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Response and recovery of Baltic Sea blue mussels from exposure to pharmaceuticals

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ABSTRACT: Physiological responses to, and recovery from, exposure to 3 concentrations of a pharmaceutical mixture (diclofenac and propranolol) were examined experimentally in Baltic Sea blue mussels *Mytilus edulis trossulus* collected with increasing distance to a wastewater treatment plant (WTP) outlet. Respiration, absorption efficiency and consumption were measured, and also combined into scope for growth (SFG). The response and recovery patterns varied both between exposure concentrations and sampling site within the bay. After exposure, mussels exposed to the highest concentration (2000 µg l^{-1}) in general had lower SFG, and mussels from 2 (out of 3) sites exposed to the medium concentration (200 µg l^{-1}) had higher SFG than the controls. In general, mussels from the 2 sites nearest the WTP recovered from the exposure response, while individuals collected further from the WTP outlet were more affected by the exposure and did not recover to the same extent. The response pattern of consumption was mainly affected by exposure concentration, whereas respiration was affected by all 3 factors (concentration, time of measurement, sampling site). Absorption efficiency was not affected at all. The differences in responses and recovery patterns could possibly be explained by the mussels sampled closer to the WTP having a history of higher food availability, improving their general health status, and/or a history of pre-exposure to natural disturbances, as well as to the test substances, via the WTP effluent. Pre-exposure to stressors could have both positive and negative impact on a community by increasing the resilience towards some stressors, but may also reduce the adaptability when facing other stressors.

KEY WORDS: *Mytilus edulis trossulus* · Baltic Sea · Physiology · Pollutants · Disturbance recovery · Pre-exposure · Effluent gradient

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INTRODUCTION

The responses of organisms to contaminant exposure have been studied for many years. The understanding of how species, populations and communities recover after withdrawal of exposure is, however, limited (Clements & Rohr 2009). At the community level, the ability to recover from a disturbance, i.e. resilience, can be challenged by chronic exposure to stressors and varies with the biodiversity, so that a gradual biodiversity loss increases vulnerability to perturbations (Clements & Rohr 2009). Loss of keystone species has an especially large impact on the stability of a system, as it can alter ecosystem functions (Gunderson 2000).

The Baltic Sea ecosystem has a low biodiversity with low functional redundancy, where each species is of high importance (Elmgren & Hill 1997). The sea is subjected to both natural and anthropogenic stress (Elmgren 2001, Wulff et al. 2001), and 2 of the most common stressors acting simultaneously on the Baltic coastal ecosystems are eutrophication and exposure to toxicants (e.g. Jansson & Dahlberg 1999, Elmgren 2001, Wulff et al. 2001, Sundbäck et al. 2010). The

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Baltic Sea is the final recipient for wastewater from a large catchment area, and about 50% of the total wastewater treatment plant (WTP) effluents are discharged directly into its shallow areas (HELCOM 2004), where the Baltic Sea blue mussel *Mytilus edulis trossulus*, together with perennial algae, have essential stabilising functions (Kautsky 1995, Norling 2009). The blue mussels both filter large volumes of water and create habitats for other species. Their filter-feeding behaviour and long life cycle, however, can subject blue mussels to both high and long exposure to disturbances such as eutrophication and pollutants. Mussels' high exposure to pollutants, in combination with being sedentary, are why they have been used in various mussel-watch programmes (e.g. Goldberg et al. 1978). Eutrophication could result in increased amounts of algae, i.e. higher particle concentrations, which has been shown to reduce mussels' filtration (Widdows et al. 1979), and could negatively affect mussels by insufficient respiration and feeding.

A group of environmental stressors that has gained increasing attention are pharmaceutical substances. They are found in the environment and are designed to have biological effects, which potentially make them harmful to non-target organisms. Studies on the effects of pharmaceuticals often focus on individual substances (Fent et al. 2006), although they normally appear in mixtures (Cleuvers 2003). Multiple stressors, including pollutants such as pharmaceuticals, but also e.g. nutrients, hypoxia, turbidity, and altered habitat and hydrological regimes, can impact organisms' health and performance through single, cumulative, synergistic or antagonistic processes (Myers 1995, Adams 2005, Todgham & Stillman 2013). Multiple stressors' combined effects can narrow an organism's window of optimal biological performance, and even shift their metabolic performance from normal function to an energetic compensation or conservation strategy if the stress levels are high or long-lasting (Sokolova et al. 2012). The response to multiple disturbances is also influenced by fluctuations in the environment and the history of stress to which a population has been exposed (Thrush et al. 2008). To understand the overall impacts of exposure to both single and multiple stressors, it is thus important to comprehend both the response to exposure, and how populations recover after removal of the stress.

In a first attempt to study the Baltic Sea blue mussels' recovery potential and degree of resilience towards exposure to pharmaceutical substances, a laboratory experiment was conducted. Mussels were sampled with increasing distance to a WTP outlet, exposed to a mixture of the human pharmaceuticals diclofenac and propranolol for 3 wk, and then tested for their physiological response and subsequent recovery from the exposure. In previous studies, Baltic Sea blue mussels were negatively affected by exposure to both diclofenac and propranolol, distributed separately as well as in combination (Ericson et al. 2010, Oskarsson et al. 2014). A history of pre-exposure can induce better capabilities of acclimation or adaptation and subsequently lead to e.g. lower exposure responses (Khan et al. 2011, Bach & Dahllöf 2012) and faster recovery from additional exposure (e.g. Thrush et al. 2008). In this study we therefore hypothesised that (1) blue mussels exposed to short-term pharmaceutical mixtures are negatively affected, but have the ability to recover after removal of the exposure, and that (2) blue mussels sampled closer to the WTP respond and recover differently compared to mussels sampled further away.

MATERIALS AND METHODS

Sampling sites and test organism

The test organisms, Baltic Sea blue mussels *Mytilus edulis trossulus*, were collected in August 2009 at 3 sites (Sites 1, 2 and 3) with increasing distance from a municipal WTP in the inner part of the Himmerfjärden bay, Baltic Proper, Sweden (Fig. 1). Baltic Sea blue mussels are specific to the Baltic Sea and constitute an introgressed form of *M. trossulus* with parts of the *M. edulis* genome (Väinölä & Strelkov 2011). Mussels were collected by SCUBA divers at 6–7 m depth at sites with similar wave exposure, a water temperature of 11°C and salinities varying between 5.4 and 5.8 PSU (Fig. 1). Epibiota was removed from the mussel shells by gently scraping the shells with a spatula. Mussels of equal size (2.5 ± 0.5 cm) were acclimated in a climate chamber (11°C, 6.1 PSU, following the natural light regime) for 5–8 d before the experiment started.

The Himmerfjärden bay is a 30 km long coastal area, 60 km south of Stockholm. It elongates in a north–southward direction, with an average depth of 17 m and a surface salinity of approximately 0.5 PSU below the 6–7 PSU of the adjacent open sea (Elmgren & Hill 1997). The drainage area covers 1286 km^2 (Savage & Elmgren 2004), which together with the WTP creates gradients of nutrients (dissolved inorganic nitrogen) and chl *a* of increasing levels closer to the WTP outlet in the Himmerfjärden bay (VAS-

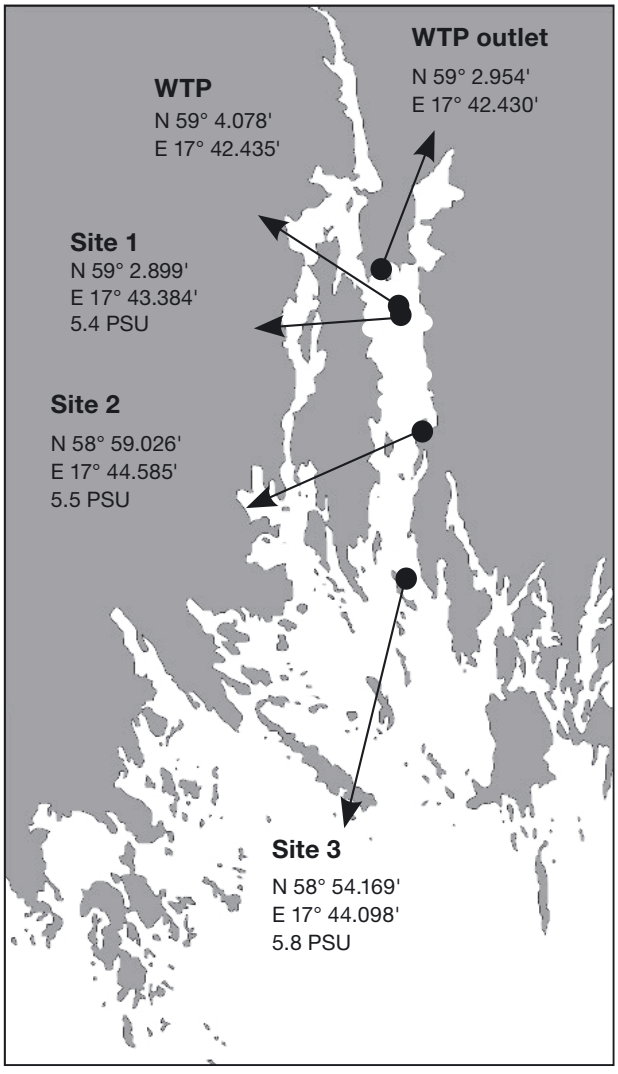

Fig. 1. Location (WGS84 coordinates) of wastewater treatment plant (WTP), WTP outlet and sampling sites in the Himmerfjärden bay (Sweden), and salinity at the time of sampling. Distance between WTP outlet and Site 1 is 0.1 km, between Sites 1 and 2 is 7.9 km, and between Sites 2 and 3 is 8.1 km

rådet 2013, Zakrisson et al. 2014), as well as salinity and oxygen gradients of increasing levels towards the coastline and the open sea (VAS-rådet 2013, Zakrisson & Larsson 2014).

The Himmerfjärden bay has been closely monitored over the last 30 yr, since the WTP came into use (VAS-rådet 2013), and the WTP effluent has been confirmed to contain metals (Swedish Environmental Protection Agency 2013) and a wide range of pharmaceutical substances (Długołęcka 2007). The total load of 70 quantified pharmaceuticals in the WTP effluent in the sampled bay is estimated to be 1.51 kg d^{-1}, including diclofenac and propranolol in concentrations of 0.475 and 0.102 µg l^{-1}, respectively (Długołęcka 2007). Upstream of the bay is a commercial WTP connected to a pharmaceutical industry, which likely also contributes to the bay's total load of pharmaceutical residues. Pharmaceuticals, including diclofenac and propranolol, have been detected in gradients of decreasing concentrations with increasing distance to other WTP outlets with similar settings through the Stockholm archipelago (Table S1 in the Supplement at www.int-res.com/articles/suppl/m526p089_supp.pdf) (Wahlberg et al. 2010), and were assumed to be distributed in a similar way in the Himmerfjärden bay.

Test substances and solvents

The pharmaceutical mixture used in this study consisted of the non-steroidal anti-inflammatory drug (NSAID) diclofenac and the non-selective β-blocker propranolol, both of which are highly prescribed (e.g. Bendz et al. 2005, Andersson et al. 2006). Despite relatively fast degradation rates (half-life of approx. 0.25–5 d for diclofenac, under lamplight [Andreozzi et al. 2003], and approx. 0.6–17 d for propranolol, under sunlight [Andreozzi et al. 2003, Robinson et al. 2007]), the parental compounds of both diclofenac and propranolol have been repeatedly detected in the environment (e.g. Andreozzi et al. 2003, Andersson et al. 2006, Długołęcka 2007, Wahlberg et al. 2010, Falås et al. 2012), and sometimes in higher concentrations in WTP effluent than in influent (Wahlberg et al. 2010). The substances have also been observed to affect non-target species (De Lange et al. 2006, Triebskorn et al. 2007), including coastal Baltic Sea organisms such as blue mussels (Ericson et al. 2010), amphipods and macroalgae (Eriksson Wiklund et al. 2011, Oskarsson et al. 2012, 2014).

A 50/50 mixture of diclofenac and propranolol was used as test substance with the following nominal concentrations of each substance: 0, 10, 100 and 1000 µg l^{-1}, resulting in total exposure concentrations of 0 µg l^{-1} (control), 20 µg l^{-1} (low), 200 µg l^{-1} (medium) and 2000 µg l^{-1} (high). The chemicals were obtained from Sigma-Aldrich. Diclofenac was dissolved in dilute bisodium carbonate solution and the pH was adjusted to 7.1 by the addition of dilute phosphoric acid. Propranolol was dissolved in dilute phosphoric acid and the pH was adjusted to 7.1 by the addition of bisodium carbonate solution. All solutions were prepared and diluted with buffer so that the final concentrations of sodium, phosphate and car-

bonate ions were identical. The solvents were also added to the controls. The use of buffers to dissolve the pharmaceuticals leads to no or insignificant adsorption to the surfaces of the exposure and experimental vessels (Palmgren et al. 2006). Previous experiments have shown that this procedure results in reliable exposure conditions with no effect of the solvent on the test organism (Ericson et al. 2010, Eriksson Wiklund et al. 2011, Oskarsson et al. 2012, 2014).

Experimental setup

The experiment was conducted in glass aquaria with 10 mussels in each and 1 l sand-filtered seawater, collected at the Askö Laboratory (Stockholm University Baltic Sea Center), close to the mussel sampling sites. Mussels were divided into size categories with regard to length (anterior–posterior axis: 2–2.19, 2.2–2.49, 2.5–2.79 or 2.8–2.9 cm), and an equivalent number of mussels per size category were distributed in each experimental glass aquarium to ensure an equal size distribution among the sites and replicates (Fig. S1 in the Supplement). The mussels were exposed to the pharmaceutical mixture during 3 wk, followed by 2 wk in the same experimental containers but without exposure (the recovery period). All 4 treatments (control, low, medium and high) included 7 replicates (n = 7), for each of the 3 sites, comprising a total of 84 experimental aquaria. Test organisms were left in their respective aquaria for acclimation for 24–48 h prior to exposure. The experiment was conducted in a climate chamber at constant temperature and salinity (11°C, 6.1 PSU), following the natural light regime. All aquaria were individually aerated with thin plastic tubes to ensure sufficient oxygen supply (levels of O_2 measured during the experiment ranged from 8.3–8.4 mg O_2 l^{-1}) and were randomly placed in the climate chamber. Semi-static systems were used and the water exchanged every 3–4 d. Food (a microalgae solution of *Thalassiosira weissflogii* [Instant Algae, Reed Mariculture], approx. 3.2×10^7 cells l^{-1}, corresponding to approx. 4.36 mg dry wt l^{-1}) and pharmaceutical substances were added to the water of each aquarium following each water exchange. The same amount of microalgae solution was also provided during the acclimation and in filtration experiments during scope for growth (SFG) measurements (described in the next subsection), and was chosen to ensure a high clearance rate and to prevent production of pseudofaeces (Widdows et al. 1979). The distributed food corresponded to the amounts of microalgae distributed in other studies (Maire et al. 2007, Prevodnik et al. 2007). Particle concentrations (cells l^{-1}) were attained by a particle counter and size analyser (Beckman Coulter Z2).

Experimental endpoints

The physiological status of the mussels following exposure to the pharmaceutical mixtures, and subsequently in the recovery period, was assessed using the experimental endpoints respiration, absorption efficiency and consumption rate, which were also combined as the energy budget scope for growth (SFG). The measurements were conducted after 3 wk of exposure and after the 2 wk recovery period. All endpoints were measured on the same day, and were conducted in the same glass aquaria as used for the exposure and recovery. Prior to measurements, the water was exchanged, and new test substances were added following the water exchange after the exposure period to prevent recovery during the measurements performed. The experimental endpoints were normalised to the total mussel dry weight of each aquarium at the end of the experiment. Mussel dry weight was obtained by drying the mussels for 48 h at 60°C at the end of the experiment.

Respiration (R, J g^{-1} dry wt h^{-1}) was determined as the change in dissolved O_2 concentration (mg O_2 l^{-1}) in the water over a time (t) of approximately 3–4 h, where $c(t_0)$ was the initial O_2 concentration at time t_0 and $c(t_1)$ was the final O_2 concentration at time t_1, assuming 14 J mg^{-1} O_2 for the estimation of SFG (Eq. 1):

$$R = \frac{\left[c(t_0) - c(t_1)\right]}{t} \times 14 \quad (1)$$

Oxygen measurements were conducted similarly to previous studies (Ericson et al. 2010), with aquarium water surfaces covered between measurements to avoid diffusion of dissolved O_2 over the water surface, control aquaria with no mussels to correct for changes in dissolved O_2 concentration caused by factors other than mussel respiration, and using an oxygen electrode (Unisense) connected to a picoamperemeter (PA 2000, Unisense).

Clearance rate (CR, l g^{-1} dry wt h^{-1}, Eq. 2), determined as the volume of water cleared of particles per biomass per hour was established following the respiration measurements, and performed similarly to previous studies (Ericson et al. 2010).

$$\mathrm{CR} = \frac{\left[\ln c(t_0) - \ln c(t_1)\right] \times V}{t} \quad (2)$$

Differences in algae concentrations for each aquarium were determined by a particle counter (Beckman Coulter Z2) between addition of microalgae, $c(t_0)$, and after 15–20 min, $c(t_1)$, where V was the volume of water and t was the time elapsed. Consideration was taken of possible sedimentation of the algae by control aquaria with no mussels. Microalgae solution prepared similarly as for feeding during the exposure (see 'Experimental setup' above) was used during the CR measurements. CR was multiplied by initial algae concentration (mg dry wt l^{-1}) and an energy equivalent of 21 J for the estimation of the consumption (C, J g^{-1} dry wt h^{-1}, Eq. 3):

$$C = \mathrm{CR} \times c(t_0) \times 21 \quad (3)$$

Absorption efficiency (AE) was thereafter measured similarly to previous studies (Ericson et al. 2010), combining the ash-free dry weight to dry weight ratio of food (F), i.e. the microalgae, and faeces (E) (Eq. 4):

$$\mathrm{AE} = \frac{(F-E)}{((1-E)\times F)} \quad (4)$$

SFG (J g^{-1} dry wt, Eq. 5) was calculated similarly to previous studies (Ericson et al. 2010):

$$\mathrm{SFG} = \mathrm{AE} \times C - R \quad (5)$$

The experimental aquaria were routinely controlled for dead individuals. Dead mussels were removed immediately when detected.

Recovery was assessed as difference in SFG and its components between the 2 measurements, i.e. the reversion from an initial exposure effect. Full recovery, as in the return to the mussels' original condition, was not assessed, but the 2 wk period without exposure was considered sufficient to quantify the mussels' potential for recovery.

Statistical analysis

To test the effects of the 3 factors: (1) 'concentration'; (2) time of 'measurement' (i.e. after exposure, t_1, versus after recovery, t_2); and (3) sampling 'site', a repeated-measures general linear model was used, using SAS software version 9.2. Measures were repeated over time within each aquarium, using measurement, site and concentration as fixed factors (y: Measurement, Site, Concentration, Measurement × Site, Measurement × Concentration, Site × Concentration, Concentration × Site × Measurement; Repeated: time; Subject: aquaria). Data fulfilled the criteria of the model. A summary of the main effects is presented in Table 1, while a summary of the p-values of the pairwise comparisons within each site, originating from the same model, is presented in Table S2 in the Supplement. Recovery was also determined from the general linear model, as differences in pairwise comparisons of the values of each variable, of each particular concentration and site at t_1 and t_2. Mortality and differences in dry weight were tested by ANOVA. Results are presented as mean ± SE. Differences between treatments were considered statistically significant at $p < 0.05$, while close-to-significant differences ($p < 0.1$) are also reported and discussed.

RESULTS

Responses to pharmaceutical exposure

SFG was significantly affected by pharmaceutical concentration ($p < 0.001$), as were 2 of its components, respiration and consumption (Table 1). Two general response patterns were found: (1) mussels exposed to the highest concentration (2000 µg l^{-1})

Table 1. Main effects from linear repeated-measures analysis (α = 0.05) of measurement, site and concentration on scope for growth (SFG), respiration rate, consumption and absorption efficiency (AE) in *Mytilus edulis trossulus*. ns: not significant

Effect	SFG			Respiration			Consumption			AE		
	df	*F*	p	df	*F*	p	df	*F*	p	df	*F*	p
Measurement	1	0.73	ns	1	84.82	<0.001	1	0.71	ns	1	8.74	0.004
Site	2	0.52	ns	2	33.43	<0.001	2	0.04	ns	2	0.91	ns
Concentration	3	12.29	<0.001	3	3.2	0.03	3	17.96	<0.001	3	0.85	ns
Measurement × Site	2	0.01	ns	2	0.09	ns	2	0.12	ns	2	0.06	ns
Measurement × Concentration	3	8.55	<0.001	3	2.4	ns	3	6.96	<0.001	3	2.23	ns
Site × Concentration	6	1.36	ns	6	2.4	0.03	6	2.24	0.04	6	0.22	ns
Measurement × Site × Concentration	6	1.63	ns	6	0.99	ns	6	1.52	ns	6	1.45	ns
Denominator df	140			143			144			141		

had lower SFG than the controls ($p < 0.001$), whereas (2) mussels exposed to the medium level of concentration (200 µg l^{-1}) showed the opposite pattern, with higher SFG than the controls ($p < 0.001$).

Pairwise comparisons within each site revealed the same pattern, with lower SFG in mussels exposed to the high concentration, compared to the respective controls, from 2 of the 3 sites, Site 1 ($p = 0.04$) and Site 2 ($p = 0.03$) (Table S2 in the Supplement at www.int-res.com/articles/suppl/m526p089_supp.pdf). For mussels exposed to the medium concentration, SFG was higher compared to the respective controls for Site 3 ($p < 0.001$) and Site 1 (although not significantly, $p = 0.06$) (Fig. 2, Table S2).

The response patterns of the individual components of the SFG were similar to the SFG, particularly for consumption, while respiration was affected by all 3 factors: concentration, time of measurement and sampling site ($p = 0.03$, $p < 0.001$, and $p < 0.001$, for Sites 1, 2, and 3, respectively; Table 1). Mussels from Site 3 were more affected by the exposure than mussels from Sites 1 and 2, as both respiration and consumption were also affected in the low and medium concentrations (Fig. 3A,B, Table S2). AE was not at all affected by the pharmaceutical exposure (Fig. 3C, Table S2).

After the exposure period, SFG of the control from Site 3 seemed lower than the controls from the other 2 sites, although not significantly. However, the SFG of the control from Site 3 was significantly lower after the exposure period than after the recovery period ($p = 0.03$; Table S2), as a result of 1 of the 7 replicates having a negative SFG due to an extremely low consumption rate (data not shown).

Recovery from pharmaceutical exposure

There was no general pattern of recovery in terms of difference in response between t_1 and t_2. The recovery pattern differed between measured variables, sites and treatments. Mussels exposed to the highest pharmaceutical concentration increased in SFG after the recovery, while mussels exposed to the medium concentration showed a decrease (Fig. 2), i.e. they evened out compared to their respective controls. This was displayed as a significant interaction between concentration and time of measurement ($p < 0.001$; Table 1). This interaction pattern was consistent between sites, where it was significant in pairwise comparisons for the high concentration at Site 1 ($p = 0.02$), and close to significant for Site 3 ($p = 0.06$), as well as for the medium concentrations at Site 1 ($p = 0.04$) and Site 3 ($p = 0.03$) (Fig. 2, Table S2). The same pattern was also demonstrated for consumption (Fig. 3B, Table S2), but not for respiration (Fig. 3A, Table S2). The recovery potential was also displayed as fewer significant differences in SFG and consumption between treatments and the control at t_2 compared to t_1 (Figs. 2 & 3B, Table S2). Mussels from Site 3 that were exposed to the high concentration had reduced ability to recover from the exposure, a result of a decreased respiration and consumption compared to the control at t_2 (Fig. 3A,B, p =

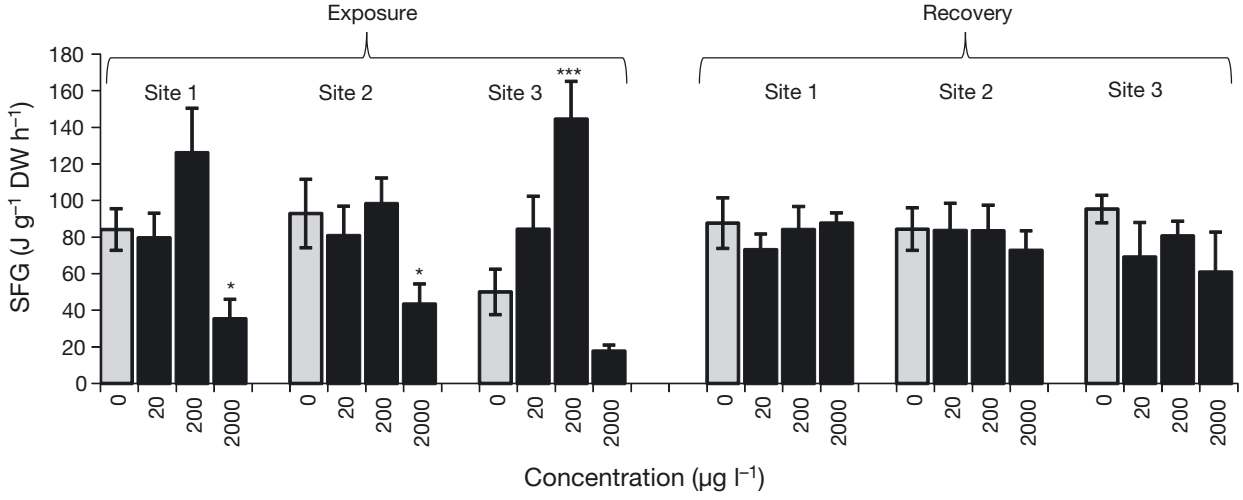

Fig. 2. Scope for growth (SFG, average ± SE) for mussels *Mytilus edulis trossulus* from 3 sites, exposed to diclofenac$_{50\%}$/propranolol$_{50\%}$ mixtures for 3 wk, then recovery for 2 wk. Significant differences between exposure treatments and control at each site (grey bars) are indicated by *$p < 0.05$ and ***$p < 0.001$. For differences between sites and sampling occasion and interactions between factors, see Table 1 (main article) and Table S2 (Supplement)

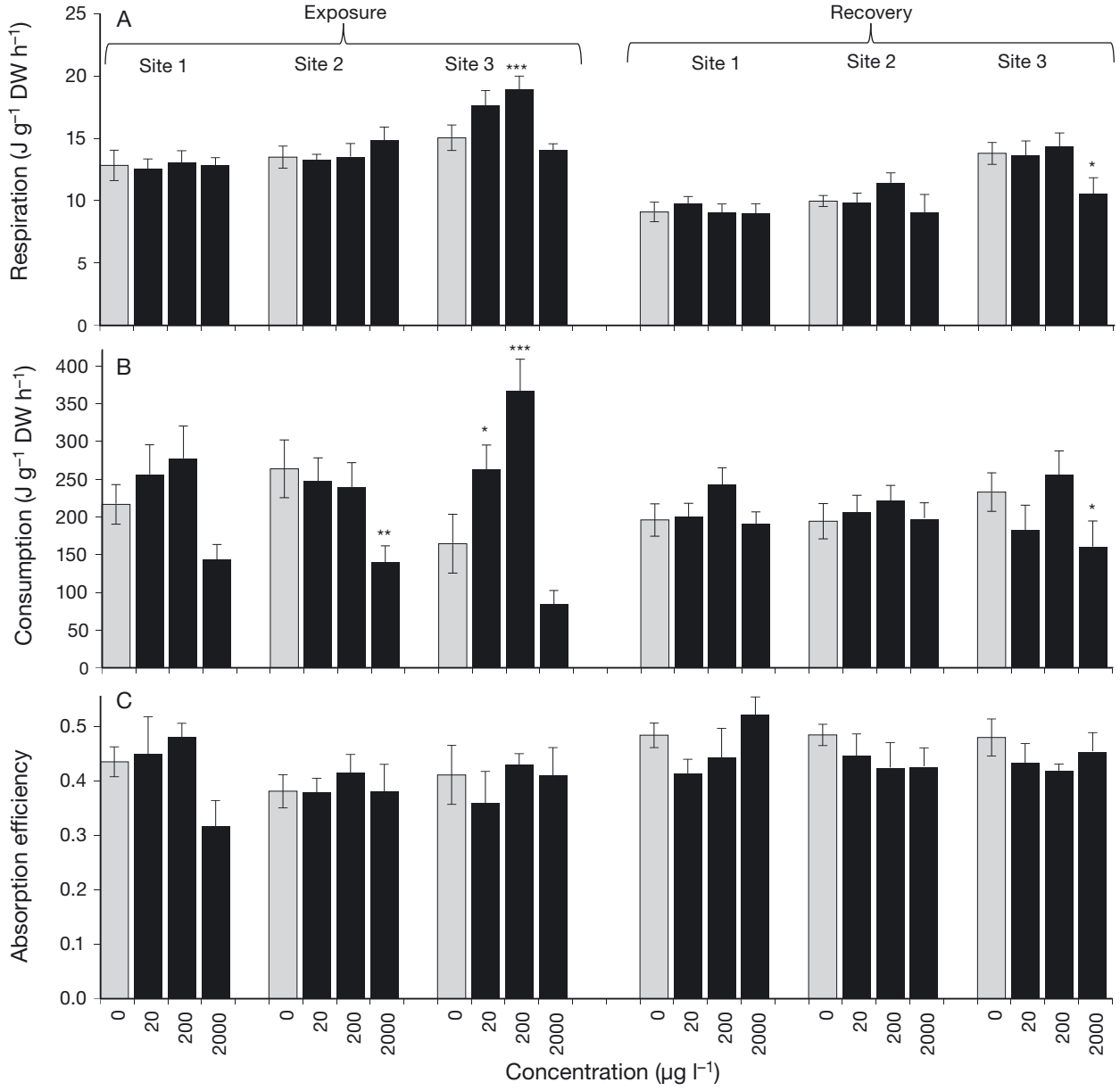

Fig. 3. (A) Respiration, (B) consumption, and (C) absorption efficiency for mussels *Mytilus edulis trossulus* from 3 sites, exposed to diclofenac$_{50\%}$/propranolol$_{50\%}$ mixtures for 3 wk, then recovery for 2 wk (data are average ± SE). Significant differences between exposure treatments and control at each site (grey bars) are indicated by $^{*}p < 0.05$, $^{*}p < 0.01$, and $^{***}p < 0.001$. For differences between sites and sampling occasion and interactions between factors, see Table 1 (main article) and Table S2 (Supplement)

0.02 and p = 0.04 respectively; Table S2). The AE was not affected during the recovery period (Fig. 3C).

Mortality and biomass at end of exposure

The mortality in the experiment was low (1.8%), and did not differ between treatments. No mussels from Site 1 died, and there were no interaction effects in mortality between time of measurement (t_1 and t_2) and site. After the experiment, the average biomass (±SE) of mussels from Site 1 (0.60 ± 0.013 mg dry wt) was higher than Site 2 (0.50 ± 0.012 mg dry wt) and Site 3 (0.34 ± 0.010 mg dry wt) ($p < 0.001$ for both, respectively), and the average biomass of mussels exposed to the medium concentration was lower than in the 2 other treatments ($p < 0.05$). No differences in biomass were detected between mussels exposed to different concentrations within a site (Fig. S2 in the Supplement).

DISCUSSION

In this study, Baltic Sea blue mussels were hypothesised to be negatively affected by short-term exposure to pharmaceutical mixtures, but to have the ability to recover after removal of the exposure, and that the blue mussels sampled closer to the WTP would respond and recover differently compared to mussels sampled further away. Both hypotheses were corroborated, to various extents. Blue mussels were physiologically stressed by exposure to the mixture containing diclofenac and propranolol, and had the ability to recover, and mussels sampled farthest from the WTP outlet (Site 3) seemed to be most affected by the pharmaceutical exposure.

Lower SFG compared to controls implies a reduced fitness of the exposed mussels (ICES 2006), which indicates a toxic effect from the exposure that resulted in less energy available for stress response. The increased SFG in mussels exposed to the medium concentration in this study was mainly caused by the increased consumption rate (Fig. 3B; Table S2 in the Supplement), which seemed to be related to maintenance and stress-induced responses, and not growth, as the biomass after the experiment was lower in mussels exposed to this concentration. Cellular stress response mechanisms require energy (Guttman 1994, Hoffmann & Parsons 1991), and exposure to contaminants can disturb the energy balance of an organism (Sokolova et al. 2012). A higher caloric intake could also per se induce or increase oxidative stress (Finkel & Holbrook 2000, Speakman & Mitchell 2011). In the shorter perspective, increased SFG indicates healthier mussels, but it may be a nonlinear response with a favourable biological reaction to low-level exposure of a stressor. As low-level exposure also can activate detoxification mechanisms (Fulda et al. 2010), chronic low-level exposures may also reduce SFG or fitness (Calow 1989). Stress responses are often nonlinear and may shift from different states (Muradian 2001, Scheffer et al. 2001, van Nes & Scheffer 2004, Wu et al. 2011, Vandenberg et al. 2012). Such a shift could explain the increased SFG in the medium concentration, followed by the reduced SFG in the high concentration in mussels from Site 3. Such patterns have also previously been observed for low-level exposures in blue mussels (e.g. Strömgren 1982, Eertman et al. 1995, Ericson et al. 2010). Although not significant, a similar pattern of activation in the medium concentration and reduction in the high concentration can be suspected also for mussels from Site 1. Nonlinear stress responses and synergistic effects of multiple exposures like these make predictions of responses to pharmaceutical exposure ambiguous, which may have severe management implications (Groffman et al. 2006). Studies of bioenergetic effects of environmental stressors and their consequences for fitness have been suggested to provide a suitable framework for integrating physiology and functional ecology. This can increase the understanding of driving forces and limitations of environmental adaption, and improve assessments of ecological risk of multiple stressors (Sokolova 2013).

There are several indications that mussels sampled closer to the WTP (Sites 1 and 2) had a higher capability of coping with the exposure, and can thus be regarded as more resilient compared to the mussels sampled further from the WTP outlet (Site 3). Mussels from Sites 1 and 2 were both less affected by the exposure, and recovered when the stress was removed, compared to the mussels from Site 3. The overall respiration after the recovery period was lower in all treatments, including the controls, indicating an effect of maintenance, which may occur when keeping field-sampled organisms in the lab for longer periods. However, the respiration (and consumption) in mussels from Site 3 that were exposed to the highest concentration was still lower than the control, while previous exposure effects in all other treatments from all the sites disappeared, i.e. recovered. The absence of mortality and higher biomass at the end of the experiment among mussels from Site 1 further suggests that they were more resilient than mussels from the other sites. The more pronounced response, the slower recovery and lower biomass in mussels from Site 3 at the end of the experiment indicates a lower adaption ability, and hence lower resilience towards pharmaceutical exposure. This is analogous with a similar study with Baltic Sea blue mussels, where mussels from a clean site were moved to contaminated areas and exposed along with local reference mussels (Turja et al. 2014). The dislocated mussels (from the clean site) required a longer time to recover compared to their respective local reference mussels.

Two potential reasons for the observed differences in responses of mussels from different sites can be coupled to the gradient of WTP effluent in the Himmerfjärden bay, where the mussels were sampled. One possible reason is the higher nutrient and chl *a* concentrations in the inner part of Himmerfjärden (VAS-rådet 2013, Zakrisson et al. 2014), which results in a larger food availability at Sites 1 and 2, than further out in the bay (Site 3). A sufficient food supply may have influenced the mussels' initial health con-

ditions, in terms of larger lipid reserves, a phenomenon observed in a similar study performed in the same area (M. Grahn et al. unpubl.). Thus, a better initial health status of mussels from Sites 1 and 2 may have contributed to a better ability to cope with the pharmaceutical exposure, compared to mussels from Site 3. Another, or complementary, explanatory factor for the observed response differences may be the pharmaceutical substances, including the substances used in the present study, that are present in the WTP effluent (Długołęcka 2007), causing different levels of pre-exposure among mussels from the 3 sites. Pre-exposure to natural and anthropogenic stressors can lead to better acclimation (Slocum & Mendelssohn 2008), lower stress responses (Khan et al. 2011, Bach & Dahllöf 2012), and higher resistance and resilience towards additional stress (Kaufman 1982, Kiffney & Clements 1996, Carroll et al. 2007), and may thus increase the organisms' fitness in variable environments (Bijlsma & Loeschcke 2005). An indication of acclimation in mussels from Sites 1 and 2 is their higher energy status after the exposure, giving them a higher flexibility and a wider stress-tolerance window, as this can be shifted by adaptation, acclimation or acclimatisation (Sokolova et al. 2012). Previous studies have shown local differences in physiological responses to contaminant exposure in Baltic Sea blue mussels (Prevodnik et al. 2007, Lilja et al. 2008). Adaption to local conditions has also been displayed as differences in gene expression in Baltic Sea flounder *Platichthys flesus* (Larsen et al. 2007), and differences in the genome of both Baltic Sea blue mussel (M. Grahn et al. unpubl.) and three-spined stickleback *Gasterosteus aculeatus* (Lind & Grahn 2011). It is, however, not likely that the mussels from the different sampling sites in the present study represent different populations, as the dispersal of blue mussel larvae can be substantial (Johannesson & André 2006). Rather, if pre-exposure to pharmaceuticals is the main factor for the observed effects, an acclimation or local adaption through selection within each generation at the different sites seems to have occurred within the Himmerfjärden bay.

The ability to recover from a 3 wk exposure to a pharmaceutical mixture suggests a plasticity of the Baltic Sea blue mussels. It also shows that the negative effects from this relatively short exposure on the mussels' energy budget seem to be short term without lasting long-term effects. Mussels are tolerant organisms that normally cope well with changing conditions (Newell 1989, Widdows & Salkeld 1993). Still, our results are of particular interest, as the Baltic Sea blue mussel has been shown to be more sensitive to disturbances than blue mussels from more saline conditions (Tedengren & Kautsky 1987). The blue mussel was also found to be the most sensitive species in a multi-species experiment with coastal organisms exposed to propranolol (Oskarsson et al. 2014). The long life cycle, sedentary life style and capacity to filter vast volumes of water (Kautsky 1982, Vuorinen et al. 2002) all result in the Baltic Sea blue mussel being both an important and relevant study organism of the effects of chronic exposure to pollutants in the Baltic Sea. As the blue mussel is the most significant filter feeder in the Baltic Sea, changes in the mussel population would influence the entire ecosystem. Its ability to withstand short-term disturbances, its resilience, is thus of great importance.

The effects observed in this study were the result of exposure to higher concentrations than detected in WTP effluents. However, being pseudo-persistent substances, pharmaceuticals can constitute a chronic exposure to organisms in aquatic environments, especially in areas close to WTP outlets. Due to the life strategy of blue mussels, they can be exposed during extended periods of time. In addition, diclofenac and propranolol belong to substance groups (NSAIDs and β-blockers) that include several different substances with the same or similar mode of action. It is therefore possible, or even plausible, that several of the substances within each group can affect non-target organisms in the same or similar manner. Several pharmaceuticals can consequently affect an organism with larger combined effects than the respective single substance. The concentration of each separate substance could then be regarded as a part of the total concentration of the substance group. Compared to previous single-substance exposures of Baltic Sea blue mussels to diclofenac and propranolol (Ericson et al. 2010, Oskarsson et al. 2014), there seems to be an increased effect on SFG (and its components) from the exposure to mixtures of the 2 substances (Ericson et al. 2010). In the present study, significant effects on SFG were found after 3 wk of exposure to a total combined concentration of 200 µg l^{-1}, while previous studies of exposure to diclofenac and propranolol applied separately revealed effects predominantly after exposure concentrations of 1000 µg l^{-1} and higher (Ericson et al. 2010, Oskarsson et al. 2014). These results emphasise the relevance of studies of mixtures, and indicate the possibility of larger effects from multiple stressors also in the environment.

The amount of diclofenac or propranolol in the exposure water or organisms was not determined in this experiment. In a study with similar settings, the nominal compared to the measured concentrations in

the water varied on average ca. 20% just before the water exchange (Eriksson-Wiklund et al. 2011), and both substances have been shown to be taken up by, and bind to, Baltic Sea blue mussel tissue (Amini et al. 2009, Ericson et al. 2010, Oskarsson et al. 2014), after exposure via the water or possibly via ingested microalgae. Propranolol has been shown not to bind to algae to a large extent, and is therefore possibly taken up mainly by mussels directly from the water, rather than via consumed food (Ismail et al. 2014). It is likely that the pharmaceutical concentrations varied slightly between each water renewal with this experimental setup; however, the repeated additions of new substances following each water exchange assured relatively constant concentrations throughout the exposure.

This study illustrates that chronic exposure of aquatic organisms to differences in food availability and/or sublethal concentrations of contaminants can affect their ability to cope with additional stress. Acclimation, adaptation or tolerance is associated with elevated fitness costs (e.g. Calow 1991, Xie & Klerks 2004, Kwok et al. 2009), which increases susceptibility to other stressors (Clements 1999, Bach & Dahllöf 2012). In the long run, this could have more severe effects on the ecosystem. The implications of the present findings may be greater in the Baltic Sea than in other seas, due to the low species diversity of the area, and the low genetic diversity of many Baltic Sea organisms (Johannesson & André 2006). Increased acclimation and recovery potential in pre-exposed populations may on the one hand contribute positively to the resilience and the diversity of the coastal communities of the Baltic Sea, but could also result in increased susceptibility to other contaminants, and hence more severe effects on the ecosystem.

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