Supplementary

Environmental safety of nanocellulose: an *in vivo* acute study with marine mussels *Mytilus galloprovincialis*

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Additional description of methods

Carboxylesterase (CbE) activity was measured using 25 µL of undiluted gills and 25 µL of digestive gland sample (10-fold diluted) mixed with 200 µL of a solution containing ρ-nitrophenyl acetate (ρNPA) or ρ-nitrophenyl butyrate (ρNPA) as substrates at 1 mM final concentration in well. For both substrates reading was performed at 405 nm for 5 minutes (ε = 18,0 mM- 1*cm-1) according to the method of Hosokawa and Satoh [1] adapted by Solé *et al.* [2]. Commercial recombinant human hCE1 (E0162) was used as positive control.

The activity of GST was analyzed according to the method of Habig *et al*. [3]. In each well 25 µL of undiluted sample (gills) or 10-fold diluted (digestive gland) was added to 200 µL of reaction mixture (containing in well: 1 mM GSH, 1 mM CDNB). Positive control of commercial GST (G6511) was used. Reading was performed 340 nm for 3 minutes. The activity was calculated by using ε = 9,6 $mM^{-1}cm^{-1}$.

CAT activity was measured according to Aebi *et al*.[4]. In each well, 10 µL of diluted sample (4-fold for gills and 10-fold for digestive gland) were added to 200 µL of reaction mixture containing H_2O_2 (50 mM final well concentration). Commercial purified catalase (Sigma ref.

SRE0041) was used as positive control. The reading was performed at 240 nm for 1 minute using ε = 40 $M^{-1}*cm^{-1}$.

The activity of GR was measuring placing 20 µL of undiluted sample and 200 µL of a mixture containing 1 mM GSSG and 0.5 mM NADPH as final concentrations. Commercial GR (Sigma ref. G3664) was used as positive control. The reading was performed at 340 nm for 3 minutes according to the method of Calberg and Mannervik[5] with ε = 6.22 $mM^{-1}*cm^{-1}$.

The GPX activity was measured using both cumene hydroperoxide (CHP) and H2O2 as substrates adapting the method of Günzler and Flohé[6]. For both gills and digestive gland tissues, in each well 10 µL of undiluted sample were placed and then 200 µL of reaction mixture (0.1 mM NADPH, 1.6 mM GSH and 1 U/mL GR as final concentrations) when using CHP as substrate or 200 µL reaction mixture containing 0.125 mM NADPH, 2.075 mM GSH, 1 U/mL GR and 1.038 mM NaN_3) when using H2O2 as substrate. After 2 minutes of incubation, 30 µL of CHP (final concentration 0.625 mM) or 30 µL of H_2O_2 (0.2 mM) were added. An extract of fish liver (*Sparus aurata*) was used as positive control. For both substrates, the reading was done at 340 nm for 3 minutes. GPx activity was calculated with ε = 6.22 $mM^{-1}*cm^{-1}$.

The total protein content in each tissue was done using the Bradford Method[7]. A 7-point bovine serum albumin (BSA) (0.05 – 0.5 mg mL^{-1}) was used for protein content calculation. 10 µL of samples and standards were mixed with 200 µL of Bradford reagent BioRad and after 15 minutes agitation the absorbance was read at 595 nm and protein content expressed in mg mL^{-1}.

The lipid peroxidation levels (LPO) were measured in terms of malondialdehyde (MDA) equivalents using 200 µL of undiluted samples mixed with 650 µL of 1-methyl- 2-phenylindole in methanol:acetonitrile (1:3) and 150 µL of 37 % HCl. Six point standards (0.1- 12.5 µM) were made, with 1,1,3,3 tetramethoxypropane. Simultaneously, samples and standards were vortexed and then kept in a water bath at 45°C for 40 minutes. After that, the reaction was stopped in ice for 10 min, then centrifuged at 10,000 rpm for 10 minutes. A 200 ul aliquot of the supernatant of sample and standards was read at 586 nm.

Integration of Results

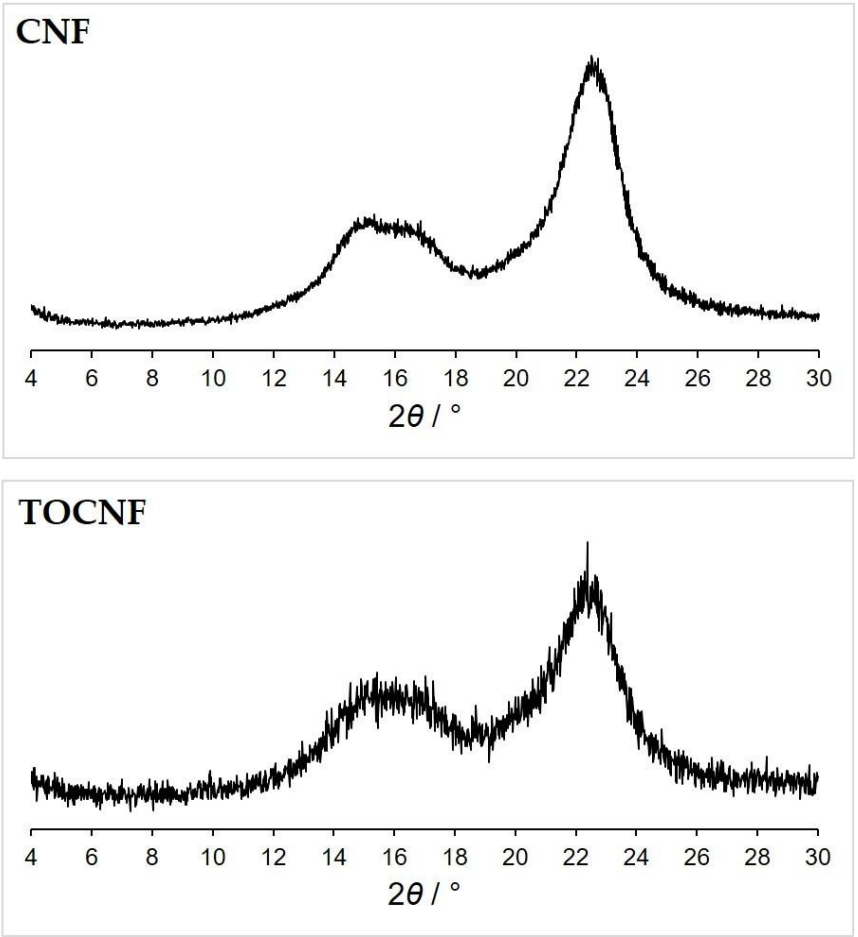

Figure S1. XRD diffractograms of CNF and TOCNF, recorded for the calculation of the Cristallinity Index.

Table S1. pH and salinity values of CNF and TOCNF (1mg L^{-1}) water suspensions over time (t0= right after dispersion, t1= 24h, t2= 5 weeks t3=6 weeks. Data shown as mean values ± standard deviation (n=3 independent measurements).

pH	**NSW**	**ASW**	**NSW + CNF**	**ASW+ CNF**	**NSW + TOCNF**	**ASW + TOCNF**
t0	8.2 ± 0.01	8.1 ± 0.06	8.1 ± 0.01	8.1 ± 0.03	8.1 ± 0.14	8.1 ± 0.05
t1	7.9 ± 0.06	8.1 ± 0.03	8.0 ± 0.05	8.1 ± 0.05	7.8 ± 0.05	8.2 ± 0.01
t2	7.9 ± 0.06	8.1 ± 0.04	7.7 ± 0.03	7.7 ± 0.04	7.8 ± 0.05	8.1 ± 0.05
t3	7.9 ± 0.06	8.1 ± 0.01	7.8 ± 0.04	7.9 ± 0.04	7.8 ± 0.02	8.0 ± 0.02
Salinity (‰)	**NSW**	**ASW**	**NSW + CNF**	**ASW+ CNF**	**NSW + TOCNF**	**ASW + TOCNF**
t0	41 ± 0.01	41 ± 0.00	41 ± 0.01	41 ± 0.01	41 ± 0.01	41 ± 0.01
t1	41 ± 0.00	39 ± 0.01	41 ± 0.01	39 ± 0.01	41 ± 0.01	39 ± 0.01
t2	41 ± 0.00	40 ± 0.01	40 ± 0.01	39 ± 0.01	40 ± 0.01	39 ± 0.01
t3	40 ± 0.01	39 ± 0.00	41 ± 0.01	39 ± 0.01	40 ± 0.01	41 ± 0.01

Table S2. Neutral red retention time (NRRT) assay in hemocytes of mussels exposed to 1 µg L^{-1} and 1 mgL^{-1} of CNF and TOCNF. Data are shown as % of destabilized cells out of 100 scored.

Concentrations tested		**1µg L^{-1}**		**1mg L^{-1}**			
Tissues	**Hemolymph**						
Time		15 min	30 min	45 min	15 min	30 min	45 min
NRRT	Control	19 ± 6.24	35 ± 2.65	43 ± 2	33.7 ± 3.1	44 ± 2.6	63.7 ± 5
	CNF	30 ± 1	69 ± 5.29	90 ± 3	36.3 ± 3.5	60 ± 8.2	75 ± 3.6
	TOCNF	25 ± 3.61	76 ± 2.65	92 ± 3.61	39.7 ± 3.1	64.7 ± 4.9	61 ± 1

Table S3. P-glycoprotein efflux pump activity expressed as Arbitrary Units of Fluorescence (AUF) in gills of mussels exposed to 1 µg L^{-1} and 1 mg L^{-1} of CNF and TOCNF and known inhibitor Verapamil (1µM).

Concentrations tested		**1µg L^{-1}**	**1mg L^{-1}**
Tissues		**Gill**	**Gill**
P-gp activity	Control	47698 ± 11457.92	22763 ± 4407.45
	CNF	90053.4 ± 12791.09	75673 ± 16437.38
	TOCNF	90864.4 ± 18386.12	67399 ± 8294.02
	VERAPAMIL	89368.2 ± 14676.93	54381 ± 16299.19

Table S4. Activity of ASCH-ChE in hemolymph, gills and digestive glands of mussels exposed to 1 µg L^{-1} and 1 mg L^{-1} of CNF and TOCNF. The activity is expressed as $nmolmin^{-1}mgprotein^{-1}$. The number of biological replicates was n=6 for gills, n= 4 for digestive gland and n= 6 for hemolymph

Concentration tested		**1µgL^{-1}**			**1mgL^{-1}**		
Tissues		**Gill**	**Digestive gland**	**Hemolymph**	**Gill**	**Digestive gland**	**Hemolymph**
ChE ASCh	Control	3.29 ± 1.6	1.778 ± 0.46	14.47 ± 1.94	3.89 ± 0.8	1.58 ± 0.8	18.95 ± 4.4
	CNF	2.99 ± 0.36	1.646 ± 0.513	12.79 ± 2.09	2.59 ± 0.21	1.54 ± 0.64	20.25 ± 3.23
	TOCNF	2.74 ± 1.3	1.492 ± 0.208	11.55 ± 2.67	2.98 ± 0.4	1.66 ± 0.53	13.45 ± 1.31

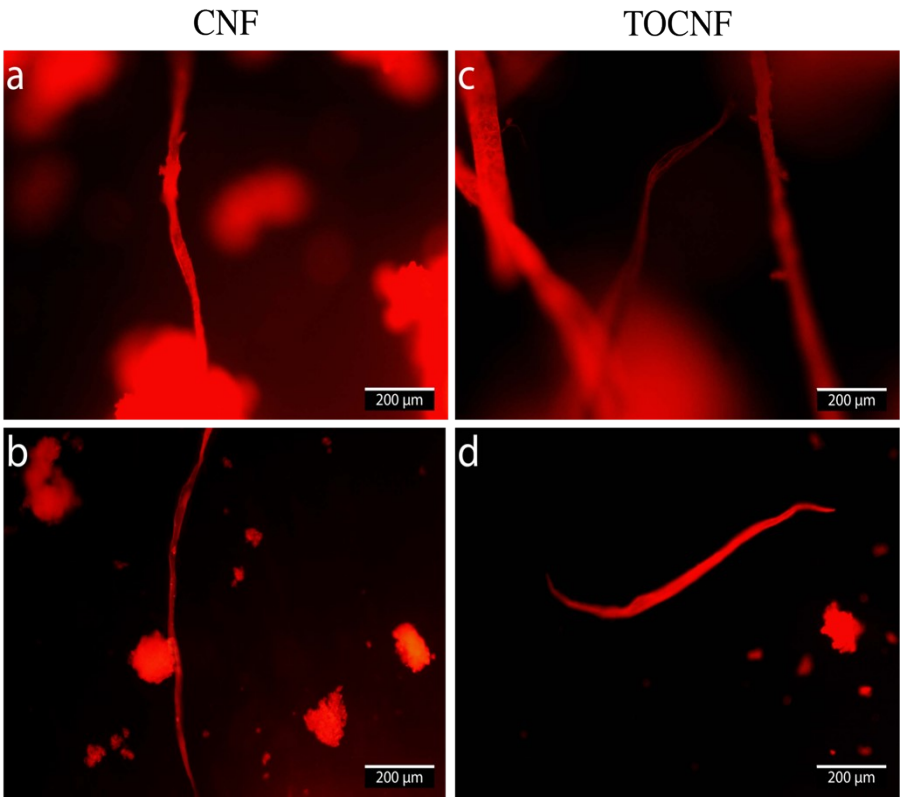

Figure S2. Fluorescence microscopy images of CNF and TOCNF labelled with RhB before and after incubation in NSW at 1mg mL^{-1} (a) pre-incubation CNF (b) after 24h of incubation (c) pre-incubation TOCNF (d) after 24h of incubation.

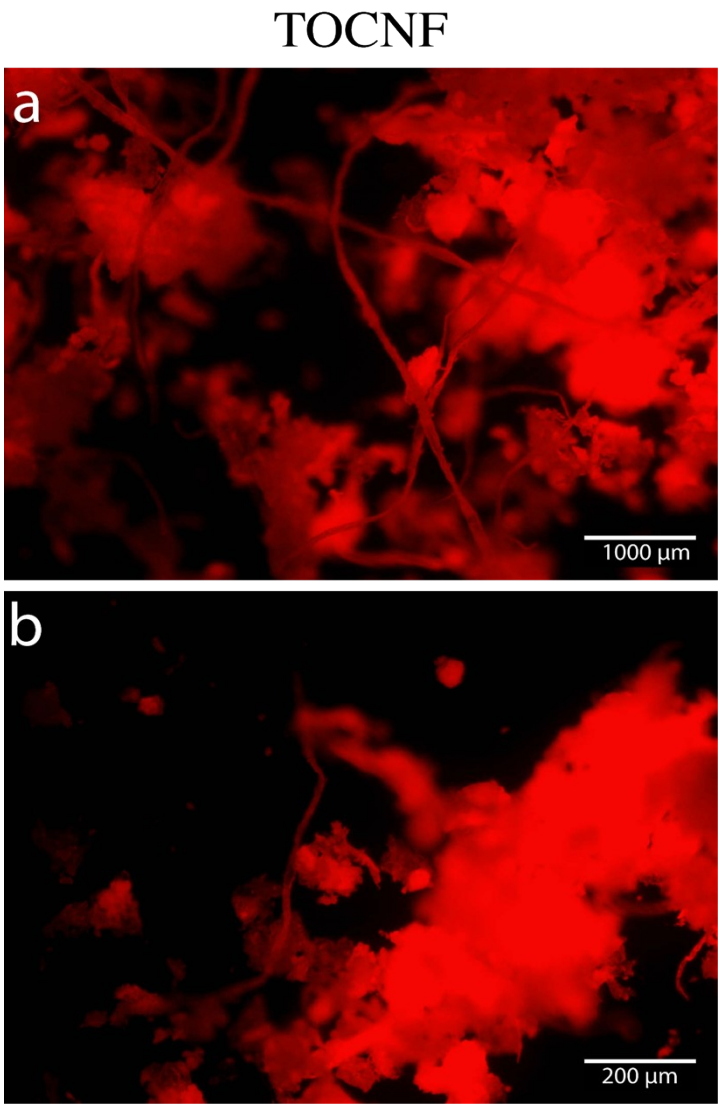

Figure S3. Fluorescence microscopy images of dry TOCNF stained with RhB and TOCNF stained with RhB after 24h of incubation in NSW (1mg mL^{-1}). (a) TOCNF (b) TOCNF after 24h incubation.

References

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