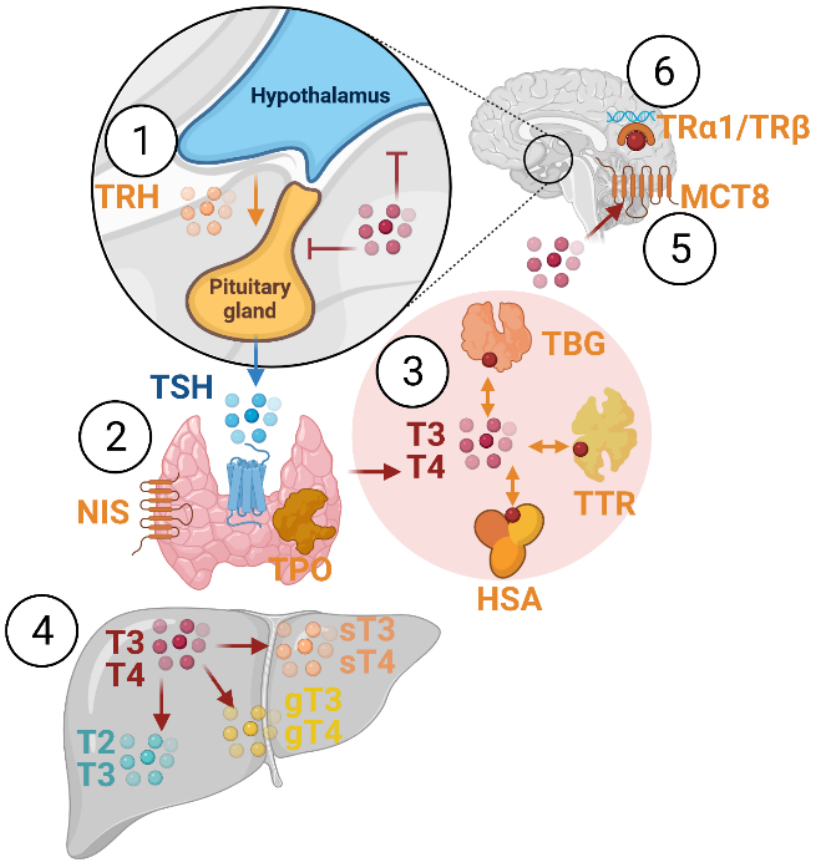
STUDY REPORT

for the Zebrafish Eleutheroembryo Thyroid Assay (ZETA) – Part 1

EURL ECVAM validation study of a battery of mechanistic methods relevant for the detection of chemicals that can disrupt the thyroid hormone system



© BioRender.com

2022

This study report has been prepared within the context of a collaboration agreement signed in 2019 with the Joint Research Centre (JRC) Directorate for Health, Consumers and Reference Materials (Chemicals Safety and Alternative Methods Unit F3 / EURL ECVAM), for the validation of mechanistic methods to identify potential modulators of thyroid hormone signalling. For information on the methodology and quality underlying the data presented in this report, users should contact the referenced source.

This study report describes the experimental design and includes data generated in Part 1 of the validation study. The method was developed by the Institute of Environmental Assessment and Water Research (IDAEA-CSIC), Spain and was implemented and experimentally assessed by EU-NETVAL laboratory IZSLER, Italy.

Contact information

Dr.ssa Silvia Dotti
Centro di Referenza Nazionale per i Metodi Alternativi, Benessere e Cura degli Animali da Laboratorio
Laboratorio di Controllo di Prodotti Biologici, Farmaceutici e Convalida di Processi Produttivi
Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna
Via Bianchi, 9 25124 Brescia,
Italia

Email: silvia.dotti@izsler.it

www.izsler.it

REPORT IZSLER FACILITY

ACTIVITY CARRIED OUT CONCERNING THE VALIDATION OF THE 7a ZETA ASSAY

	Study Director *(Approval)*	**Quality Assurance** *(Verified)*	**Test Facility Manager** *(Acknowledgement)*
Signed			
Date	03 FEB 2022	04 feb 2022	

Table of contents

1. Study scope

After the full description of the method Zebrafish Eleutheroembryo Thyroid Assay (ZETA) in a standard operating procedure, five valid runs were performed in order to assess the robustness and reliability of the method. This study was performed for PART 1 of the EURL ECVAM coordinated Thyroid Validation Study.

2. Information on Test and Control items

2.1. Control items

Control Item	Name	CAS No.	Water Solubility mg/L	Supplier	Lot Number	Purity	Expiry date	Physical state	H statement	Storage
Positive Control	Methimazole (MMI)	60-56-0	2750	Sigma (Merck) 301507	WXBC8588V	≥99%	/	solid	H317, H361	RT
Solvent Control	Fish water plus solvent	NR	NR	NR	NR	NR	NR	Liquid	NR	NR
Negative Control	Fish water (FW)	NR	NR	NR	NR	NR	NR	Liquid	NR	NR

NR: not relevant

2.2. Test item

Test item name	CAS No.	Water Solubility mg/L	Supplier	Lot Number	Purity	Expiry date	Physical state	H statement	Storage
$KClO_4$	7778-74-7	15000	Sigma (Merck) 460494	MKCD3612	≥99.99%	/	solid	H271 H302	RT

Due to the solubility of $KClO_4$ it was necessary to use the solvent Dimethylsulfoxide (DMSO).
The concentration corresponding to the Highest Test Item concentration (HTC) was determined, according to SOP.GLP/29.00, from existing data (Thienpont at al., 2011).
Three concentrations of $KClO_4$, that correspond to the HTC and two further concentrations 100 fold of the HTC were tested as below:

- 1st concentration: HTC corresponding to 11000 µM
- 2nd concentration: 1:100 of HTC corresponding to 110 µM
- 3rd concentration: 1:10000 of HTC corresponding to 1.10 µM

The three control solutions were prepared according to SOP.GLP/29.00:

- Methimazole was used as positive control (PC) item.
- DMSO was used as solvent control (SC).
- Fish water was used as negative control (NC).

3. The test system

Zebrafish are housed and maintained as described in SOP.GLP/28.00. For the ZETA, eggs are produced and collected as described in SOP.GLP/29.00. At 24 hours post-fertilization (hpf) the viability was checked by stereomicroscopy and at 54 hpf was evaluated the hatching rate.

3.1. Quality check

The suitability of the eggs was assessed based on the viability at 24 hpf which was ≥ 70% and on the hatching rate at 54 hpf ≥ 30%.

4. Equipment

Equipment	Supplier	Model
Incubator (28°C±1) equipped for dark/light cycle (10/14)	VWR	INCU-Line® IL 240CR PREMIUM
Glass ware (beakers, graduated cylinders and graduated pipettes)	Glass ware: Simax Graduated Cylinders: Simax Graduated Pipettes: Sorfa Life Science	n/a
24-well plates with lid	SPL Life Sciences	Cell Culture Plate
Depression slides	Sigma - Aldrich	Brand ™ Cavity Slides 76x26mm 1.2
Freezer (-20°C)	Ocean	W510
Baskets for whole-mount immunofluorescence on 24-well plates (to be constructed as described in Annex 1)	In house constructed	In house constructed
Stereomicroscope	Zeiss	SteREO Discovery.V8
Fluorescence microscope with a 10X magnification and a filter block capable of reading Alexa Fluor® 555 fluorescence	Nikon	Eclipse TE2000-S
Digital CCD camera to acquire images and corresponding software (see below)	Nikon	Digital Sight
Image analysis software (ImageJ, NIH - https://imagej.nih.gov/ij/)	https://imagej.net	Image J
Entomological pin	Omnes Artes	S56 - Label pins

n/a: not applicable

5. Materials

Reagent	CAS number	Supplier	Catalogue number
Acetone	67-64-1	Carlo Erba	508201
Alexa Fluor® 555 goat anti-rabbit IgG	n/a	Life Technologies	A21429
Albumin, bovine serum (BSA)	9048-46-8	Sigma-Aldrich	A2514
Collagenase	9001-12-1	Sigma-Aldrich	C9891
Dimethylsulfoxide (DMSO)	67-68-5	Sigma-Aldrich	D8418
Normal goat serum (NGS)	n/a	Life Technologies	81893
Hydrogen peroxide (H_2O_2)	67-64-1	Sigma-Aldrich	32201-M
Potassium hydroxide (KOH)	1310-58-3	Sigma-Aldrich	P5958
Methanol (100%)	67-56-1	Sigma-Aldrich	32213 –M
Methyl cellulose	9004-67-5	Sigma-Aldrich	M0387
Phosphate buffered saline (PBS)	34487-61-1	Made in house	n/a
Paraformaldehyde (PFA)	30525-89-4	Made in house	n/a
Rabbit Anti-Thyroxine BSA serum	n/a	MP Biomedicals	SKU 08658501
Triton X-100	9002-93-1	Sigma-Aldrich	n/a
Tween-20	9005-64-5	Sigma-Aldrich	P1379
Ultrapure water	7732-18-5	Made in house	n/a

n/a: not applicable

6. Study schedule

Three independent and valid run were performed for the three concentrations of KClO4 test item.
The method consists of two parts:

1. The first part is the exposure of the ZF embryos to the selected chemicals and subsequent fixation and freezing.
2. The second part involves the immunofluorescence analysis of previously incubated ZF embryos.

6.1. Chronological order

1. Part 1 – Exposure and fixation

Time (day / hpf, if appropriate)	Activity
-1	Breeding
0	Fertilised egg production; Collection of eggs.
1 (24 hpf)	Egg viability check
2 – 3 (54 hpf)	Prepare stock solutions; Solubility check; Check hatching rate; Prepare working solutions, start exposure.

Time (day / hpf, if appropriate)	Activity
4	Exposure of embryos
5 (120 hpf)	End exposure; Start fixation
6	End fixation; dehydration; Storage -20°C.

2. Part 2 – Whole-mount immunofluorescence and subsequent analysis

Time (day)	Activity
1	Rehydration of embryos; Incubation with primary antibody
2	Incubation with secondary antibody
3	Image acquiring / analysis

6.2. Exposure of ZF embryos

The exposure of ZF embryos was performed according to SOP.GLP/29.00.

For each run the ZF embryos are distributed over the 24-well plate as follows:

- 6 ZF embryos in $KClO_4$ at concentration 1 ($KClO_4$.1- HTC)
- 6 ZF embryos in $KClO_4$ at concentration 2 ($KClO_4$.2 - 1:100 of HTC)
- 6 ZF embryos in $KClO_4$ at concentration 3 ($KClO_4$.3 – 1:10000 of HTC)
- 6 ZF embryos in fish water as negative control (NC)
- 6 ZF embryos in Methimazole as positive control (PC)
- 6 ZF embryos in fish water plus DMSO as solvent control (SC)

7. Results

The images obtained as a result of immunolabeling were analyzed using the ImageJ software. A fundamental step for the analysis involved estimating the fluorescence of the image background, obtained by analyzing the Average Pixel Intensity (API), that is the threshold value of the background itself, calculated individually for each photograph in the areas of the image that did not contain any immunolabeled objects.

The software also provides other parameters that allow a detailed analysis of the image and whose numerical values are shown in the tables below:

- Area: surface of the image selected and reported in square pixels;
- Mean: average of the gray value of each pixel of the selected area;
- Minimum (Min): minimum gray value within the selected area;
- Maximum (Max): maximum gray value within the selected area;
- Integrated Density (ID): sum of the pixel values in the image or selection, equivalent to the product of Area and Mean.
- RawIntDen: integrated raw density value.

In order to calculate the integrated density (ID) of immunofluorescence *T*4 in the thyroid follicles, the API was subtracted from each pixel of the image.
Here below will be indicated the results related to the area of the thyroid follicles for each concentration ($KClO_4$.1, $KClO_4$.2, $KClO_4$.3, NC, PC e SC), making a distinction between the three different runs.

7.1. First run

7.1.1. KClO4.1 – HTC (11000 µM)

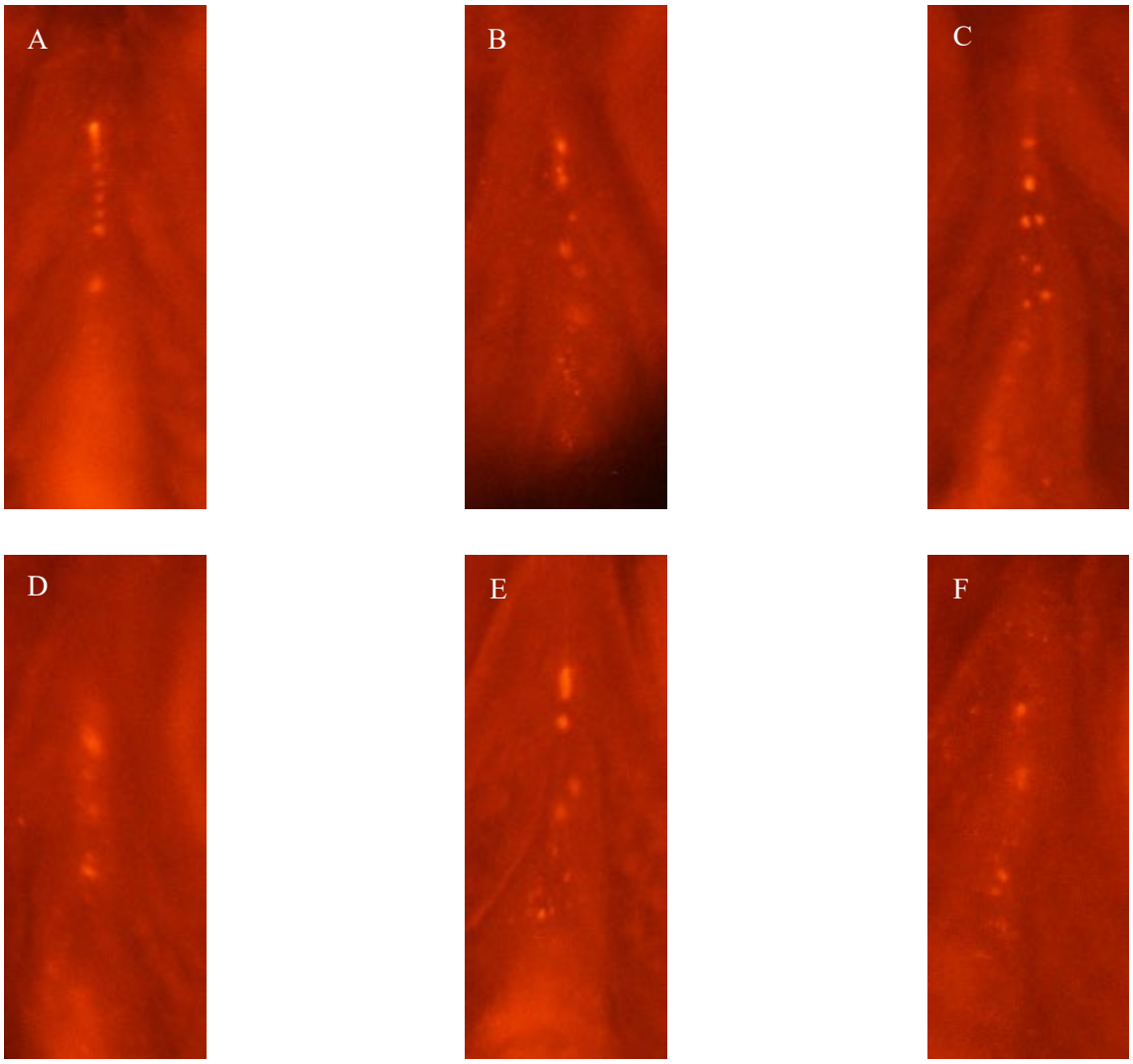


Image	Area	Mean	Min	Max	IntDensity	RawIntDen
A	1025	51.157	41	81	52436	52436
B	1229	50.525	43	80	62095	62095
C	909	50.768	39	80	46148	46148
D	792	53.586	47	69	42440	42440
E	846	54.703	45	74	46279	46279
F	468	53.626	46	71	25097	25097

Average of the ID values	45749,17

7.1.2. KClO4.2 – 1:100 of HTC (110 µM)

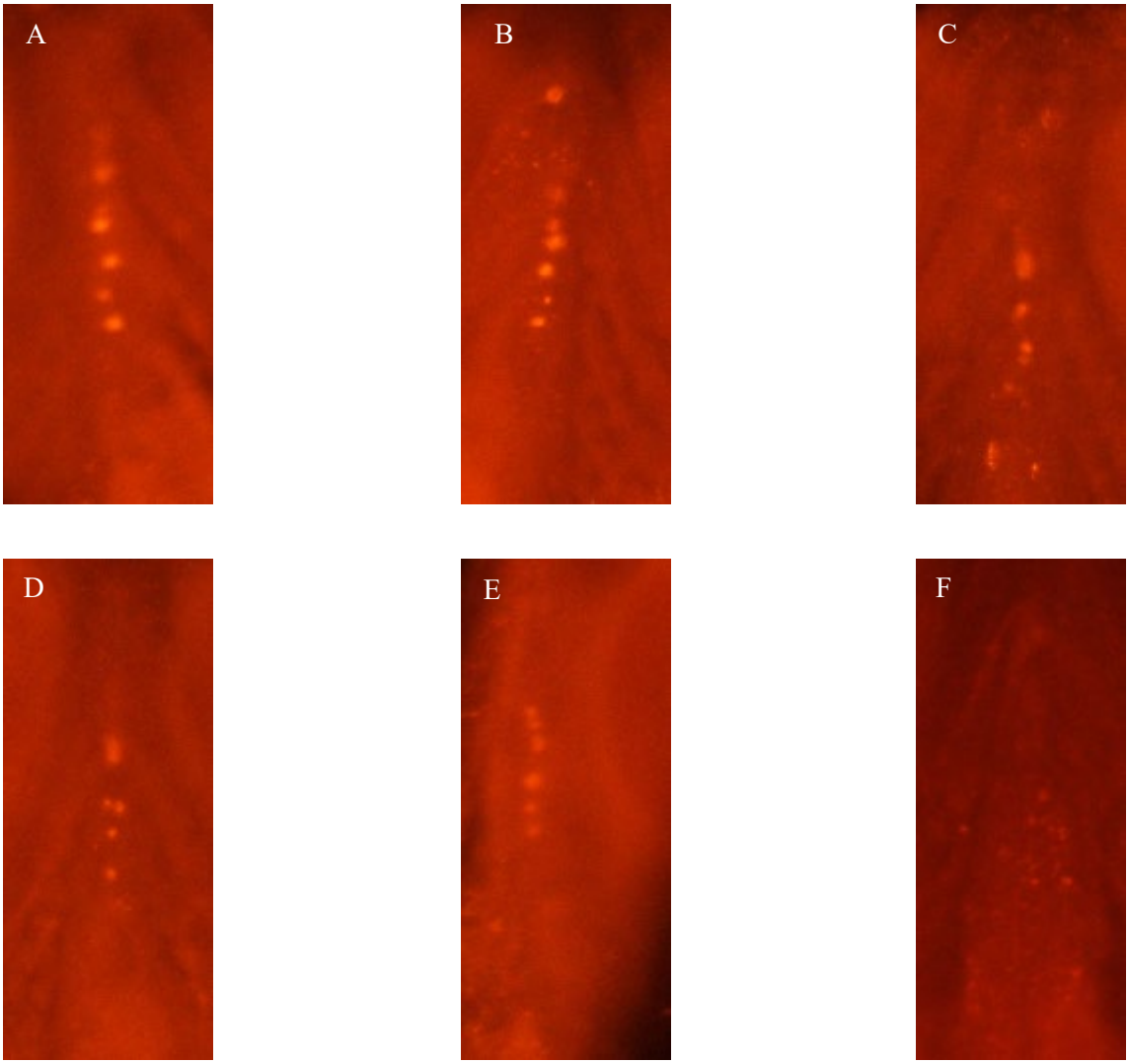


Image	Area	Mean	Min	Max	IntDensity	RawIntDen
A	1515	55.673	45	78	84344	84344
B	1346	54.710	43	80	73640	73640
C	1761	47.537	38	89	83713	83713
D	941	46.344	38	64	43610	43610
E	1377	48.709	42	63	67072	67072
F	518	38.270	34	52	19824	19824

Average of the ID values	62033,83

7.1.3. KClO4.3 – 1:10000 of HTC (1.10 µM)

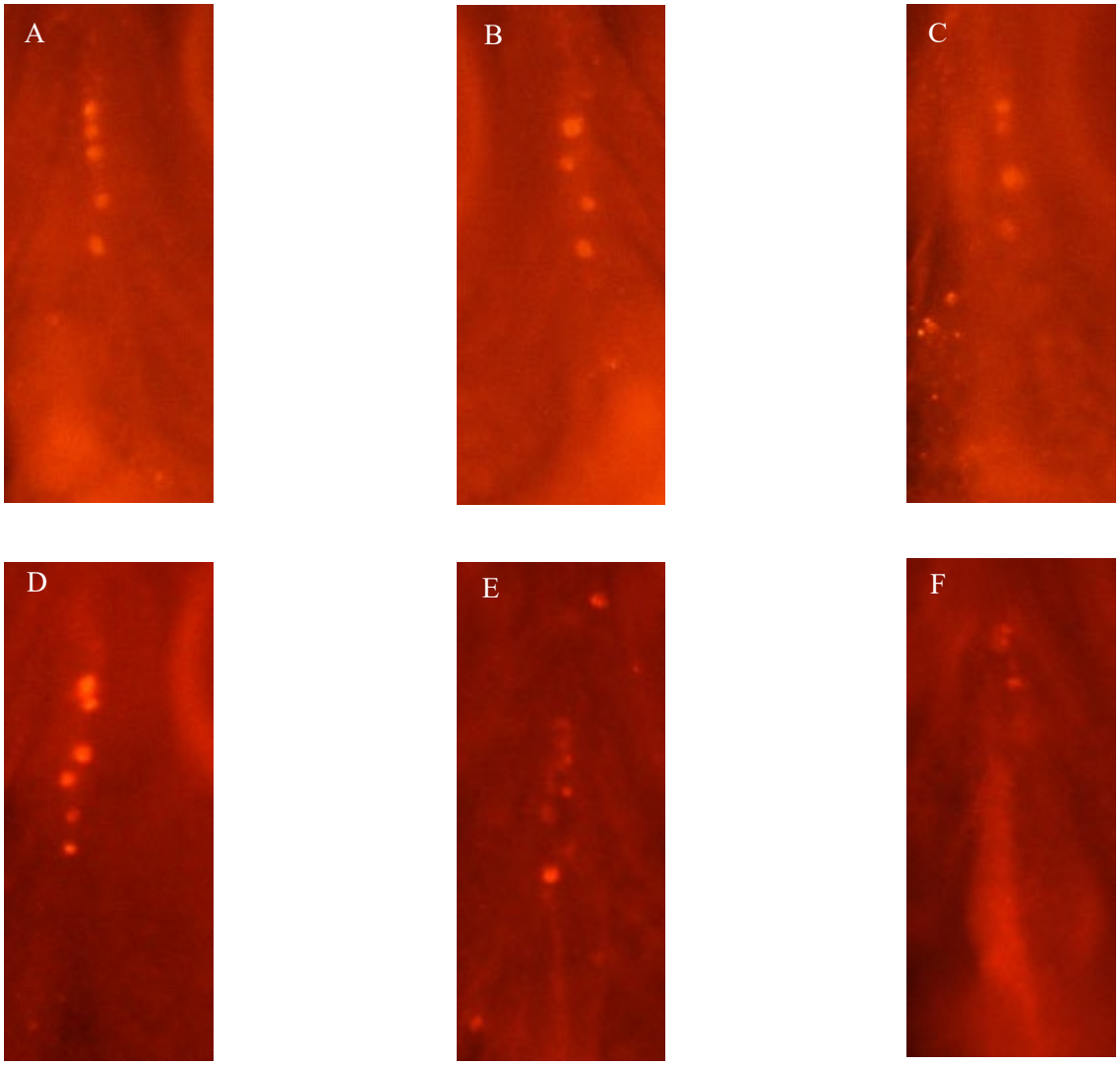


Image	Area	Mean	Min	Max	IntDensity	RawIntDen
A	959	53.153	45	71	50974	50974
B	951	53.200	45	66	50593	50593
C	935	53.117	46	84	49664	49664
D	1591	62.613	46	86	99618	99618
E	1669	43.479	33	76	72567	72567
F	1415	38.901	33	59	55045	55045

Average of the ID values	63076,83

7.1.4. Negative control – FW

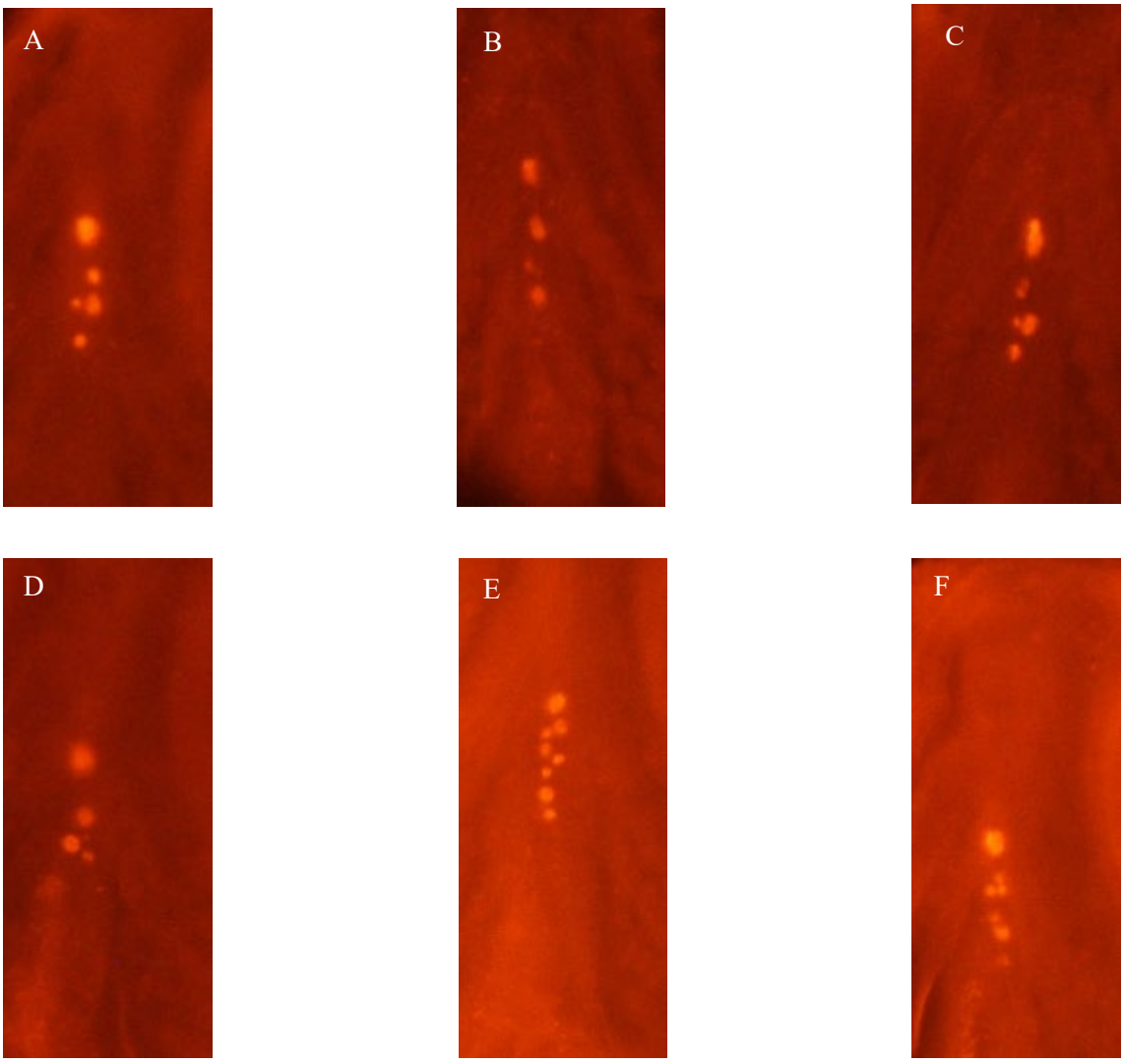


Image	Area	Mean	Min	Max	IntDensity	RawIntDen
A	1438	64.715	45	92	93060	93060
B	1220	48.914	35	75	59675	59675
C	1541	59.276	40	94	91344	91344
D	1468	50.073	36	70	73507	73507
E	1589	56.137	44	73	89201	89201
F	1306	61.625	49	86	80482	80482

Average of the ID values	81211,5

7.1.5. Positive control – MMI

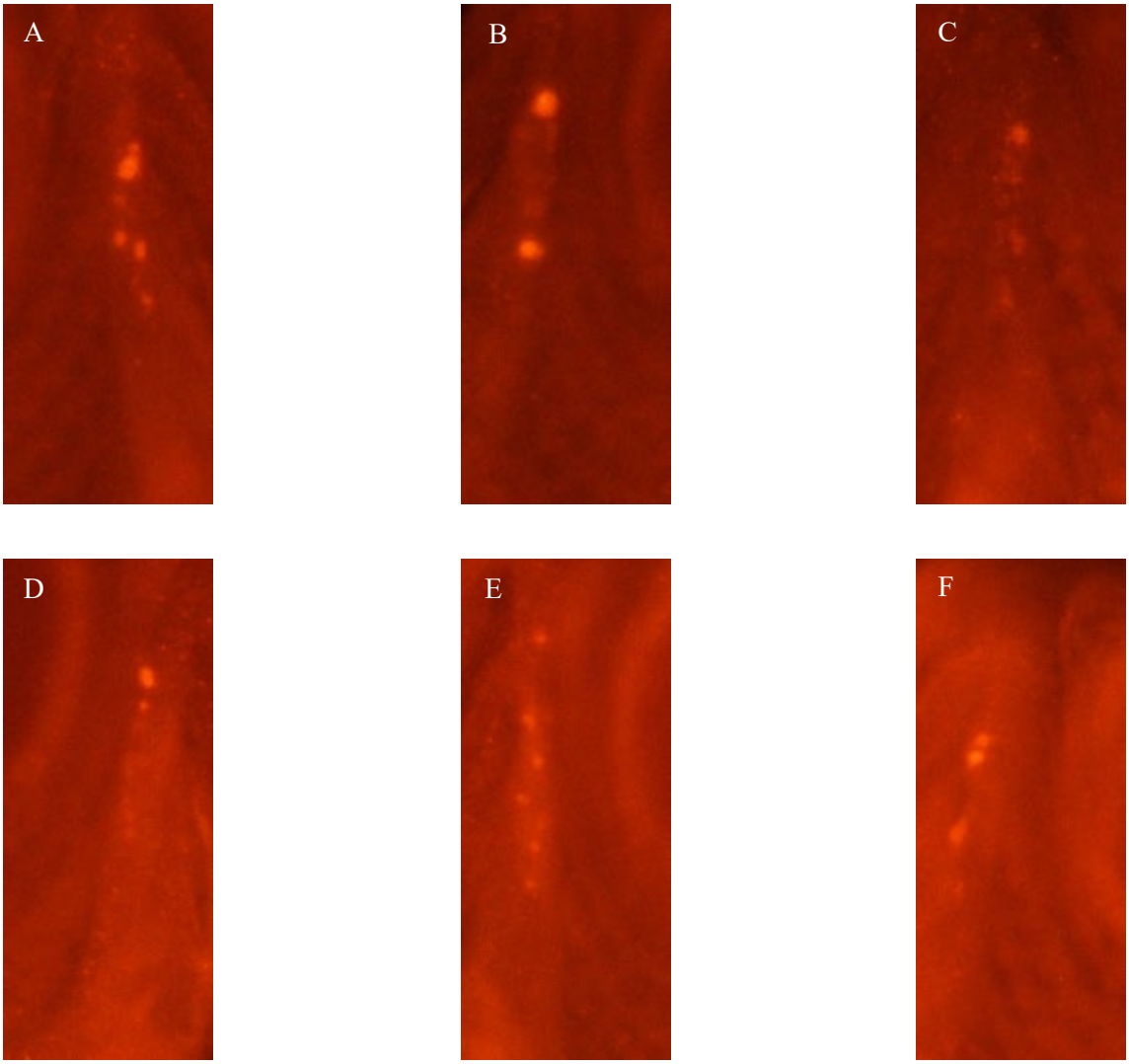


Image	Area	Mean	Min	Max	IntDensity	RawIntDen
A	1500	51.100	37	80	76650	76650
B	998	61.928	40	90	61804	61804
C	1071	38.418	31	58	41146	41146
D	605	51.443	43	73	31123	31123
E	1036	49.467	44	60	51248	51248
F	1174	53.973	44	77	63364	63364

Average of the ID values	54222,5

7.1.6. Solvent control - DMSO in fish water

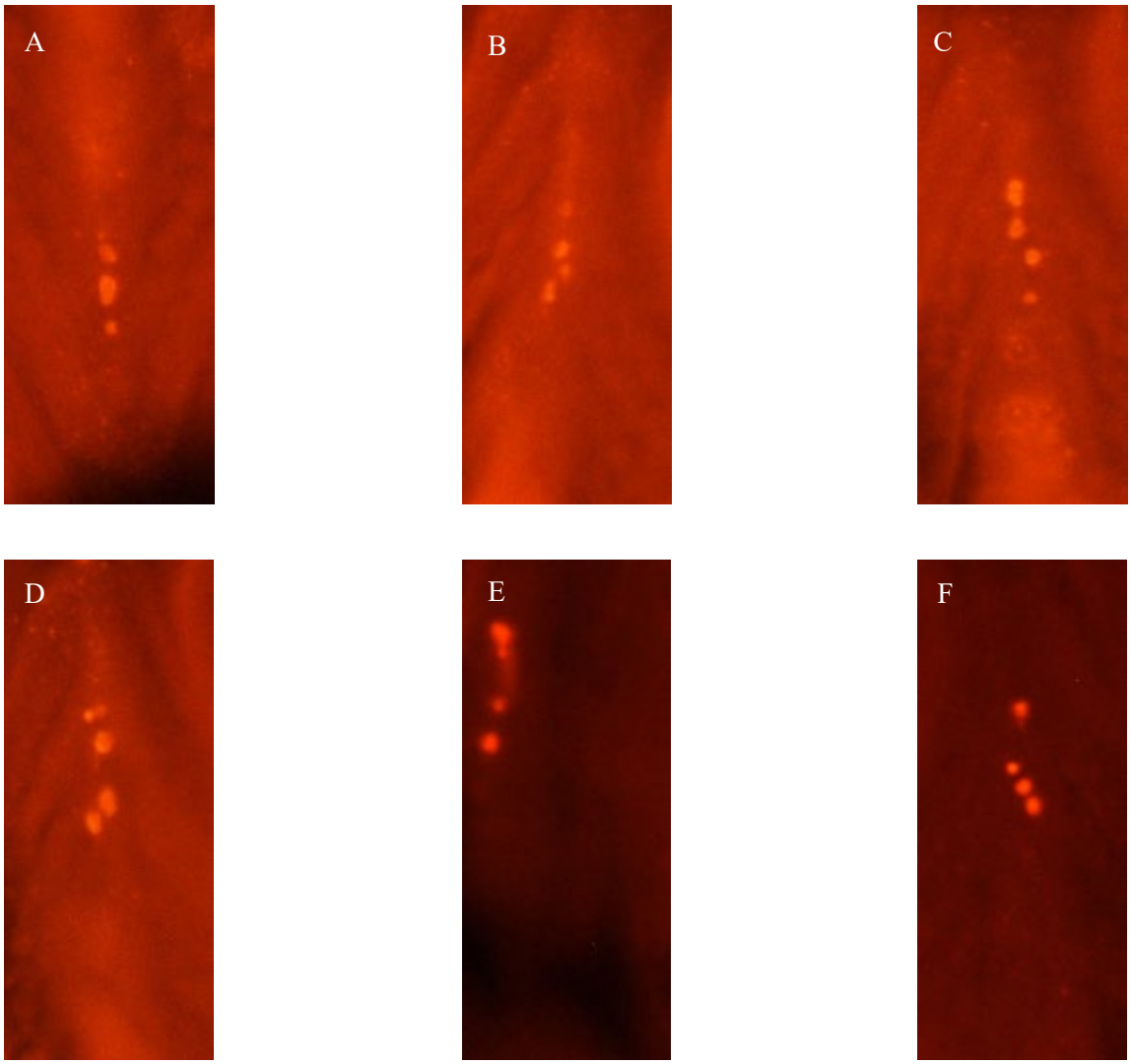


Image	Area	Mean	Min	Max	IntDensity	RawIntDen
A	985	56.074	44	77	55233	55233
B	1039	53.522	46	70	55609	55609
C	1165	57.936	44	74	67495	67495
D	1585	56.796	42	79	90022	90022
E	1816	54.386	33	92	98765	98765
F	853	64.556	41	96	55066	55066

Average of the ID values	70365

7.2. Second run

7.2.1. KClO4.1 – HTC (11000 µM)

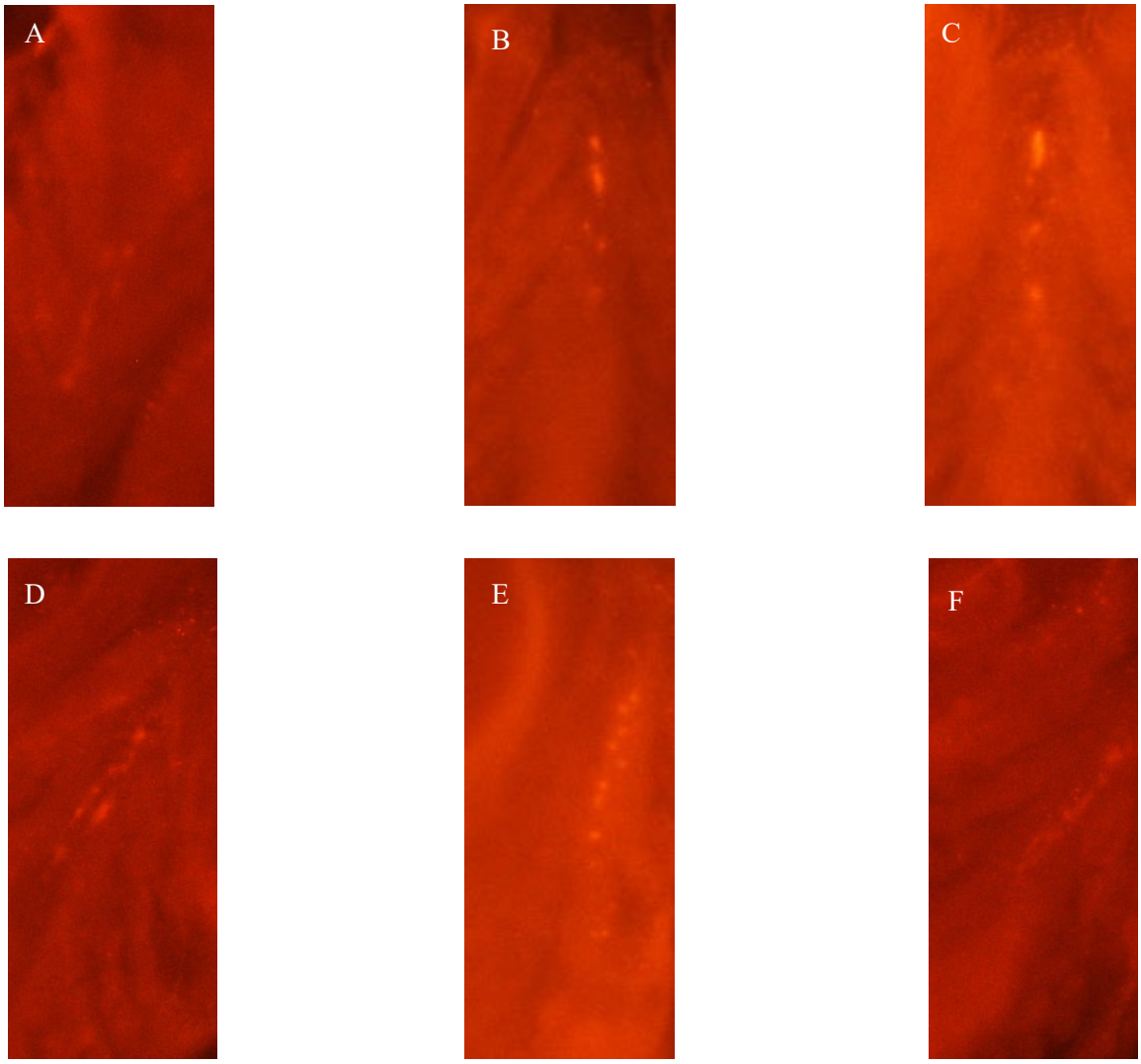


Image	Area	Mean	Min	Max	IntDensity	RawIntDen
A	565	39.998	36	45	22599	22599
B	685	49.352	41	70	33806	33806
C	729	55.938	48	73	40779	40779
D	629	46.491	40	59	29243	29243
E	791	51.660	48	58	40863	40863
F	691	41.394	37	48	28603	28603

Average of the ID values	32648,83

7.2.2. KClO4.2 – 1:100 of HTC (110 µM)

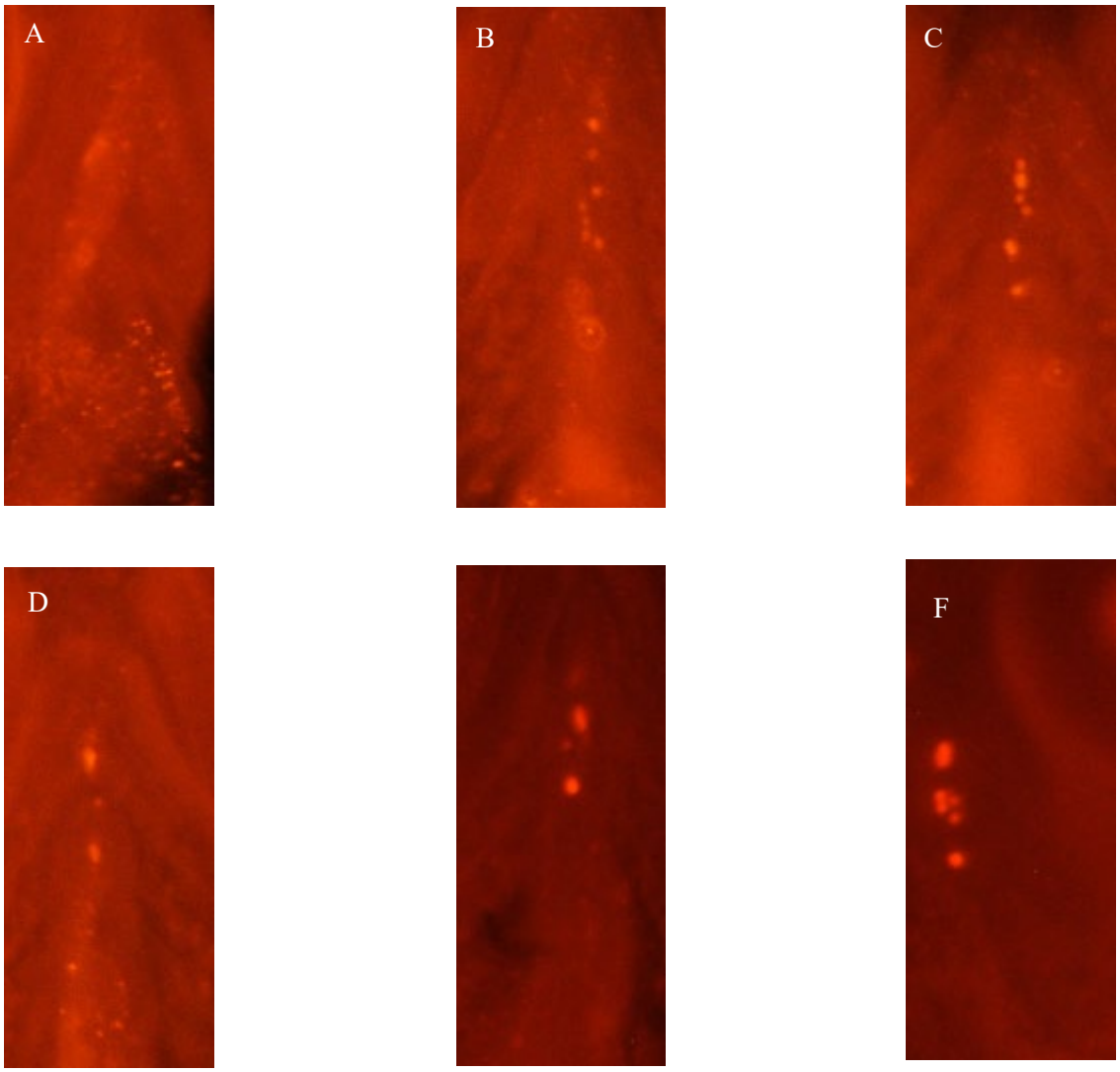


Image	Area	Mean	Min	Max	IntDensity	RawIntDen
A	913	50.275	45	60	45901	45901
B	627	48.600	41	70	30472	30472
C	1029	51.652	38	75	53150	53150
D	674	53.491	41	77	36053	36053
E	922	48.513	30	83	44729	44729
F	1589	52.544	33	80	83492	83492

Average of the ID values	48966,17

7.2.3. $KClO_4$.3 – 1:10000 of HTC (1.10 µM)

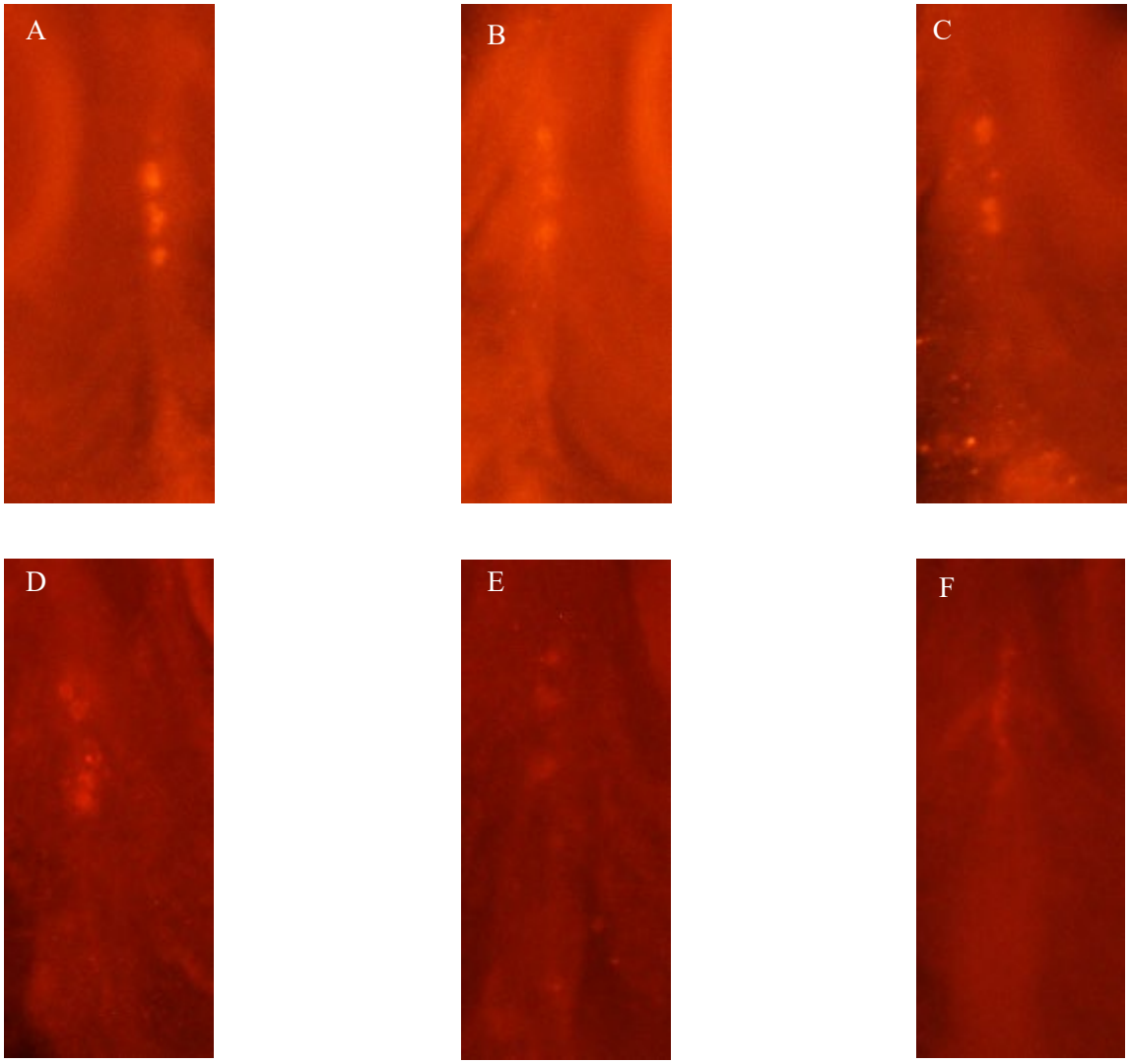


Image	Area	Mean	Min	Max	IntDensity	RawIntDen
A	1480	52.874	43	69	78254	78254
B	1288	52.842	49	60	68060	68060
C	1072	50.651	43	61	54298	54298
D	1512	46.041	39	68	69614	69614
E	1208	36.059	31	44	43559	43559
F	1888	37.166	33	46	70169	70169

Average of the ID values	63992,33

7.2.4. Negative control – FW

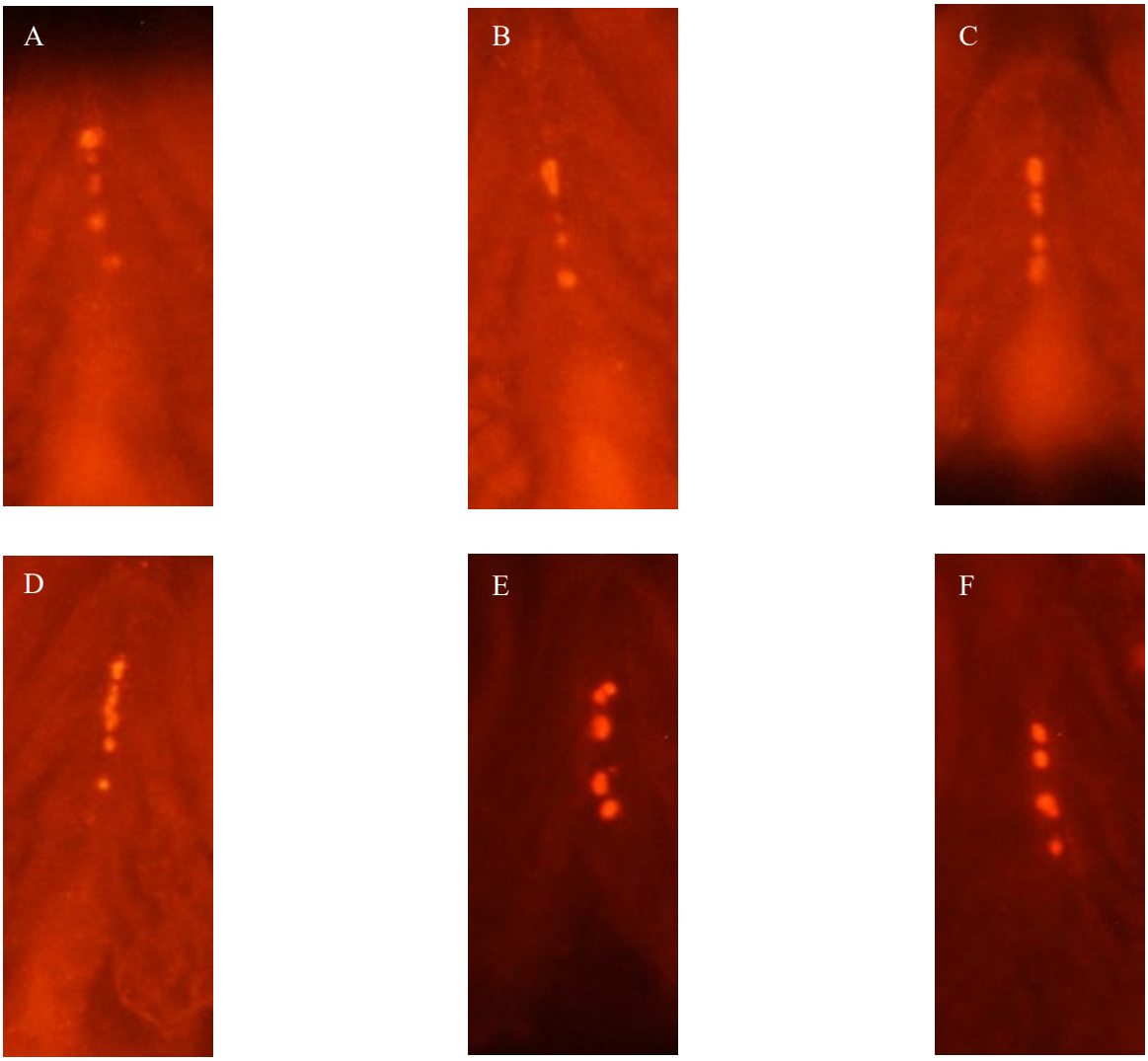


Image	Area	Mean	Min	Max	IntDensity	RawIntDen
A	1286	55.456	45	80	71316	71316
B	1102	54.912	43	73	60513	60513
C	1460	58.775	45	78	85812	85812
D	1331	58.177	40	90	77434	77434
E	1580	68.455	40	99	108159	108159
F	1117	68.156	44	91	76130	76130

Average of the ID values	79894

7.2.5. Positive control – MMI

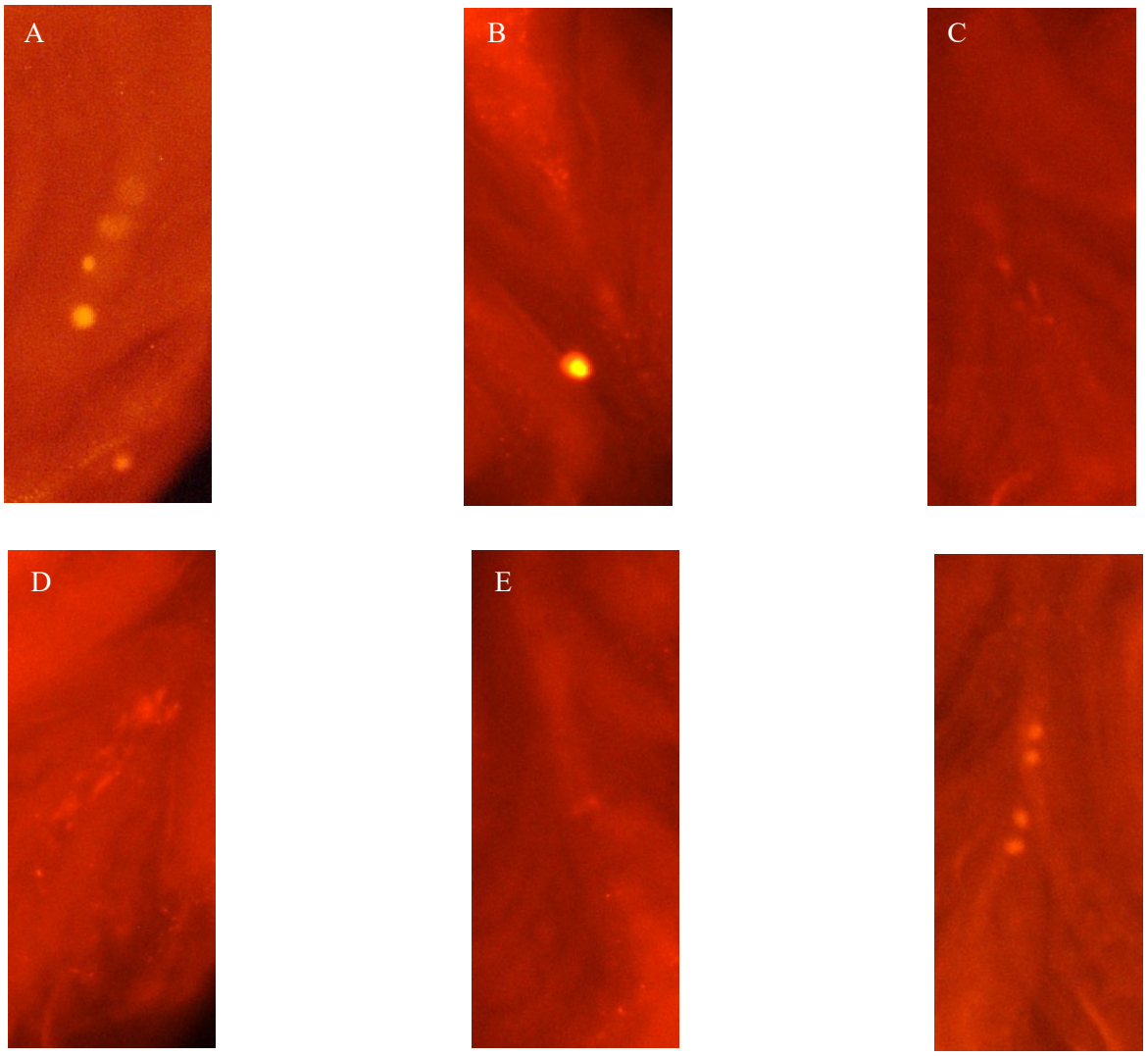


Image	Area	Mean	Min	Max	IntDensity	RawIntDen
A	1330	58529	42	88	77843	77843
B	145	43.628	40	47	6326	6326
C	285	41.242	36	48	11754	11754
D	616	53.834	49	62	33162	33162
E	323	41.625	38	44	13445	13445
F	695	56.268	48	67	39106	39106

Average of the ID values	30272,7

7.2.6. Solvent control - DMSO in fish water

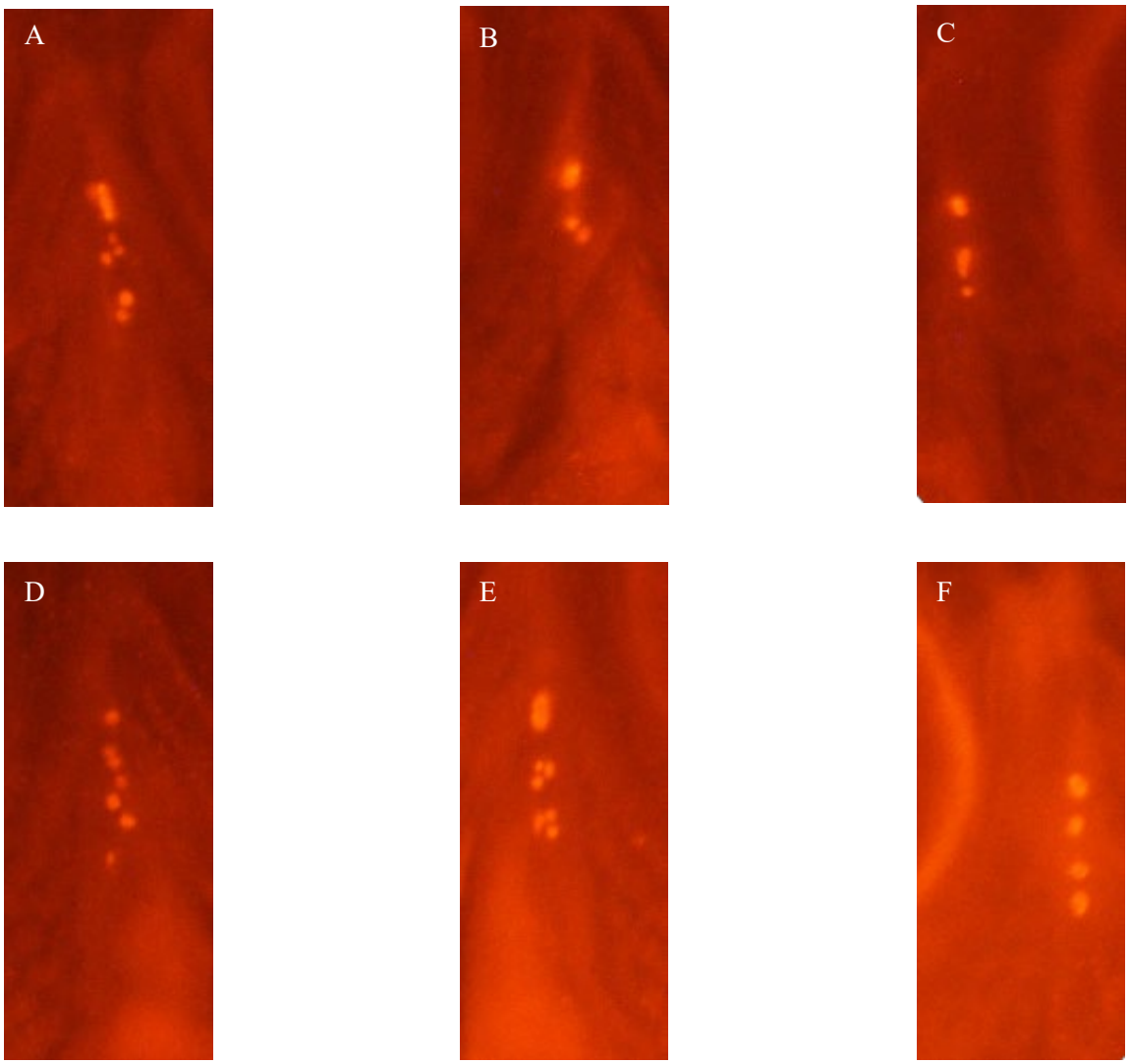


Image	Area	Mean	Min	Max	IntDensity	RawIntDen
A	1735	58.100	41	84	100803	100803
B	1110	63.361	49	89	70331	70331
C	823	63.674	48	85	52404	52404
D	1368	50.770	38	73	69454	69454
E	1159	60.963	50	72	70656	70656
F	1532	58.347	50	72	89387	89387

Average of the ID values	75505,83

7.3.Third run

7.3.1. KClO4.1 – HTC (11000 µM)

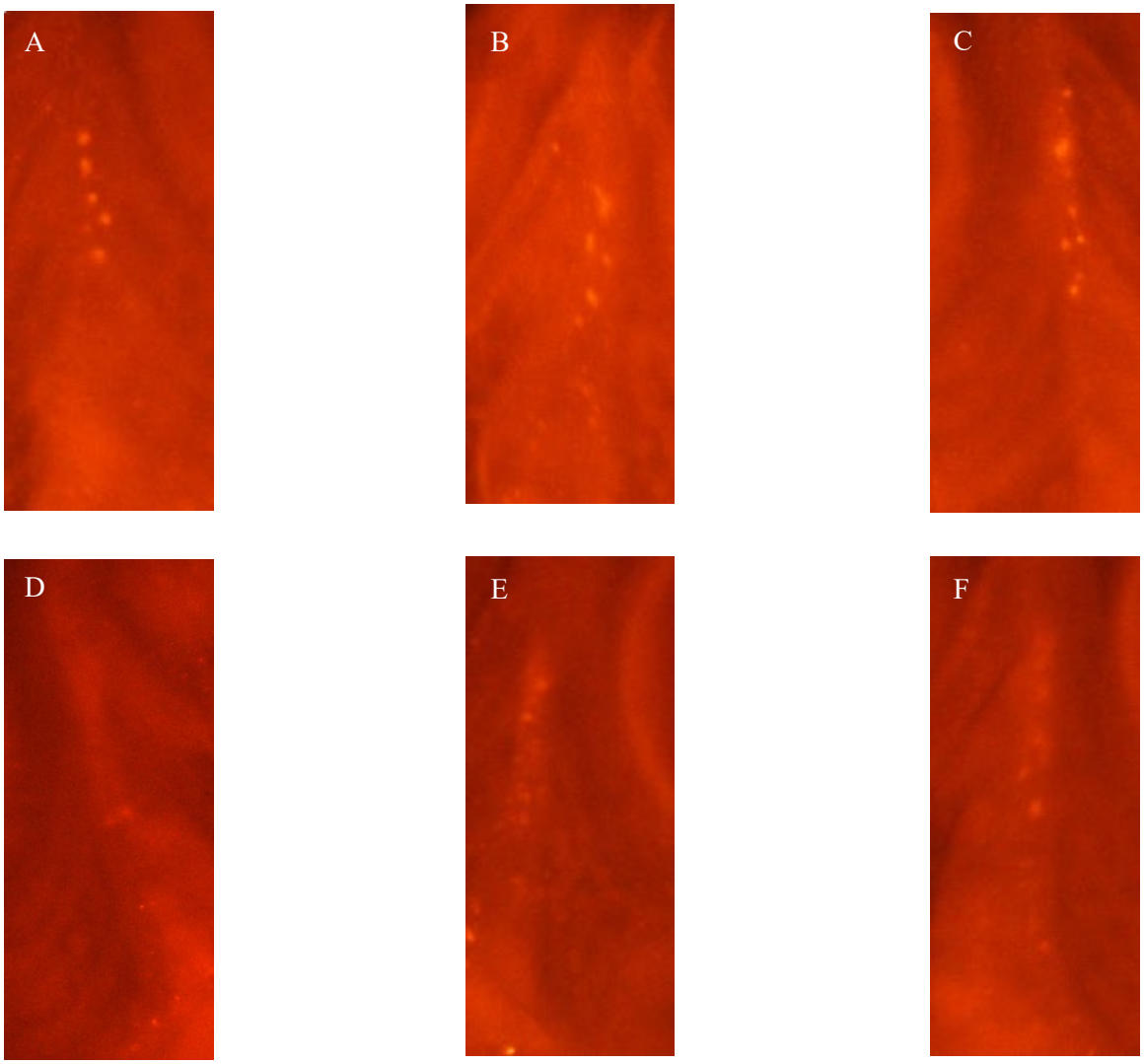


Image	Area	Mean	Min	Max	IntDensity	RawIntDen
A	751	50.690	44	64	38068	38068
B	1137	53.668	48	66	61020	61020
C	1279	52.260	45	70	66841	66841
D	1266	55.826	49	73	70676	70676
E	722	45.807	41	56	33073	33073
F	714	46.195	42	53	32983	32983

Average of the ID values	50443,5

7.3.2. KClO4.2 – 1:100 of HTC (110 µM)

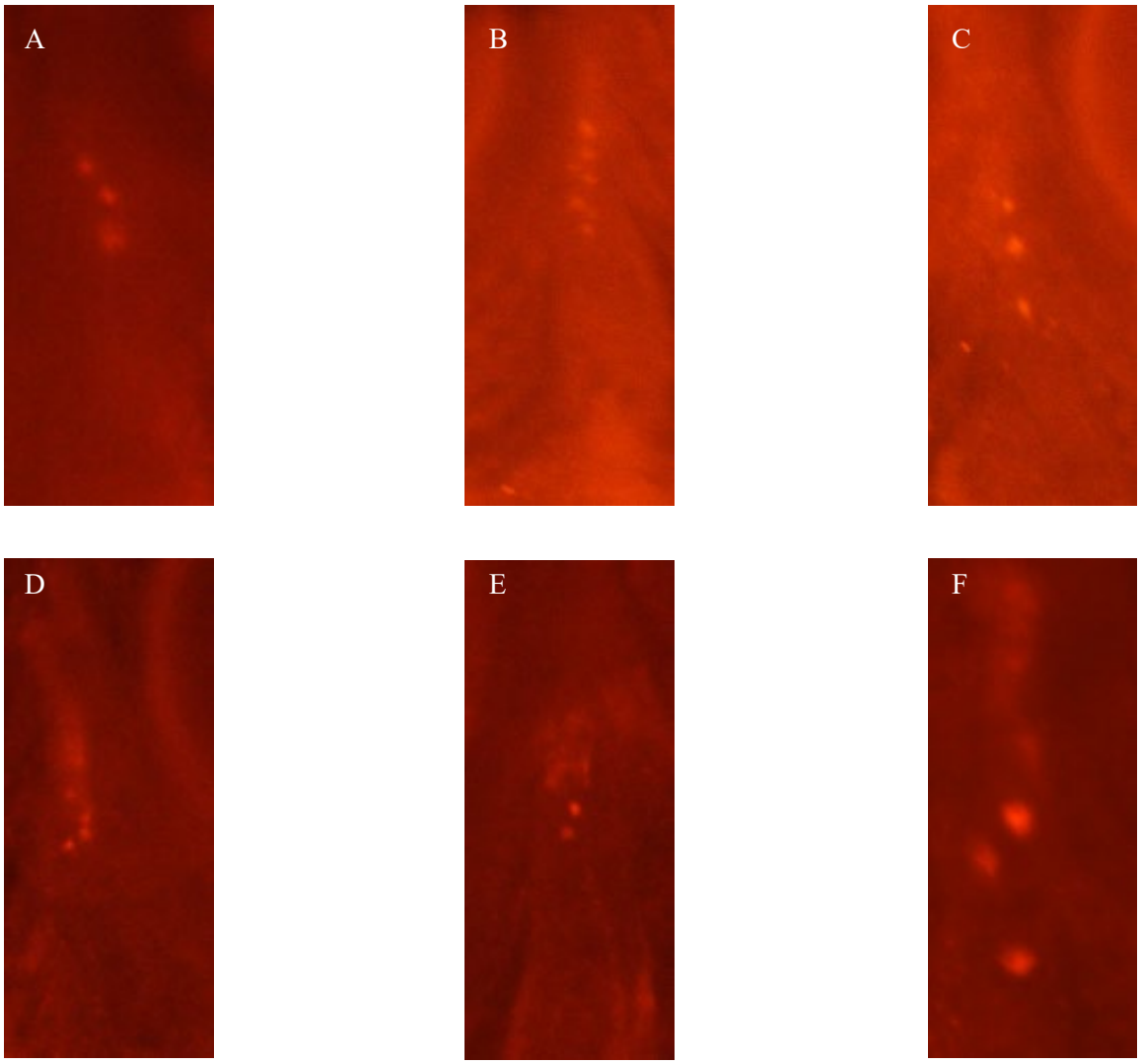


Image	Area	Mean	Min	Max	IntDensity	RawIntDen
A	1235	38.772	32	53	47884	47884
B	1034	46.076	41	54	47643	47643
C	1089	50.720	44	64	55234	55234
D	989	41.887	35	59	41426	41426
E	562	40.278	34	61	22636	22636
F	2618	45.319	35	76	118645	118645

Average of the ID values	55578

7.3.3. $KClO_4$.3 – 1:10000 of HTC (1.10 µM)

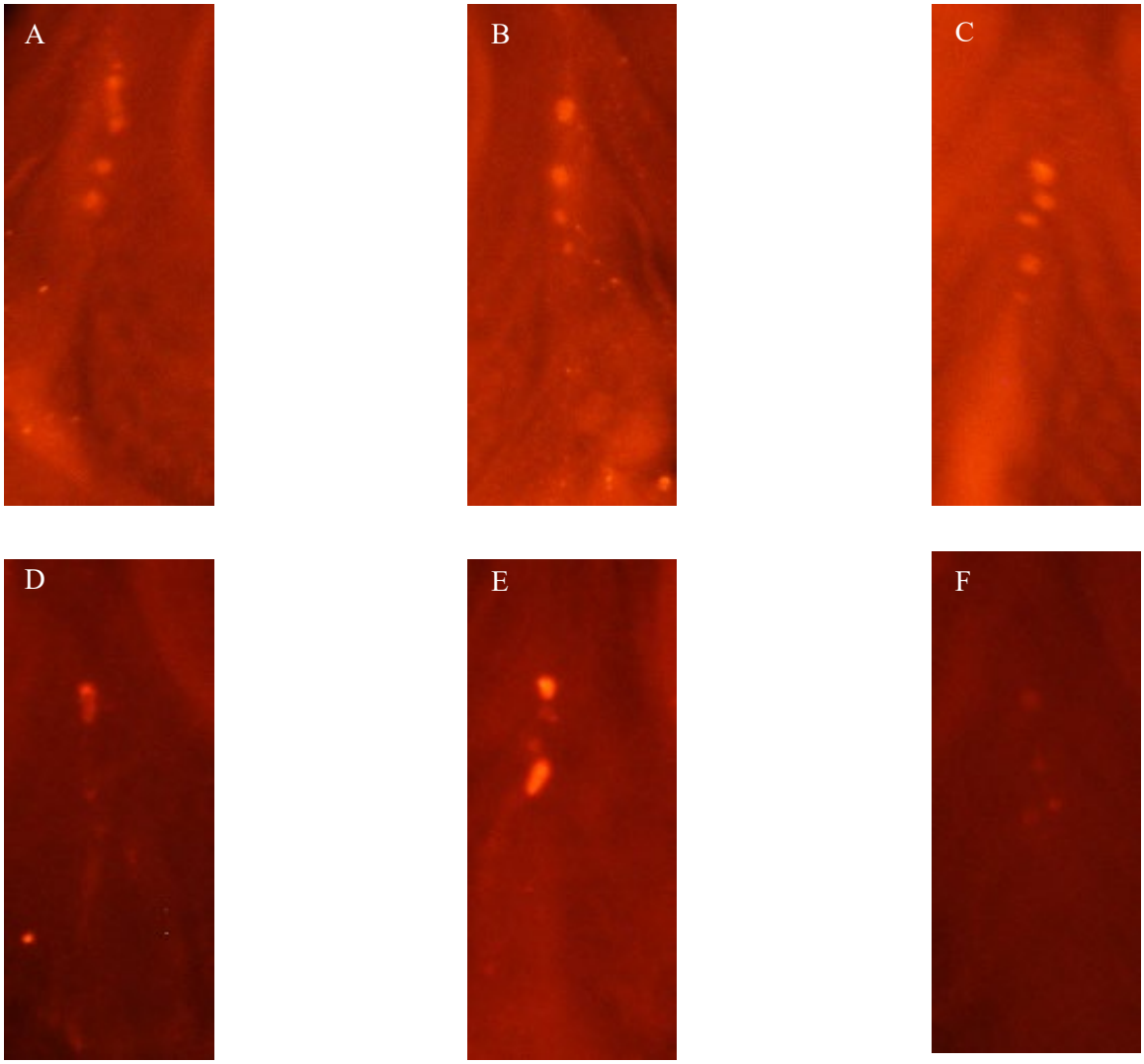
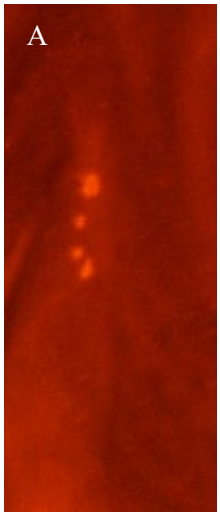
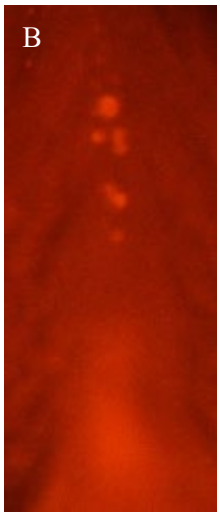


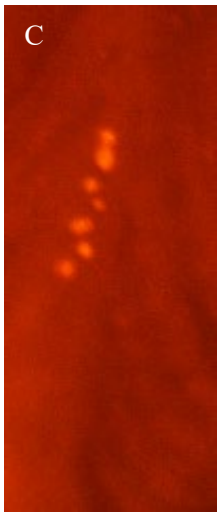
Image	Area	Mean	Min	Max	IntDensity	RawIntDen
A	1601	48.406	40	67	77498	77498
B	1115	53.355	44	67	59491	59491
C	1285	51.166	42	69	65748	65748
D	993	43.011	28	98	42710	42710
E	1558	58.789	37	95	91594	91594
F	1119	28.225	24	38	31584	31584

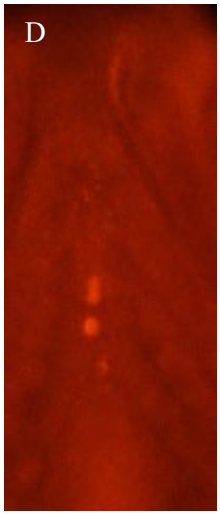
Average of the ID values	61437,5

7.3.4. Negative control – FW









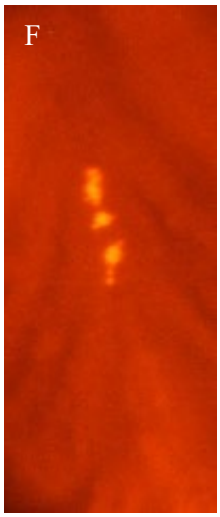


Image	Area	Mean	Min	Max	IntDensity	RawIntDen
A	854	53.378	42	79	45585	45585
B	1218	60.210	46	76	73336	73336
C	1279	52.384	41	72	66999	66999
D	1622	58.325	27	92	94603	94603
E	1365	66.173	45	86	90326	90326
F	1551	51.964	31	91	80596	80596

Average of the ID values	75240,8

7.4.5. Positive control – MMI

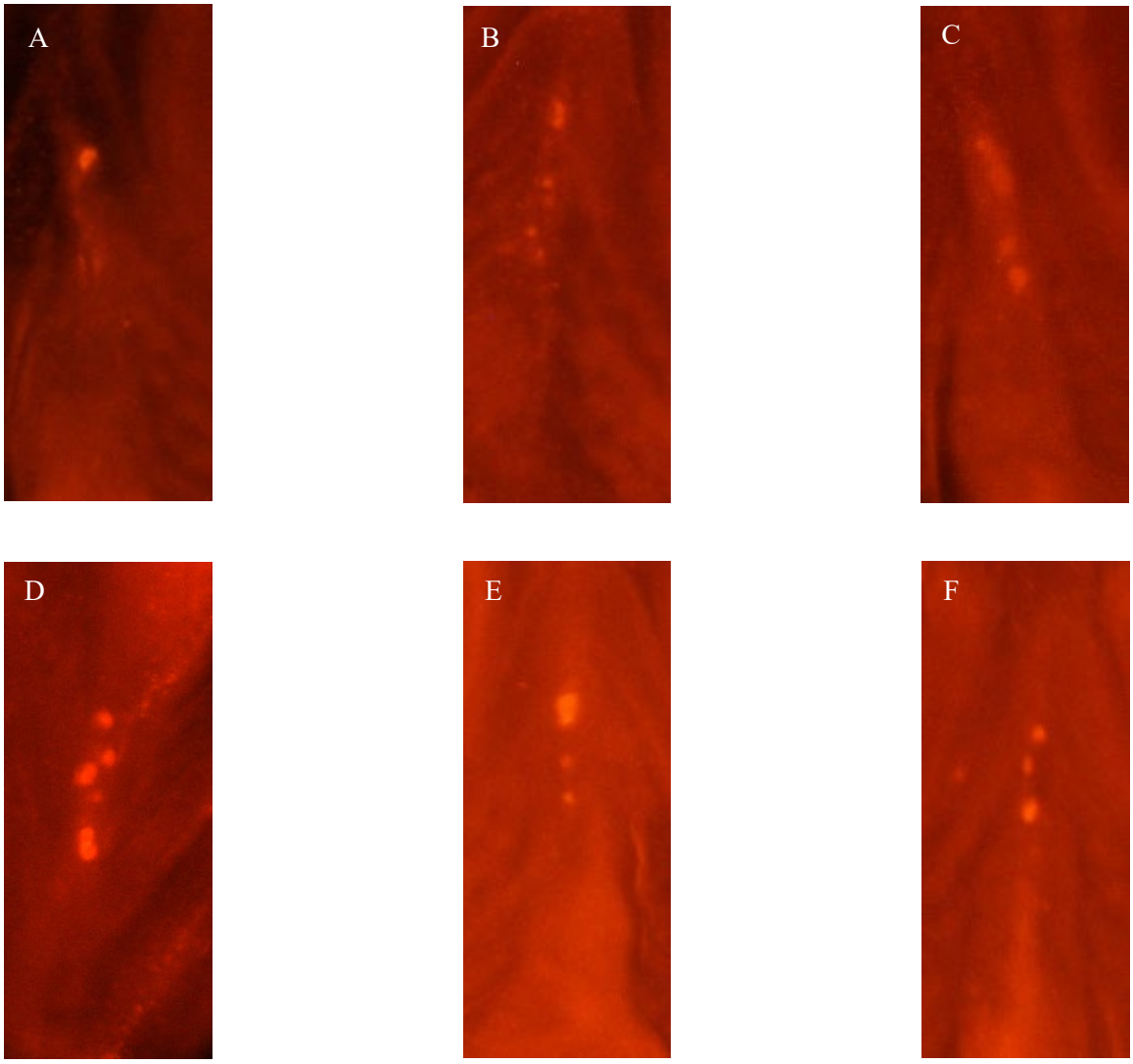


Image	Area	Mean	Min	Max	IntDensity	RawIntDen
A	999	42.405	30	80	42363	42363
B	1074	42.919	35	59	46095	46095
C	1192	47.388	42	56	56486	56486
D	1527	60.078	45	83	87747	87747
E	972	56.524	45	71	54941	54941
F	906	50.019	38	72	45317	45317

Average of the ID values	55491,5

7.4.6. Solvent control - DMSO in fish water

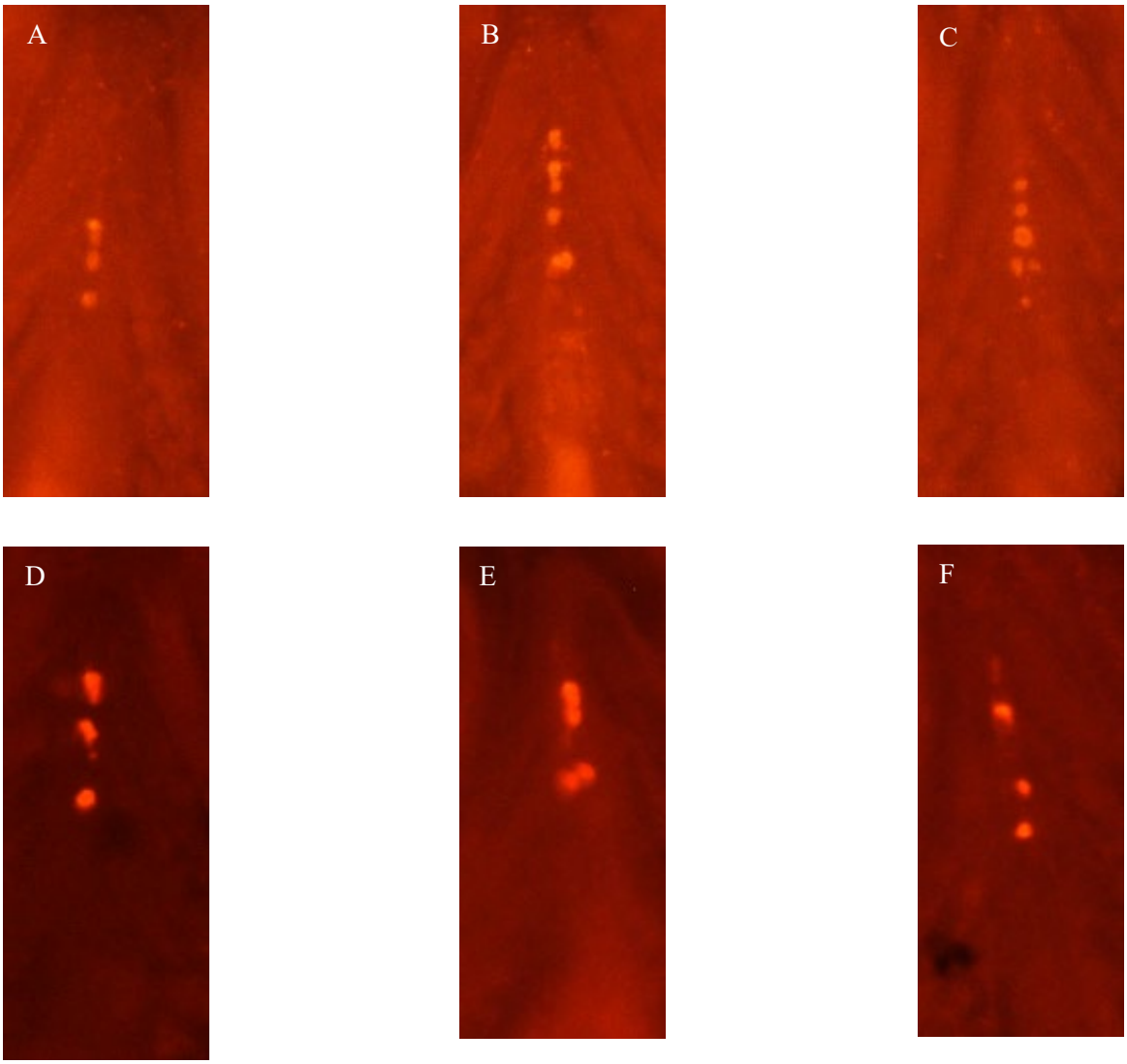


Image	Area	Mean	Min	Max	IntDensity	RawIntDen
A	884	57.121	42	76	50495	50495
B	1267	44.714	35	57	56653	56653
C	1889	56.997	42	81	107668	107668
D	730	47.333	36	69	34553	34553
E	946	55.759	47	71	52748	52748
F	1003	72.363	56	92	72580	72580

Average of the ID values	62449,5

7.4.Forth run

7.4.1. KClO4.1 – HTC (11000 µM)

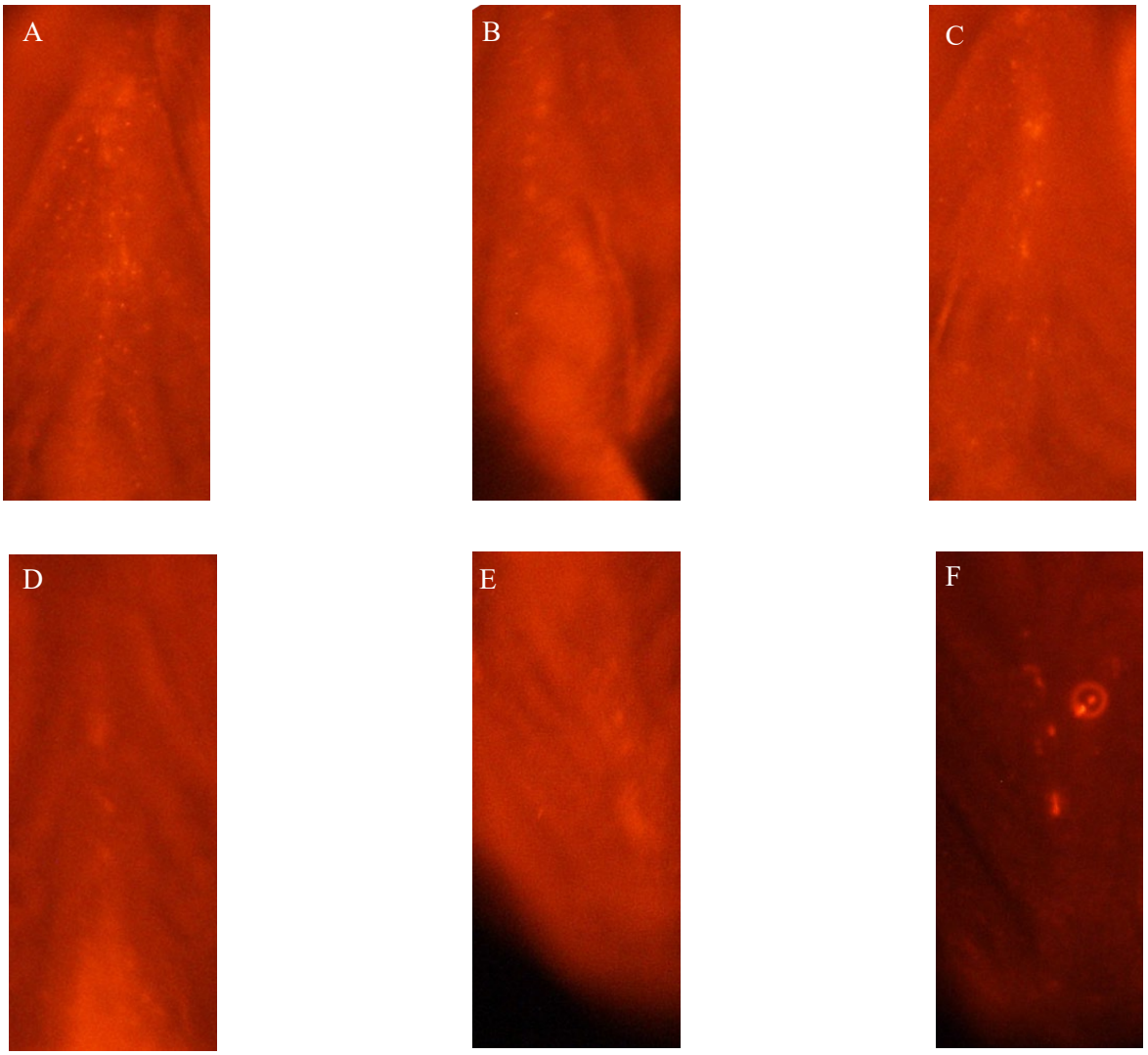


Image	Area	Mean	Min	Max	IntDensity	RawIntDen
A	720	51.294	48	64	36932	36932
B	1081	46.786	43	54	50576	50576
C	580	52.881	49	62	30671	30671
D	639	40.856	37	45	26107	26107
E	838	51.381	48	58	43057	43057
F	629	46491	40	59	29243	29243

Average of the ID values	36097,67

7.4.2. KClO4.2 – 1:100 of HTC (110 µM)

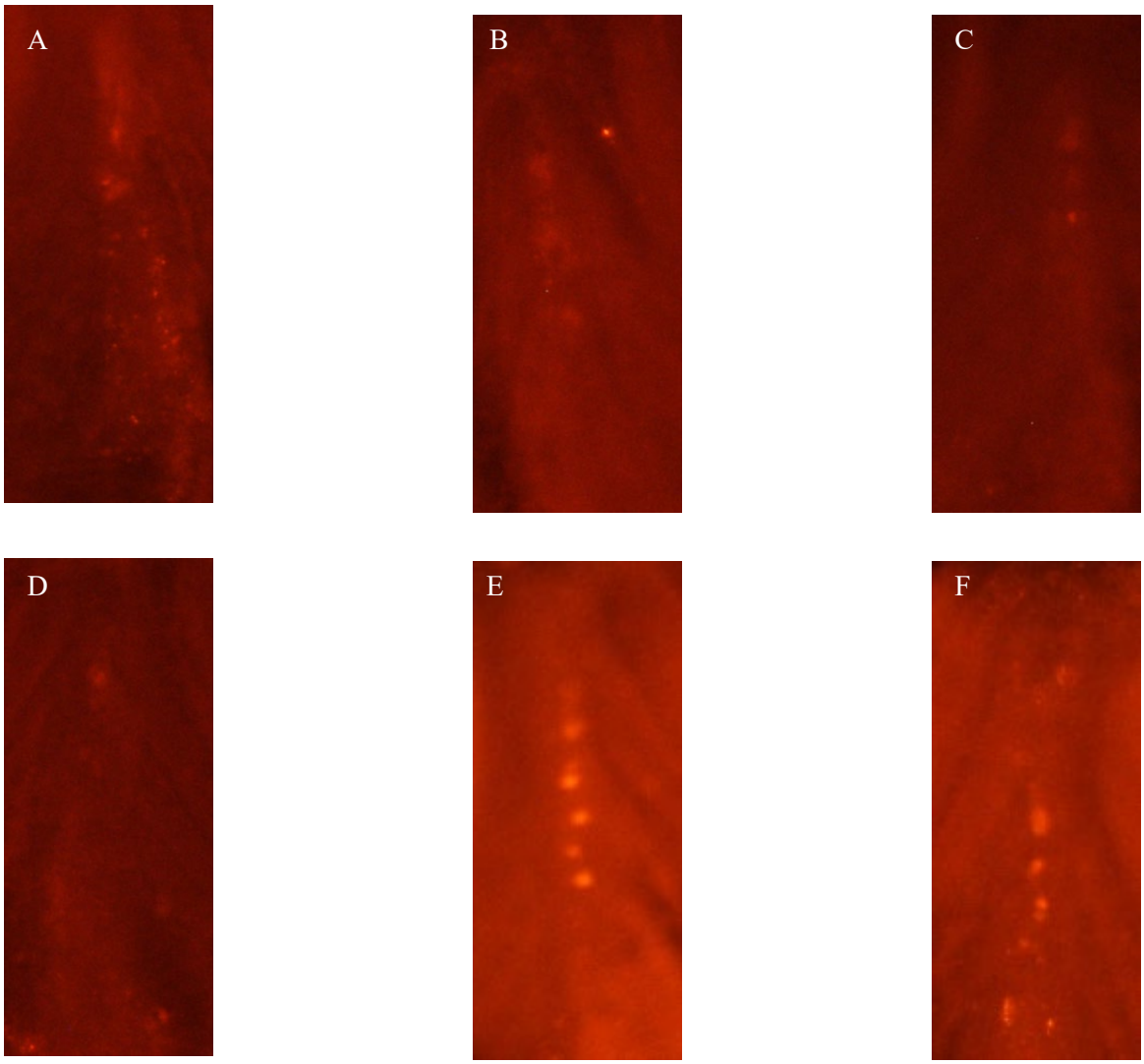


Image	Area	Mean	Min	Max	IntDensity	RawIntDen
A	650	40.365	35	54	26237	26237
B	631	35.680	32	41	22514	22514
C	280	31.618	27	45	8853	8853
D	394	34.931	29	65	13763	13763
E	1515	55.673	45	78	84344	84344
F	1761	47.537	38	89	83713	83713

Average of the ID values	39904

7.4.3. $KClO_4$.3 – 1:10000 of HTC (1.10 µM)

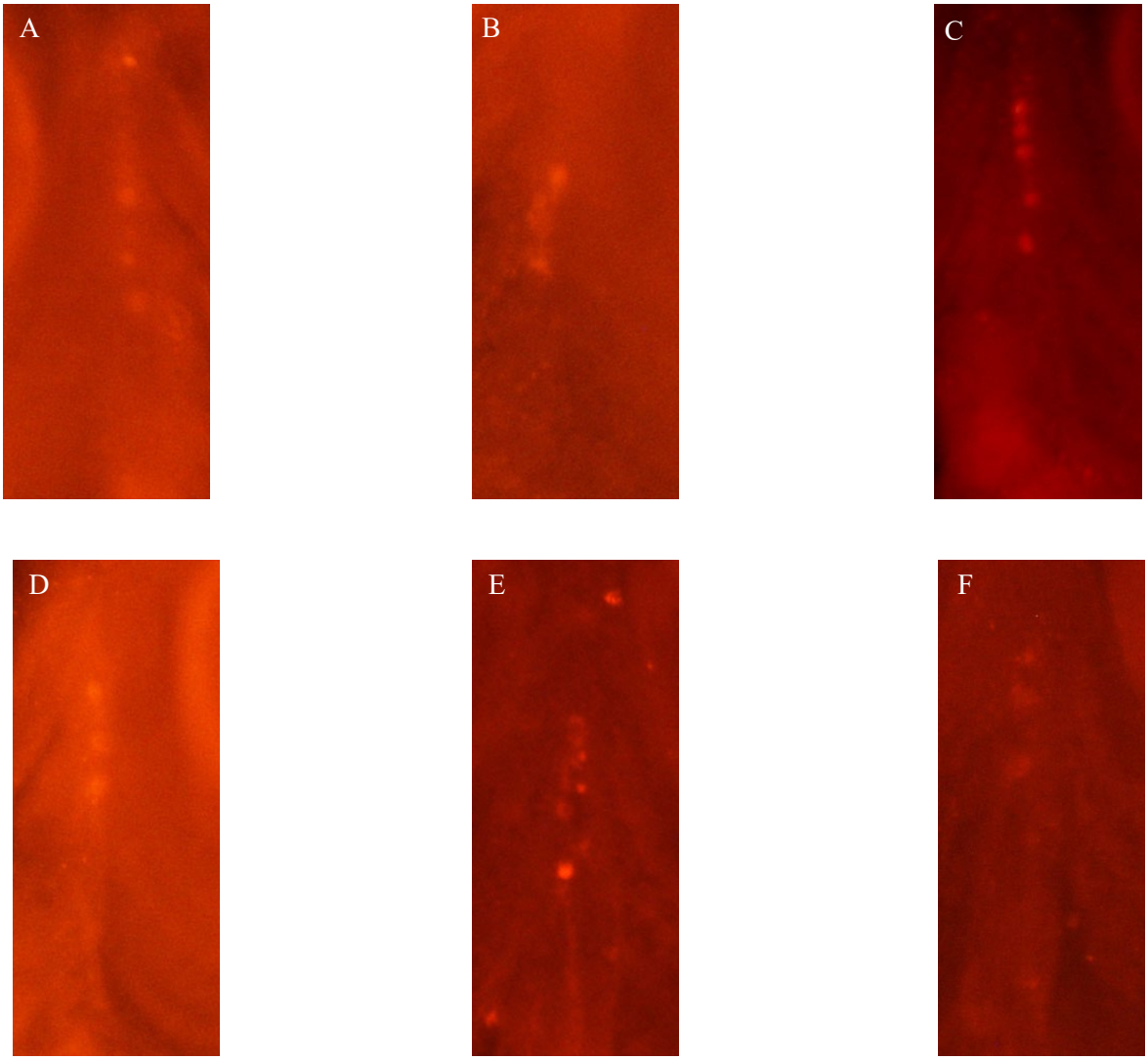
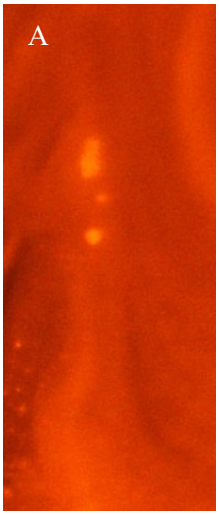
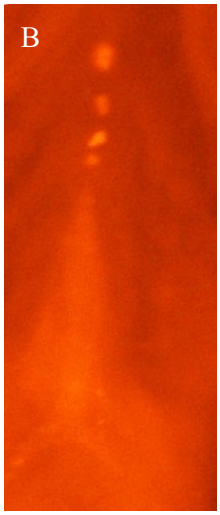


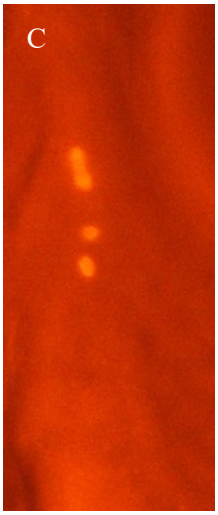
Image	Area	Mean	Min	Max	IntDensity	RawIntDen
A	789	47.098	42	62	37160	37160
B	994	47.157	42	57	46874	46874
C	959	53.153	45	71	50974	50974
D	1288	52.842	49	60	68060	68060
E	1669	43.479	33	76	72567	72567
F	1208	36.059	31	44	43559	43559

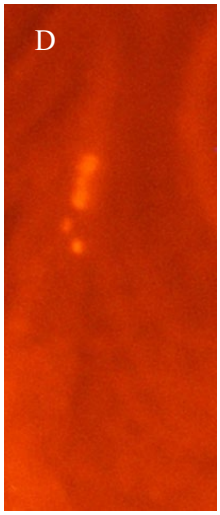
Average of the ID values	53199

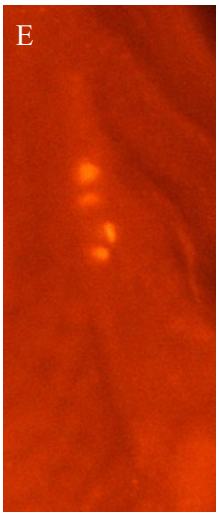
7.4.4. Negative control – FW











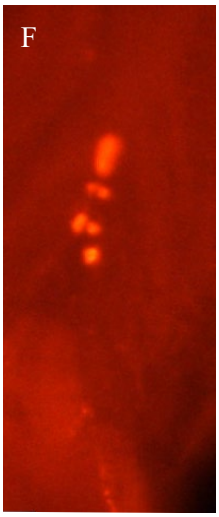


Image	Area	Mean	Min	Max	IntDensity	RawIntDen
A	932	62.055	52	76	57835	57835
B	1000	52.038	44	69	52038	52038
C	1434	62.441	50	77	89541	89541
D	1369	56.583	47	70	77462	77462
E	969	62.568	51	84	60628	60628
F	1677	66.661	49	95	111790	111790

Average of the ID values	74882,33

7.4.5. Positive control – MMI

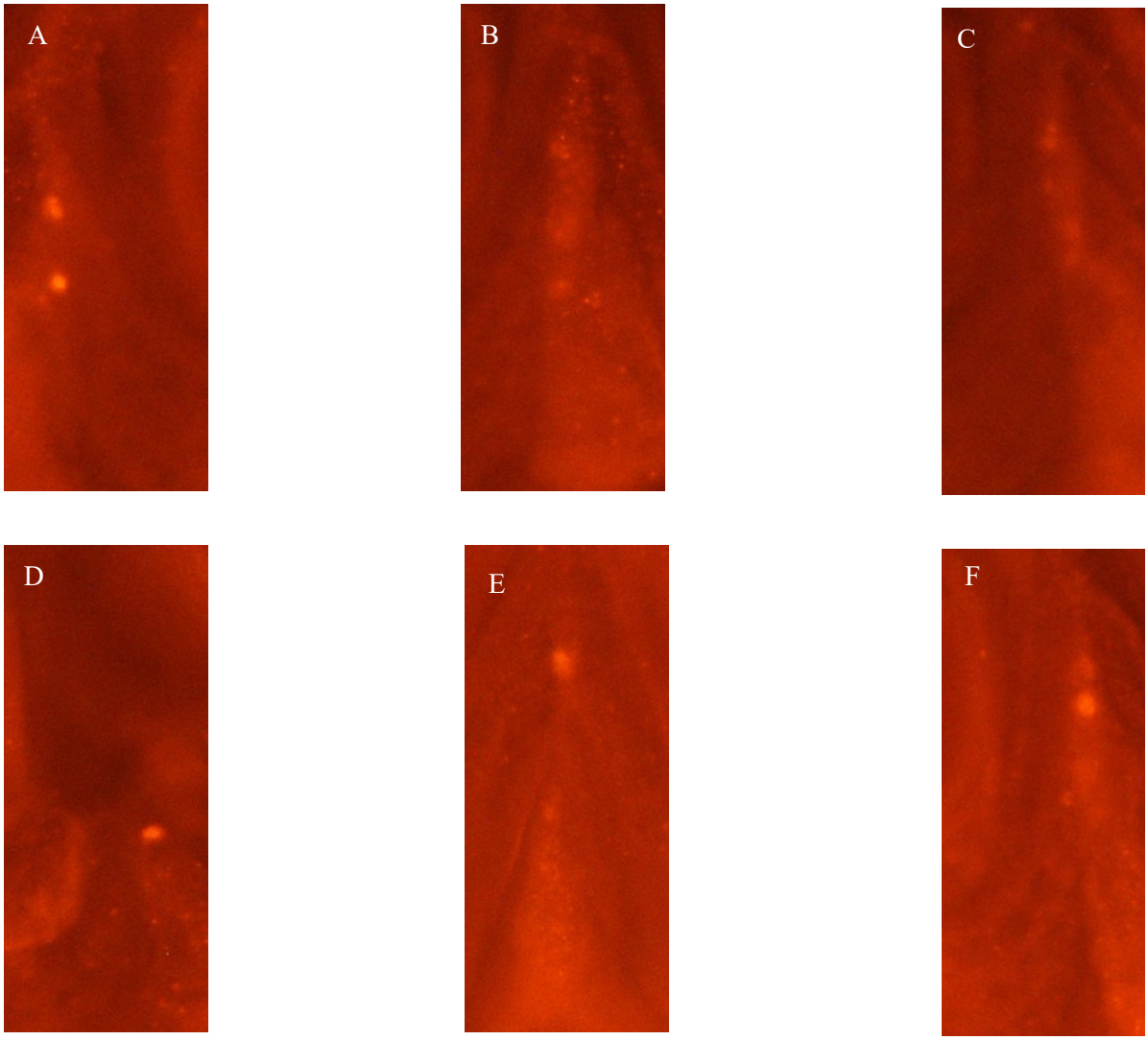
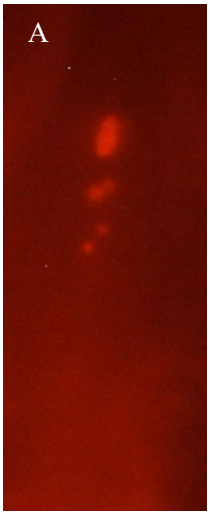
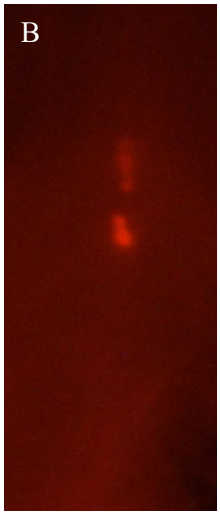


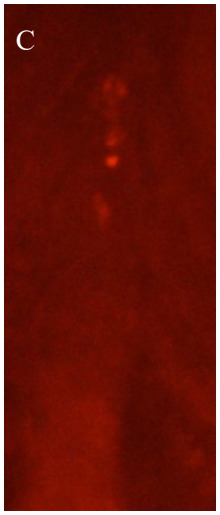
Image	Area	Mean	Min	Max	IntDensity	RawIntDen
A	959	56.894	42	89	54561	54561
B	1318	44.020	39	65	58018	58018
C	881	41.969	38	48	36975	36975
D	300	56.780	41	77	17034	17034
E	516	51.109	44	64	26372	26372
F	1634	48.918	42	72	79932	79932

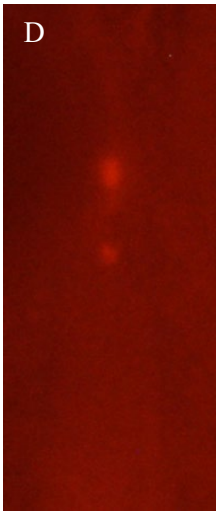
Average of the ID values	45482

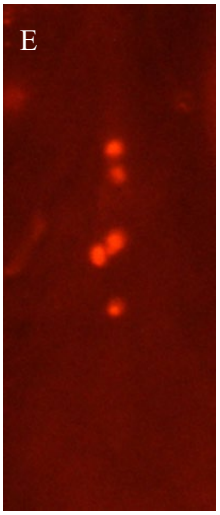
7.4.6. Solvent control - DMSO in fish water











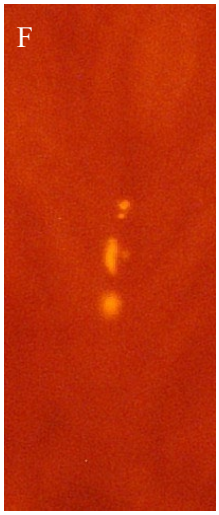


Image	Area	Mean	Min	Max	IntDensity	RawIntDen
A	1738	40.516	28	57	70416	70416
B	1060	39.455	28	65	41822	41822
C	772	33.492	25	65	25856	25856
D	1308	36.128	29	49	47256	47256
E	1457	58.496	36	87	85229	85229
F	868	67.482	47	87	58574	58574

Average of the ID values	54858,83

7.5. Fifth run

7.5.1. KClO4.1 – HTC (11000 µM)

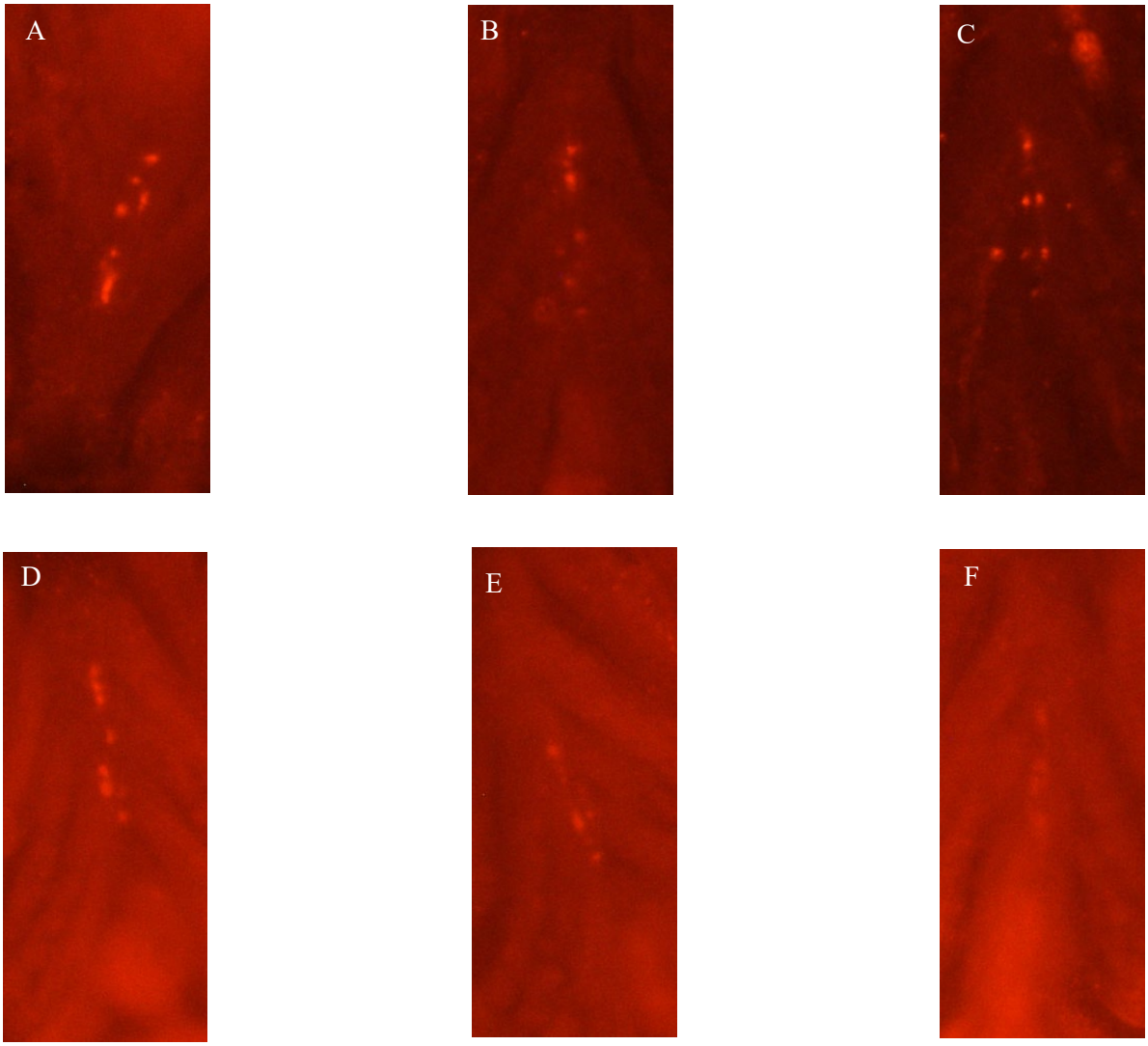


Image	Area	Mean	Min	Max	IntDensity	RawIntDen
A	1010	50.742	37	74	51249	51249
B	571	43.625	33	68	24910	24910
C	599	46.471	34	83	27836	27836
D	719	48.281	40	60	34714	34714
E	787	41.399	34	55	32581	32581
F	560	42.070	39	47	23559	23559

Average of the ID values	32474,83

7.5.2. KClO4.2 – 1:100 of HTC (110 µM)

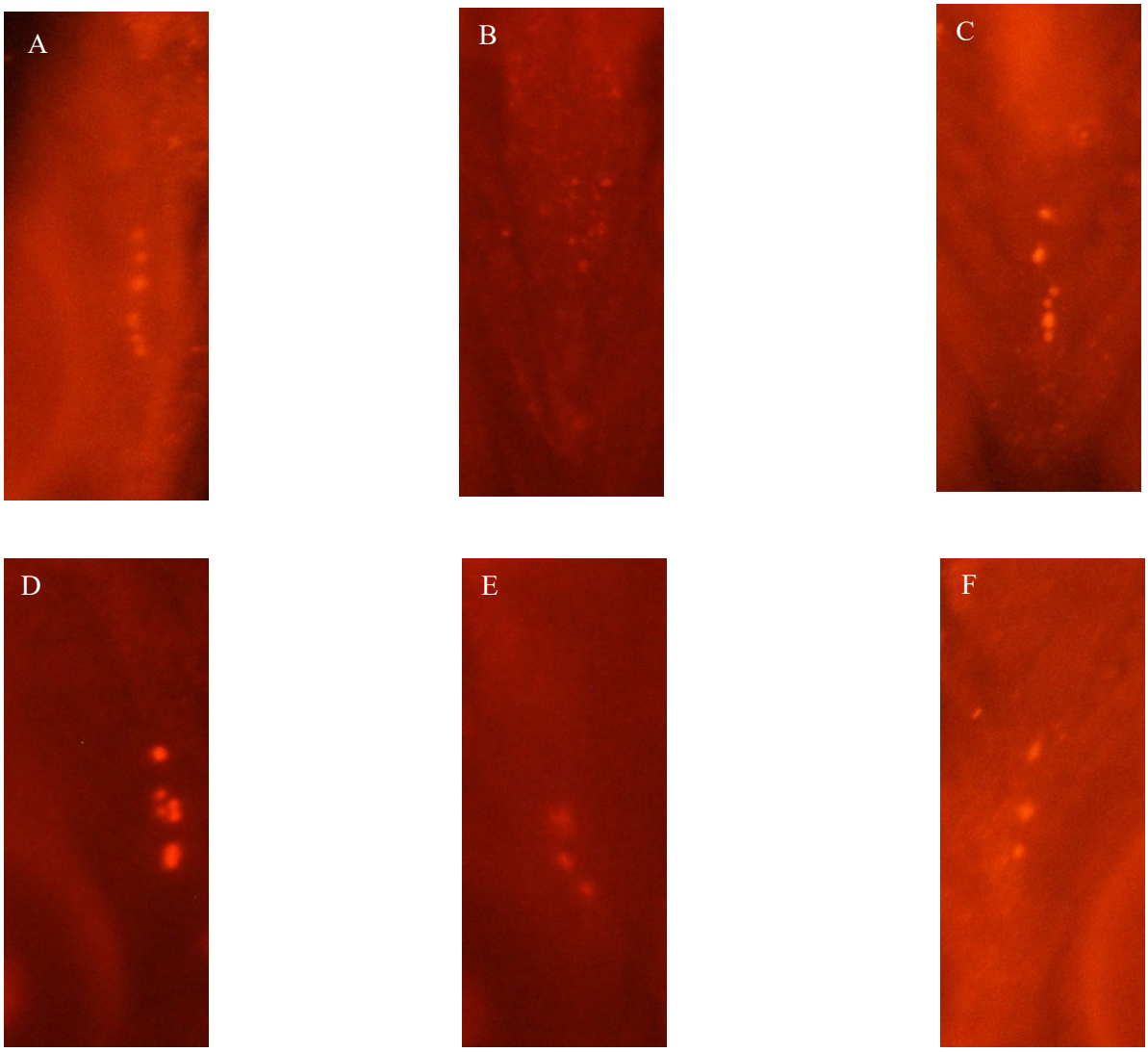


Image	Area	Mean	Min	Max	IntDensity	RawIntDen
A	1377	48.709	42	63	67072	67072
B	518	38.270	34	52	19824	19824
C	1029	51.652	38	75	53150	53150
D	1589	52.544	33	80	83492	83492
E	1235	38.772	32	53	47884	47884
F	1089	50.720	44	64	55234	55234

Average of the ID values	54442,67

7.5.3. $KClO_4$.3 – 1:10000 of HTC (1.10 µM)

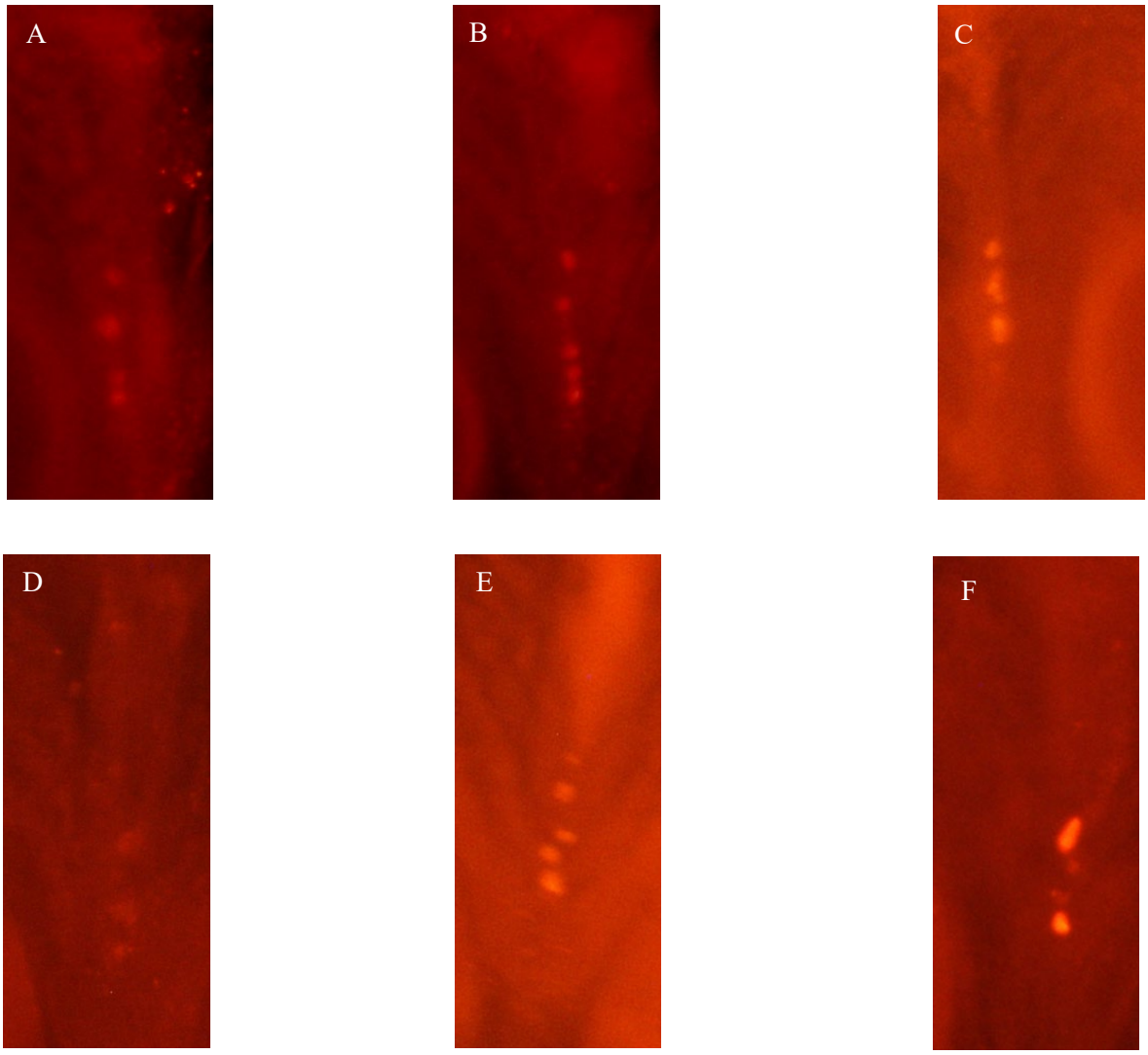
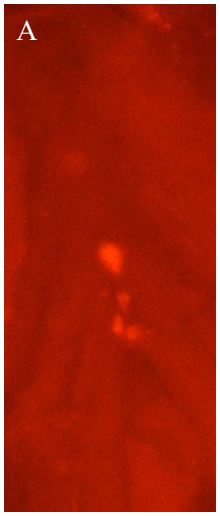
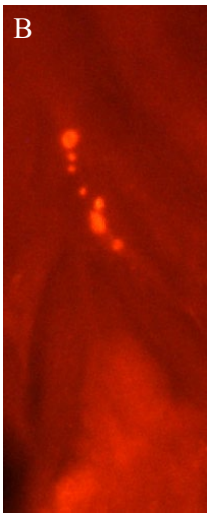


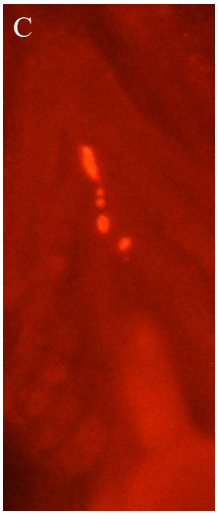
Image	Area	Mean	Min	Max	IntDensity	RawIntDen
A	935	53117	46	84	49664	49664
B	959	53153	45	71	50974	50974
C	1072	50651	43	61	54298	54298
D	1208	36059	31	44	43559	43559
E	1285	51166	42	69	65748	65748
F	1558	58789	37	95	91594	91594

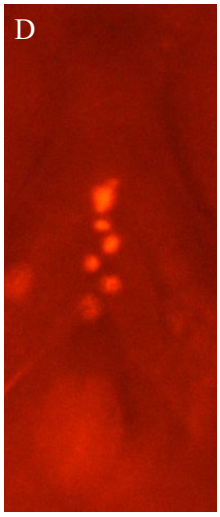
Average of the ID values	59306,17

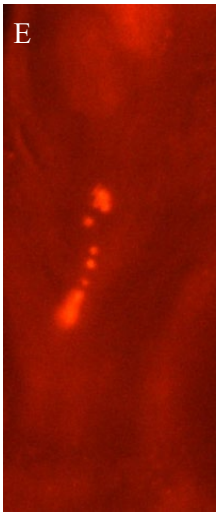
7.5.4. Negative control – FW











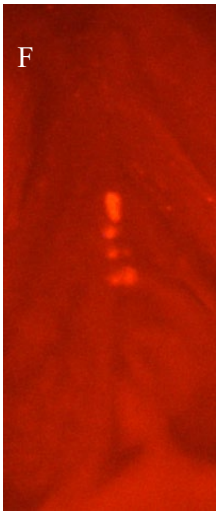
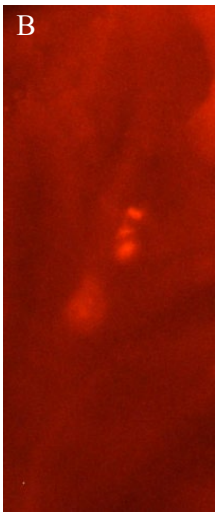
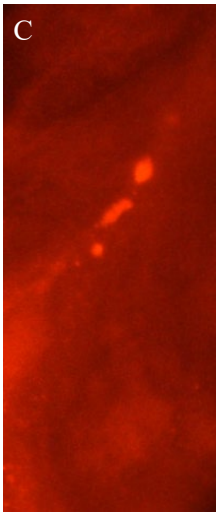


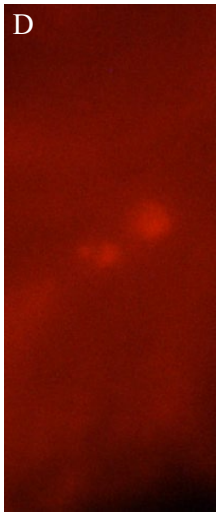
Image	Area	Mean	Min	Max	IntDensity	RawIntDen
A	644	67.559	56	85	43508	43508
B	1049	64.006	46	86	71142	71142
C	1002	60.813	43	81	69935	69935
D	1873	57.743	39	83	108153	108153
E	1555	59.684	42	79	92809	92809
F	1177	56077	41	72	66003	66003

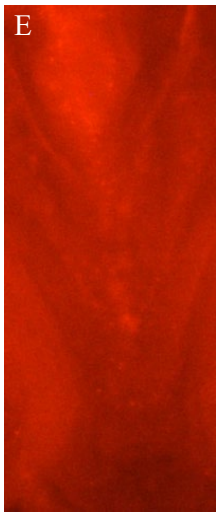
Average of the ID values	75258,33

7.5.5. Positive control – MMI









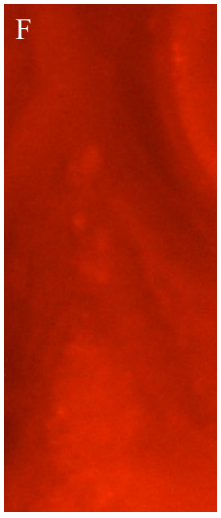


Image	Area	Mean	Min	Max	IntDensity	RawIntDen
A	264	66.375	48	82	17523	17523
B	1077	51.208	42	71	55151	55151
C	960	61.207	45	75	58759	58759
D	1915	39.530	32	51	75700	75700
E	634	44326	40	55	28103	28103
F	693	41.144	38	46	28513	28513

Average of the ID values	43958,2

7.5.6. Solvent control - DMSO in fish water

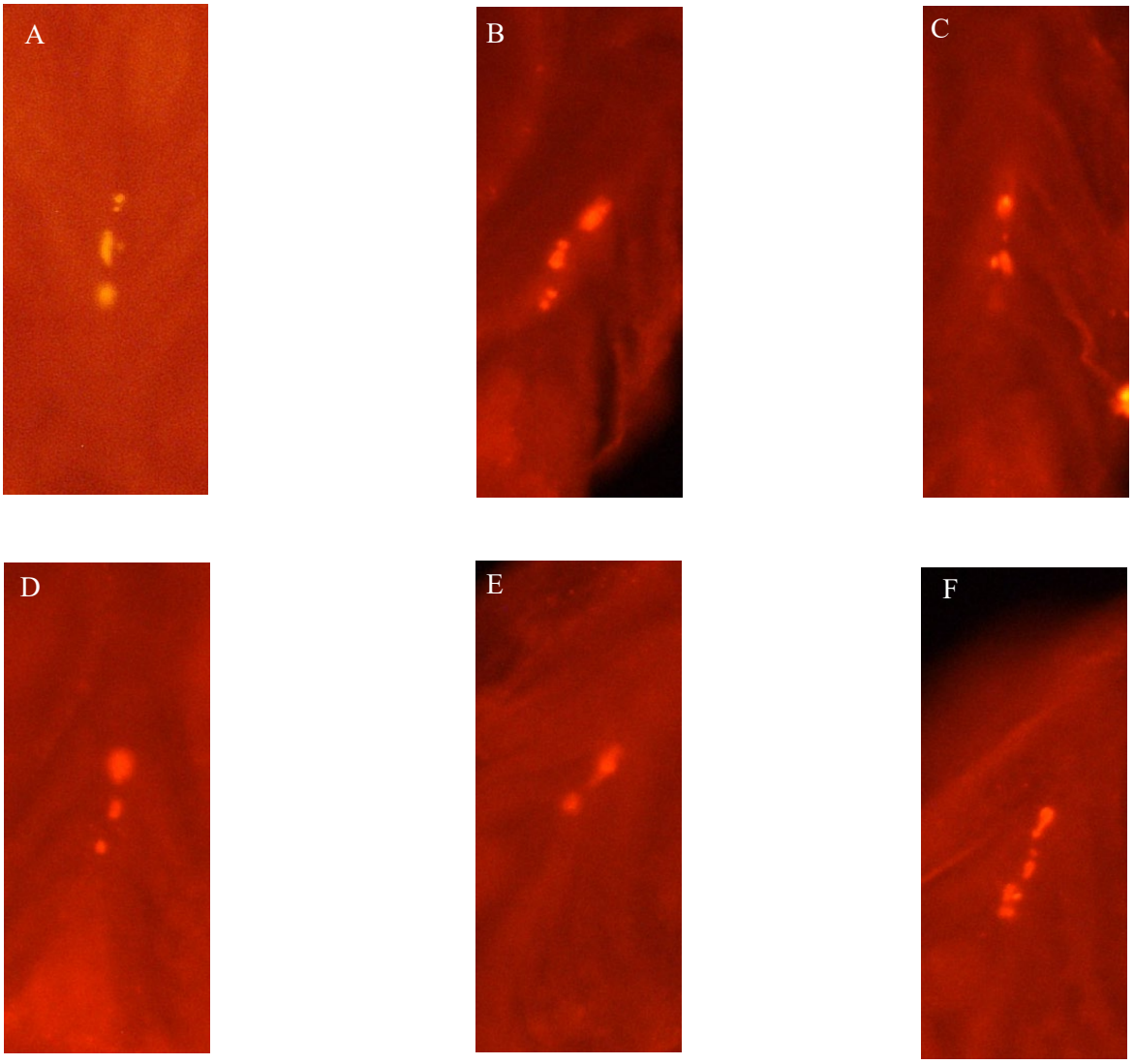


Image	Area	Mean	Min	Max	IntDensity	RawIntDen
A	868	67.482	47	87	58574	58574
B	1228	68.414	52	85	84012	84012
C	866	63.711	46	94	55174	55174
D	1223	55.528	42	65	79911	79911
E	1416	51.915	39	71	73511	73511
F	1143	65.332	51	83	74674	74674

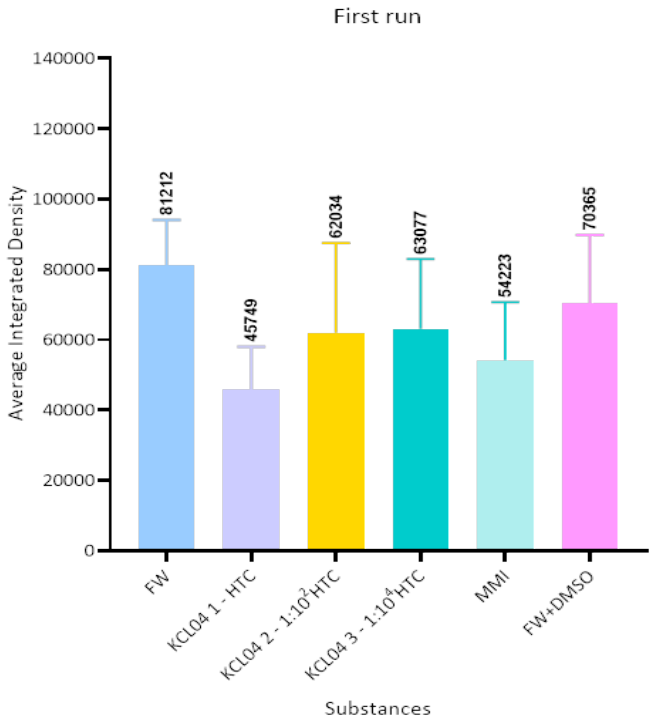
Average of the ID values	70976

8. Graphs

Here below are shown the graphs relating to each of the runs performed. For a better understanding, it should be noted that only the data relating to the average of the integrated density (ID) relative to the thyroid follicles will be used. The average ID, presented in the previous tables, were obtained by averaging the ID values of all the larvae exposed to the same item in the same run.
In the following graphs the abscissa shows the results of the 6 different solutions used, while on the y axis it is possible to observe the numerical value referred of the Average IDs.

8.1. First run

Test item	Average integrated Density
FW	81211,5
$KClO_4$.1-HTC	45749,17
$KClO_4$.2 – 1:100 of HTC	62033,83
$KClO_4$ .3 – 10000 of HTC	63076,83
MMI	54222,5
FW+DMSO	70365

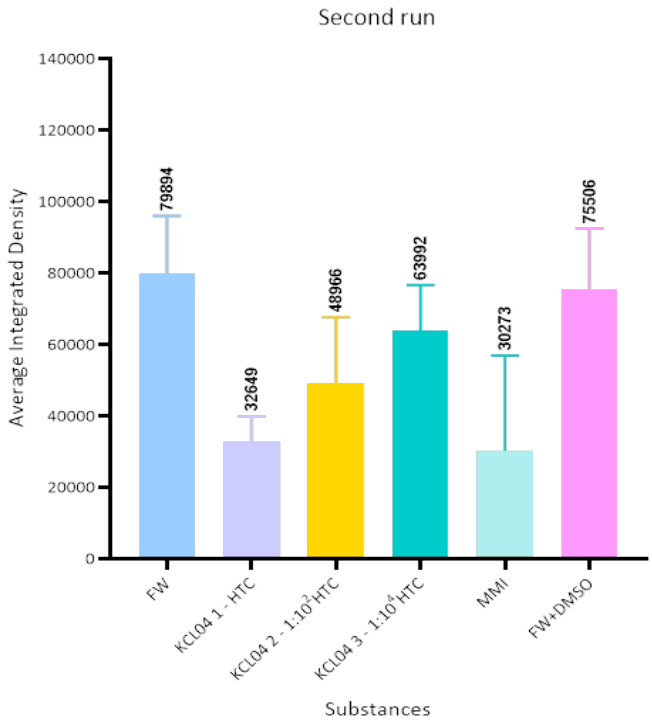


Graph 1: The graph shows the relative Average IDs of the larvae used in the first run.

Observing the Graph 1, it is possible to see how the average of the IDs referred to the FW negative control was the highest as well as it is also possible to see an effect on this value following exposure to the MMI positive control. The solvent control, on the other hand, appears to have a value that lies between the two controls mentioned above. As for the three concentrations of KClO4, it can be seen that the reported values are inversely proportional to the concentration of the substance, thus showing an increase in the average IDs parallel to the dilution of $KClO_4$.

8.2. Second run

Test item	Average integrated Density
FW	79894
$KClO_4$.1-HTC	32648,83
$KClO_4$.2 – 1:100 of HTC	48966,17
$KClO_4$ .3 – 10000 of HTC	63992,33
MMI	30272,7
FW+DMSO	75505,83



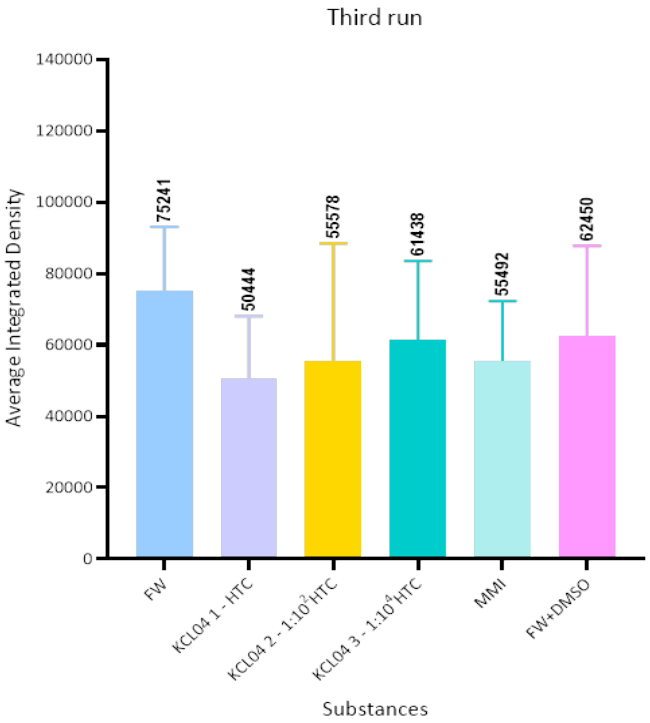
Graph 2: The graph shows the relative Average IDs of the larvae used in the second run.

In Graph 2, the higher value refers to the negative control FW, followed by the solvent control, FW+DMSO, which is represented by an intermediate value between the FW and the positive control MMI, whose value, in accordance with expectations, is the lowest among the three. As regards the three tested concentrations, it can be seen how the average ID values increase as the concentration of KClO4 decreases.

8.3. Third run

Test item	Average integrated Density
FW	75240,83
$KClO_4$.1-HTC	50443,5
$KClO_4$.2 – 1:100 of HTC	55578
$KClO_4$ .3 – 10000 of HTC	61437,5
MMI	55491,5
FW+DMSO	62449,5

Looking Graph 3 it can be seen that the average ID values relating to the FW, negative control, and the MMI, positive control, are in line with expectations, as well as the solvent control whose average value is between the two mentioned above. In addition to what has just been reported, it can be noted that the Average IDs values relating to the three concentrations of KClO4 are



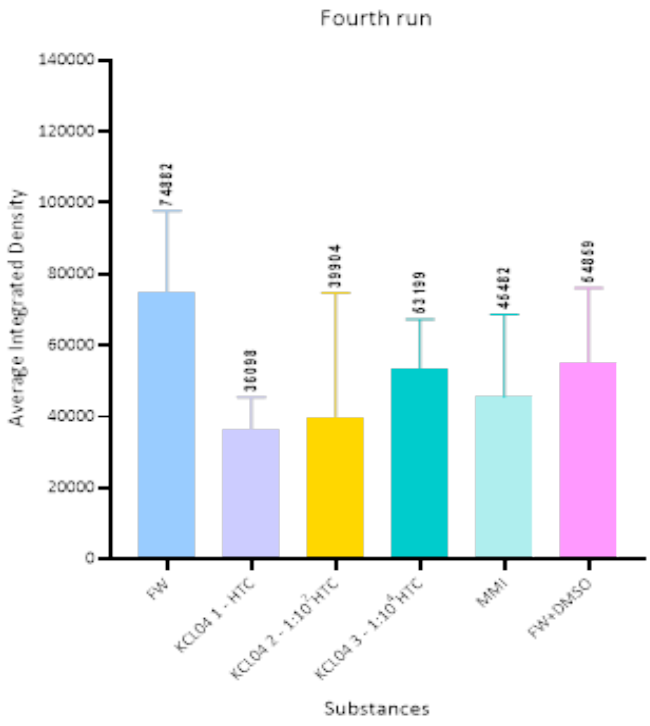
Graph 3: The graph shows the relative Average IDs of the larvae used in the third run.

characterized by an increase as the concentration of the substance in the solutions used decreases.

8.4. Fourth run

Test item	Average integrated Density
FW	74882,33
$KClO_4$.1-HTC	36097,67
$KClO_4$.2 – 1:100 of HTC	39904
$KClO_4$ .3 – 10000 of HTC	53199
MMI	45482
FW+DMSO	54858,83

Observing the Graph 4, you can see that the value relative to FW is the highest, followed by the solvent control and the positive one. Again, the ID values for the concentrations under consideration are inversely proportional to the concentration of the test item. In this regard, it should be noted the important difference found between ID values referring to KClO4.2 – 1:100 of HTC and those of KClO4 .3 – 10000 of HTC.

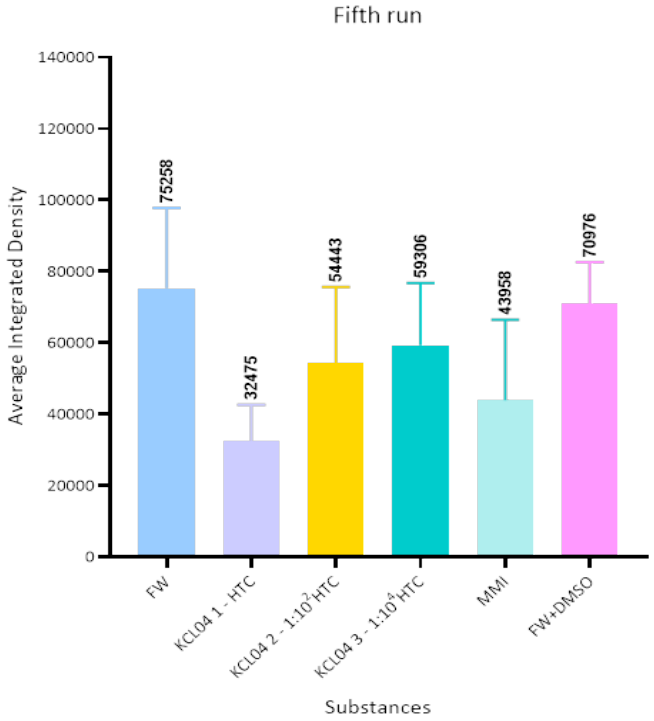


Graph 4: The graph shows the relative Average IDs of the larvae used in the fourth run.

8.5. Fifth run

Test item	Average integrated Density
FW	75258,33
$KClO_4$.1-HTC	32474,83
$KClO_4$.2 – 1:100 of HTC	54442,67
$KClO_4$ .3 – 10000 of HTC	59306,17
MMI	43958,2
FW+DMSO	70976

In this last run, like in the others, it is possible to see, Graph 5, how the MMI positive control turned out to be the lowest, the FW negative control was the highest, while the solvent control stood at an intermediate value between the two mentioned above. With regard to the average values of the IDs relating to the 3 concentrations



Graph 5: The graph shows the relative Average IDs of the larvae used in the fifth run.

of KClO4 tested as indicated, the highest value is that relating to KClO4 .3 - 10000 of HTC, while the lowest is referred to the highest concentration used, KClO4. 1-HTC.

8. Conclusion

The Zebrafish Eleutheroembryo Thyroid Assay (ZETA) allows to detect and quantify, through the use of specific antibodies marked with fluorescent substances, the deposit of intrafollicular content of thyroxine (IT4C), a physiologically relevant endpoint, because, in case of disturbance of the thyroid gland function, the production of this hormone is modulated (Raldúa & Babin, 2009; Thienpont et al., 2011). The ID is a parameter that provides the best indication of the IT4C. Indeed, this parameter allows you to combine information relating to the average intensity of the fluorescent signal with immunolabeling allowing a measurement of the intrafollicular *T*4.The data obtained following the analysis using the ImageJ software were interpreted using a qualitative / quantitative approach in which the immunolabeling allows a detection of T4 while the average intensity, in association to the development of the area of follicles matured during exposure allows a quantification of the IT4C.

Following the bond between the antibodies and the *T*4 hormone, the signal emitted in the larvae exposed to the negative control FW is characterized by a high intensity, and it is therefore possible to hypothesize that the thyroid has not suffered any adverse effects.

The ID values recorded in the specimens of *Danio rerio* exposed to MMI are lower than those related to the negative control FW, and are the result of a decrease in the emitted fluorescence, which is therefore weakened and altered. Moreover, it is important so underline a decrease of T4 immunoreactivity in larvae treated with the three different concentrations of $KClO_4$, compared to controls.

The ZETA assay, given the characteristics exposed so far, has both advantages and disadvantages. The main limitation of this method lies in the fact that, although it allows the identification of substances capable of inducing an alteration in the endocrine function of the thyroid, it does not provide any information about the mechanism of action through which the compound in question exerts this effect. Despite this it is an efficient qualitative/quantitative approach both from an economic and a time point of view as three days of exposure are sufficient to visualize the reduction of the intrafollicular content of *T*4 at the level of the thyroid follicles of the zebrafish, and therefore to obtain reliable information about a potential thyroid interference for a large number of substances, tested simultaneously on different larvae. Furthermore, since this is an assay in which the exposure of the larvae ends at five day post fertilization, it is one of the alternative methods since, according to the Directive 2010/63/EU, *Danio rerio* embryos within 120 hpf are not included in the protected animal models.

7. References

Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes, 276 20 (2010).

Raldúa, D., & Babin, P. J. (2009). Simple, rapid zebrafish larva bioassay for assessing the potential of chemical pollutants and drugs to disrupt thyroid gland function. *Environmental science & technology, 43*(17), 6844–6850.

Regulation (EC) No. 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), (2006).

Thienpont, B., Tingaud-Sequeira, A., Prats, E., Barata, C., Babin, P. J., & Raldúa, D. (2011). Zebrafish eleutheroembryos provide a suitable vertebrate model for screening chemicals that impair thyroid hormone synthesis. *Environmental science & technology, 45*(17), 7525–7532.