Supplementary material and methods

Chemical compounds analyses

2',7'-difluoro-3',6'-dihydroxy-3-oxo-N-propyl-3H-spiro[isobenzofuran-1,9'-xanthene]-5-carboxamide
1H NMR (DMSO, 500 MHz): δ 10.78 (bs, 1H), 8.82 (t, J = 5.6 Hz, 1H), 8.47 (s, 1H), 8.26 (dd, J = 8.1 Hz, J = 1.6 Hz, 1H), 7.39 (d, J = 8.2 Hz, 1H), 6.89 (d, 7.6 Hz, 2H), 6.58 (d, J = 11.3 Hz, 2H), 1.60-1.55 (m, 2H), 0.92 (t, J = 7.4 Hz, 3H); tR = 4.03 min, RP-HPLC purity >90%, MS (ESI) m/z: 454.0 (M+H)+

3',6'-dihydroxy-3-oxo-N-propyl-3H-spiro[isobenzofuran-1,9'-xanthene]-5-carboxamide (5-PFA): 1H NMR (DMSO, 500 MHz): δ 10.16 (bs, 1H), 8.81 (t, J = 5.4 Hz, 1H), 8.46 (d, J = 0.5 Hz, 1H), 8.24 (dd, J = 8.1 Hz, J = 1.6 Hz, 1H), 7.37 (d, J = 8 Hz, 1H), 6.59-6.53 (m, 4H), 3.29-3.25 (m, 2H), 1.59-1.55 (m, 2H), 0.92 (t, J = 7.4 Hs, 3H); 13C NMR (DMSO, 125 MHz): δ 168.2, 164.5, 159.6, 154.5, 151.8, 136.4, 134.7, 127.2, 126.4, 124.2, 123.2, 112.7, 109.1, 102.3, 41.2, 22.3, 11.5. tR = 3.79 min, RP-HPLC purity >90%, MS (ESI) m/z: 418.0 (M+H)+.

Propyl-3',6'-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-5-carboxylate & propyl-3',6'-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-6-carboxylate (5,6-PEF): For isomer 5: tR = 4.96 min, RP-HPLC purity >95%, HR-MS for C24H18O7: calcd 418.1053, found 418.1012; For isomer 6: tR = 4.82 min, RP-HPLC purity >95%, HR-MS for C24H18O7: calcd 418.1053, found 418.1033.

3-oxo-5-(3-propylthioureido)-3H-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl diacetate (5-PFDAT): Yellow solid; yield: 2.4 mg (18% over 2 steps); tR = 5.37 min, RP-HPLC purity ≥ 87%, HR-MS for C28H24N2O7S: calcd 532.1304, found 532.1310

2',7'-difluoro-3-oxo-5-(propylcarbamoyl)-3H-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl diacetate (Triembarine DA) Yellow solid; 4.5 mg (76% over 2 steps); tR = 5.51 min, RP-HPLC purity ≥95%, HR-MS for C28H21F2NO8: calcd 537.1235, found 537.1236.

3-oxo-5-(propylcarbamoyl)-3H-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl diacetate (5-PFDA) Yellow solid; 3.2 mg (60% over 2 steps); tR = 5.21 min, RP-HPLC purity ≥89%, HR-MS for C28H23NO8: calcd 501.1424, found 501.1424.

3-oxo-5-(propoxycarbonyl)-3H-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl diacetate (5-PFDAE): Yellow solid; 4.2 mg (70%); tR = 6.28 min, RP-HPLC purity ≥95%, HR-MS for C28H22O9: calcd 502.1264, found 502.1261.

3',6'-diacetoxy-2',7'-difluoro-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-5-carboxylic acid (5-COGDA): Yellow solid; 10 mg (83%); tR = 5.10 min, RP-HPLC purity ≥98%, HR-MS for C25H14F2O9: calcd 496.0606, found 496.0623.

2',7'-difluoro-3-oxo-5-(propylcarbamoyl)-3H-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl diacetoxymethyl ester (Triembarine AM): tR = 5.33 min, RP-HPLC purity >95%, HR-MS for C31H21F2NO13: calcd 653.0981, found 653.0979.

Spectroscopic properties
Absorption spectra were recorded in 1 cm path length cuvette (Hellma, 104.002B-QS) on a SPECORD®-205 spectrometer from Analytik Jena. Extinction coefficients of fluorescein, 5-carboxyfluorescein (5-CF), 5-carboxyOregon green (5-COG), *N*-propylOregon Green 5-amido (Triembarine) and *N*-propylfluorescein 5-

amido (5-PFA) were measured in 0.1 M NaOH solution, pH 12.7. The extinction coefficients of pro-fluorophores, *N'*-propylfluorescein diacetate 5-thiourea (5-PFDAT), *O*-propylfluorescein diacetate 5-ester (5-PFDAE), *N*-propylfluorescein diacetate 5-amido (5-PFDA), 5-carboxyOregon Green diacetate (5-COGDA), *N*-propylOregon Green diacetate 5-amido (Triembarine DA) and *N*-propylOregon Green acetoxymethylester 5-amido (Triembarine AM) were measured in PBS buffer (Euromedex, ET330), 0.6 % glutaraldehyde (Sigma-Aldrich, G7776), pH 7. Fluorometric measurements were made using fluorescence grade quartz cuvette (Hellma, 109.004F-QS) and FluoroLog®-3 spectrofluorometer from Horiba Jobin Yvon equipped with sample stirring. The quantum yields (QY) of 5-CF, 5-COG, Triembarine and 5-PFA were measured with samples at 1 µM (absorbance $_{488\ nm} \leq 0.2$) in 0.1 M NaOH, pH 12.7. Every sample and standard (fluorescein) was prepared freshly before use. In this study, fluorescein in 0.1 M NaOH, pH 12.7 was the reference system with a known quantum yield value given by Magde et al. ($\Phi = 0.925$) [4]. Quantum yield measurements were done using standard absorption and emission spectrometers. Emission spectra range was between the wavelengths 498 - 600 nm and excitation wavelength was 488 nm for samples and fluorescein. The refractive indices of samples and fluorescein solutions were the same since they were prepared in the same buffer. Under these conditions, quantum yields were calculated by using this equation: $\Phi^i = (F^i f_s / F^s f_i)^* \Phi^s$

Where Φ^i and Φ^s are the photoluminescence QY of the sample and standard (fluorescein), respectively. F^i and F^s are the integrated intensities (areas) of sample and fluorescein spectra, respectively. f_i and f_s are the absorption factor of sample and fluorescein, respectively ($f_{i\ or\ s} = 1\text{-}10^{-A}{}_{i\ or\ s}$, where A = absorbance) [5].

Supplementary figures legends

Supplementary figure 1: Different bacteria imaging with new pro-fluorophore.

The pictures, overlay with brightfield images, show viable or dead waterborne *Enterobacter cloacae* **(a)**, *Chryseobacterium indologenes* **(b)**, *Pseudomonas aeruginosa* **(c)**, and *Bacillus subtilis* (ATCC 6633) **(d)**, incubated with 5-CFDA or Triembarine AD or Triembarine AM (15 µM) for 30 min at 37 °C. Bacteria were washed before imaged with a Leica DM5500 microscope, 100x objective. Scale bars: 10 µm.

Supplementary figure 2: Triembarine background extinction by different contrast agents.

(a) Representative pictures of viable *E. coli* (EC18) immobilized on beads with anti–EC18 antibodies and stained with Triembarine DA (15 µM) for 30 min at 37 °C. After washes and contrast agent adding (1x PBS: not treated with contrast agent but PBS, Trypan blue, Coomassie blue or Bromophenol blue), beads were imaged with a Leica DM5500 microscope, 63x objective. Scale bare = 10µm. **(b)** Measurements of the median background in fields of interest near the beads after treatment with each contrast agent. N=100 for each condition *p<0.0001. **(c)** Absorption spectrum of contrast agents.

Supplementary Table 1. Physicochemical and spectroscopic properties of fluorescein and Oregon Green derivatives probes.

Cpd	HPLC, T_r (min)[a]	Formula	MW calculated	$\lambda_{Abs\ max}$ (nm)	ε ($M^{-1}.cm^{-1}$)	λ_{ex}[b] (nm)	λ_{em}[b] (nm)	Φ[b]
Fluorescein[d]	ND	$C_{20}H_{12}O_5$	332.31	491[b]	87458 ± 8271[b]	490 ± 0.3	512± 0.5	0.925[e]
5-CF[d]	ND	$C_{21}H_{12}O_7$	376.32	493[b]	76474 ± 4009[b]	491 ± 0.7	517 ± 0.6	0.963 ± 0.004
5-COG[d]	ND	$C_{21}H_{10}F_2O_7$	412.30	493[b]	83136 ± 4452[b]	491 ± 1.2	517 ± 0.4	0.912 ± 0.011
Triembarine	4.03	$C_{24}H_{17}F_2NO_6$	453.39	495[b]	74835 ± 4762[b]	493 ± 0.6	518 ± 1.1	0.902 ± 0.013
5-PFA	3.79	$C_{24}H_{19}NO_6$	417.41	495[b]	73490 ± 8454[b]	493 ± 0.3	518 ± 0.4	0.835 ± 0.002
5-PFDAT	5.37	$C_{28}H_{24}N_2O_7S$	532.56	ND	ND	ND	ND	ND
5-PFDAE	6.28	$C_{28}H_{22}O_9$	502.13	295[c]	7903 ± 761[c]	ND	ND	ND
5-PFDA	5.21	$C_{28}H_{23}NO_8$	501.48	291[c]	9284 ± 758[c]	ND	ND	ND
5-COGDA	5.10	$C_{25}H_{14}F_2O_9$	496.37	300[c]	6979 ± 282[c]	ND	ND	ND
TriembarineDA	5.51	$C_{28}H_{21}F_2NO_8$	537.12	303[c]	9784 ± 351[c]	ND	ND	ND
TriembarineAM	5.33	$C_{31}H_{21}F_2NO_{13}$	653.09	302[c]	11165 ± 334[c]	ND	ND	ND

[a] The detection was performed at 220 nm (see experimental section for HPLC conditions), purity>95% for all compounds.
[b] The spectroscopic parameters of fluorescent probes were measured in NaOH solution (0.1 M, pH 12.7)
[c] The absorption parameters were measured in PBS buffer (pH 7), 0.6% glutaraldehyde.
[d] Commercially available [e] Standard value of fluorescein quantum yield given by Madge *et al.* (2002)
ND: not determined

Abbreviations: 5,6-CF, 5,6-carboxyfluorescein; 5,6-PEF, *O*-propylfluorescein-5,6-ester; 5-PEFDA, *O*-propylfluorescein diacetate 5-ester; 5-FITC, Fluorescein 5-isothiocyanate; 5-PITCF, N'-propylfluorescein 5-thiourea; 5-PITCFDA, *N'*-propylfluorescein diacetate 5-thiourea; 5-CFSE, 5-carboxyfluorescein succinimidyl ester; 5-COGSE, 5-carboxyOregon Green succinimidyl ester; 5-PAF, *N*-propylfluorescein 5-amido; Triembarine, *N*-propylOregon Green 5-amido; 5-PAFDA, *N*-propylfluorescein diacetate 5-amido; TriembarineDA, *N*-propylOregon Green diacetate 5-amido; TriembarineAM, *N*-propylOregon Green acetoxymethylester 5-amido; 5-COG, 5-carboxyOregon green; 5-COGDA, 5-carboxyOregon green diacetate.