Supplementary materials for

Predicting Wastewater Effects on Antibiotic Fluorescence from Laboratory DOM Quenching Behavior by Fingerprinting and Machine Learning

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Text S1. Machine Learning Analysis

19-feature matrix was constructed for each antibiotic (n=27), including: (1) intensity features (raw and log-transformed fluorescence at pH 5, 7, 9; maximum intensity; pH sensitivity ratio IntpH9/IntpH5), (2) PARAFAC component scores (C1-C3 at each pH), and (3) spectral complexity (number of emission peaks). Features were z-score standardized before analysis. [1]

Random Forest classifiers (TreeBagger, MATLAB R2023b) were trained using 100 decision trees with bootstrap aggregation, following. [2,3] Leave-one-out cross-validation (LOOCV) was employed given the sample size, where each antibiotic was iteratively held out for prediction while the remaining 26 were used for training. [4] Two classification tasks were performed: (1) pH Response (Enhanced/Quenched/Stable based on pH ratio >2 and <0.5), and (2) Detection Feasibility (Easy/Conditional/Difficult based on peak count and maximum intensity thresholds.

Permutation-based feature importance was calculated from out-of-bag error increases. [4] The Detection Risk Index (DRI) was developed as a weighted composite score that integrates three fluorescence-derived parameters: maximum intensity (w = 0.5), spectral window coverage (w = 0.3), and number of detectable peaks (w = 0.2). This weighting scheme emphasizes the primary influence of signal intensity on EEM-based detectability, while also accounting for the additional roles of spectral complexity and peak multiplicity. DRI values, which range from 0 to 1, were subsequently used to classify all 27 antibiotics into three risk categories: Low Risk (DRI $\geq$ 0.557), Medium Risk (DRI 0.396-0.557), and High Risk (DRI < 0.396). Model performance was evaluated using accuracy, precision, recall, and F1-score derived from confusion matrices. [5,6]

Table S1. Lists of 27 kinds of antibiotics

NO	Antibiotic	No	Antibiotic
1	Tetracycline	15	Azithromycin
2	Doxycycline	16	Tylosin
3	Chlortetracycline	17	Gentamicin
4	Norfloxacin	18	Neomycin
5	Ciprofloxacin	19	Metronidazole
6	Levofloxacin	20	Ornidazole
7	Enrofloxacin	21	Chloramphenicol
8	Amoxicillin	22	Florfenicol
9	Ampicillin	23	Vancomycin
10	Cephalexin	24	Polymyxin B
11	Sulfadiazine	25	Clindamycin
12	Sulfadimethoxine	26	Trimethoprim
13	Sulfanilamide	27	Rifampicin
14	Erythromycin		

Table S2. PARAFAC component scores for all 27 antibiotics at pH 5, 7, and 9.

Antibiotic	pH5			pH7			pH9		
	C1	C2	C3	C1	C2	C3	C1	C2	C3
Tetracycline	0.16749	0.29191	0.41013	1.66384	1.05519	3.57138	4.13038	0	9.35109
Doxycycline	0.14868	0.18760	0.07924	0	14.5210	0	24.5749	6.59467	10.8537
Chlortetracycline	4.33048	0	0.13125	273.107	41.1709	0	1063.54	81.161	0
Norfloxacin	351.731	9.22386	0	2769.35	0	0	0	197.794	0
Ciprofloxacin	394.560	0	0	4660.22	512.317	0	32.3911	874.276	11.9580
Enrofloxacin	495.147	45.9987	0.91021	3526.52	339.257	0	0	6.07754	575.614
Levofloxacin	0	482.444	0	109.904	6134.21	0	6.10412	1.16912	586.818
Amoxicillin	0	0.00019	0.10067	0	0.43110	2.59441	0.83968	0.00777	0.50056
Ampicillin	0.0002	0	0.00007	1.00893	0.94239	1.86330	0.79347	0.993702	0
Cephalexin	0.43007	0	0.00938	9.47679	37.1620	0.16079	12.3378	0	2.66185
Sulfadimethoxine	0.15962	0.14831	0.23705	1.94376	0.21349	1.17794	0.48611	0.36982	0.59690
Sulfadiazine	0.20117	2.22386	0.13413	0.22602	0	1.34568	0.41622	0.46298	0.16120
Sulfanilamide	3.37696	0	146.623	8.03046	0	2248.61	0	10.4640	0
Erythromycin	0	0.02049	0.03465	0.02545	0.0032	0.0627	0.1197	0.0006	0.0197

					7	3	1	7	7
Azithromycin	1.98540	0	0.02889	0	0.00712	0.29039	0.72641	0.12228	0.09103
Tylosin	0.42325	0	0.17988	0.01377	0.01677	0.00660	0.41464	0.48747	0.19591
Gentamicin	0	0.00002	0.00111	0.03799	0	0.033814	0.139801	0.208473	0.069294
Neomycin	0	0.0000203	0.01119	0.00500	0	0.00192	0.45274	0.21198	0.00670
Metronidazole	0.00067	0.0000967	0.00019	0.07659	0.10137	0.11828	1.14744	0	0
Ornidazole	0.00088	0	0.00014	0.01569	0	0.00264	0.30960	0.43391	0.04106
Chloramphenicol	0	0.00000414	0.0000656	0.00767	0.00184	0.00837	0.07001	0.12675	0.02861
Florfenicol	0	0.0000368	0.033018	0	0.05967	1.01335	0.10217	0.15110	0.07160
Vancomycin	0.00712	0	0.255385	0	0.07366	1.5127	0	0.44074	0.17751
PolymyxinB	0	0.0000194	0.00106	0.00040	0.02346	0.20287	0.14454	0.15217	0.06117
Clindamycin	0	0.0003	0.05517	0	0.00281	0.73451	0.32084	0.03494	0.02215
Trimethoprim	0.342299	0	0.13820	0	4.90112	3.42422	0	0.81863	0.59194
Rifampicin	0	0.0000148	0.00021	0.01130	0.14456	0.00682	0.06425	0	0.69637

Table S3. pH Quenching effects of antibiotic-DOM mixture in pure-water

pH	Intensity of Mixture without HA	Mixture intensity with HA (5mg/L)	Quenching (%)
5	22,355 ± 559	13,779 ± 247	+38.4 ± 2.8
7	15,662 ± 156	14,849 ± 74	+5.2 ± 2.5
9	16,458 ± 326	15,243 ± 457	+7.4 ± 4.3

Table S4. Comparison of antibiotic-DOM quenching parameters with published literature values.

Antibiotic	Parameter	This study	Literature value	Reference	Key difference
Ciprofloxacin (CIP)					
	K_{sv} (L/mg)	-0.0446 (enhancement)	$K_b < 1$ (static quenching)	[7]	Literature measures HA fluorescence quenched by CIP at pH 6-7.4, ionic strength, while this study measured CIP fluorescence with HA addition in pure water at pH 7.
	R^2	0.3469	Linear S-V plot reported	[7]	No significant S-V relationship in present study

	Mechanism	No significant interaction	Static quenching; ΔH = -9.5 to -27.6 kJ/mol	[7]	Literature measures antibiotics to HA while this study HA to antibiotic
	Binding Site	-	Aromatic ring adjacent to two N - group	[8]	Consistent with identification of CIP binding domain
	Ex/Em (nm)	270-345/395-455	275/445 (zwitterionic, pH 7.4)	[9]	Consistent spectral positions
Chlortetracycline (CTC)					
	K_{sv} (L/mg)	0.1212	OTC: K_{sv} = 3.22-12.78 × 10^3 L/mol (HA quenched by OTC)	[10]	The researcher used structural analog OTC; measured HA fluorescence quenched by antibiotic nearly constant pH 4-6, maximum around 6–8, decreases near pH 10. This study focuses only pH 7, so consistent with mid-range binding.
	R^2	0.0148	$R^2 > 0.95$ (OTC-HA system)	[10]	Poor linearity in present study suggests mixed mechanisms
	Quenching % (pH 7)	-5.1 ± 1.2% (enhancement)	OTC: 0-41.8% quenching of DOM	[11]	Literature found HA quenching by OTC, while this study measured CTC slight quenching by HA
	Binding stoichiometry	-	1:1 (OTC:HA)	[10]	Saturation plateau at 5-10 mg/L HA supports binding site model
	Mechanism	Non-significant, mixed	Static quenching: H-bond + van der Waals	[10]	CTC-HA may involve both static and dynamic pathways
Sulfanilamide (SAN)					
	K_{sv} (L/mg)	0.2003	K_{sv} (SD) < K_{sv}(OTC); K_b = 2.05-5.53 × 10^3 L/mol	[11]	different systems and pH/ionic strength
	R^2	0.0856	Linear S-V (SD-HA)	[10]	Good linearity in both studies supports dynamic quenching

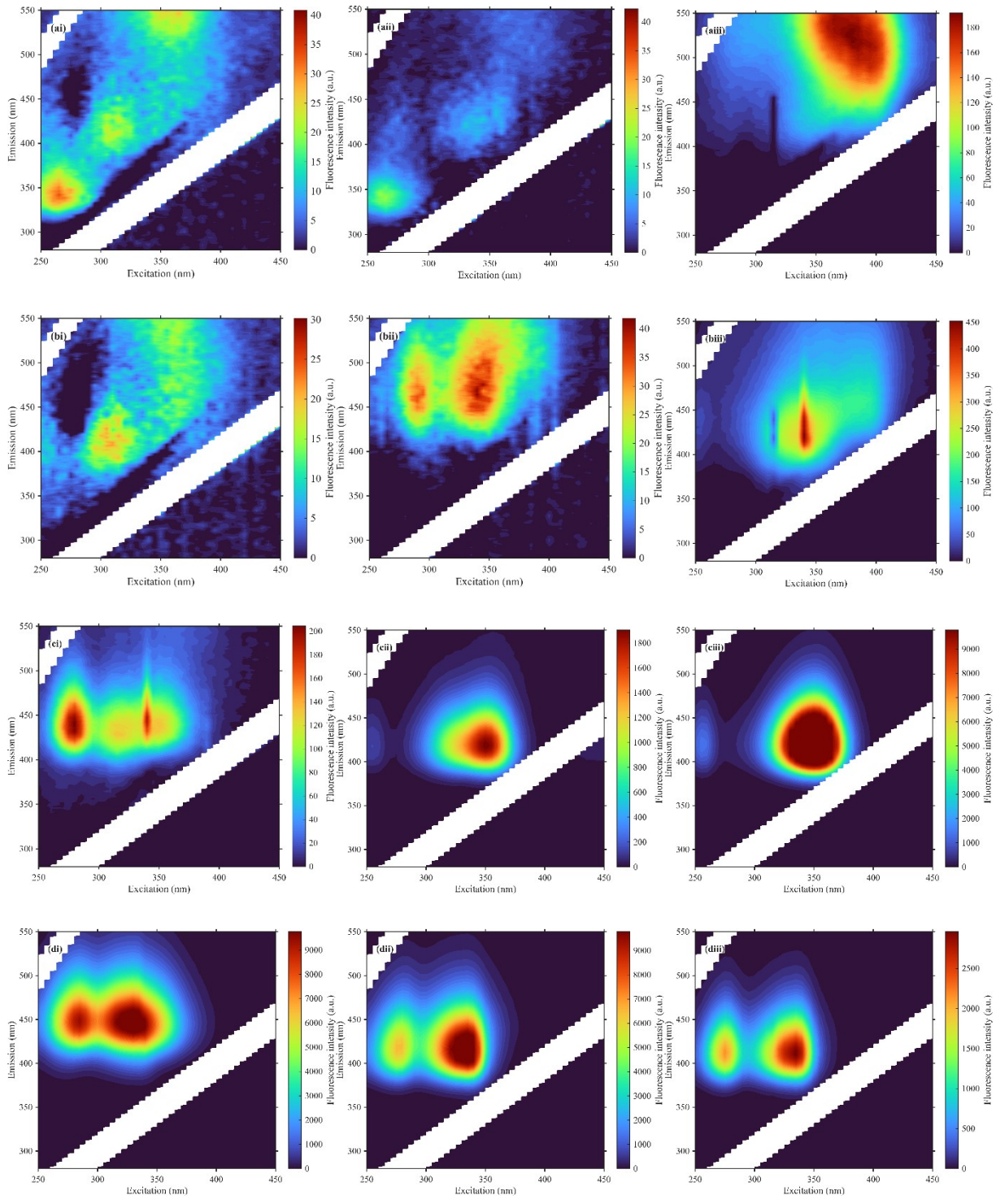
	Quenching %	12.6 ± 1.3 quenching	SD: 0-32% quenching of DOM components	[11]	Variable pH response consistent with literature
	Mechanism	Dynamic quenching (significant)	Static quenching (SD-HA)	[10]	Sulfadiazine (SD), a structurally related sulfonamide, SAN mostly neutral while SDZ partly deprotonated. This supports stronger hydrogen bonding for neutral SAN and more static complexation for SDZ.
Cephalexin (CEP)					
	K_{sv} (L/mg)	-0.0678 (enhancement)	log K_c = 5.33-8.48 (pH-dependent, Ryan-Weber model)	[12]	The present study found CEP fluorescence enhanced by HA at pure water pH 7, no background salts. The recent study reported synthetic wastewater, various ionic strengths; found binding insensitive to Na^+/Ca^{2+} but strongly pH-dependent.
	R^2	0.7117	$R^2 > 0.90$ (Ryan-Weber fits)	[12]	Good fit in both studies
	Mechanism	Fluorescence enhancement	π-π interactions; pH-dependent binding	[12]	Both studies identify π-π bonding as key interaction
	pH dependence	Enhancement increases from pH 5 to pH 9	log K_c decreases from 8.48 (pH 4) to 5.33 (pH 7)	[12]	Consistent with CEP-DOM interaction is pH-dependent

Notes:

This study measures antibiotic fluorescence in the presence of increasing HA concentrations (0-10 mg/L) at pH 7.

Literature values often measured HA/DOM fluorescence quenched by antibiotic addition (reverse direction).

K_{sv} values are not directly comparable across studies due to differences in measurement direction, DOM source, HA concentration range, ionic strength, and units (L/mg vs. L/mol)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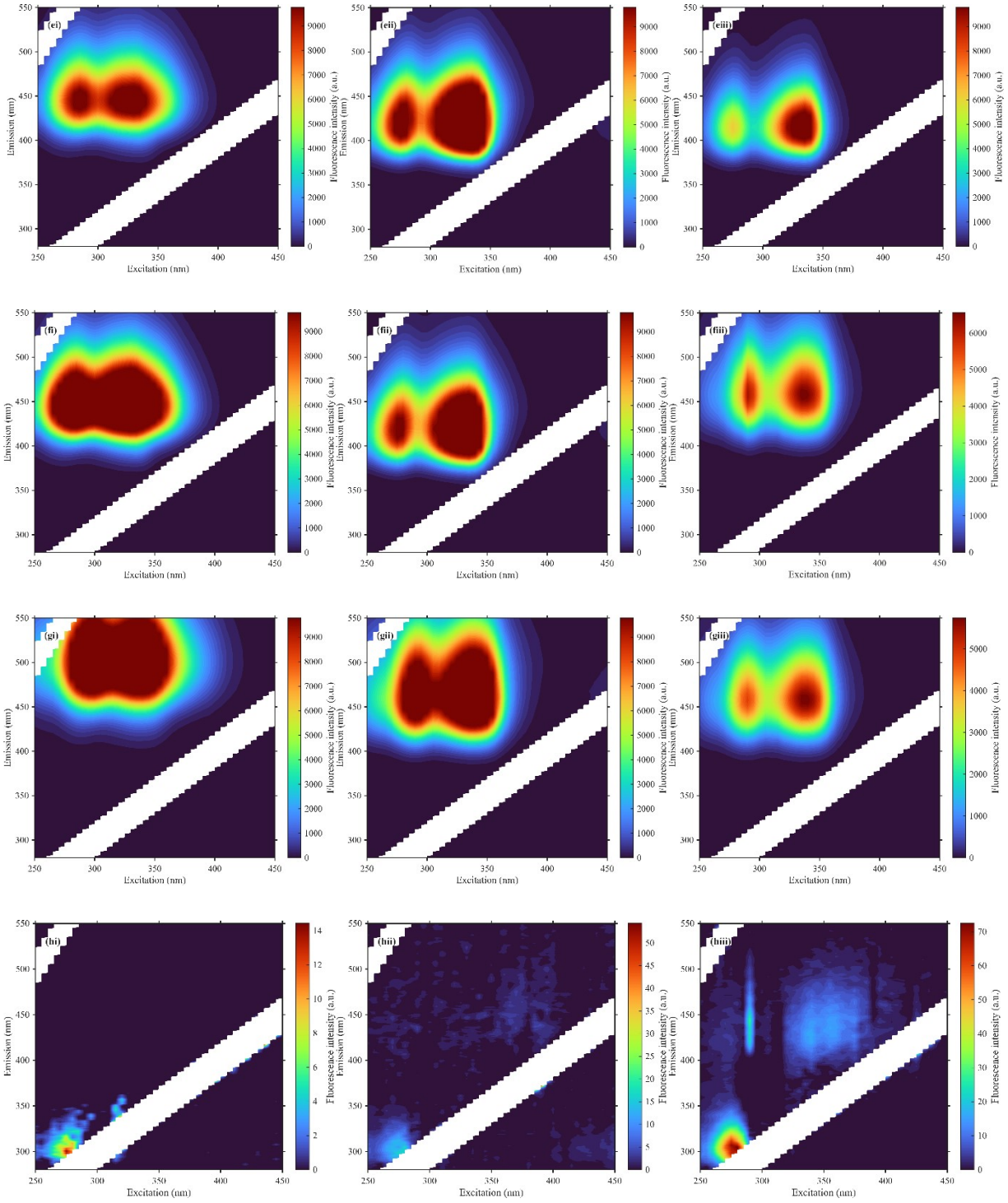
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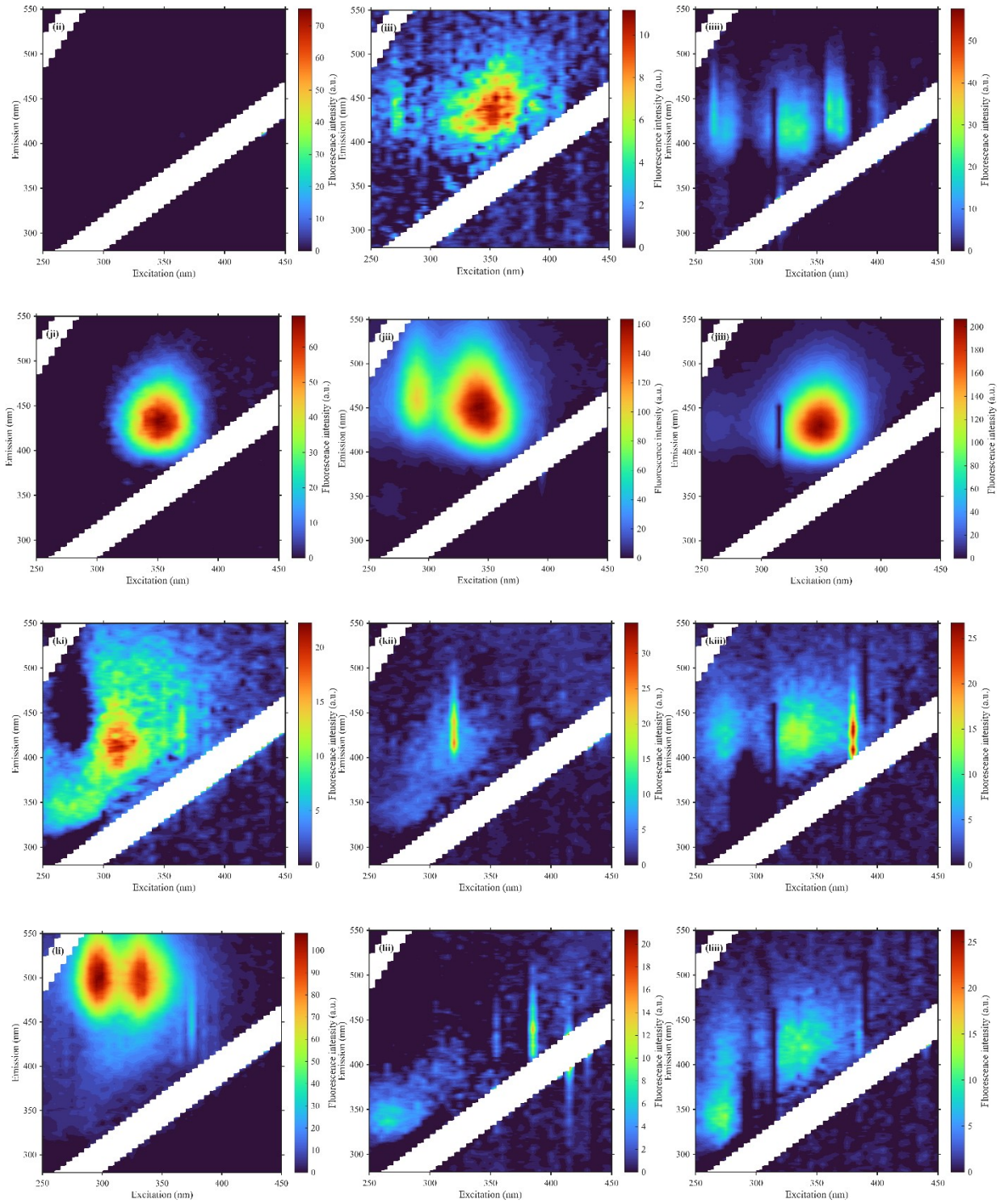
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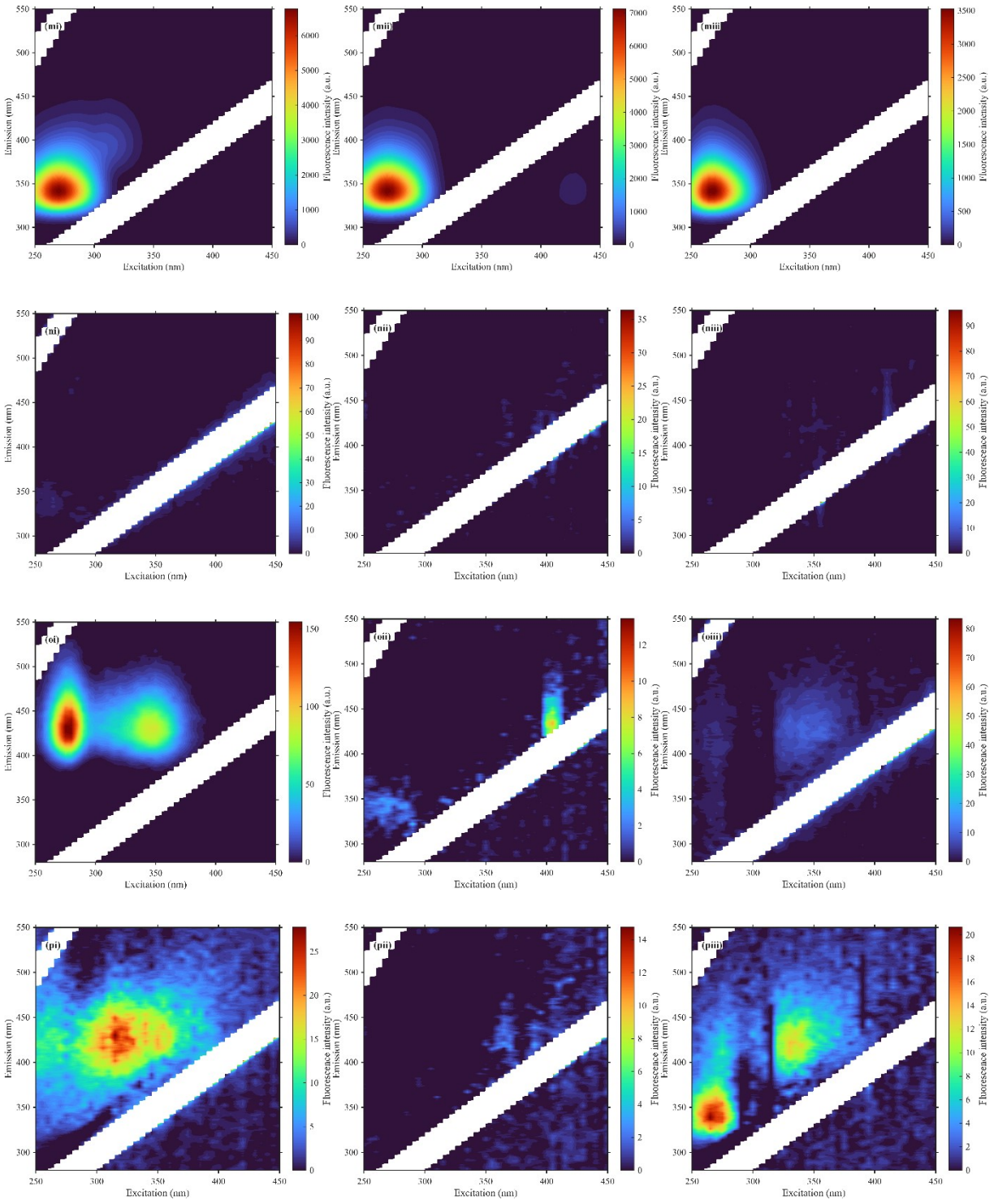
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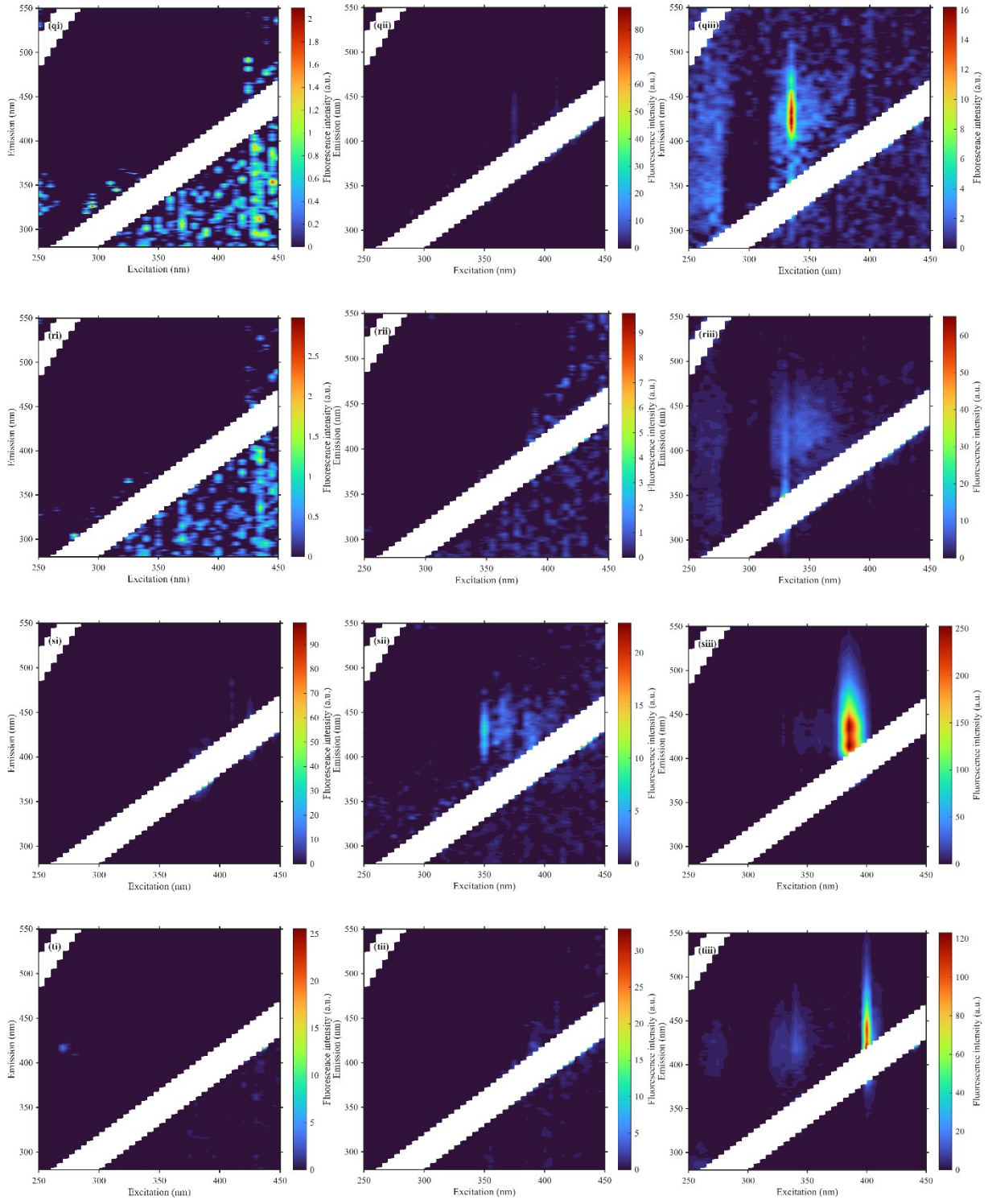
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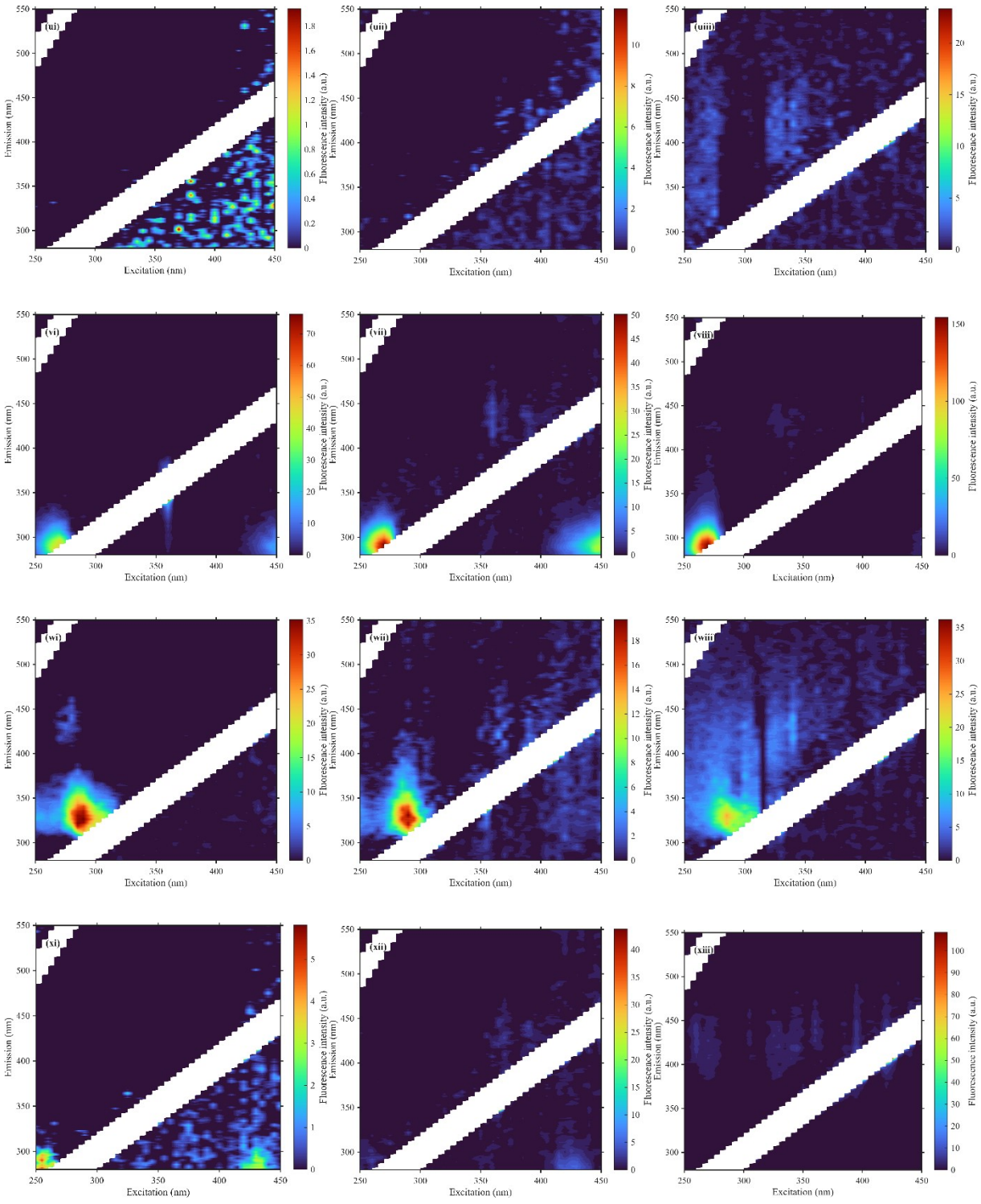
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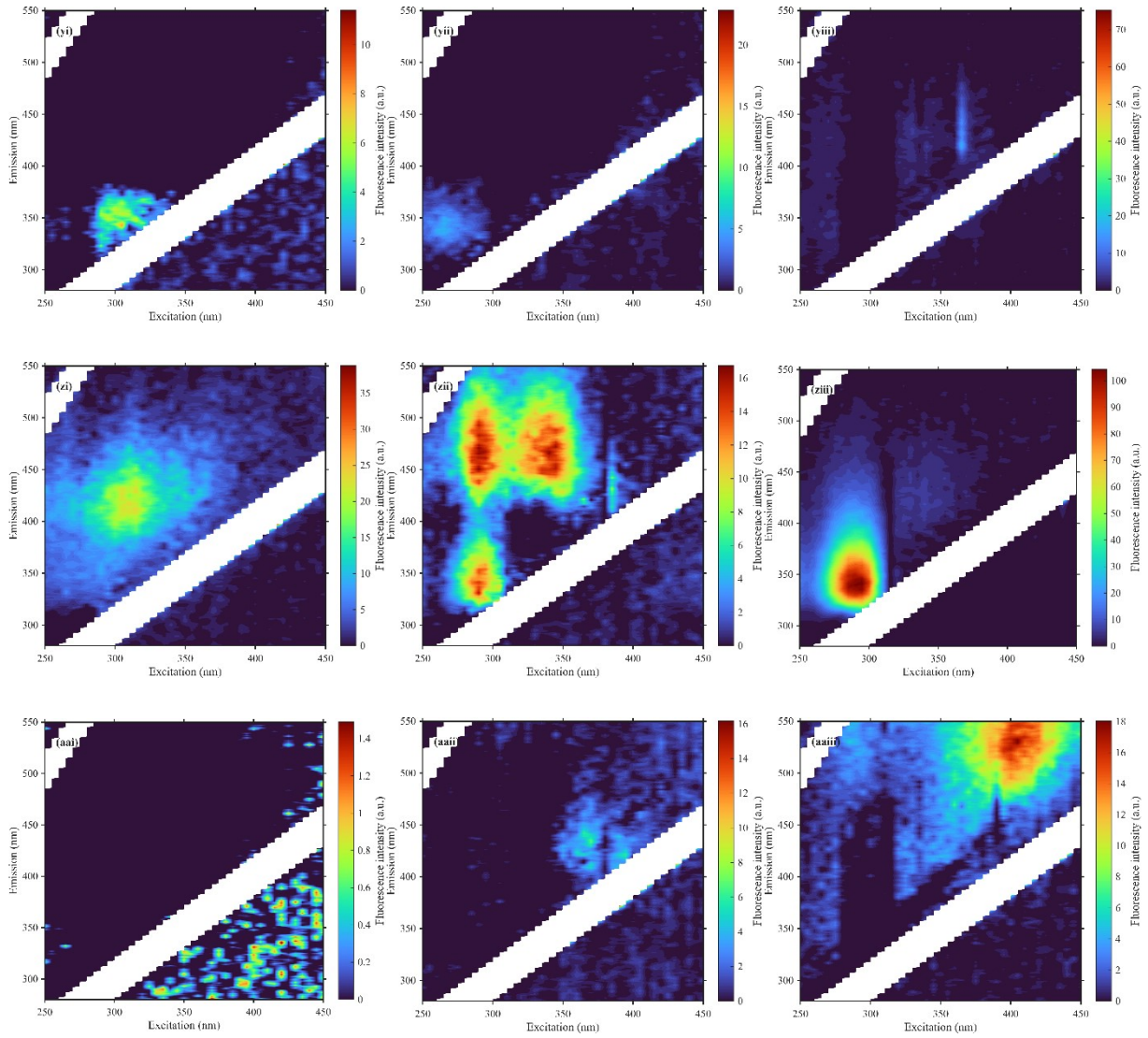

Figure S1. pH-dependent fluorescence overview of (ai) Tetracycline at pH 5 (aii) Tetracycline at pH 7 (aiii) Tetracycline at pH 9 (bi) Doxycycline at pH 5 (bii) Doxycycline at pH 7 (biii) Doxycycline at pH 9 (ci) Chlortetracycline at pH 5 (cii) Chlortetracycline at pH 7 (ciii) Chlortetracycline at pH 9 (di) Norfloxacin at pH 5 (dii) Norfloxacin at pH 7 (diii) Norfloxacin at pH 9 (ei) Ciprofloxacin at pH 5 (eii) Ciprofloxacin at pH 7 (eiii) Ciprofloxacin at pH 9 (fi) Enrofloxacin at pH 5 (fii) Enrofloxacin at pH 7 (fiii) Enrofloxacin at pH 9 (gi) Levofloxacin at pH 5 (gii) Levofloxacin at pH 7(giii) Levofloxacin at pH (hi) Amoxicillin at pH 5 (hii) Amoxicillin at pH 7 (hiii) Amoxicillin at pH 9 (ii) Ampicillin at pH 5(iii) Ampicillin at pH 7 (iiii) Ampicillin at pH 9 (ji) Cephalexin at pH 5(jii) Cephalexin at pH 7 (jiii) Cephalexin at pH 9 (ki) Sulfadimethoxine at pH 5 (kii) Sulfadimethoxine at pH 7 (kiii) Sulfadimethoxine at pH 9 (li) Sulfadiazine at pH 5 (lii) Sulfadiazine at pH 7 (liii) Sulfadiazine at pH 9 (mi) Sulfanilamide at pH 5 (mii) Sulfanilamide at pH 7 (miii) Sulfanilamide at pH (ni) Erythromycin at pH 5 (nii) Erythromycin at pH 7 (niii) Erythromycin at pH 9 (oi) Azithromycin at pH 5 (oii) Azithromycin at pH 7 (oiii) Azithromycin at pH 9 (pi) Tylosin at pH 5 (pii) Tylosin at pH 7 (piii) Tylosin at pH 9 (qi) Gentamicin at pH 5 (qii) Gentamicin at pH 7 (qiii) Gentamicin at pH 9 (ri) Neomycin at pH 5 (rii) Neomycin at pH 7 (riii) Neomycin at pH 9 (si) Metronidazole at pH 5 (sii) Metronidazole at pH 7 (siii) Metronidazole at pH 9 (ti) Ornidazole at pH 5 (tii) Ornidazole at pH 7 (tiii) Ornidazole at pH 9 (ui) Chloramphenicol at pH 5 (uii) Chloramphenicol at pH 7 (uiii) Chloramphenicol at pH 9 (vi) Florfenicol at pH 5 (vii) Florfenicol at pH 7 (viii) Florfenicol at pH 9 (wi) Vancomycin at pH 5 (wii) Vancomycin at pH 7 (wiii) Vancomycin at pH 9 (xi) PolymyxinB at pH 5 (xii) PolymyxinB at pH 7 (xiii) PolymyxinB at pH 9 (yi) Clindamycin at pH 5 (yii)

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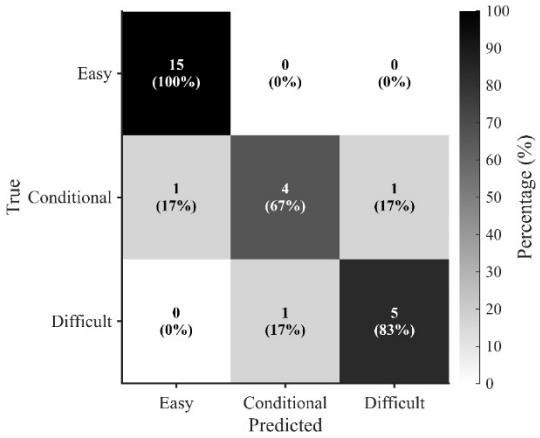


Figure S2. Confusion matrix for detection feasibility classification

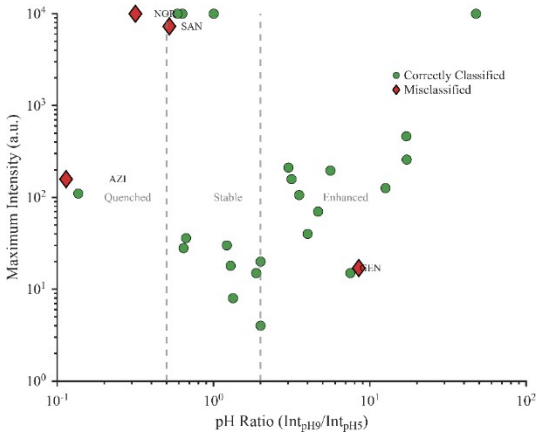
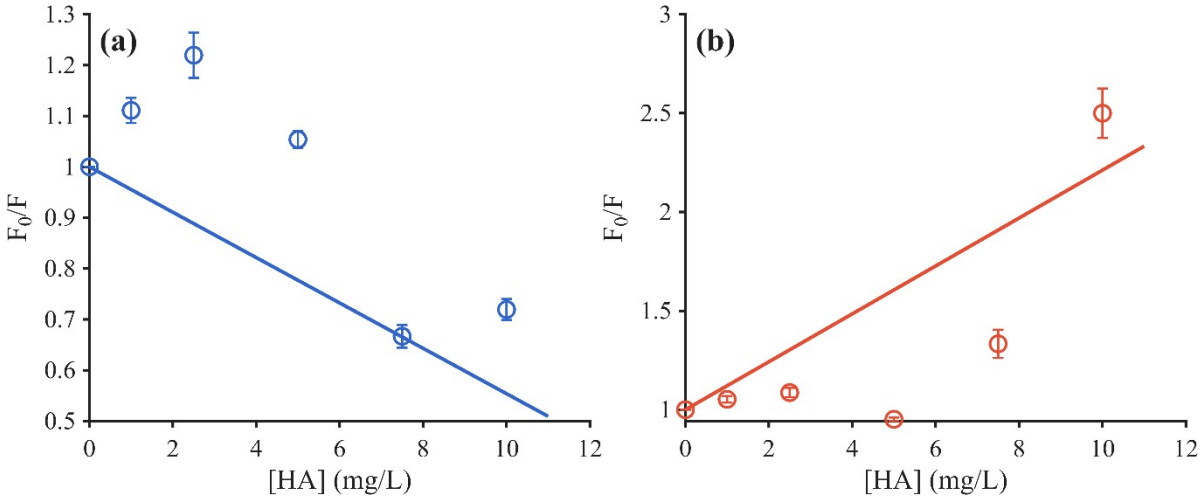


Figure S3. Misclassification analysis versus pH Ratio



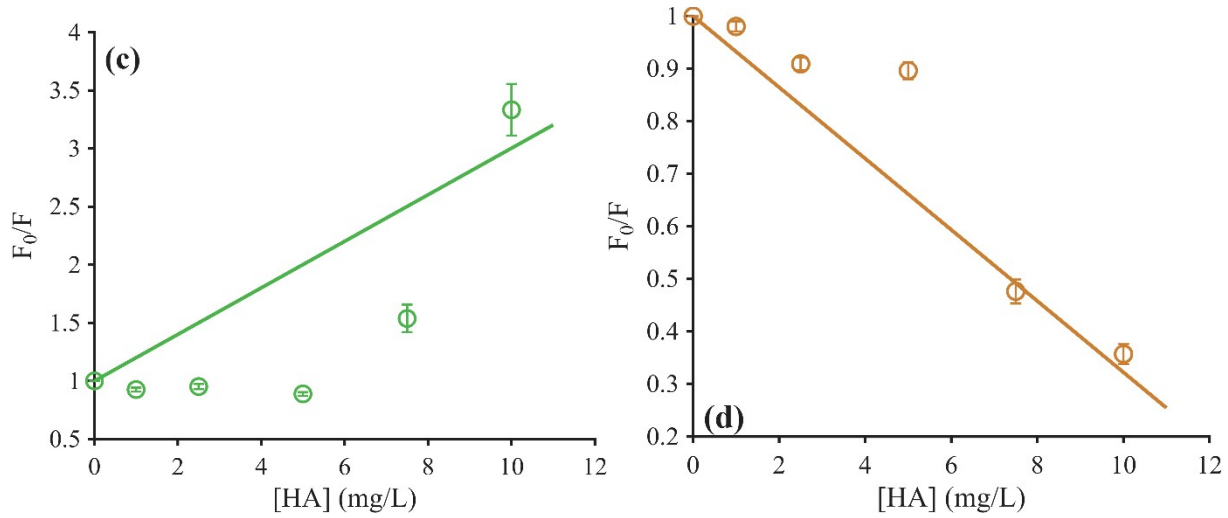
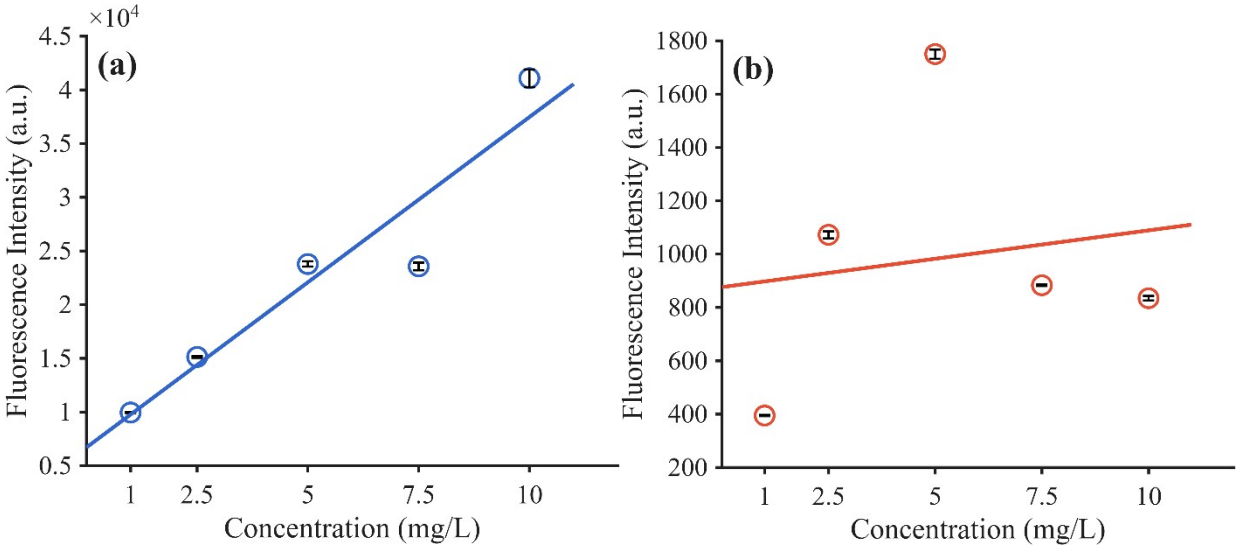


Figure S4. Stern-Volmer plot of Antibiotic-humic acid interactions (a) CIP; (b) CTC; (c) SAN; (d) CEP



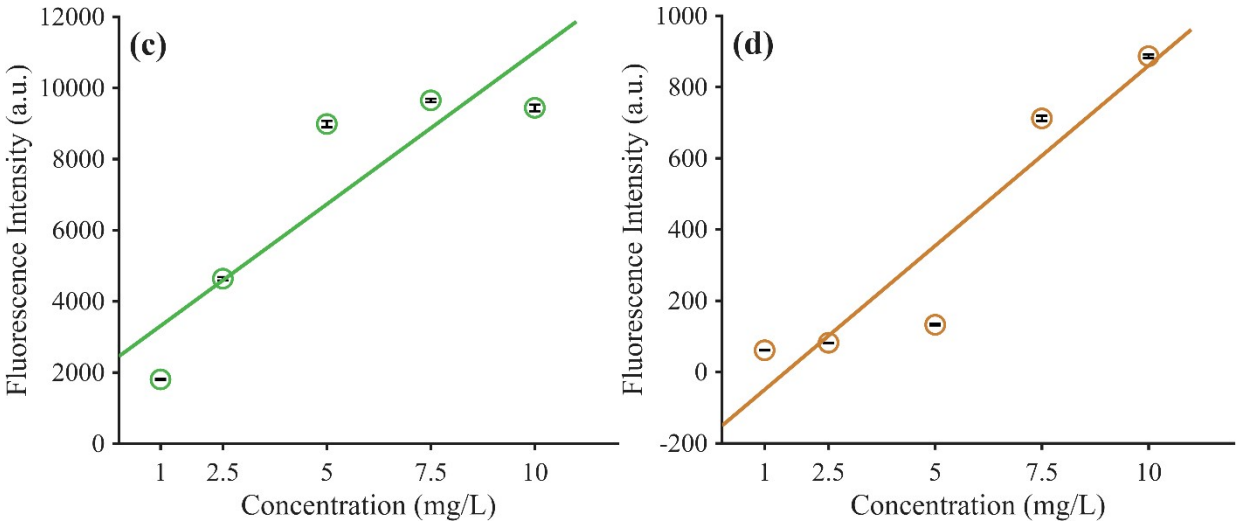


Figure S5. Calibration in wastewater of (a) CIP; (b) CTC; (c) SAN; (d) CEP

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