylphenol standard and a sample extract by liquid injection GC-MS

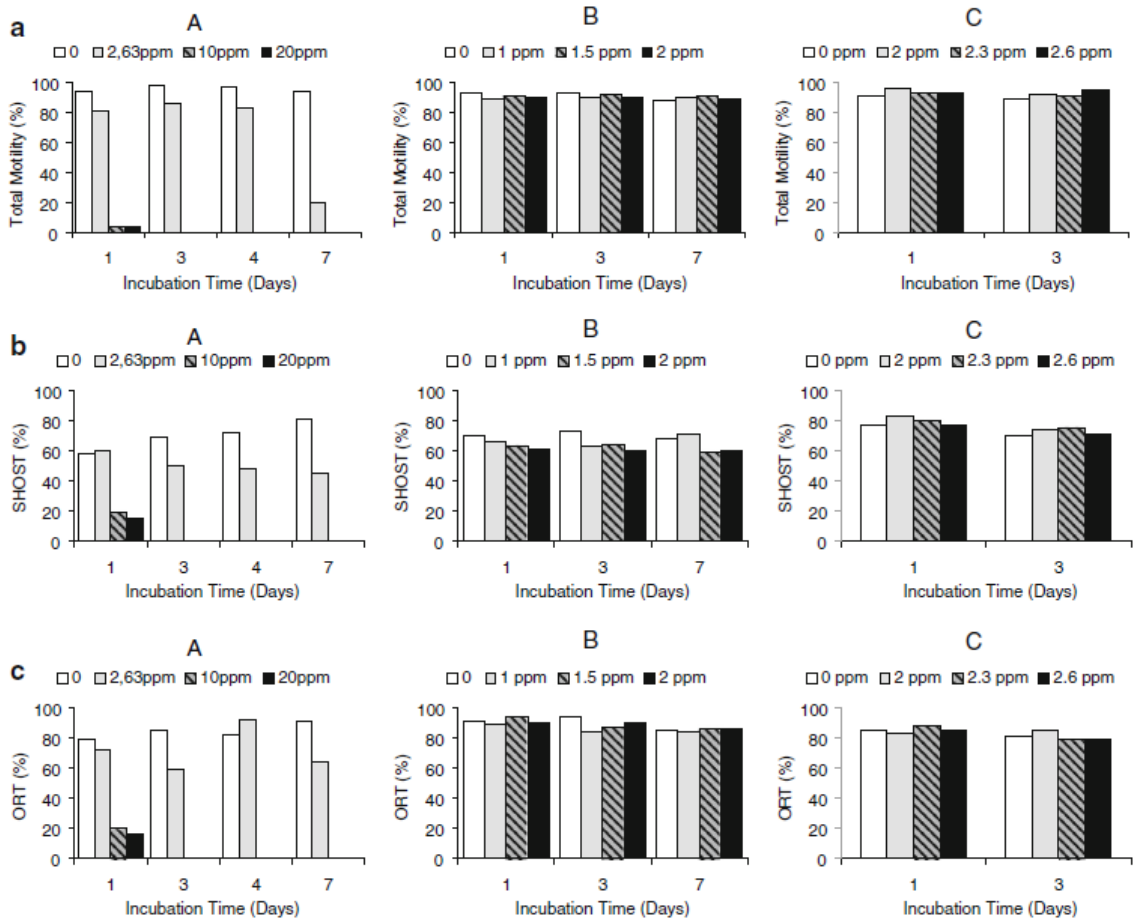

Fig. 2 a Total motility evaluated by CASA system in seminal samples incubated with different concentrations of nonylphenol: (A) high concentrations; (B) mid-range concentrations; (C) low concentrations; n = 8. b Functional integrity of the sperm membrane evaluated by short hypoosmotic swelling test (sHOST) in seminal samples incubated with different concentrations of nonylphenol: (A) high concentrations; (B) mid-range concentrations; C) low concentrations; n = 8. c Functional integrity of the sperm acrosome evaluated by short osmotic resistance test (sORT) in seminal samples incubated with different concentrations of nonylphenol: (A) high concentrations; B) mid-range concentrations; C) low concentrations; n = 8

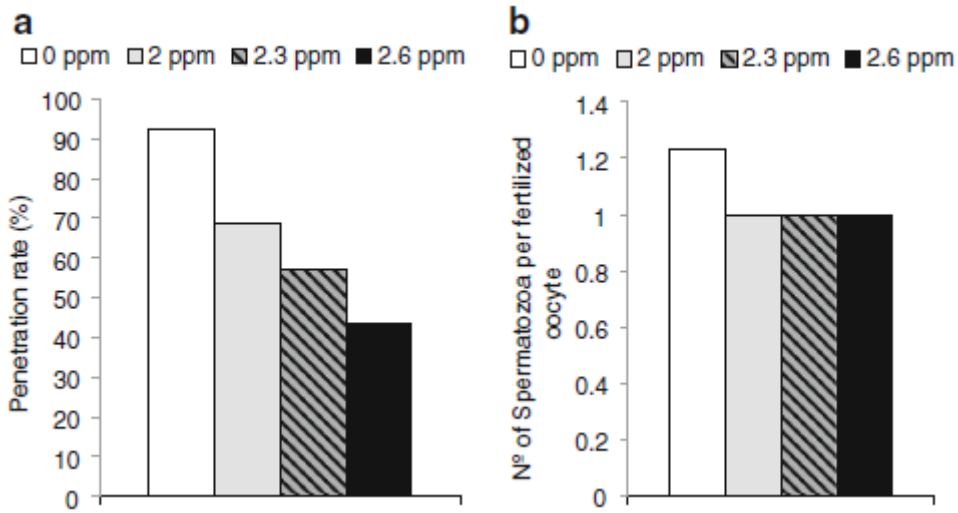

Fig. 3 Sperm penetration rate (a) and number of spermatozoa per fertilized oocyte (b) at 48 h in seminal samples incubated with different concentrations of nonylphenol (mid-range concentrations); n = 5

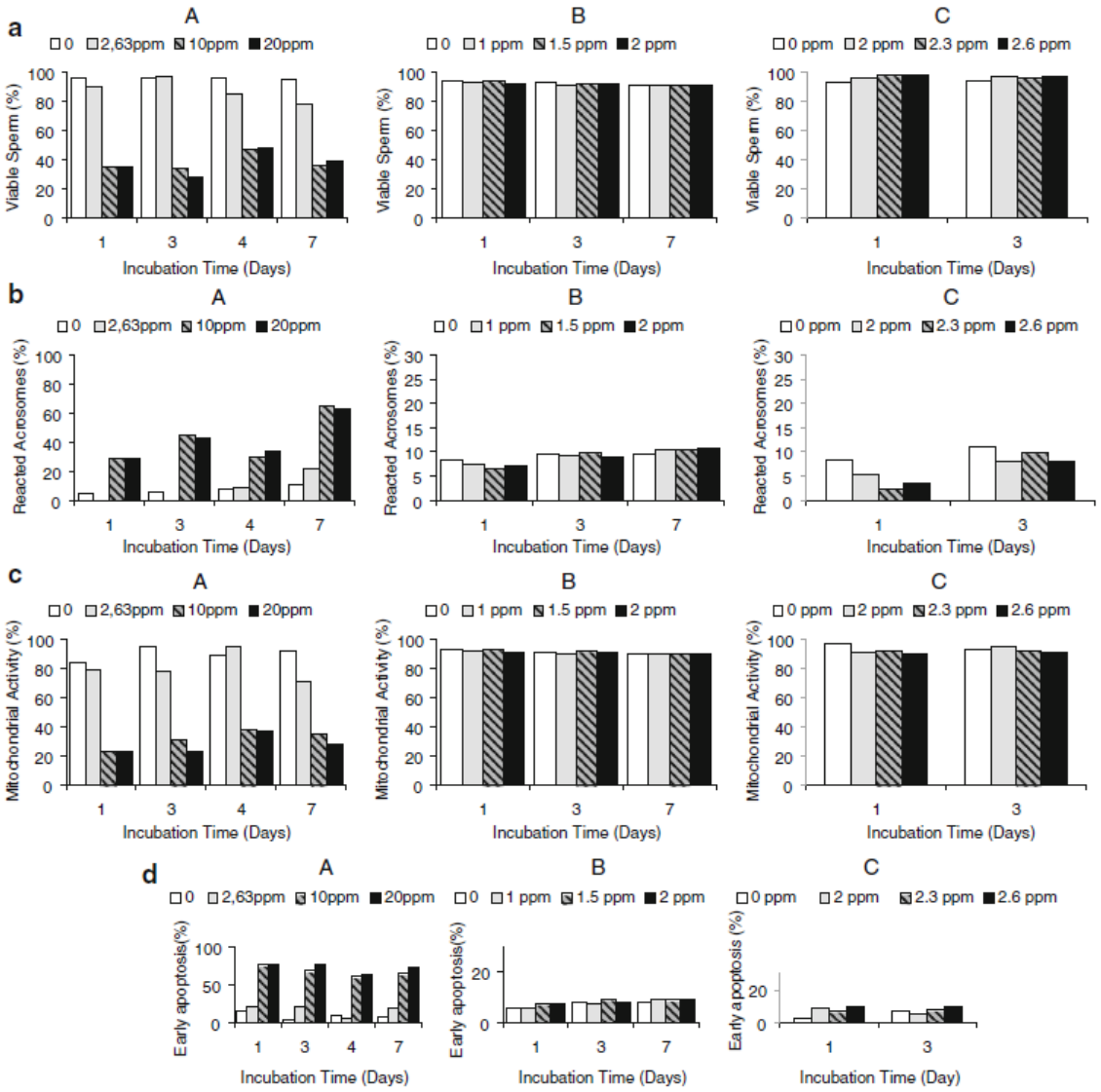

Fig. 4 a Plasma membrane integrity evaluated by flow cytometry with propidium iodide stain in seminal samples incubated with different concentrations of nonylphenol: (A) high concentrations; (B) mid-range concentrations; (C) low concentrations; n = 8. b Acrosome status evaluated by flow cytometry with FITC-PNA stain in seminal samples incubated with different concentrations of nonylphenol: (A) high concentrations; (B) mid-range concentrations; (C) low concentrations; n = 8. C Mitochondrial activity evaluated by flow cytometry with MitoT stain in seminal samples incubated with different concentrations of nonylphenol: (A) high concentrations; (B) mid-range concentrations; (C) low concentrations; n = 8. d Early apoptosis evaluated by flow cytometry with YoPro1 stain in seminal samples incubated with different concentrations of nonylphenol: (A) high concentrations; (B) mid-range concentrations; (C) low concentrations; n = 8