http://doi.org/10.7427/DDI.25.02

2. In vivo eco-toxicological assessment of some cationic and amphoteric surfactants in transgenic *Caenorhabditis elegans*

CICÎRMA Marius[1,2*], de POMERAI David [3]

[1]School of Life Science, University of Nottingham, Nottingham NG7 2RD.
[2]Danube Delta National Institute for Research and Development, Street Babadag 165, Tulcea 820112, Romania
[3]University of Edinburgh, New College, Mound Place, Edinburgh EH1 2LX, UK.

**Address of author responsible for correspondance: Marius Cicîrma, Danube Delta National Institute for Research and Development, Street Babadag 165, Tulcea 820112, Romania, e-mail: marius.cicirma@ddni.ro*

Abstract: *Caenorhabditis elegans* is a free-living nematode that is exposed naturally to multiple surfactants contaminating fresh water and soil. Their breakdown products can act as Endocrine Disruptor Chemicals (EDCs), which interfere with hormonal signalling in vertebrates and invertebrates. Vitellogenin (yolk protein) synthesis is induced by oestrogens and related hormones *in vivo,* and is widely utilized as a biomarker for EDCs at sub-lethal concentrations in various test species, including *C. elegans*. The study shows that *C. elegans* feeding activity is inhibited at high concentrations of both amphoteric [cocamidopropyl betaine (CAPB) and cocamidopropyl hydroxysultaine (CAPHS)], and cationic [Hyamine 1622] surfactants. Molecular assays measuring induction of stress-responsive Green Fluorescent Protein (GFP) reporter transgenes confirmed the activation of *vitellogenin*-2 (*vit*-2) and antioxidant/phase II defences during larval development when using synchronized populations. Among known EDCs, Bisphenol A (BPA) strongly induced *vit-2* at 48 and 72 h but had less effect on the stress-reporters, whereas 17α-Ethinylestradiol (EE_2; active ingredient of contraceptive pills) down-regulated *vit-2* after 24 h but upregulated it dose-dependently after 48 h and 72 h, and also strongly induced *sod-4* and *gst-1* stress-reporters. CAPB and CAPHS induced *vit-2* expression rather weakly at 10^{-2} mg.l^{-1} after 48 and 72 h, but had more dramatic effects on stress-reporter expression. Hyamine 1622 at 10^{-3} mg.l^{-1} down-regulated *vit-2* at early time points and also down-regulated the stress-reporters (though *gst*-1 was upregulated at 24 h). Males are rare in *C. elegans* populations, but both EDC and surfactant treatments were associated with subtle modifications of male tail morphology.
Keywords: Cationic/amphoteric surfactants; feeding inhibition; reporter transgenes; stress inducibility; vitellogenin expression; *Caenorhabditis elegans*

AIMS

1. Measuring the effects of test surfactants on *C. elegans* life history parameters, including feeding, growth and developmental rate (time from egg to egg-bearing adult).

2. Determination of possible oestrogenic effects of surfactants and known EDCs (Bisphenol A and Ethinylestradiol) on the level or timing of *vit*-2 production in hermaphrodites and on the expression of GFP-reporter genes for phase II detoxification and oxidative stress enzymes.

INTRODUCTION

Caenorhabditis elegans is a small nematode that can be maintained at low cost and handled using standard *in vitro* techniques. Unlike toxicity testing using cell cultures, *C. elegans* toxicity assays provide data from a whole animal with intact and metabolically active digestive, reproductive, endocrine, sensory and neuromuscular systems (Hunt, 2016). The nematode *Caenorhabditis elegans* has emerged as an important animal model in various fields including neurobiology, developmental biology, and genetics. Characteristics of this animal model that have contributed to its success include its genetic manipulability, invariant and fully described developmental program, well-characterized genome, ease of maintenance, short and prolific life cycle, and small body size. These same features have led to an increasing use of *C. elegans* in toxicology, both for mechanistic studies and high-throughput screening approaches (Leung et

al., 2008). Transgenic *C. elegans* strains carrying stress-inducible *lacZ* or GFP reporter genes have proved particulary useful for rapid screening of multiple environmental samples (Anbalagan et al., 2013, 2012; De Pomerai et al., 2010; Guven, Duce, & de Pomerai, 1994).

Endocrine-disrupting chemicals are hormone-like agents present in the environment that alter the endocrine system of wildlife and humans. Perhaps the most problematic group of EDCs are the so-called estrogen mimics, which have been blamed for widespread population crashes among freshwater fish. These agents include dioxins (Ohtake et al., 2003) pesticides, and plasticisers such as phthalates or nonylphenol ethoxylates. However it is worth noting that a superficially similar effect of the pesticide DDT on alligator populations was in fact caused by the powerful anti-androgenic action of one particular DDT metabolite (DDE). Most EDCs have potencies far below those of the natural female hormone, 17β-estradiol (E2), when acting through the classic estrogen receptors (ERs) (Quesada et al., 2002). 17α-Ethinylestradiol (EE_2) is a synthetic hormone derived from the natural hormone, estradiol (E2). EE_2 is a bioactive estrogen, utilized as a pharmaceutical in the contraceptive pill as well as in aquaculture. It is highly resistant to biodegradation, and although it is inactivated metabolically prior to excretion, it may become reactivated through bacterial action in sewage treatment facilities and hence escape into the aquatic environment. EE_2 is known to induce alterations in sex determination pathways, delay sexual maturity, and decrease the secondary sexual characteristics of exposed organisms, even at very low concentrations (ng/l), by mimicking its natural analogue, 17β-estradiol (E2) (Aris, Shamsuddin, & Praveena, 2014). Other classic EDCs include Bisphenol A (BPA) and related chemicals. In a recent review, Rochester & Bolden (2015) surveyed 32 studies (25 *in vitro* only, and 7 *in vivo*) of the effects of BPA as compared to E2. The majority of these studies examined the activities of Bisphenols (BPs), which are broadly comparable to both natural (E2) and synthetic (EE_2) estrogens in terms of their effects on a range of endpoints. Paradoxically, whereas BPs on their own show estrogenic activity in human breast cancer cell line MCF-7 , they become anti-estrogenic when combined with natural 17β-estradiol (Kitamura et al., 2005), altering the course of development in mice (Howdeshell & Vom Saal, 2000). Even if these chemicals do not overtly alter gonadal sex, there are indications that several EDCs might demasculinize male-specific behaviors that are essential for attracting a mate. In so doing, these chemicals may reduce the likelihood that these males reproduce and also affecting their epigenome (Bhandari et al., 2015). Histological and gene expression studies revealed that BPA and BPA + E2 exposures promoted the establishment of spermatogenesis whereas E2 alone delayed it in rats (Brouard, Guénon, Bouraima-Lelong, & Delalande, 2016).

In the case of *C. elegans* the vast majority of individuals in any population are XX hermaphrodites, producing sperm as L4 larvae but eggs (which are usually self-fertilized by the stored sperm) in young adults. However, a small minority (c. 0.2%) of these nematodes are XO males (arising by non-disjunction of the X chromosome), which produce sperm throughout adult life and possess a distinctive tail morphology consisting of a posterior fan supported by rays – which is used to clasp hermaphrodites during mating. Heat shock or outcrossing with mutant *him* (*h*igh *i*ncidence of *m*ales) strains can be used to increase the proportion of males in a population. Bisphenol A (BPA) from plastics and Ethinylestradiol (EE_2) from contraceptive pills are well known Endocrine Disruptor Chemicals (EDCs), both of which have wide-ranging effects on C*aenorhabditis elegans* (Allard & Colaiácovo, 2011; Hoshi, Kamata, & Uemura, 2003; Zhou et al., 2016) and on other nematodes (Höss & Weltje, 2007).

Surfactants are important ingredients of personal care products and household products. The main effect of these compounds is to decrease the surface tension of solvents and thereby change many properties such as contact angle, foaming ability etc. Their behavior in the environment and biodegradability was characterized upon their molecular charge as: anionic, cationic, nonionic and amphoteric. The coexistence of other ingredients in commercial products may also affect the properties of surfactants (Staszak, Wieczorek, & Michocka, 2015), but once they reach the environment, they end up dispersed in different compartments such as soil, water or sediment (Ivanković & Hrenović, 2010). There is only a low potential for acute effects on aquatic species due to the release of secondary or tertiary sewage effluents containing the breakdown products of laundry detergents; however, untreated or primary-treated effluents containing cleaning products may pose a greater problem (Lenntech, 2015). Low levels of surfactants and other detergents may also increase the uptake of other pollutants. Invertebrates, especially in their juvenile stages, are extremely sensitive to detergents: concentrations below 0.1 $mg.l^{-1}$ interfere with growth and development in some species. Research on the interactions between chemicals such us surfactants and proteins, and their effects on membrane permeability, may explain some of the biological actions of detergents in living organsisms. In natural waters, surfactants are usually partially degraded, and a maximum permissible concentration of 0.5 $mg.1^{-1}$ would probably be harmless under most

conditions (Abel, 1974). However, some of these agents have been classified as environmentally friendly, such as Cocamidopropyl betaine (CAPB), (Hoeman & Culbertson, 2008), though their sublethal effects on organisms are not well characterised.

MATERIAL AND METHODS

MATERIALS

Test compounds

Surfactants were kindly provided by Dr Stefania Ivan Gheorghe of the National Research and Development Institute for Industrial Ecology — ECOIND, Bucharest, Romania. Using *Caenorhabditis elegans*, we have assessed the toxicity and estrogen-mimic potential of five selected compounds: namely, two amphoteric surfactants – Cocamidopropyl betaine (commercial name AMFODAC LB, 34.6 %), and Cocamidopropyl hydroxysultaine (CAPHS, 34.6%), both from Sasol Italy S.p.A. (Milan Italy) – as well as the cationic surface-tension agent Hyamine 1622 (benzethonium chloride monohydrate; >96%, from Sigma-Aldrich, Poole, UK), and, as positive controls, both Bisphenol A (BPA, ≥99%) and 17α-Ethinylestradiol (EE_2, ≥98%) – also from Sigma-Aldrich Ltd. BPA was dissolved in 100% ethanol as a stock solution and stored at 4°C.

Bacterial strain

E. coli OP50 (wild type)

C. elegans strains

Strain CF1553 (*sod-3*::GFP) and RT-130 pwls 23 (*vit-2*::GFP) were supplied by the Caenorhabditis Genetics Centre (CGC, University of Minnesota, Twin Cities, USA – funded by the NIH National Centre for Research Resources). Other strains were supplied as integrated promoter::GFP fusions by the Baillie Genome GFP Project (Simon Fraser University, Burnaby, Vancouver, Canada; Hunt-Newbury et al., 2007) each containing c. 3 kb of upstream promoter sequence: these are BC20316 (*gst*-1::GFP) and BC20333 (*sod*-4::GFP). Fusion gene arrays were integrated by X-irradiation, and stable transgenic lines were out crossed 4 x.

METHODS

Feeding inhibition assays

In order to assess the general toxicity of surfactants in *C. elegans*, feeding inhibition assay (Jones & Candido, 1999) were performed using N2 (wild type, data not shown) and RT-130 strain. Nematodes were washed extensively with K medium (53 mM NaCl, 32 mM KCl; Williams & Dusenbery 1990), in equal aliquots, (5000 worms per well in 6-well plates) were mixed with a dense suspension of food bacteria [*E. coli* OP50, (at an initial optical density of 1.5) at 550 nm], plus a full set of test concentrations and solvent and/or K medium controls for each detergent, in a final volume of 1.5 ml. All assays were performed in quadruplicate. Plates were placed in a sealed humidified box and incubated at 20°C for 72 h. The contents of each well were transferred into Eppendorf tubes and kept on ice to allow worms and faecal pellets to settle, then 1.0 ml of each supernatant was withdrawn carefully and the OD measured at 550 nm in a plastic cuvette. Controls without worms present (NWC) were also included. All test results were normalised against the solvent controls (K medium plus bacteria OD 1.5) at each time point at 20°C.

Synchronizing worm cultures

Worms were grown on NGM agar plates and washed off using ice-cold K medium, then washed with K medium and transferred into 50 ml Falcon tubes and placed on ice for 20 minutes. After settling, worms were washed twice in fresh K medium and centrifuged for 4 minutes at 1600 x g. The worm pellet was resuspended in 10 ml of ice-cold alkaline hypochlorite solution (1% v/v NaOCl in 0.5 M KOH) to break up the worms and release the eggs. The tube was vortexed for 10 minutes, centrifuged for 3 minutes (as above) , and the egg pellet resuspended in 30-40 ml K medium, then washed 3 times to remove residual traces of hypochlorite. The collected eggs were dispensed into 6 well plates in K medium (150 μl of egg suspension per well), then incubated overnight at 25°C with no food to allow hatching and synchronization of the starved larvae at the early L1 stage. On the second day, equal aliquots of L1 worms from each strain were dispensed into 24-well plates along with the test chemicals and appropriate controls, with four replicates in different plates for each test.

Assessment of growth and development

L1 larval cultures were grown at 20°C in the presence or absence of surfactant (10^{-2} mg.l^{-1} CAPB, CAPHS or Hyamine) or EDCs (10^{-6} mg.l^{-1} BPA or 10^{-4} m.g.l^{-1} EE_2). Worm lengths were measured using a calibrated eyepiece graticule after 72 h (before eggs appeared), using 15 measurements per condition (data not shown). After 96 h, eggs appeared inside the gonads of many treated worms, and some hatched L1 or even L2 larvae were also apparent Photographs were taken using an Olympus BH-2 fluorescence microscope coupled to a camera system HIROCAM (High Resolution Optics Camera) with TS View Digital imaging software.

GFP reporter assays.

Synchronized L1 larvae of the GFP reporter strains were incubated at 20°C in a total volume of 300 µl in sealed 24-well microplates (Nunc Ltd, Rosskilde, Denmark), and the contents of each well were transferred at 24 h time intervals into black non-fluorescent U-bottomed 96-well microplates. After standing on ice for 15 min, GFP expression was quantified in the worm pellet using a Perkin-Elmer Victor 1420 Multilabel plate reader using narrow band-pass filters at 485 nm (excitation) and 525 nm (emission), as previously described (Anbalagan et al., 2012). Data are shown for two oxidative stress genes (*sod*-3::GFP, CF1553; *sod*-4::GFP, BC20333) and a phase II detoxification gene (*gst*-1:GFP, BC20316). After reading, worm suspensions were returned to their parent 24-well plates for further incubation or examined under a low-power inverted microscope to estimate mortality before disposal.

A *C. elegans* reporter strain RT-130 (*vit*-2::GFP) was previously developed by X-ray bombardment and outcrossed twice. This strain was grown on NGM agar plates at 15°C, synchronized as above, and about 1000 L1 larvae exposed to surfactants or EDCs at concentrations ranging between 10^{-2} and 10^{-7} mg.l^{-1}, together with controls, in sealed 24-well microplates as above. GFP fluorescence was measured (as above) in larvae after 24 h (L2-L3), after 48 h (L4 plus young adults), and after 72 h and 96 h (adults; data not shown for 96 h, because of high mortality under some conditions). After reading, worm suspensions were returned to their parent 24-well plates for further incubation, or examined (as above) under a low-power inverted microscope.

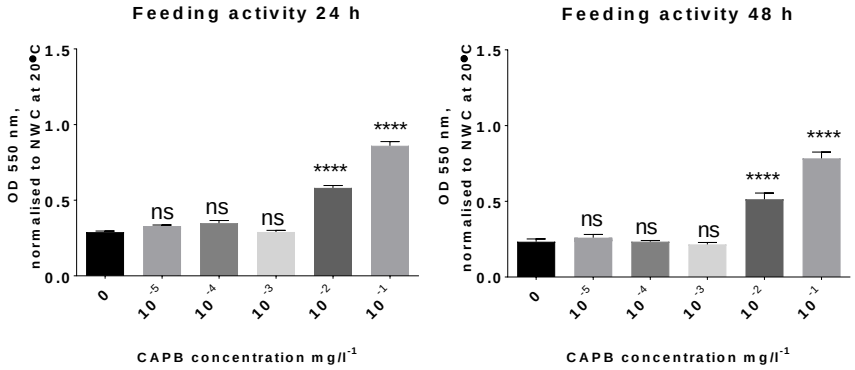
Statistics

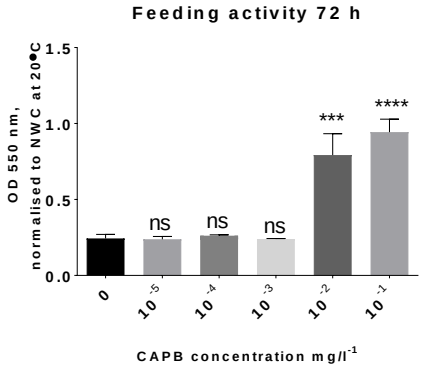
For GFP expression (which varies between repeat runs), mean fluorescence readings were normalised as the ratio of GFP fluorescence in treated samples to that in solvent controls with no surfactant or EDC present (hence controls are always 1.0); these data are presented in arbitrary relative fluorescence units (RFU ± SEM). All quantitative dose-response data were analysed by one-way ANOVA with Dunnett's *post hoc* multiple comparisons test against solvent controls at the same time point. Other comparisons within data sets were made using one-way ANOVA with Bonferroni's *post hoc* multiple comparisons test. Straightforward comparisons between control and test conditions used a two-tailed Student's t test. All measurments were analysed in GraphPad Prism 7.04.

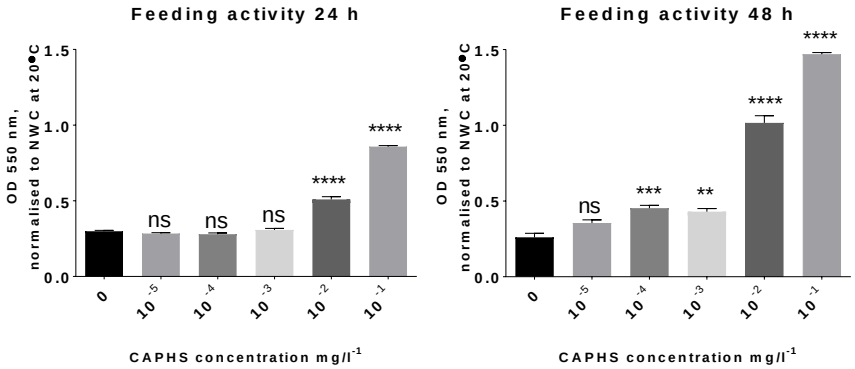
RESULTS

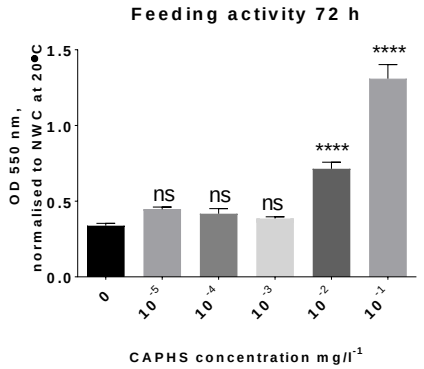
Feeding inhibition assays

Caenorhabditis elegans can respond to a variety of stressors (alcohols, heavy metals, sulhydryl-reactive compounds, salicylate and heat) by reducing or ceasing pharyngeal pomping, and this can be readily measured using a simple feeding inhibition assay (Jones & Candido, 1999). We evaluated the feeding activity of RT 130 worms in the presence of eather cationic or amphoteric surfactants. The optical density of bacteria used as a food source was measured by spectrophotometry (λ=550 nm) after 24, 48 and 72 hours, and increased significantly at test concentrations of 10^{-2} mg.l^{-1} and 10^{-1} mg.l^{-1}, suggesting that feeding of the mixed population was inhibited dose dependently by these agents (Figure 1). Concentrations of 10^{-3} mg.l^{-1} or less had no significant effect on feeding, and although Hyamine 1622 inhibited feeding strongly at 10^{-1} mg.l^{-1} after 48 h, no such effect was seen after 24 or 72 h (Figure 1). At least in part, this may reflect the fact that worm mortality increased at higher surfactant concentrations and over longer exposure times (data not shown).


Feeding activity 24 h
Feeding activity 48 h
OD 550 nm, normalised to NWC at 20°C
1.5
1.0
0.5
0.0
ns
ns
ns
****
****
0
10 -5
10 -4
10 -3
10 -2
10 -1
CAPB concentration mg/l-1


Feeding activity 72 h
OD 550 nm, normalised to NWC at 20°C
1.5
1.0
0.5
0.0
ns
ns
ns
***
****
0
10 -5
10 -4
10 -3
10 -2
10 -1
CAPB concentration mg/l-1


Feeding activity 24 h
Feeding activity 48 h
OD 550 nm, normalised to NWC at 20°C
1.5
1.0
0.5
0.0
ns
ns
ns
**
***
****
****
0
10 -5
10 -4
10 -3
10 -2
10 -1
CAPHS concentration mg/l-1


Feeding activity 72 h
OD 550 nm, normalised to NWC at 20°C
1.5
1.0
0.5
0.0
ns
ns
ns
****
****
0
10 -5
10 -4
10 -3
10 -2
10 -1
CAPHS concentration mg/l-1

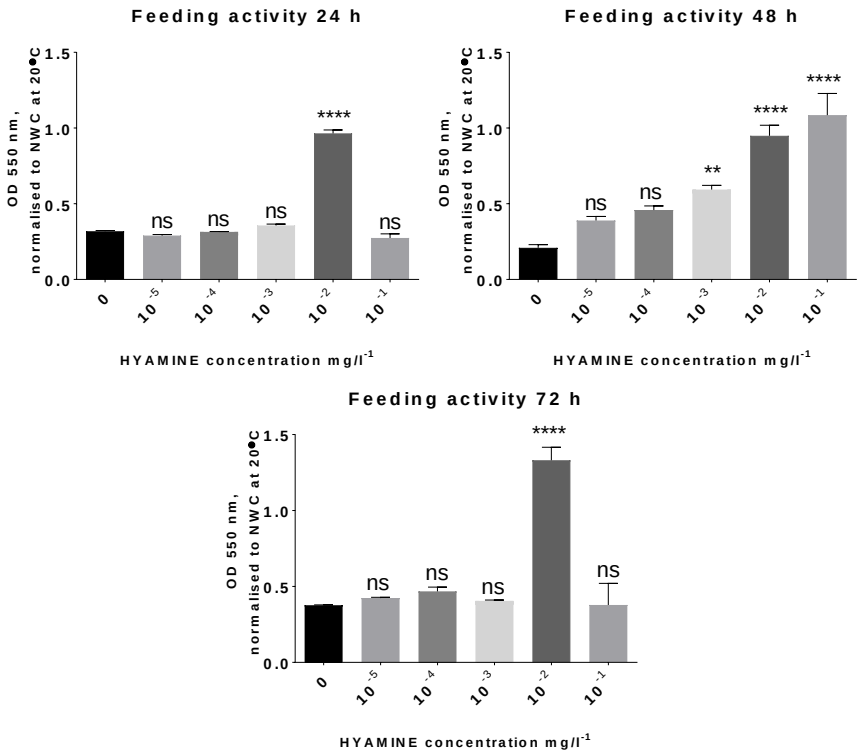


Figure 1. Feeding inhibition assay. These assays were performed at 20 °C as described in Methods, using 5000 RT-130 worms and *E. coli* OP 50 bacteria as food [OD 550 nm = 1.5] for up to 72 h.

Light microscopic examination of synchronized cultures of RT-130 worms suggested, paradoxically, that submaximal concentrations of either surfactants (10^{-2} or 10^{-3} mg.l^{-1}) or known EDCs (10^{-5} mg.l^{-1} BPA or 10^{-6} mg.l^{-1} EE_2) could accelerate the growth and development of synchronized RT-130 worms. In control cultures, the adult worms remained thin and devoid of eggs (Figure 2). By contrast, in the treated cultures, adult worms were much thicker, eggs appeared after about 80 h (but not with EE_2), and by 96 h newly hatched larvae (L1s, and even L2s in the case of 10^{-3} mg.l^{-1} Hyamine 1622) were abundant. Worm 'bagging' was also observed extensively under both treatment and control conditions, a point that will be covered briefly in the Discussion below

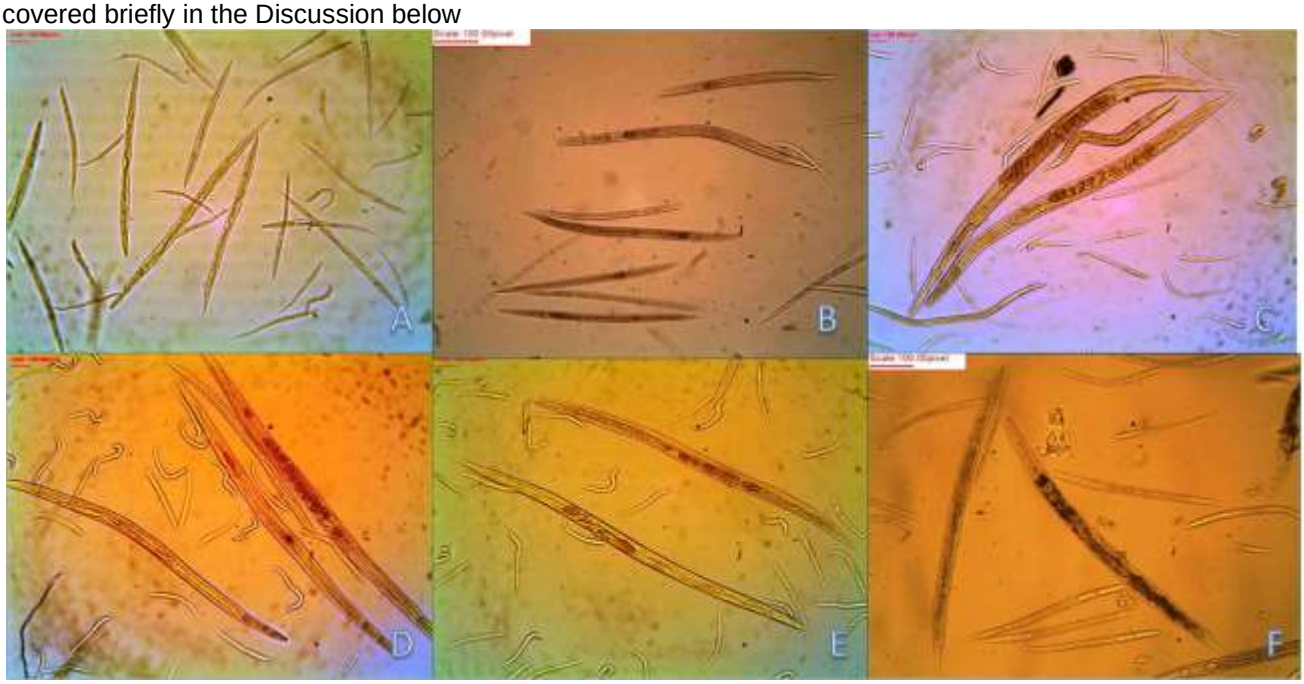
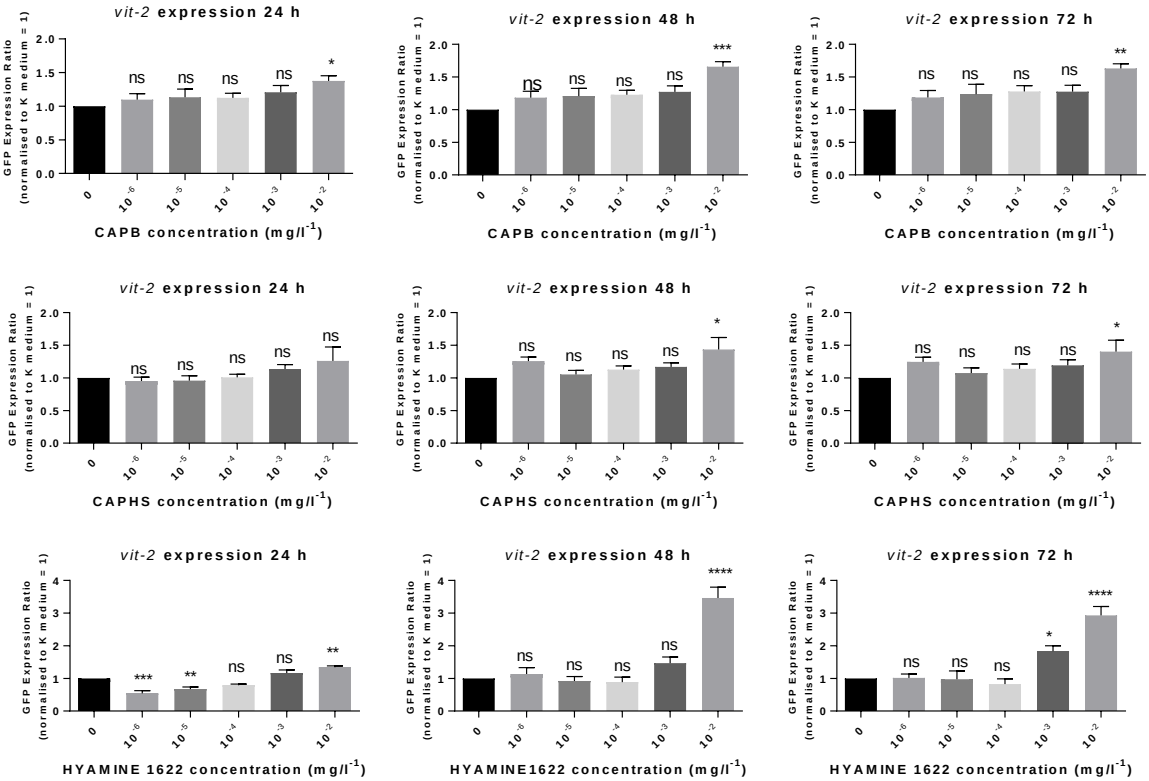


Figure 2. Appearance of eggs and larvae after 96 h in synchronized RT-130 populations.
A – Control: no eggs, only adults and late larvae. **B** – Adults exposed to 10^{-6} mg.l^{-1} EE_2: no eggs, adults larger and fatter than controls. **C -** BPA 10^{-5} mg.l^{-1}: adults with abundant eggs in gonad, plus hatched L1

larvae; **D -** CAPB 10^{-2} mg.l^{-1}: presence of eggs and L1 larvae, as in **C**. **E**- CAPHS 10^{-2} mg.l^{-1}**:** presence of eggs and L1 larvae; **F** – 10^{-3} mg.l^{-1} Hyamine 1622: fewer eggs in gonad, apparent disruption of vulva, but some larvae at larger L2 stage. Photographs taken with HiROCAM (High Resolution Optics Camera), using TS View Digital imaging software;

This impression of increased growth (Figure 2) was reinforced by length measurements in cultures of synchronized RT-130 worms. We observed apparent promotion of growth by surfactants and by EDCs at certain concentrations over a 72 h period (prior to the appearance of eggs or hatched larve), using the same cultures shown in Figure 2. Treated worms reached mean lengths of 500-600 µm as compared to only 400 µm for controls. Although these length differences between treated and control worms were highly significant ($p < 0.0001$; unpaired Student t test) for all treatments shown in Figure 2, they were based on limited length data (n = 15 per condition) and hence are not presented here.

The nematode *Caenorhabditis elegans* contains a small family of vitellogenin genes that are expressed abundantly, but only in the intestine of adult hermaphrodite worms (Spieth et al., 1985). Both BPA and EE_2 can induce vitellogenin synthesis in the germ cells of *C. elegans* (Hoshi et al., 2003), and vitellogenesis is regulated both transcriptionally and postranscriptionally in response to environmental and reproductive cues (Depina et al., 2011). We therefore examined the effects of these EDCs and of surfactants on vitellogenin expression in transgenic RT-130 (*vit*-2::GFP) worms, as shown in Figure 3. After 24 h, *vit*-2 expression was enhanced only at the highest test concentrations (10^{-2} mg.l^{-1}) of CAPB ($p < 0.05$) and Hyamine 1622 ($p < 0.01$), whereas EE_2 significantly reduced *vit*-2 expression at most test concentrations ($p = 0.001$ to 0.0001). After 48 h, *vit*-2 expression was significantly raised at the highest concentrations (10^{-2} mg.l^{-1}) of CAPB ($p < 0.001$), CAPHS ($p < 0.05$) and Hyamine 1622 ($p < 0.0001$), also by BPA at all test concentrations ($p < 0.0001$) and by EE_2 at concentrations of 10^{-4} mg.l^{-1} and above. Finally, at 72 h, the picture was basiclly unchanged for CAPB and CAPHS, and there was significant upregulation of *vit*-2 expression by Hyamine 1622 at 10^{-3} mg.l^{-1} ($p < 0.05$) as well as 10^{-2} mg.l^{-1} ($p < 0.001$). For BPA, the increase in *vit*-2 expression was clearly dose dependent ($p < 0.0001$; non-significant at 10^{-7} mg.l^{-1}), whereas EE_2 induced *vit*-2 only marginally at 10^{-3} mg.l^{-1} ($p < 0.05$) but strongly at 10^{-2} mg.l^{-1} ($p < 0.0001$). Increased *vit-2*::GFP expression induced by surfactants or EDCs up to 72 h (Figure 3) could account in part for the more rapid appearance of eggs and hatched larvae after 96 h (Figure 2).



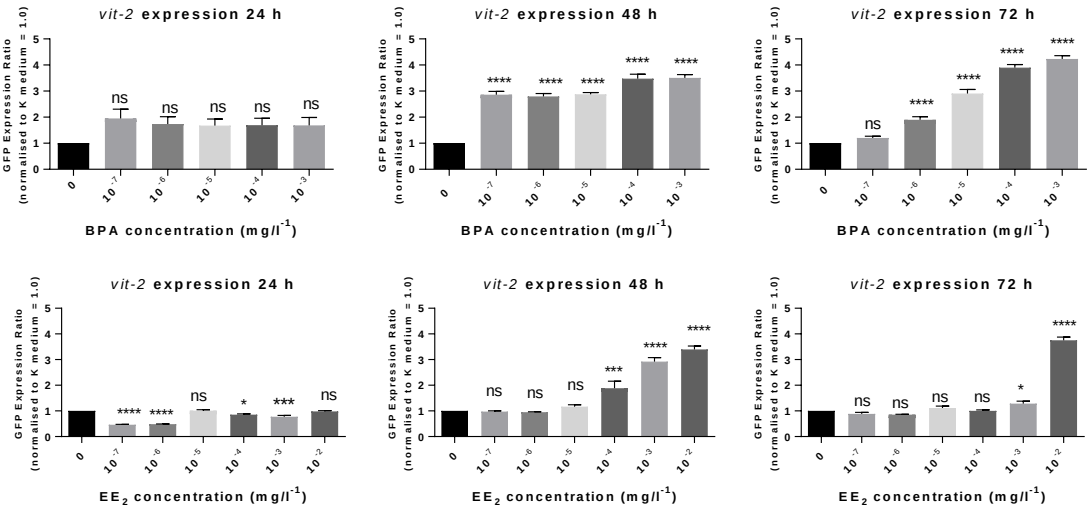
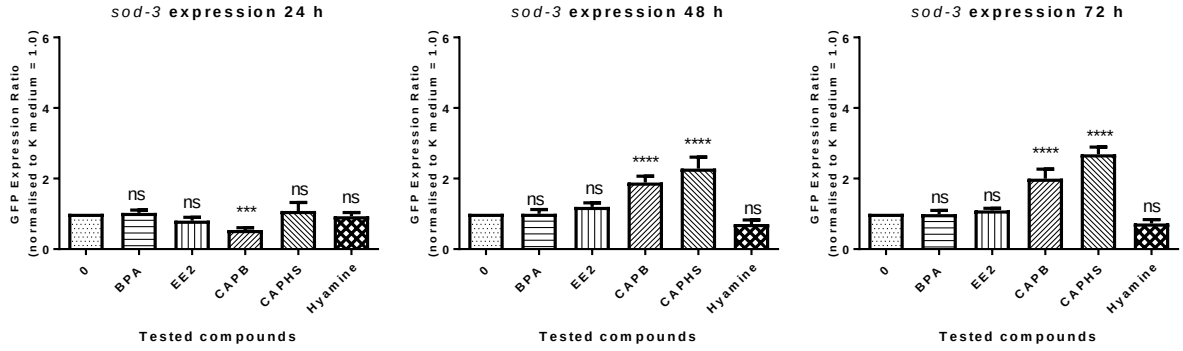


Figure 3. Induction of *vit*-2 gene expression by surfactants and EDCs. RT130 *vit-2*::GFP worms were exposed to surfactants (top 3 rows) or EDCs (bottom 2 rows) for 72 h at 20°C, and GFP fluorescence measured as described in Methods. Untreated controls (ER = 1) are shown as black bars on left of each panel, with test concentrations increasing from left to right. Significant differences from controls (ER = 1) are shown by asterisks: * = p<0.05; ** = p<0.01; *** = p<0.001; **** = p<0.0001; ns = not significant.

Synchronized cultures of 3 stress-responsive transgenic GFP reporter strains (CF1553, *sod*-3::GFP; BC20333, *sod*-4::GFP; and BC20316, *gst*-1::GFP) were exposed to the highest test concentrations of each surfactant and of both EDCs for up to 72 h at 20°C, with GFP measurements every 24 h (Figure 4). Two of these genes are involved in dealing with superoxide radicals during oxidative stress (the mitochondrial *sod*-3 and extracellular *sod*-4 superoxide dismutases), and one functions in phase II detoxification (the *gst*-1 glutathione-S-transferase). Expression of *sod*-3 was essentially unchanged at 24 h, apart from a significant decrease ($p < 0.001$) for CAPB. After 48 and 72 h, significant induction was observed for CAPB and CAPHS ($p < 0.0001$), but not for the other agents tested. For *sod*-4, both BPA and EE_2 significantly up-regulated expression after 24 h ($p < 0.0001$), and this remained the case for EE_2 at 48 and 72 h – with even higher levels of expression – but not for BPA, where GFP fluorescence fell back to control levels. For the surfactants, both CAPB and CAPHS induced *sod*-4 slightly after 24 h ($p < 0.05$) and more significantly after 48 and 72 h ($p = 0.01$ to 0.0001). As for *gst*-1, expression was increased significantly after 24 h ($p = 0.001$ to 0.0001) by BPA, CAPHS and Hyamine 1622 (the only effect recorded for this agent), and by EE_2, BPA (not at 48 h), CAPB and CAPHS after 48 and 72 h. Despite its marked effects on *vit*-2 expression (Figure 3) and on the appearance of eggs and larvae (Figure 2), Hyamine 1622 had the least effect of all our test agents on stress-reporter gene expression (Figure 4).



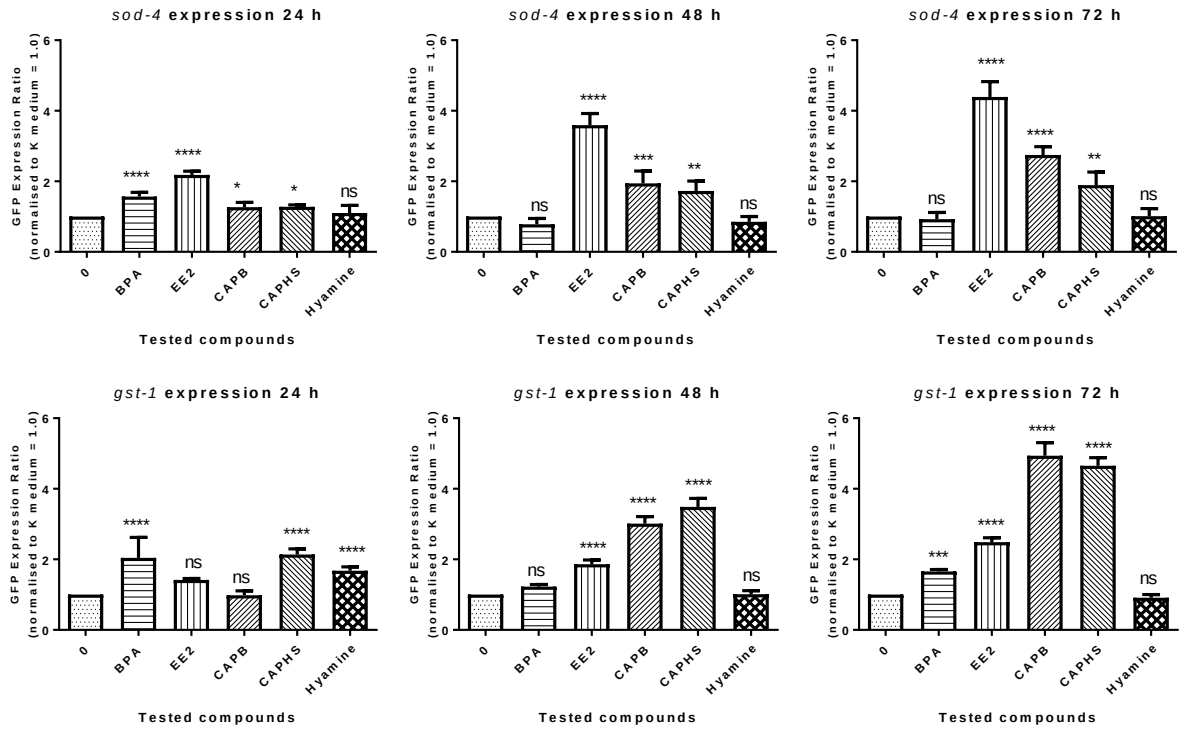


Figure 4 Oxidative stress and phase II genes induced by detergents and EDCs. CF1553 (*sod-3*::GFP), BC20333 (*sod-4*::GFP) and BC20316 (*gst-1*::GFP) worms were exposed to intermediate concentrations of EDCs (BPA 10^{-5} mg.l^{-1}, EE_2 10^{-6} mg.l^{-1}), or high concentrations of surfactants (CAPB and CAPHS 10^{-2} mg.l^{-1}; Hyamine 1622 10^{-3} mg.l^{-1}) for 24, 48 or 72 h at 20°C, and GFP fluorescence measured as described in Methods. Speckled bars show untreated controls on the left of each panel. Top row *sod-3*::GFP expression; middle row, *sod-4*::GFP; bottom row, *gst-1*::GFP. Exposure time increases from left to right . Significant differences from controls (ER = 1) are shown by asterisks: * = p<0.05; ** = p<0.01; *** = p<0.001; **** = p<0.0001; ns = not significant.

Sexual dimorphism in *C. elegans* is most apparent in the tail region, where XX hermaphrodites narrow to a sharp point (Figure 5A), whereas XO males possess a distinctive blunt-pointed fan supported by rays and distinctive spicules used during copulation (Figure 5C). Unfortunately, males are very rare (about 0.2%) in populations of wild-type *C. elegans*, and our attempts to increase the prevalence of males in cultures of RT-130 worms – either through heat-shock or by outcrossing with various *him* strain males – were unsuccessful. It must be stressed that these images are at best indicative, and that the numbers of males observed were to low to ascribe any statistical significance. For the same reason, we were unable to determine whether EDCs or surfactants could induce *vit-2* expression in the intestine or gonads of male worms. Previous studies with EDCs have shown disruption of male tail morphology, and this is confirmed by Figure 5B in the case of 10^{-3} mg.l^{-1} BPA (essentially an intersex tail, with a hermaphrodite point and partial male fan), and also by the subtly altered male tail morphology resulting from treatment with lower concentrations of BPA (10^{-5} mg.l^{-1}; Figure 5D) or EE_2 (10^{-2} mg.l^{-1} and 10^{-5} mg.l^{-1}; Figures 5E and I). Given the induction of *vit*-2::GFP expression by surfactants (above) as well as EDCs, it is natural to ask whether the former could also cause feminization of male worms. Figures 5F (CAPB, 10^{-2} mg.l). 5G (CAPHS, 10^{-2} mg.l^{-1}) and 5H (Hyamine 1622, 10^{-3} mg.l^{-1}) all suggest mild disruption of male tail anatomy, with altered spicules, missing or thinner rays, and a more rounded fan. Though not definitive, these changes are suggestive of a subtile feminizing effect of these surfactants on male *C. elegans*, similar to that caused by EDCs.

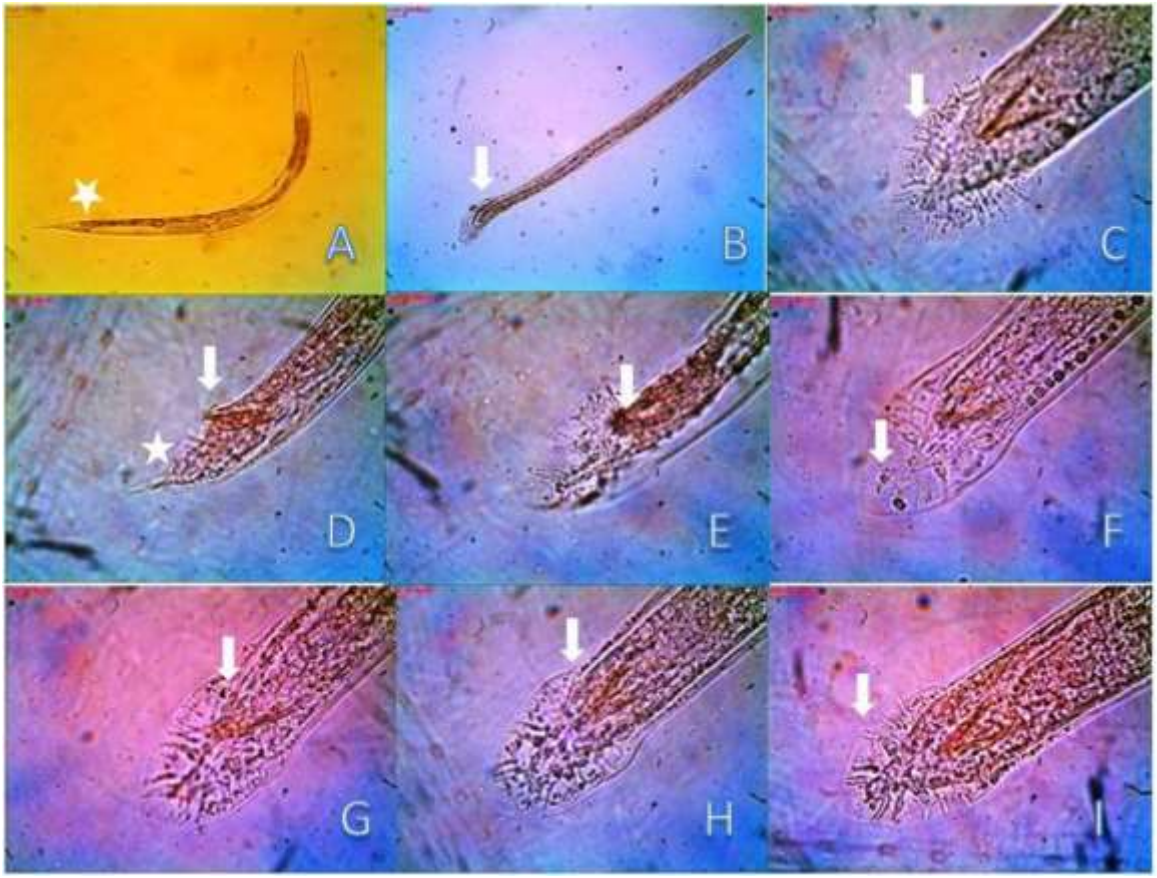


Figure 5. Altered tail anatomy in RT130 worms after exposure to EDCs or surfactants. RT130 worms were exposed to surfactants or EDCs for 96 h and rare males examined for abnormal tails using HiROCAM and TS View Digital imaging software. **A,** normal adult hermaphrodite tail (star); **B,** modified tail of male worm treated with 10^{-3} mg.l^{-1} BPA (arrow); **C,** normal male tail of a control adult with distinctive sensory rays and copulatory aparatus (spicules, arrow); **D,** disruption of adult male tail after treatment with 10^{-5} mg.l^{-1} BPA – the tail extremity is heprmaphrodite-like (star), with altered spicule (arrow); **E, a**dult male treated with 10^{-2} mg.l^{-1} EE_2 showing altered spicule (arrow); **F,** adult male treated with 10^{-2} mg.l^{-1} CAPB shows fine tail rays with some missing (arrow) and altered spicule morphology; **G,** adult male treated with 10^{-2} mg.l^{-1} CAPHS showing rounded fan, absence of some sensory rays, and an overgrown spicule (arrow); **H,** adult male treated with Hyamine 1622 10^{-3} mg.l^{-1} showing rounded fan; and altered spicule (arrow); **I a**dult male treated with **EE_2** 10^{-5} mg.l^{-1} showing fine rays (arrow) and rounded fan.

DISCUSSIONS

C. elegans feeding behavior is regulated by extrinsic and intrinsic cues such as serotonergic fat regulation and lipid metabolism (Srinivasan et al., 2008). Environmental conditions modulate energy balance in *C. elegans* through the *daf-7*/TGF-β signaling system that regulates fat metabolism and feeding behaviour, involving a compact neural circuit that allows for integration of multiple inputs and the flexibility for differential regulation of outputs(Greer, Pérez, Van Gilst, Lee, & Ashrafi, 2008). Metabotropic glutamate receptors modulate one such pharyngeal microcircuit controlling pharyngeal muscles and feeding (Dillon et al., 2015). Toxicants present in the fluid bathing worms are able to influence feeding activity through these neural circuits. However, it is important to assess worm mortality at the end of any feeding inhibition assay, as dead or moribund worms do not consume food bacteria, leading to artefactually higher optical densities. Although many panels in Figure 1 appear to show classic dose-responsiveness with increasing concentrations of surfactants, our observations suggest that worm mortality was a major factor at the highest test concentrations, especially over longer time-periods.

This confounding factor may help explain why surviving worms were able to grow and mature faster at intermediate concentrations of all 3 surfactants and both EDCs (Figure 2), even though less food was consumed overall (Figure 1). Also noted in surfactant- and EDC-treated cultures were numerous 'bagged' worms, where larvae hatch inside a dead or moribund adult, often induced by bacterial toxicity (Mosser, Matic, & Leroy, 2011). The adaptive value of bagging in the life history of *C. elegans* is to prevent starvation of the larvae by providing nutrition from the dead adult (Chen & Caswell-Chen, 2003). The

increasing frequency of this phenotype is time-dependent in the case of controls and both dose- and time-dependent in the presence of surfactants. The highest test concentrations markedly affected worm movement, feeding, morphology and behavior. Young larvae were particularly susceptible to these high concentrations during early exposure, whereas bagging larvae might be able to survive much longer through resistance and adaptation mechanisms. During the first 18 hours of exposure at high test concentrations, adults aften appeared paralyzed (rare twitching suggested they were still alive), with hypertrophied eggs and a loss of hydrostatic pressure. No pharyngeal pumping activity was observed in such adults, which may in part account for the high level of feeding inhibition observed (Figure 1). Preliminary length measurements (data not shown) suggest that both surfactants and EDCs can also enhance growth, producing eggs and larvae precociously as compared to untreated controls (Figure 2). Similar results have previously been reported for cultures of *C. elegans* treated with 10 $mg.l^{-1}$ of several surfactants (Pluronics F-68, F-127, F-38, L-35; Tween-20 and Triton X-100), all of which caused significant stimulation of growth over 3 days (Mutwakil, Steele, Lowe, & dePomerai, 1997). Our data suggest that surviving worms respond to surfactant stress by accelerating growth, oogenesis and reproduction, possibly because surfactants act as estrogen mimics (like BPA and EE_2). A likely corollary of this is precocious vitellogenin synthesis, needed to produce yolk for the eggs.

This prediction is vindicated by the *vit*-2::GFP expression data shown in Figure 3, which clearly demonstrate that all five test agents upregulate vitellogenin production, though the effects of CAPB and CAPHS are rather small and confined to the highest test concentration (10^{-2} $mg.l^{-1}$). As might be expected, the strongest effects are seen with the two EDCs (BPA and EE_2), with Hyamine 1622 showing intermediate effects. The precise mechanisms linking endocrine hormonal signals (probably steroids derived from cholesterol) with vitellogenin gene transcription and translation remain to be clarified, but previous reports show that a variety of EDCs cause multiple effects on the physiology of *C. elegans*, including upregulation of vitellogenin gene expression (reviewed by Hoss & Weltje in 2007). Oxidative stress is associated with age-related decline of biological functions; reactive oxygen species (ROS) often produce harmful effects on cells or organisms, but recent studies have shown that they are also important in regulating lifespan and other biological processes (Sasakura et al., 2017a, 2017b). Superoxide dismutases (SOD) are enzymes that catalytically remove the superoxide radical ($*O_2^-$; a key ROS) and protect organisms from oxidative damage during normal aging. SOD-3 is a mitochondrial superoxide dismutase, and its expression is induced in intestinal tissue by the DAF-16 transcription factor (which plays a central role in ageing in *C. elegans*), e.g. following exposure to pathogenic bacteria (Chávez, Mohri-Shiomi, Maadani, Vega, & Garsin, 2007) . Xenobiotic stress responses involve various combinations of genes from several large families, including 80 phase I cytochrome P450 (*cyp*) genes and 40 phase II glutathione-S-transferase (*gst*) genes.

Mutations in the *C. elegans m*ale-*ab*normal gene *mab*-3 cause aberrant male tail morphology (affecting mainly the V ray cell lineages) and upregulate vitellogenin expression in the male intestine (where the *vit* genes are normally repressed), but have no obvious effect in hermaphrodites. The *C. elegans mab*-3 gene is itself negatively regulated by the zinc-finger transcription factor TRA-1A, encoded by the *tra*-1 sex-determination gene – which is active in hermaphrodites but not in males (Yi, Ross, & Zarkower, 2000). The *mab*-3 gene is therefore expressed in males only, where it acts as a DM-class (*doublesex*/*mab*-3) transcription factor to repress the expression of vitellogenin genes in the intestine (Shen & Hodgkin, 1988; Yi et al., 2000). This provides a direct molecular link between sex-specific differentiation (e.g. of male tail structures; Figure 5C) and vitellogenin regulation in the intestine of L4 and adult worms (Figure 3).
A crucial question to be addressed in future studies of surfactant and EDC effects on RT-130 worms is whether these agents can induce *vit-2*::GFP expression specifically in intestine of XO males. This experiment will require populations of RT-130 worms greatly enriched in males, which unfortunately we were unable to achieve during the course of this study, despite attempting both heat-shock and outcrossing with various *him* strain males. If *C. elegans vit*-2 (or other *vit*) genes are indeed inducible by surfactants in males, then this would establish these widely used chemicals as *bona fide* EDCs (albeit much weaker than BPA or EE_2), and should properly lead to establishment of strict environmental emission limits comparable to those already in force for known EDCs.

AKNOWLEDGEMENTS

This study was funded by the University of Bucharest, through a 6-month EU-funded ERASMUS-program scholarship, enabling MC to undertake research for a dissertation thesis in the laboratory of DdeP in the School of Life Science at the University of Nottingham (UK). Many thanks to Declan Brady and Dr Andrew

Phiri for help in culture maintenance and laboratory practice, to Damilia Moise for picture editing, and to Dr Stefania Ivan Gheorghe for supplying the surfactants.

Compliance with Ethical Standards:
Author Marius Cicirma declares that he has no conflict of interest. Author David de Pomerai declares that he has no conflict of interest.
Ethical approval : All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.
Ethical approval: This article does not contain any studies with human participants performed by any of the authors.

REFERENCES

Abel, P. D., 1974. Toxicity of synthetic detergents to fish and aquatic invertebrates. *Journal of Fish Biology*, *6*(3), 279–298. https://doi.org/10.1111/j.1095-8649.1974.tb04545.x

Allard, P., & Colaiácovo, M. P., 2011. Mechanistic insights into the action of Bisphenol A on the germline using C. elegans. *Cell Cycle*, *10*(2), 183–184. https://doi.org/10.4161/cc.10.2.14478

Anbalagan, C., Lafayette, I., Antoniou-Kourounioti, M., Gutierrez, C., Martin, J. R., Chowdhuri, D. K., & De Pomerai, D. I., 2013. Use of transgenic GFP reporter strains of the nematode Caenorhabditis elegans to investigate the patterns of stress responses induced by pesticides and by organic extracts from agricultural soils. *Ecotoxicology*, *22*(1), 72–85. https://doi.org/10.1007/s10646-012-1004-2

Anbalagan, C., Lafayette, I., Antoniou-Kourounioti, M., Haque, M., King, J., Johnsen, B., Pomerai, D. De., 2012. Transgenic nematodes as biosensors for metal stress in soil pore water samples. *Ecotoxicology*, *21*(2), 439–455. https://doi.org/10.1007/s10646-011-0804-0

Aris, A. Z., Shamsuddin, A. S., & Praveena, S. M., 2014. Occurrence of 17α-ethynylestradiol (EE2) in the environment and effect on exposed biota: A review. *Environment International*, Vol. 69, pp. 104–119. https://doi.org/10.1016/j.envint.2014.04.011

Bhandari, R. K., Deem, S. L., Holliday, D. K., Jandegian, C. M., Kassotis, C. D., Nagel, S. C., Rosenfeld, C. S., 2015. Effects of the environmental estrogenic contaminants bisphenol A and 17α-ethinyl estradiol on sexual development and adult behaviors in aquatic wildlife species. *General and Comparative Endocrinology*, Vol. 214, pp. 195–219. https://doi.org/10.1016/j.ygcen.2014.09.014

Brouard, V., Guénon, I., Bouraima-Lelong, H., & Delalande, C., 2016. Differential effects of bisphenol A and estradiol on rat spermatogenesis' establishment. *Reproductive Toxicology*, *63*, 49–61. https://doi.org/10.1016/j.reprotox.2016.05.003

Chávez, V., Mohri-Shiomi, A., Maadani, A., Vega, L. A., & Garsin, D. A., 2007. Oxidative stress enzymes are required for DAF-16-mediated immunity due to generation of reactive oxygen species by Caenorhabditis elegans. *Genetics*, *176*(3), 1567–1577. https://doi.org/10.1534/genetics.107.072587

Chen, J., & Caswell-Chen, E. P., 2003. Bagging as a part of the C. elegans life cycle. *International Worm Meeting*. Retrieved from http://www.wormbase.org/db/misc/paper?name=WBPaper00019121

De Pomerai, D., Anbalagan, C., Lafayette, I., Rajagopalan, D., Loose, M., Haque, M., & King, J., 2010. High-throughput analysis of multiple stress pathways using GFP reporters in C. elegans. *WIT Transactions on Ecology and the Environment*, *132*, 177–187. https://doi.org/10.2495/ETOX100171

Depina, A. S., Iser, W. B., Park, S. S., Maudsley, S., Wilson, M. A., & Wolkow, C. A., 2011. Regulation of Caenorhabditis elegans vitellogenesis by DAF-2/IIS through separable transcriptional and posttranscriptional mechanisms. *BMC Physiology*, *11*(1). https://doi.org/10.1186/1472-6793-11-11

Dillon, J., Franks, C. J., Murray, C., Edwards, R. J., Calahorro, F., Ishihara, T., O'Connor, V., 2015. Metabotropic glutamate receptors: Modulators of context-dependent feeding behaviour in C. elegans. *Journal of Biological Chemistry*, *290*(24), 15052–15065. https://doi.org/10.1074/jbc.M114.606608

Greer, E. R., Pérez, C. L., Van Gilst, M. R., Lee, B. H., & Ashrafi, K., 2008. Neural and Molecular Dissection of a C. elegans Sensory Circuit that Regulates Fat and Feeding. *Cell Metabolism*, *8*(2), 118–131. https://doi.org/10.1016/j.cmet.2008.06.005

Guven, K., Duce, J. A., & de Pomerai, D. I., 1994. Evaluation of a stress-inducible transgenic nematode strain for rapid aquatic toxicity testing. *Aquatic Toxicology*, *29*(1–2), 119–137. https://doi.org/10.1016/0166-445X(94)90052-3

Hoeman, K. W., & Culbertson, C. T., 2008. A novel, environmentally friendly sodium lauryl ether sulfate-, cocamidopropyl betaine-, cocamide monoethanolamine-containing buffer for MEKC on microfluidic devices. *Electrophoresis*, *29*(24), 4900–4905. https://doi.org/10.1002/elps.200800463

Hoshi, H., Kamata, Y., & Uemura, T., 2003. Effects of 17beta-estradiol, bisphenol A and tributyltin chloride

on germ cells of Caenorhabditis elegans. *The Journal of Veterinary Medical Science / the Japanese Society of Veterinary Science*, *65*(8), 881–885. https://doi.org/10.1292/jvms.65.881

Höss, S., & Weltje, L., 2007. Endocrine disruption in nematodes: Effects and mechanisms. *Ecotoxicology*, Vol. 16, pp. 15–28. https://doi.org/10.1007/s10646-006-0108-y

Howdeshell, K. L., & Vom Saal, F. S., 2000. Developmental exposure to bisphenol A: Interaction with endogenous estradiol during pregnancy in mice. *American Zoologist*, *40*(3), 429–437. https://doi.org/10.1093/icb/40.3.429

Hunt-Newbury R, Viveiros R., Johnsen R., Mah A., Anastas D., Fang L., Halfnight E., Lee D., Lin J., Lorch A., McKay S., Okada H. M., Pan J., Schulz A. K, Tu D., Wong K., Zhao Z, Alexeyenko A., Burglin T., Sonnhammer E., Schnabel R., Jones S. J, Marra M. A, Baillie D.L, Moerman D.G., 2007. High-Throughput In Vivo Analysis of Gene Expression in *Caenorhabditis elegans. PLoS Biol*, 5(9):237

Hunt, P. R., 2016. The C. elegans model in toxicity testing. *Journal of Applied Toxicology*, n/a-n/a. https://doi.org/10.1002/jat.3357

Ivanković, T., & Hrenović, J., 2010. Surfactants in the environment. *Arhiv Za Higijenu Rada i Toksikologiju*, Vol. 61, pp. 95–110. https://doi.org/10.2478/10004-1254-61-2010-1943

Jones, D., & Candido, E. P. M., 1999. Feeding is inhibited by sublethal concentrations of toxicants and by heat stress in the nematode Caenorhabditis elegans: Relationship to the cellular stress response. *Journal of Experimental Zoology*, *284*(2), 147–157. https://doi.org/10.1002/(SICI)1097-010X(19990701)284:2<147::AID-JEZ4>3.0.CO;2-Z

Kitamura, S., Suzuki, T., Sanoh, S., Kohta, R., Jinno, N., Sugihara, K., Ohta, S., 2005. Comparative study of the endocrine-disrupting activity of bisphenol A and 19 related compounds. *Toxicological Sciences*, *84*(2), 249–259. https://doi.org/10.1093/toxsci/kfi074

Leung, M. C. K., Williams, P. L., Benedetto, A., Au, C., Helmcke, K. J., Aschner, M., & Meyer, J. N., 2008. Caenorhabditis elegans: An emerging model in biomedical and environmental toxicology. *Toxicological Sciences*, Vol. 106, pp. 5–28. https://doi.org/10.1093/toxsci/kfn121

Mosser, T., Matic, I., & Leroy, M., 2011. Bacterium-induced internal egg hatching frequency is predictive of life span in *Caenorhabditis elegans* populations. *Applied and Environmental Microbiology*, *77*(22), 8189–8192. https://doi.org/10.1128/AEM.06357-11

Mutwakil, M., Steele, T. J. G., Lowe, K. C., & dePomerai, D. I., 1997. Surfactant stimulation of growth in the nematode Caenorhabditis elegans. *Enzyme and Microbial Technology*, *20*(96), 462-470 ST-Surfactant stimulation of growth in.

Ohtake, F., Takeyama, K. ichi, Matsumoto, T., Kitagawa, H., Yamamoto, Y., Nohara, K., Kato, S., 2003. Modulation of oestrogen receptor signalling by association with the activated dioxin receptor. *Nature*, *423*(6939), 545–550. https://doi.org/10.1038/nature01606

Quesada, I., Fuentes, E., Viso-León, M. C., Soria, B., Ripoll, C., & Nadal, A., 2002. Low doses of the endocrine disruptor bisphenol-A and the native hormone 17beta-estradiol rapidly activate transcription factor CREB. *The FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *16*(12), 1671–1673. https://doi.org/10.1096/fj.02-0313fje

Rochester, J. R., & Bolden, A. L., 2015. Bisphenol S and F: A systematic review and comparison of the hormonal activity of bisphenol a substitutes. *Environmental Health Perspectives*, Vol. 123, pp. 643–650. https://doi.org/10.1289/ehp.1408989

Sasakura, H., Moribe, H., Nakano, M., Ikemoto, K., Takeuchi, K., & Mori, I., 2017a. Lifespan extension by peroxidase/dual oxidase-mediated ROS signaling through pyrroloquinoline quinone in C. elegans. *Journal of Cell Science*, (July), jcs.202119. https://doi.org/10.1242/jcs.202119

Sasakura, H., Moribe, H., Nakano, M., Ikemoto, K., Takeuchi, K., & Mori, I., 2017b. Lifespan extension by peroxidase and dual oxidase-mediated ROS signaling through pyrroloquinoline quinone in *C. elegans*. *Journal of Cell Science*, *130*(15), 2631–2643. https://doi.org/10.1242/jcs.202119

Shen, M. M., & Hodgkin, J., 1988. mab-3, a gene required for sex-specific yolk protein expression and a male-specific lineage in C. elegans. *Cell*, *54*(7), 1019–1031. https://doi.org/10.1016/0092-8674(88)90117-1

Spieth, J., Denison, K., Kirtland, S., Cane, J., & Blumenthal, T., 1985. The C. elegans vitellogenin genes: Short sequence repeats in the promoter regions and homology to the vertebrate genes. *Nucleic Acids Research*, *13*(14), 5283–5295. https://doi.org/10.1093/nar/13.14.5283

Srinivasan, S., Sadegh, L., Elle, I. C., Christensen, A. G. L., Faergeman, N. J., & Ashrafi, K., 2008. Serotonin Regulates C. elegans Fat and Feeding through Independent Molecular Mechanisms. *Cell Metabolism*, *7*(6), 533–544. https://doi.org/10.1016/j.cmet.2008.04.012

Staszak, K., Wieczorek, D., & Michocka, K., 2015. Effect of sodium chloride on the surface and wetting properties of aqueous solutions of cocamidopropyl betaine. *Journal of Surfactants and Detergents*, *18*(2). https://doi.org/10.1007/s11743-014-1644-8

Williams, P. L., & Dusenbery, D. B., 1990. Aquatic toxicity testing using the nematode, Caenorhabditis

elegans. *Environmental Toxicology and Chemistry*, *9*(10), 1285–1290. https://doi.org/10.1002/etc.5620091007

Yi, W., Ross, J. M., & Zarkower, D., 2000. mab-3 is a direct tra-1 target gene regulating diverse aspects of C. elegans male sexual development and behavior. *Development*, *127*(20), 4469–4480.

Zhou, D., Yang, J., Li, H., Cui, C., Yu, Y., Liu, Y., & Lin, K., 2016. The chronic toxicity of bisphenol A to Caenorhabditis elegans after long-term exposure at environmentally relevant concentrations. *Chemosphere*, *154*, 546–551. https://doi.org/10.1016/j.chemosphere.2016.04.011

Received: 17.12.2019
Revised: 05.02.2020