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BIOCONCENTRATION AND DEPURATION OF ^{14}C-LABELED 17α-ETHINYL ESTRADIOL AND 4-NONYLPHENOL IN INDIVIDUAL ORGANS OF THE MARINE BIVALVE *MYTILUS EDULIS* L.

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Abstract
Endocrine-disrupting compounds (EDCs), including 17α-ethinyl estradiol (EE2) and 4-nonylphenol (4-NP), enter coastal environments primarily in effluents of wastewater treatment facilities and have become ubiquitous in marine surface waters, sediments, and biota. Although EE2 and 4-NP have been detected in marine shellfish, the kinetics of bioconcentration and their tissue distribution have not been thoroughly investigated. The authors performed bioconcentration and depuration experiments in the blue mussel, *Mytilus edulis*, with 3.37 nM EE2 (0.999 μg/L) and 454 nM 4-NP (100.138 μg/L). Mussels and seawater were sampled throughout a 38-d exposure and a 35-d depuration period, and 6 tissues were individually assayed. Uptake of EE2 and 4-NP was curvilinear throughout exposure and followed a similar uptake pattern: digestive gland > gill ≥ remaining viscera > gonad > adductor > plasma. Depuration varied, however, with half-lives ranging from 2.7 d (plasma) to 92 d (gill) for EE2 and 15 d (plasma) to 57 d (gill) for 4-NP. An innovative modeling approach, with 3 coupled mathematical models, was developed to differentiate the unique roles of the gill and plasma in distributing the EDCs to internal tissues. Plasma appears pivotal in regulating EDC uptake and depuration within the whole mussel.
Keywords: Uptake, Depuration, Endocrine-disrupting compound (EDC), Mussel tissue, Organ
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INTRODUCTION

In 1999, the US Geological Survey (USGS) revealed that US rivers and drinking water supplies are contaminated with numerous pharmaceuticals and commonly used organic chemicals, several of which are endocrine-disrupting compounds (EDCs) [1]. The presence of EDCs is alarming because minute concentrations of these chemicals can interfere with the endocrine system of humans and other animals, adversely affecting development, reproduction, and incidence of cancer [2,3] and possibly resulting in population-level and ecosystem-level effects [4]. These chemicals enter the marine environment through several routes. Some are excreted by humans after taking medications (e.g., birth control compounds including 17α-ethinyl estradiol [EE2]), and others are found in commercial detergents (e.g., 4-nonylphenol [4-NP]). Thus, it is not surprising that relatively high concentrations are present in the effluent from wastewater treatment facilities [5].

Since the USGS report, focused surveys conducted throughout the United States, Europe, South America, and Asia have revealed that EDC contamination is widespread in marine waters

and sediments [6,7]. Concentrations have been reported as high as 34 ng/L in water and more than 100 ng/g dry weight in sediments for EE2 [8], and 2.5 □g/L in water and more than 20 000 ng/g dry weight in sediments for 4-NP [7]. Concentrations of EDCs in bivalves are widely variable (e.g., 4-NP ranged from 1.5 ng/g to 5190 ng/g dry wt, depending on the geography and proximity to urban areas [7]). In addition, 4-NP has been detected in shellfish meat in Italy [9], Germany [10], and Taiwan [11]. In those studies, dietary intake of 4-NP contaminated shellfish was shown to be a significant source of 4-NP exposure to humans, contributing up to 44% of total 4-NP exposure. These studies clearly indicate that significant bioconcentration of EDCs occurs in marine bivalves, posing risks to marine life and possibly to humans through shellfish consumption.

Of the many marine organisms potentially exposed to EDCs, bivalve mollusks are known to be sensitive. Estrogen and estrogen mimics (e.g., EE2) have caused reproductive and developmental delays as well as the formation of ovo-testis and skewed sex ratios in several species of bivalves [12,13]. In a series of studies by Nice et al., exposure to 1 □g/L and 100 □g/L 4-NP was found to cause developmental impairments and reduced fecundity in the Pacific oyster *Crassostrea gigas* [14,15]. In the blue mussel, *Mytilus edulis*, exposure to 5 ng/L to 50 ng/L EE2 or estradiol resulted in altered gene expression of egg yolk proteins (vitellogenin and vitelline), testis-specific kinase, and the putative mussel estrogen receptor genes, suggesting adverse impacts to reproductive physiology [16–18]. Field studies have suggested that 4-NP accumulation is associated with hastened gonadal maturation and development in mussels [19]. In addition to EDCs, these bivalves face multiple challenges, including climate change and pollution-related stressors. *Mytilus edulis* has already experienced climate-related contraction of its geographical range on the east coast of the United States [20]. Additional stress caused by EDCs may further threaten this species. Because of the economic and ecological importance of marine mussels, understanding the effects of endocrine-disrupting compounds on the reproductive health of these organisms is vital for the environmental protection of coastal communities.

Endocrine-disrupting compounds present an obstacle for effective monitoring programs because environmental concentrations are often below analytical detection limits (sub-ng/L levels) [12–17]. However, bivalves are able to bioconcentrate EDCs, offering a means for overcoming this limitation. They also integrate exposure over time, even when water concentrations are temporally variable. Nevertheless, the distribution of EE2 and 4-NP in individual organs of bivalves has not been examined, nor has tissue that bioconcentrates EE2 and 4-NP to the greatest extent been identified. Assaying this tissue in monitoring programs may be a far more sensitive tool than analyzing whole-body tissues. In addition, tracking the bioconcentration and depuration of EE2 and 4-NP in individual tissues would give us important insights into the internal processing and turnover rates of these compounds and help to identify potential target tissues for adverse effects. With these goals in mind, we set out to investigate, through experimentation and mathematical modeling, the uptake and depuration of EE2 and 4-NP within the major tissues of the blue mussel *M. edulis* L.

We chose EE2 and 4-NP as model estrogenic EDCs because of the pervasiveness of their use, their occurrence in marine environments worldwide, and their potency. An alkylphenol, 4-NP is a non-ionic surfactant produced commercially for use as a detergent [7,21]. In addition, other alkylphenol ethoxylate surfactants are degraded to 4-NP in wastewater treatment facilities, making 4-NP the most prominent alkylphenol found in sewage effluent and sludge [22]. 17α-Produced primarily as a human contraceptive, EE2 is the most potent synthetic estrogen [8,21].

Because of the frequent and high level of occurrence of 4-NP and the high potency of EE2, these 2 compounds often contribute a large percentage of the unnatural estrogenic activity (as measured by in vitro assays) detected in marine environments [5]. Each compound has distinctly different physical characteristics (Table 1), although their somewhat low octanol–water partition coefficient (K_{OW}) values would suggest relatively low bioconcentration factors (580 times for EE2, 2900 times for 4-nonylphenol) [23], in contrast to values reported in the field and in laboratory exposure studies (e.g., 1400–4400 times higher than water concentrations) [6,24].

MATERIALS AND METHODS

Mussel collection

More than 150 mussels, *M. edulis* (45.6–51.8 mm length), were collected at low tide in November 2013 from a wooden breakwater on Spectacle Island (Boston Harbor, MA, USA), returned to the laboratory, and cleaned of epibionts. Mussels were maintained in 5-μm filtered seawater, collected from Dorchester Bay, Boston Harbor, and acclimated at 11 °C, 35 practical salinity units (PSU), and a 10:14-h light:dark cycle for 12 d prior to starting the experiment (actual measured values: 11.2 ± 0.7 °C, 33 ± 1 PSU, 9.07 ± 0.45 mg/L dissolved oxygen, equivalent to >100% oxygen saturation; n = 45 measurements).

EE2 and 4-NP dosing

On the day of the first dosing, 50 mussels were apportioned to each of 3 19-L glass aquaria: EE2, 4-NP, and control. Each aquarium was filled with 15 L of 5-μm filtered seawater. To maintain a constant ratio of seawater to mussel (300 mL/mussel), the volume of seawater in each aquarium was adjusted as mussels were sampled during the ensuing 38-d exposure period but held constant (6 L) during the subsequent 35-d depuration.

During the exposure period, aquaria were spiked with stock solutions made from radiolabeled EE2 (17α-[4-^{14}C]ethinyl estradiol [ARC 1894-50 μCi] and 4-NP [ring-^{14}C(U)] [ARC 0900-50 μCi]; American Radiolabeled Chemicals). Primary stock solutions (as purchased) were diluted with ethanol and adjusted with cold compounds to obtain working stocks (specific activities of 55 nCi/nmol EE2 and 0.79 nCi/nmol 4-NP), which were directly spiked into the aquaria, bringing the final concentrations up to 3.37 nM EE2 (= 0.999 μg/L) and 454 nM 4-NP (= 100.138 μg/L). These chosen EE2 and 4-NP concentrations were relatively high compared with most levels measured environmentally because of the specific activity of the commercially available radiolabeled material and the need to have an acceptable radioactive count in our seawater and tissue samples. The control aquarium was spiked with a comparable volume of the ethanol carrier. Aquaria were fitted with airstones, and airtight plexiglass lids with exhaust lines connected to activated charcoal scrubbers.

During the acclimation, exposure, and depuration periods, aquarium water was changed at 3-d to 4-d intervals. Mussels were fed 2 h prior to the water change with *Isochrysis galbana* and *Thalassiosira weissflogii* (approximately 2.7×10^7 cells/mussel). Water quality was periodically monitored in the control (nonradioactive) tank: temperature and dissolved oxygen were monitored using a Yellow Spring Instruments model 55/12 FT portable dissolved oxygen meter, and salinity was monitored with a Milwaukee salinity refractometer. Parameters remained well within optimal range throughout the present study (temperature, 11.2 ± 0.7 °C; salinity, 33 ± 1 PSU; dissolved oxygen, 9.07 ± 0.47 mg/L or approximately 100% oxygen saturation; n = 45).

Mussel and seawater sampling

Prior to and after each water change at 3 d to 4 d, 1 mL of seawater was taken from each aquarium, in duplicate. Water samples were also collected directly before and after feeding to monitor possible changes in EDC concentration as a result of the algal food. Additional water

samples were taken periodically at shorter intervals to better determine the rate of loss of the radiolabeled compounds over the interval between water changes. Each 1-mL water sample was added to 10 mL of Hionic scintillation fluid (Perkin Elmer), stored in the dark overnight, and counted on a Perkin Elmer Tri-Carb 3110TR liquid scintillation counter (10-min counts, background-subtracted, corrected for color and quench). Analytical detection limits for seawater, plasma, and tissue digestates (3 Φ) were 0.024 pmoles of EE2 and 0.002 nmoles of 4-NP.

Four mussels were removed from each of the 3 aquaria on days 0, 3, 7, 10, 17, 24, 31 and 38 (exposure period) and on days 40, 43, 50, 57 and 73 (35-d depuration period). Each mussel was rinsed in nonlabeled seawater, its length was measured, and it was dissected into 6 tissues: cell-free blood plasma, posterior adductor muscle, gill, digestive gland, gonad, and remaining viscera. After cutting the posterior adductor muscle, mantle cavity seawater was drained and whole blood was obtained from the central extrapallial space and centrifuged (16 000 *g*, 3 min) to separate hemocytes and plasma. Plasma samples (1 mL) were added to 10 mL of Soluscint XR (National Diagnostics) and processed as described for the seawater samples. Gill, central mantle (primarily gonad), posterior adductor muscle, digestive gland, and remaining tissues were dissected, placed in preweighed 50-mL Falcon tubes, and frozen at –80 °C. The central mantle was primarily composed of gonadal tissue, so it is referred to as "gonad" in the present study.

Tissue solubilization and ^{14}C counting

Tissues were solubilized using Solusol (National Diagnostics). Because of the different masses of each tissue, different amounts of Solusol were added (300 µL for hemocytes, 1 mL for gill and posterior adductor muscles, 2 mL for mantle and digestive gland, 10 mL for remaining viscera). Various volumes of 15% benzoyl peroxide (1–2 mL/mL of Solusol) and distilled water were added to samples as necessary to decolorize and to resolubilize precipitated salts. The final clear digestates were diluted as necessary to reduce quenching, and portions were added to 10 mL of Soluscint XR (National Diagnostics). All of the tissue digestate solutions were counted for each tissue (none of the digestates were subsampled). Counts from multiple vials were combined to obtain the total disintegrations per minute per tissue. The disintegrations per minute values were converted to molar units based on the individual compound's specific activity (i.e., 55 nCi/nmol EE2; 0.792 nCi/nmol 4-NP) and then normalized to tissue wet weight.

Model development

Three coupled mathematical models were developed to quantify the changes in concentration of EDCs throughout the experiment in the seawater during exposure, the mussel tissues during exposure, and the mussel tissues during depuration.

Seawater model

Concentrations of EDCs in seawater are described by a 3-component model consisting of seawater concentration (C_{sw}) and 2 components acting as agents of change on the seawater concentration (C_{a1} and C_{a2}). The rate of change of the decreasing concentration of EDCs in the seawater is the result of the sum of the rates of increasing concentrations of EDCs in the agents of change. The rates of increase for the first agent and the second agent are µ and α, respectively. The results of this model provide a solution from which a time-integrated seawater concentration is derived. The solution allows for the interpolation of seawater concentrations throughout the course of the experiment and, thus, is an improvement over time-integrated values determined on the basis of data specifically collected at discrete points in time that may be infrequent and may contain high levels of uncertainty. The 3-component model consists of

$$\frac{dC_{sw}}{dt} = -\mu - \alpha \qquad (1)$$

$$\frac{dC_{a1}}{dt} = \mu \qquad (2)$$

$$\frac{dC_{a2}}{dgt} = \alpha \qquad (3)$$

where C_i is the concentration of EDC in component i = *sw*, *a*1, or *a*2, and t is time in days since each water change. Parameters μ and α are given by

$$\mu = A - BC_{a1}t \quad (4)$$

$$\alpha = Ce^{-Dt} \qquad (5)$$

where *A*, *B*, *C*, and *D* are parameters of this model that are fit to the seawater data. The best-fit parameters are determined by an algorithm that minimizes the root mean squared error of the solution to the above system of equations with respect to the measured values of the concentrations of the EDCs within the seawater.

The solution to the seawater model is determined using the fourth-order Runge-Kutta method (subroutine ode45 in Matlab R2013b). The solution is used to determine a single seawater concentration that best represents the exposure to the mussels. To determine this value, a time-averaged value of the seawater concentration of EDCs is calculated from the seawater function that is the solution to the seawater model: the function is numerically integrated over the duration of each spike cycle, then divided by the duration of the spike cycle. These time-integrated values are then utilized to obtain a representative seawater concentration, C_{sw}^{*}.

Mussel model: Uptake

Traditional kinetic models of uptake and depuration of compounds within an organism assume that the rate of change of the concentration within the organism is a function of 2 factors, the concentration of the compound in the medium in which the organism lives, and the concentration of the compound within the organism [25,26]. The functional form of the basic model is

$$\frac{d[x]_i}{dt} = k_{ui}[x]_{i-1} - k_{di}[x]_i \qquad (6)$$

where $[x]_i$ is the concentration of the compound in compartment i of the organism, t is time, and k_{ui} and k_{di} are the uptake and depuration rate constants of compartment i of the organism. In this model, the first i compartment is the source of compound to the ith compartment, so this model is effective for modeling transport that takes place in a linear manner. The traditional kinetic model is not appropriate here since plasma provides a source of EE2 and 4-NP to 4 compartments: digestive gland, gonad, adductor muscle, and remaining viscera.

The traditional kinetic model implies that uptake and depuration can be considered as separate processes and that both processes are exponential in nature. As such, the traditional kinetic model has embedded in the solution a limiting concentration of the compound within the organism (i.e., the compound's steady-state concentration). When the upstream concentration is

held constant, this limiting dynamic has been observed in cases where whole-body bioaccumulation is measured. In this experiment, however, the gill and plasma act as tandem upstream components, each having dynamically changing concentrations that are dependent on complex processes. The data (presented in *Results*) illustrate that the concentrations within the gill and plasma do not reach a steady state in this experiment.

According to the traditional kinetic model, the uptake for a component of a model follows exponential growth when the concentrations within the components are very low [27]. Consider $[x]_{i-1}$ and $[x]_i$ to be small values such that$[x]_i \approx [x]_{i-1}$. The traditional kinetic model is then approximated by

$$\frac{d[x]_i}{dt} = k_{ui}[x]_i - k_{di}[x]_i \qquad (7)$$

The solution to this equation is then

$$[x]_i = x_0 e^{(k_{ui} - k_{di})t} \qquad (8)$$

Our data suggest that uptake is not exponential within any of the components of the mussel in the early stages of exposure. An alternative model was therefore developed to predict the uptake of EE2 and 4-NP in the various tissues within the mussel during exposure.

The uptake of the EDCs in the components of the mussel during exposure is modeled using a coupled system of ordinary differential equations. In this system, the rate of uptake for each component is modeled as a function of the concentration of EDC in the upstream component times a rate-limiting term that is based on the concentration of EDC within the component. The processing of EE2 and 4-NP (uptake, transfer, etc.) by the mussel tissues is assumed to be the same, so a single model (Figure 1) is developed for both compounds. Six components are considered in this model: gill, plasma, digestive gland, gonad, adductor muscle, and remaining viscera. The gill is assumed to be the primary organ that interacts with the seawater and across which the EDCs are taken up. Although the mantle may also take up some EDCs, because it is also in direct contact with the seawater and like the gill is a single-celled epithelium that abuts the seawater apically and blood plasma basally, it consists of a single epithelial sheet, whereas the gill is composed of numerous cylindrical tubes approximately 20 µm to 35 µm in diameter (with a 20–35 µm seawater channel between them) [28]. The gill epithelium is also much thinner than that of the mantle. Each pair of gills (right and left) consists of 2 demibranchs (outer and inner), each of which has a descending and an ascending arm. A rough calculation indicates that the gill has at least 20 times greater surface area than the mantle epithelium. The gill should therefore take up the vast majority of EDCs, transferring these compounds to the plasma. We exclude mantle as an uptake organ in our model, although we retain it in the model as “gonad,” since gonadal tissue lies between the outer and inner mantle epithelia and makes up most of the weight of this tissue. For simplicity, we also exclude hemocytes from the present model, since the size of this compartment is very small (and thus its capacity for EDCs limited) in relation to that of the other tissues. According to the present illustrative model (Figure 1), EDCs enter the gill (most likely by diffusion) during uptake and are transferred to the plasma. The plasma then distributes the EDCs to the various internal tissues of the mussel (digestive gland, gonad, adductor muscle, and remaining viscera). During depuration, EDCs are released into the plasma by the internal tissues and transported to the gill, where they are released into the seawater.

The uptake of EDCs in the tissues is modeled so that the concentrations increase with time at rates that are coupled to the concentrations in the neighboring upstream tissues. Since the digestive gland, gonad, adductor muscle, and remaining viscera are all directly downstream of the plasma, the model allows for each tissue to draw directly from the concentration of EDC in the plasma (Equation 11). The gill is modeled as the only tissue directly linked to the seawater (Equation 9), so the differential equation describing uptake within the gill is unique. The gill equation follows a functional form that is a variation of the Michaelis-Menton kinetics utilized by Yu et al. [25] to describe accumulation. The concentration within the gill increases during exposure, although the rate of increase slows as the concentration in the gill increases. The other tissues behave similarly (i.e., the concentration of EDCs also increases over time but at decreasing rates); however, this decrease is coupled directly with the concentrations in the plasma (Figure 1). Our exposure model is described by

$$\frac{dC_1}{dt} = \frac{r_1 C_{sw}^*}{C_1 + s_1} \quad (9)$$

$$\frac{dC_2}{dt} = s_2 e^{-r_2 C_1} \quad (10)$$

$$\frac{dC_j}{dt} = s_i e^{-r_j C_2} \text{ for } j = 3..6 \qquad (11)$$

where C_i is the concentration of EDC in tissue component j (j = 1 . . 6) and r_i and s_i are parameters of the model. The index j is associated with 1) gill, 2) plasma, 3) digestive gland, 4) gonad, 5) adductor muscle, and 6) remaining viscera. The initial values of C_i for j = 1 . . 6 are 0 in the uptake model. This model is coupled to the seawater model in Equation 9 as the change in the concentration in the gill is dependent on the concentrations of EDCs in the seawater, C_{sw}^*, that are derived from the solution to the seawater model (Equations 1–3).

Mussel model: Depuration

Depuration of EDCs is modeled in each mussel using an exponential elimination model. This is the traditional kinetic model minus the uptake term. The data suggest that the concentrations within the plasma, central to the network of tissues within the mussel, drop rapidly when the mussel is exposed to contaminant-free seawater. The only tissue that is downstream of the plasma, the gill, is exposed to upstream concentrations close to 0 during the depuration phase of the experiment; because of this, the use of this simple exponential decay model effectively captures the change in concentration in those tissues. The initial conditions of the exponential decay model are determined by the predicted values of concentration within each tissue as determined by the exposure model. The depuration for each of the tissues is then modeled as the solution to the following 6 differential equations

$$\frac{dC_j}{dt} = -k_{dj} C_j \quad \text{for } j = 1..6 \qquad (12)$$

where k_{dj} is the depuration rate constant of component (i.e., tissue) j.

RESULTS

Behavior of EE2 and 4-NP in seawater

Changes in the concentrations of EE2 and 4-NP in the seawater declined over time after aquaria were spiked following each water change (Supplemental Data, Table S1; Figure 2). Seawater samples taken immediately before and after feeding indicated that the microalgae did not strip out any of the radiolabeled compounds that remain in the water, because there was no difference between the 2 sets of water samples (paired *t* test on samples that contained radioactivity: EE2, $p = 0.786$, $n = 15$; 4-NP, $p = 0.640$, $n = 15$). The decline in seawater concentrations had 3 distinct features that occurred at 3 different timescales: minutes, days, and weeks. Similar to other published studies [29], initial concentrations of both EE2 and 4-NP decreased exponentially in our seawater aquaria minutes after being spiked (Figure 2). Minimum values were reached by 18 h to 24 h postspike for both EE2 and 4-NP, after which time seawater concentrations of both EDCs rose slightly over the 3-d to 4-d period between water changes.

Only EE2 (not 4-NP) exhibited an additional trend over the 38 d of exposure. With each advancing spike cycle (water change), the concentration of EE2 taken within the first 5 min after spiking the water increased linearly over the 38-d period. This linear trend also applied to the samples taken 3 d after each spiking event, at the time just prior to the next water change; and the rate of change was very close to that of the measurements taken minutes after the spike. Fitting a linear regression to the values of EE2 concentrations measured immediately after each spike event on day 0 and on day 3 resulted in a rate of increase in EE2 of 0.0234 nM/d and 0.0240 nM/d, respectively, which amounted to a total change of approximately 27% of the target EE2 concentration over the 38-d exposure period. The 4-NP concentrations in the seawater did not appear to contain this linearly increasing trend. However, this trend may be the result of the fact that the concentration of EE2 in the seawater was 2 orders of magnitude lower than that of 4-NP. Although the observed linear change of EE2 was relatively large (27% of the target EE2 concentration), changes of the same magnitude in the 4-NP would only account for 0.2% of the target 4-NP concentration, which is well below the variance observed in the sample measurement of 4-NP, and thus would not be detected in the data set.

Additional experiments were conducted in beakers containing 400 mL of filtered seawater, both with and without a live mussel, to better characterize the behavior of EE2 and 4-NP (Supplemental Data, Table S1). Beakers containing a live mussel showed a similar exponential drop in EE2 and 4-NP concentrations as observed in the exposure aquaria. In the absence of a mussel, however, seawater concentrations of both EE2 and 4-NP remained close to the target concentrations of 3.37 nM and 454 nM, respectively, indicating that components of the mussel were responsible for the exponential drop in seawater concentrations (e.g., rapid uptake of both compounds, binding to the shell or to mucus sheets on mussel epithelia).

The best-fit parameters of the 3-component seawater model (Equations 1–3) were determined by fitting the solution to the measured concentrations in seawater. The agents of change, $a1$ and $a2$, captured the collective effects of the mussel on the EDC concentration in the seawater. The initial values of the concentration of seawater were the intended concentrations of the initial spike (454 nM for 4-NP and 3.37 nM for EE2), whereas the initial values for the 2 agents of change were both 0 nM. To account for the linearly increasing 38-d trend in the EE2 concentrations in the seawater, the linear trend was removed from the data prior to determining the best-fit parameters for the seawater model. The linear trend was then added to the solution to the model to accurately predict the seawater changes in concentrations of EE2 in the seawater. No such trend was observed in the 4-NP data, so such preprocessing and postprocessing of the data and model solution were not necessary. The best-fit parameters for the 4-NP and detrended EE2 seawater are listed in Table 2.

A time-integrated average concentration of seawater was determined using the solutions to the 3-component seawater model. The modeled average concentrations for 4-NP were found to be 155 nM and 151 nM for spike cycles that are 3 d and 4 d in duration. Because the spike cycles alternate in duration between 3 d and 4 d, the average value of $C_{sw}^{*} = 153\,\text{nM}$ was used to represent the time-integrated concentration of 4-NP over the course of the exposure period. A similar calculation was performed on the solution to the seawater model applied to the EE2 concentrations. Recall that the model for EE2 was fit to data where the linearly increasing trend observed in the data was removed. The time-integrated value determined for this solution was 1.839 nM. The linear trend that is removed from the original data to fit the seawater model is then superimposed on the time-integrated value so that it provides an EE2 concentration in the seawater that reflects the observed linearly increasing values over time. As such, the EE2 concentration in the seawater is represented as

$$C_{sw}^{*}(t) = 0.0234t + 1.839 \qquad (13)$$

This linear function is then used as a boundary condition on the exposure model for EE2.

Mussel tissue uptake

Each tissue of the mussel displayed the characteristic nonlinear increase in EE2 and 4-NP over the 38-d exposure period (the full data set is presented as Supplemental Data, Table S2; Figure 3 depicts digestive gland and gill as typical examples). Because mussels are exposed to much lower concentrations of EE2 than 4-NP (nominally 3.37 nM vs 454 nM), tissues accumulated picomoles per gram (wet wt) concentrations of EE2 compared with nanomoles per gram (wet wt) concentrations of 4-NP. Differential tissue bioconcentrations followed the same pattern for EE2 and 4-NP: digestive gland > gill ≥ remaining viscera > gonad > adductor > plasma.

Plasma contained much lower concentrations of EE2 and 4-NP (Figure 4) compared with any of the other tissues (e.g., Figure 3; Supplemental Data, Table S2). Most interesting, however, is that the plasma samples contained the same concentration of EE2 and 4-NP as the targeted seawater exposure concentration of these 2 compounds on the first sampling day (i.e., d 3 of exposure) and subsequently exhibited concentrations that were 2 to 3 times higher than the targeted seawater exposure concentrations (Figure 4). Since hemocytes were removed from the plasma samples, the high concentrations of EE2 and 4-NP in the plasma can only be explained by the binding of these EDCs to a component of the plasma itself (most likely plasma proteins).

Endocrine-disrupting compounds in individual mussel tissues were modeled using a 3-component uptake model (Equations 9–11). The parameters of the uptake model were fit to scaled average values of concentration measured in each of the mussel components at each sampled time during exposure. The scaling of the values accounts for differences observed in the mean and variance in the measurements from the different components. The scale factor for each component was set to the inverse of the maximum of the means so that the range of concentration values for all components varied between 0 and 1. The true parameter values for the model were then calculated according to the transformation of the data as dictated by the differential equations. The best-fit parameters for this model are summarized in Table 3.

The best-fit uptake model provides an excellent prediction of the measured values of EDCs in all of the tissues for both EE2 and 4-NP (Figures 3 and 4; Supplemental Data, Table S2). The total root mean squared errors for the EE2 and 4-NP models are 0.51 and 0.54, which are lower than the root of the sum of the squares of the standard deviations of the tissue data to

which the model was fit, having values of 0.79 and 0.84. The parameter values for the model indicate that there are at least 2 different factors that have an effect on the change in the concentrations of EDCs within each tissue. This model illustrates the unique role of the gill in the transport of EDCs into the mussel and the important role of the plasma in the distribution of EDCs to the tissues.

Mussel tissue depuration

The depuration of EE2 and 4-NP from mussel tissues also followed the typical exponential decay pattern (Figures 3 and 4). Depuration rate constants and half-lives were calculated using the best-fit parameter of an exponential decay model coupled with the exposure model. The depuration rates for tissue *j*, k_{dj}, are those parameters that result in a solution to the model in Equation 12 that have the lowest root mean squared error with respect to the measured concentration values of EDCs within the tissues during depuration. The initial values of the concentrations within each tissue are those predicted values of concentration determined by the exposure model at the end of the exposure period. These best-fit depuration rates are summarized in Table 4. Half-lives were calculated as $0.963/k_d$.

There are considerable differences in k_d and $t_{½}$ values among the different tissues. The gill exhibits the longest half-life of all the tissues for both EE2 and 4-NP, indicating a very slow process of depuration. Interestingly enough, the remaining viscera also have fairly long half-lives, which indicates that there is some tissue or tissue compartment in the remaining viscera that retains EE2 and 4-NP for a considerable length of time. At the other end of the spectrum, plasma exhibited relatively rapid depuration half-lives (9 d for EE2 and 15 d for 4-NP). Half-lives displayed a very similar pattern for both compounds: EE2, gill > remaining viscera > digestive gland ≥ gonad = adductor > plasma; 4-NP, gill > remaining viscera > digestive gland = gonad = adductor > plasma.

To compare our results with those of previous studies that analyzed whole-body tissues of EDCs, we “reassembled” each mussel to determine whole-body EE2 and 4-NP concentrations. Individual EDC organ bioburdens were summed for each mussel and divided by the summed total wet weight of the tissues (Figure 5). The overall pattern of bioconcentration and depuration was similar to what was observed for individual tissues. Both EDCs showed similar uptake trends where the uptake rates, r_i, for the digestive gland, gonad, adductor muscle, and remaining viscera are considerably larger than that of the plasma for both EE2 and 4-NP. Depuration half-lives were a bit more variable, with a 39.2-d half-life for EE2 and a 58.9-d half-life for 4-NP.

Similar to the reconstruction of the raw data, the solutions to the multicompartmental models were used to reconstruct the uptake of the EDCs by the whole model. The modeled results match those of the reconstructed data well (Figure 5), further validating the success of the modeling approach taken.

DISCUSSION

Measurement of total EDCs in mussel tissues: Parent compounds and metabolites

Although specific data on the metabolism and biotransformation of EE2 and 4-NP in bivalves are scarce, previous studies investigating natural steroid hormones and consistencies in the metabolism of 4-NP and EE2 across animal phyla enable predictions of how these compounds are likely metabolized, stored, and excreted in mussels. For example, studies in *Mytilus* sp. exposed to 17ß-estradiol have found that estradiol is converted almost entirely to estradiol esters [30,31], which is suggested to be a mechanism for sequestering and storing

excess steroid hormone [31]. However, this does not mean that adverse effects to estradiol exposure are not expected. Although free estradiol represented only 5% of total measured estradiol, Janer et al. [30] found a 10-fold increase in parent estradiol at their highest exposure concentration (2 µg/L), suggesting that biological effects are likely. Sulfation of estradiol has been shown to occur in other mollusks and may be another route for excretion [32]. For 4-NP, in contrast, a recent study in the snail *Lymnaea stagnalis* revealed that 42% of 4-NP found in tissues was present as the unmetabolized parent compound, whereas glucuronic acid conjugates were the primary metabolites found in tissues and feces [33]. Overall, this suggests a slow metabolism of 4-NP in mollusks. This may result from the lower activity of phase I and phase II biotransformation enzymes seen in marine invertebrates compared with mammals [32].

In the present study, we used radioactively labeled EE2 and 4-NP to trace bioconcentration of these compounds in individual mussel tissues. Since these C-14 labels would also be present in EE2 and 4-NP metabolites, we cannot distinguish between parent compounds and potential metabolites. Based on our results, we would expect that the majority of EE2 measured in mussel tissues would be in the form of estradiol esters, whereas 4-NP would likely be present as both parent compound and 4-NP glucuronic acid conjugates. Although these metabolites are not estrogenic, they can be easily converted back to estrogenic compounds through hydrolysis within the mussels themselves or during digestion of mussels by predators.

EDC bioconcentration in the digestive gland

The digestive gland holds the highest concentration of the 2 EDCs of the tissues we examined and exhibits a very strong linear correlation with the concentrations of each EDC in the whole body ($r^2 = 0.95$, $p < 0.001$). Surprisingly, the gonad was not a major concentrator of EDCs (higher concentrations were found in the digestive gland, gill, and remaining viscera). Since mussels were collected at the end of November, long after the spring and fall spawning periods, some mussels were spent, while others contained variable amounts of residual gametes. It is unlikely that the mussels would have already started the annual cycle of gametogenesis in preparation for the following spring. If we had used mussels that were fully ripe with gametes, perhaps the gonad would have been a greater sink for EDCs. Nevertheless, given the dramatic seasonal changes in the gonad wet weight as a result of the reproductive cycle, the digestive gland would likely show far less seasonal variability in EDC concentrations.

Our data show that the digestive gland constitutes approximately 5% of the total wet weight of the whole-body tissues yet amasses concentrations of EE2 that are approximately 17% higher and concentrations of 4-NP that are approximately 20% higher than the whole body. We suggest that analyzing digestive glands may be a better alternative than analyzing whole-body tissues in screening and monitoring programs. Instead of extracting a dilute analyte from a large mass of tissue, it would be far more efficient to extract a much higher concentration of analyte from a small mass of tissue, likely increasing extraction recoveries. Having smaller masses of tissue to extract would reduce the volume of solvent and the time needed for sample processing, leading to a reduction in overall cost. The digestive gland is easy to identify and cleanly dissect from surrounding tissues, so it would not significantly increase processing time. Being small, the entire digestive gland could be extracted, rather than having to subsample the mass of whole-body tissue, as is sometimes done, eliminating a major source of variability. Matrix interferences would likely be reduced since a single tissue is being extracted, eliminating those interferences contributed from other tissues and possibly reducing analytical detection limits. Experiments are currently underway in our lab to directly compare whole-body and isolated digestive gland extractions and analyses (e.g., recoveries, interferences, number of mussels needed, final analyte

volumes) to better support this suggestion.

Several studies to date have investigated the effects of EDCs on the gonadal tissue in bivalves, including studies of enzyme expression and activity [34], gonadal development and histology [15,35], expression of vitellogenin-like proteins [16], and presence of intersex condition (e.g., ovo-testis in clams [35], hermaphroditic oysters [15]). However, the present study suggests that the digestive gland may also be an important target site because of its high level of bioconcentration. Endocrine-disrupting compounds have been shown to affect the expression level of steroid metabolism enzymes within the digestive gland, including 17ß-hydroxysteriod dehydrogenase, the enzyme that catalyzes the conversion of precursor steroids to testosterone, estrone [36], and P450 aromatase, which converts testosterone to estradiol [30]. In addition, a gene expression study found that 44 genes were differentially expressed in the digestive gland of female mussels following exposure to 17ß-estradiol including genes involved in "hormone response" [37]. Although the role of "vertebrate-like" steroid hormones in molluskan endocrinology is debatable (e.g., Scott [38]), steroid hormones (e.g., 17ß-estradiol) and anthropogenically derived mimics (e.g., EE2 and 4-NP) induce effects, and in some cases *adverse* effects, in bivalves as the above studies have shown. Our demonstration that EE2 and 4-NP bioconcentrate in the digestive gland and previous studies revealing activation of steroid hormone metabolic enzymes suggest that the digestive gland may be a target site causing disruption of steroid metabolism, which then initiates effects in other tissues. Alteration in steroid hormone levels in the digestive gland may then lead to adverse impacts seen in the gonadal tissue. Alternatively, EDCs may interact directly with receptors in the gonadal tissues, causing the effects observed in previous studies. Clearly, additional studies are needed to clarify the molecular initiating events that lead to endocrine disruption in bivalves; however, the present study places renewed emphasis on the digestive gland and highlights the need to investigate effects on this tissue.

Differential EDC bioconcentration in other tissues

The gill was the second-highest bioconcentrator of EDCs among the tissues sampled, possibly linked to its likely role in directly taking up EDCs from seawater and transferring them to the blood. The gill accumulated EDCs relatively quickly, yet retained EDCs for appreciably longer time periods than any of the other tissues examined (EE2 half-life = 92 d; 4-NP = 57 d). The gill's ability to bioconcentrate EDCs likely comes from some intrinsic component that sequesters relatively high amounts of these compounds. During depuration, EDCs are likely transferred from internal tissues (digestive gland, gonad, adductor muscle, remaining viscera) to the plasma. The EDCs are then rapidly transferred from the plasma to the gill, which does not retain them in its tissues during depuration but immediately shunts them to the outside seawater. As a result, the concentration of EDCs in the plasma falls to exceedingly low levels as everything that is drawn from the internal tissues is immediately passed to the gill and discharged into the surrounding seawater. Although there is a constant flow of EDCs through the gill tissues, the overall concentration of EDCs in the gill remains relatively high, since the gill's previously sequestered EDC is only slowly depurated. It is likely that as the rate and extent of depuration in the other internal tissues slows, depuration from the gill tissue stores will become more apparent.

The concentrations of EDCs in the remaining viscera were greater than those of the gonad and posterior adductor muscle, and the half-lives of EDCs in the remaining viscera were

only exceeded by those of the gill. This suggests that we may have overlooked an important tissue for EDC storage. The bivalve kidney is a possible candidate. It is known to have storage capabilities, both in the pericardial gland portion of this renal system and in the kidney proper. However, the small size of this organ would mean that the bioconcentration of EDCs would have to be exceedingly high to have elevated the concentrations in the remaining viscera. Storage in various connective tissues of the remaining viscera is another possibility. As a relatively large compartment, connective tissues would be more likely to have the necessary storage capacity than does the kidney.

Modeling of EDCs in tissues

We have modeled our mussel system using 3 coupled mathematical models to quantify changes in the bioconcentrations of EE2 and 4-NP in individual tissues of *M. edulis* over time. It is not uncommon to model accumulation and depuration using different models that deviate from a simple kinetic modeling approach [26,39]. The models used in the present analysis are coupled so that the results of the seawater model are boundary conditions for the exposure model. Similarly, the results of the exposure model provide initial conditions for the depuration model [26,39].

Because of the observed temporal fluctuations in seawater concentrations of EDCs, we modeled the concentrations of EE2 and 4-NP in the seawater using a system of differential equations fit to the data to provide a systematic way to obtain a continuous function that describes the changes in the concentration over time. Using this function, a time-integrated value of the concentration is obtained. This value is advantageous to use because it avoids potential bias in solutions that are determined purely by scarce data.

We developed a new approach to modeling the uptake and transport of EDCs within interconnected tissues. Our modeling is an improvement over the traditional kinetic-based modeling because it captures the uptake of EDCs in each of the tissues when the concentration of EDCs within each tissue is low. Furthermore, the model incorporates plasma into the transport model and successfully fits the data from both the EE2 and the 4-NP experiments, where the concentrations of EDCs are 2 orders of magnitude different. The model integrates an increasing trend in the EE2 concentrations over the length of the exposure period. Finally, parameters of the model provide a means by which the processing of EDCs within the tissues can be differentiated.

Our multicompartmental model gave us a much better understanding of the interplay between the various tissues in the mussel. While whole-body models provide information about the gross processing of compounds such as EDCs, these models do not provide insight into how compounds are differentially distributed within the mussel or on the roles that different tissues play in the internal processing of EDCs. This differential knowledge is extremely valuable in understanding the possible impact of bioaccumulation on a species. The model we have developed provides a predictive tool that describes how the mussel processes EDCs throughout its body as well as direction for future research on EDC processing pathways.

Potential human exposure through shellfish consumption

Because of their low-dose and multigenerational effects (e.g., de Assis et al. [40]), the potential risk of EDC exposure to humans from dietary exposure has been a concern. Indeed, these chemicals have been found to be ubiquitous in food [10]. Although the finding that EE2 was present in drinking water sources was initially alarming, intake through this route was estimated to be very low, 0.013 ng/d, whereas dietary exposure was higher at 23 ng/d [41]. Several studies have investigated dietary exposure to 4-NP with ranges varying by geographic location and diets of study participants. In Germany, dietary intake of 4-NP was estimated to be

7.5 µg/d [10], whereas the range in Taiwan was 17 µg/d to 40 µg/d depending on region [11]. In another study, Ferrara et al. [9] specifically focused on seafood to determine the potential risk of EDC exposure from shellfish. In the Adriatic Sea, 4-NP in shellfish was found to contribute 6 µg/d to 13 µg/d to average seafood consumers and 48 µg/d to 87 µg/d to heavier seafood consumers. Shellfish consumption could therefore contribute a substantial amount of the daily intake of EDCs.

In the present study, we investigated the bioconcentration of EE2 and 4-NP over the course of 38 d. Although the concentrations of these chemicals did not plateau and our modeling results suggest that they will continue to increase with continued exposure, we decided to calculate the bioconcentration at 38 d using the time-integrated seawater concentrations (Table 5). Our values (103.00 for EE2 and 196.29 for 4-NP) are lower, and therefore more conservative, than published bioconcentration factors [6,24] for 2 reasons. First, our concentrations are reported as wet weight, rather than as lipid content or dry weight. Second, we calculated these values at 38 d, which were not steady-state values. Nevertheless, using the 38-d bioconcentration values, we then predicted the concentrations that would be found in shellfish given measured seawater concentrations of EE2 and 4-NP globally. Our estimates produced predicted exposures from shellfish consumption as 2.1 ng/d to 0.35 µg/d for EE2 and 0.04 ng/d to 80.5 µg/d for 4-NP (Table 5). These upper-limit values are comparable to previous studies of dietary intake in general [10,11,41] and shellfish specifically [9]. However, the question of risk to these levels remains. Values for acceptable daily intake have been developed by the World Health Organization and other authors for EE2 (see Caldwell et al. [41]), whereas the only tolerable daily intake value that we could find for 4-NP was reported from the Danish Environmental Protection Agency [42]. We found that the upper range of our predicted daily doses of EE2 and 4-NP from shellfish consumption are close to or exceed proposed acceptable daily intake (EE2) and tolerable daily intake (4-NP) values. This suggests that shellfish consumption may represent a large portion of dietary exposure to these compounds in certain areas and, when combined with other exposure sources, could pose significant risk.

CONCLUSION

Whereas most reports of EDCs in bivalve tissues have dealt with whole-body tissues, the present study has helped to characterize the bioconcentration, transport, and depuration of 2 of the most prevalent environmental EDCs, EE2 and 4-NP, in individual organs of the blue mussel *M. edulis*. All tissues bioconcentrated significant quantities of EDCs. The digestive gland, however, bioconcentrated both EDCs to a far greater extent than any of the other tissues examined. The digestive gland would therefore be the best choice of a tissue to examine in EDC monitoring programs rather than analyzing whole-body tissues. The gonad was less of a sink for EDCs than the digestive gland, gill, or remaining viscera. The long half-life of EDCs in the remaining viscera suggests that there was an important storage compartment left in the remaining viscera that we had not sampled. Modeling indicates that the blood plasma mediates the transfer of EDCs between the gill and the other organs. Depuration is slower in the gill, apparently because the gill may continually accept EDCs from the other, internal tissues, transported via the plasma, and then transfers them to the external seawater.

SUPPLEMENTAL DATA

Tables S1–S2. (60 KB XLSX; 242 KB XLSX).

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Data availability—All the data are included in 2 supplemental Excel files (Supplemental Data, Tables S1 and S2). Questions about the data can be addressed to the corresponding author (william.robinson@umb.edu).

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Figure 1. Diagrammatic representation of the pathways for the uptake and elimination of 17α-ethinyl estradiol and 4-nonophenol in the tissues of the marine mussel *Mytilus edulis*. Tissues connected by solid arrows are included in our mathematical modeling. Tissues connected by dashed arrows are potential alternative pathways (e.g., mantle

uptake) or tissues for which we currently do not have robust data (e.g., hemocytes). The overall pathway (seawater to gill to plasma to other tissues) concurs with mussel morphology.

Figure 2. Measured and modeled concentrations of 17α-ethinyl estradiol (EE2) and 4-nonylphenol (4-NP) in aquarium seawater over 3-d and 4-d periods between water changes. Dotted and dashed lines represent agents of change (*a*1, *a*2; see text). Solid lines are model fits to the raw data represented by filled squares for EE2 and filled circles for 4-NP.

Figure 3. Typical examples of the pattern of endocrine-disrupting compound concentrations in *Mytilus edulis* tissues. Mean concentrations (± standard error) of 17α-ethinyl estradiol (EE2) and 4-nonylphenol (4-NP) in digestive gland (DG) and gill tissues are presented for the 38-d exposure period (filled squares for EE2 and filled circles for 4-NP) and the 35-d depuration period (open symbols). Solid and dashed lines are model fits to the bioconcentration data during exposure and depuration, respectively.

Figure 4. Mean concentration (± standard error) of 17α-ethinyl estradiol (EE2) and 4-nonylphenol (4-NP) in *Mytilus edulis* blood plasma (cell-free) over the 38-d exposure period (filled squares for EE2 and filled circles for 4-NP) and the 35-d depuration period (open symbols). Horizontal lines depict the targeted exposure concentration of the 2 endocrine-disrupting compounds (i.e., 3.37 pmol/mL for EE2 and 454 pmol/mL for 4-NP). Solid and dotted lines are model fits to the raw bioconcentration data during exposure and depuration, respectively.

Figure 5. Mean concentrations (± standard error) of 17α-ethinyl estradiol (EE2) and 4-nonylphenol (4-NP) in *Mytilus edulis* whole bodies obtained by totaling individual endocrine-disrupting compound organ bioburdens for each mussel's tissues and dividing by the sum of the wet tissue weights of each of the tissues. Bioconcentration data (filled squares for EE2 and filled circles for 4-NP) are presented for the 38-d exposure period and the 35-d depuration period (open symbols). Solid lines and dashed lines are reassembled from the exposure and depuration model fits to the accumulation data, respectively.

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Table 1. Physical properties of 17α-ethinyl estradiol (EE2) and 4-nonylphenol (4-NP) that are relevant to bioaccumulation in marine organisms[a]

	EE2	4-NP
Molecular weight	296.40	220.35
Log K_{OW}	3.67	4.48
Aqueous solubility	11.3 mg L^{-1} (at 27 °C)	7 mg L^{-1} (at 25 °C)
Vapor pressure	1.95×10^{-9} mm Hg (at 25 °C)	0.11×10^{-5} Pa (at 20 °C)
Bioconcentration factor[b]	580	2900

[a] Log K_{OW} for 4-NP from European Union [43]; all other data from PubChem [44].

[b] Calculated from the linear regression in Meylan et al. [23].

K_{OW} = octanol–water partition coefficient.

Table 2. Best-fit parameters of the 3-component model fit to aquarium seawater concentrations of 17α-ethinyl estradiol (EE2) and 4-nonylphenol (4-NP) over the course of 3 d or 4 d between water changes[a]

Parameter	EE2 (detrended)	4-NP
A	2.361	339.96
B	2.857	3.144
C	1.572	461.73
D	1.137	1.624

[a] EE2 parameters were calculated after correcting for the slight increase in seawater concentrations over the course of the 38-d exposure. No such trend was observed for 4-nonylphenol. Parameters A, B, C, and D are applied to Equations 4 and 5.

Table 3. Best-fit parameters for the model of concentrations of 17α-ethinyl estradiol (EE2) and 4-nonylphenol (4-NP) in *Mytilus edulis* components during endocrine-disrupting compound exposures[a]

Component	Index, i	EE2		4-NP	
		r_i	s_i	r_i	s_i
Gill	1	0.0021	0.0589	0.1845	0.0526
Plasma	2	0.7340	0.0013	0.0562	0.1690
Digestive gland	3	344.05	0.1288	0.2170	4.0212
Gonad	4	327.22	0.0280	1.1542	1.8204
Adductor	5	537.77	0.0266	1.1974	1.0947
Viscera	6	305.64	0.0358	0.8211	2.0744

[a] Parameters r_1 and s_1 are applied to Equation 9; r_2 and s_2 are applied to Equation 10; and r_3 to r_6 and s_3 to s_6 are applied to Equation 11.

Adductor = posterior adductor muscle; viscera = remaining viscera after dissection of gill, whole blood, digestive gland, gonad/mantle, and posterior adductor.

Table 4. Depuration rate constants (k_d) and half-lives of 17α-ethinyl estradiol (EE2) and 4-nonylphenol (4-NP) in various tissues (components) of *Mytilus edulis*[a]

Component	Index	EE2		4-NP	
		k_{di} (d^{-1})	Half-life (d)	k_{di} (d^{-1})	Half-life (d)
Gill	1	0.0075	92	0.015	57
Plasma	2	0.26	2.7	0.12	15
Digestive gland	3	0.028	25	0.033	30
Gonad	4	0.040	17	0.022	33
Adductor	5	0.054	13	0.021	32
Viscera	6	0.022	32	0.017	48

[a] The k_{di} values were obtained using Equation 12 with concentrations at the start of depuration given by the modeled tissue concentration of each endocrine-disrupting compound on day 38 of the exposure period. Half-lives were calculated as (ln 2)/k_{di}.

Adductor = posterior adductor muscle; viscera = remaining viscera after dissection of gill, whole blood, digestive gland, gonad/mantle, and posterior adductor.

Table 5. Estimated bioconcentration of 17□-ethinyl estradiol (EE2) and 4-nonylphenol (4-NP) in shellfish meat based on measured seawater concentrations globally[a]

	EE2	4-NP
38-d tissue concentration, from Fig. 5: Reassembled mussel (nmol/g wet wt)	0.2352	30.0382
38-d tissue concentration (µg/g wet wt)	0.06971	6.6189
Time-integrated water concentration (µg/L)	0.6768	33.72
38-d bioconcentration	103.00	196.29
Seawater concentrations in marine environment (µg/L)	<0.0002–0.034	0.000002–4.10
Predicted shellfish concentrations (µg/g wet wt)	<0.000021–0.0035	0.0000004–0.805
Predicted dose in meal (i.e., 100 g)	2.1 ng to 0.35 µg	0.04 ng to 80.5 µg
Acceptable (for EE2) or tolerable (for 4-NP)	60 ng to 3.0 µg	300 µg[b]

[a] The 38-d bioconcentration was determined by dividing the 38-d wet weight tissue concentration by the integrated water concentration. Predicted shellfish tissue concentrations were determined by multiplying the range of seawater concentrations measured in the environment (EE2 from Aris et al. [8]; 4-NP from David et al. [7]) by the 38-d bioconcentration. Acceptable daily intake and tolerable daily intake values were taken from Caldwell et al. [41] for EE2 and Nielsen et al. [42] for 4-NP.

[b] The tolerable daily intake for 4-NP determined by Nielsen et al. [42] is 5 µg/kg body weight. This value was multiplied by the average adult weight (60 kg) to derive a daily intake of 300 µg.