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Les dommages à l'ADN comment les identifier et les quantifier ?

L'exemple de deux techniques

Delphine Lamirand-Plaire

26 septembre 2018

DNA damage

Genotoxic compounds → Alteration of DNA integrity → Mutations → Inheritable effects

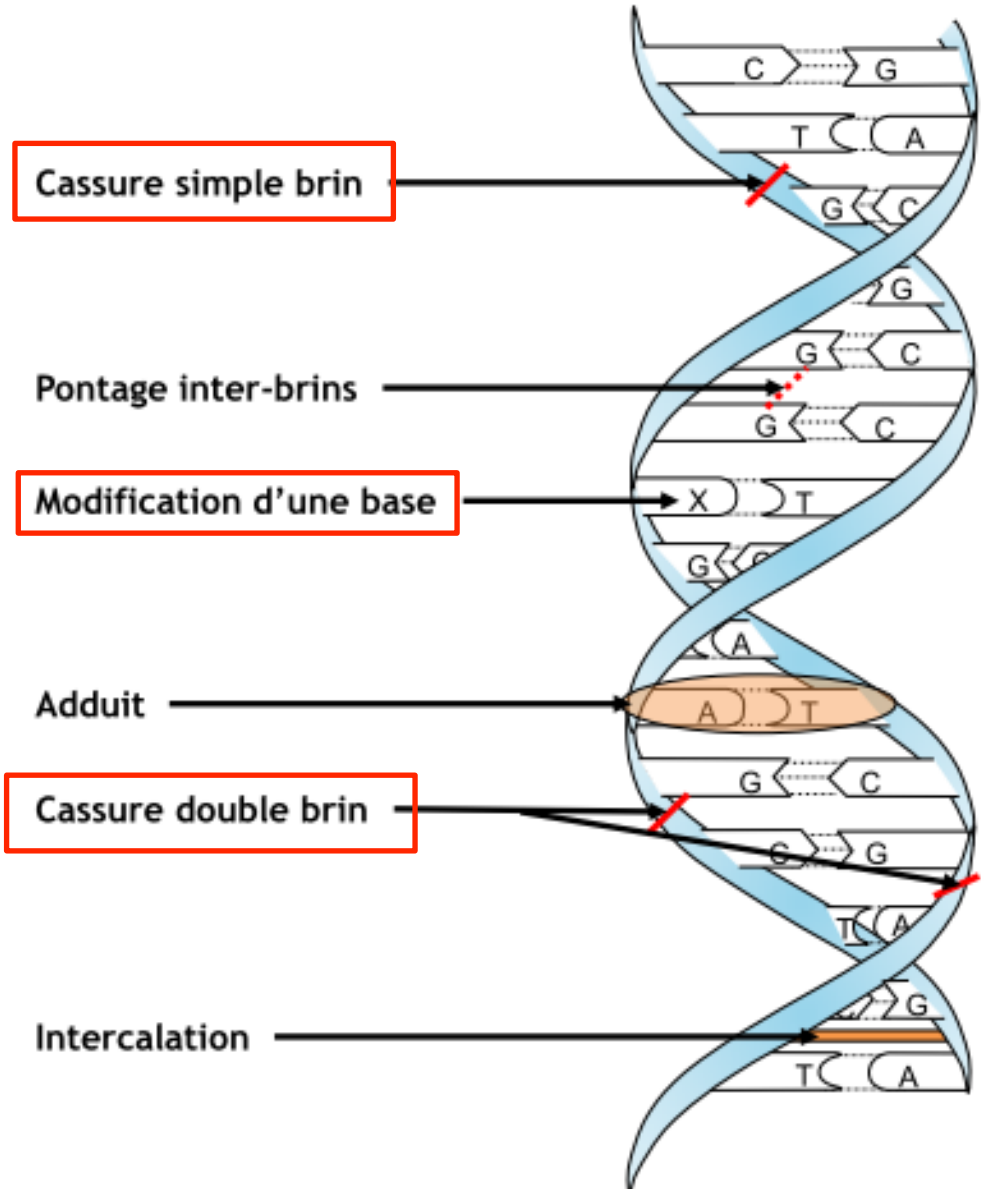
Due to the variety of genotoxic compounds present in the environment, there is a large number of possible DNA damage

DNA damage

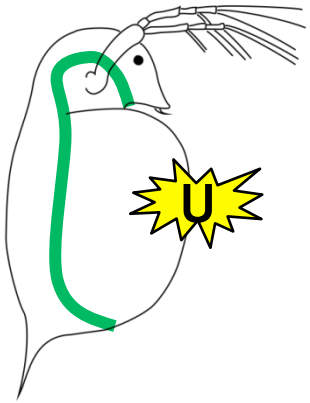
Lésions primaires

↓

Premier stade d'altération



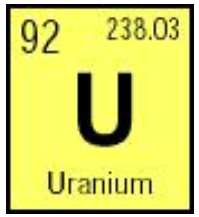
Context



Heavy metal + model organism

Uranium as pollutant

- Anthropogenic activities → modify natural U abundance in the environment



- Chimiotoxicity : heavy metal
- Radiotoxicity: alpha emitter

Consequences of DNA damage in zebrafish germinal cells on reproduction, development and behaviour

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Master degree internship (2010) at the Radioecology and Ecotoxicology Laboratory

Danio rerio as a biological model

- Organism commonly used for standard ecotoxicological tests (OCDE)

✓ Short life cycle: sexual maturity in 75 days
✓ Easy to maintain under laboratory conditions
✓ Sequenced genome

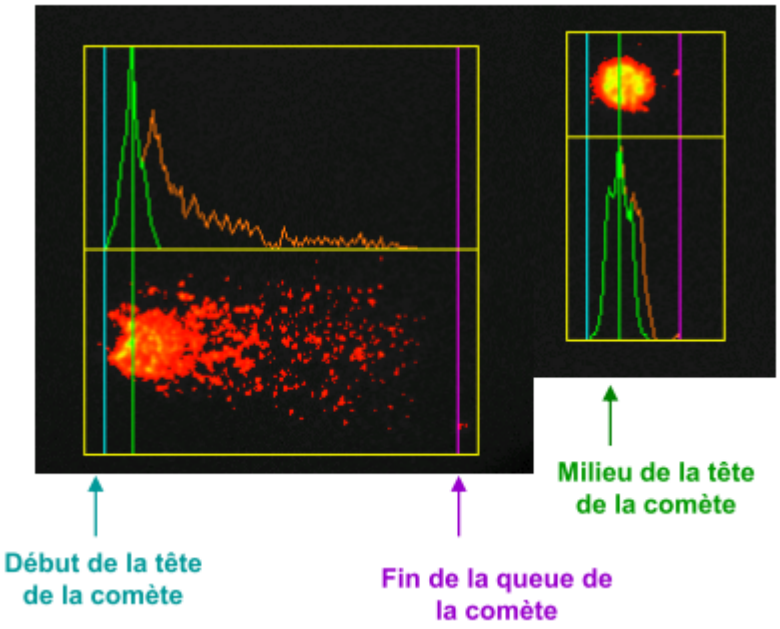
Uranium toxicity on:

- DNA in germinal cells
- Reproduction

Alkaline comet assay

- Technique mise au point par Singh *et al.* (1988) : permet de détecter les **cassures simple et double** brin d'ADN

- Méthode : Electrophorèse sur gel agarose



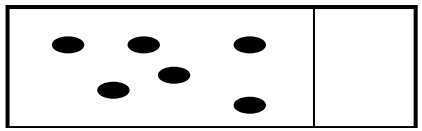
Alkaline comet assay

- Isolation des tissus : dissociation enzymatique

• Dissection des tissus et dépôt dans tampon PBS 0.01 M + EDTA à 0.02 %, maintenu à 25°C.
• Eliminer le tampon
• Ajouter 1 ml de solution de collagénase type IV (1 mg/ml) : 30 min, 20°C, 120 rpm
• Aspirer et refouler 1 ou 2 fois l'homogénat tissulaire pour dissocier les cellules.
• Prélever la suspension cellulaire et l'éjecter sur un filtre de 60 µm.
• Rincer deux fois avec du PBS 0.01 M + BSA 10 % (4°C), transvaser sur le filtre.
• Centrifuger à 110 g pendant 10 min à 8°C.
• Enlever le surnageant. Ajouter 1.5 ml de L15 + HEPES 10 mM (4°C).
• Centrifuger à 110 g pendant 10 min, à 8°C.
• Reprendre le culot dans 500 µl de L15+HEPES 10 mM (4°C).
• Incuber 25 µl de suspension cellulaire avec 25 µl de bleu Trypan pendant au moins 5 min. Déposer 40 µl sur cellule de Malassez et compter densité et viabilité cellulaire. Diluer la suspension pour avoir environ 1.35 10^6 cellules/ml.

Alkaline comet assay (Devaux *et al.*, 1997)

- Comet assay



1/ Agarose 0.8%
2/ Agarose 1% + cells > ice 2-3 min (polymerisation)
3/ Agarose 0.5% > ice

● cell

Inclusion

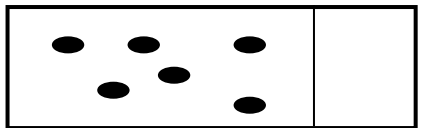
↓

Lysis (4°C, 1h)

Lysis buffer:
- Stock standard solution : NaCl 2,5M, EDTA disodique 100mM, Tris 10mM, pH 10
- Triton X100 (1% final)
- DMSO (10% finaux).

Alkaline comet assay (Devaux *et al.*, 1997)

- Comet assay



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2/ Agarose 1% + cells > ice 2-3 min (polymerisation)
3/ Agarose 0.5% > ice

cell

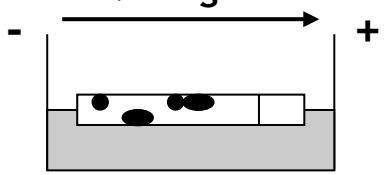
Inclusion

↓

Lysis (4°C, 1h)

↓

DNA migration



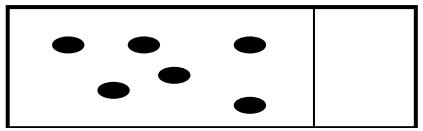
Electrophoresis

Unwind for 40 min
Electrophoresis: 24 min, 300 mA, 20 V

Unwind Buffer:
- NaOH (0,3M)
- EDTA (1mM)

Alkaline comet assay (Devaux *et al.*, 1997)

- Comet assay



1/ Agarose 0.8%
2/ Agarose 1% + cells > ice 2-3 min (polymerisation)
3/ Agarose 0.5% > ice

cell

Inclusion

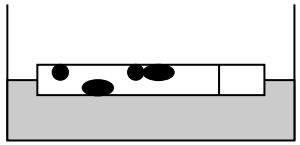
↓

Lysis (4°C, 1h)

↓

DNA migration

\- +



Unwind for 40 min
Electrophoresis: 24 min, 300 mA, 20 V

Electrophoresis

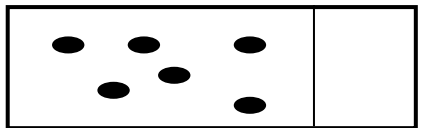
↓

Neutralization

Neutralization buffer:
Tris (0,4M), adjusted to pH 7.5

Alkaline comet assay (Devaux *et al.*, 1997)

- Comet assay



1/ Agarose 0.8%
2/ Agarose 1% + cells > ice 2-3 min (polymerisation)
3/ Agarose 0.5% > ice

cell

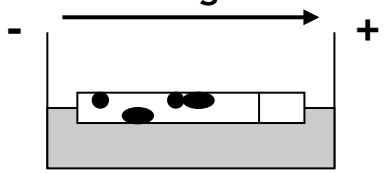
Inclusion

↓

Lysis (4°C, 1h)

↓

DNA migration



Unwind for 40 min
Electrophoresis: 24 min, 300 mA, 20 V

Electrophoresis

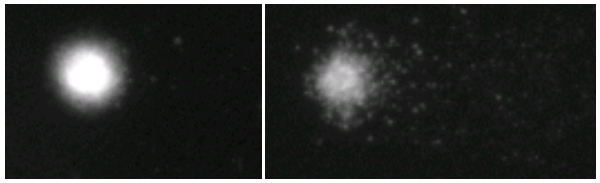
↓

Neutralization

Flooded: 2 times
Dehydrated with ethanol: 10 min

↓

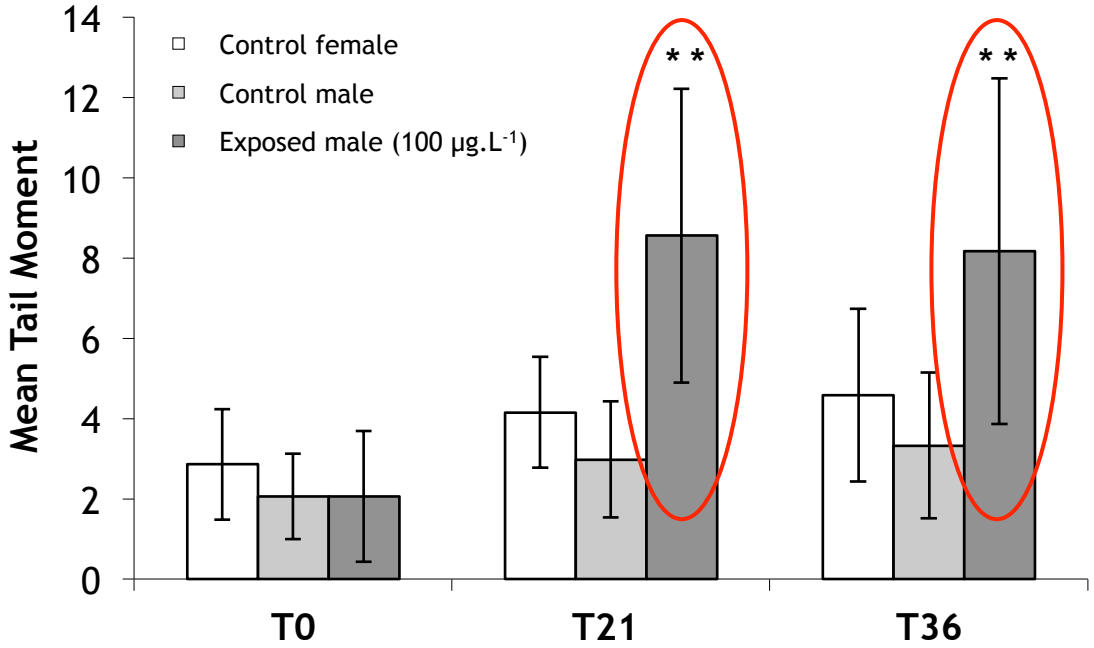
Microscopic observation
(Comet IV software)



Intact cell Damaged cell

Uranium genotoxicity

- Exposure for 21 days (100 µgU.L^{-1} for males) followed by 15 days in clean water
- « Tail Moment »: product of tail length and DNA % in the tail



Increase in DNA alterations in exposed males

No DNA repair

Daphnia magna exposed to uranium: From the organism to the molecular level

Consequences of DNA damage on life history traits and energy budget

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PhD (2010/2013) at the Radionuclide Ecotoxicology Laboratory (LECO)
Supervisor: F Alonzo
within a scientific cooperation with Bordeaux university (JP Bourdineaud)

The RAPD-PCR

Context

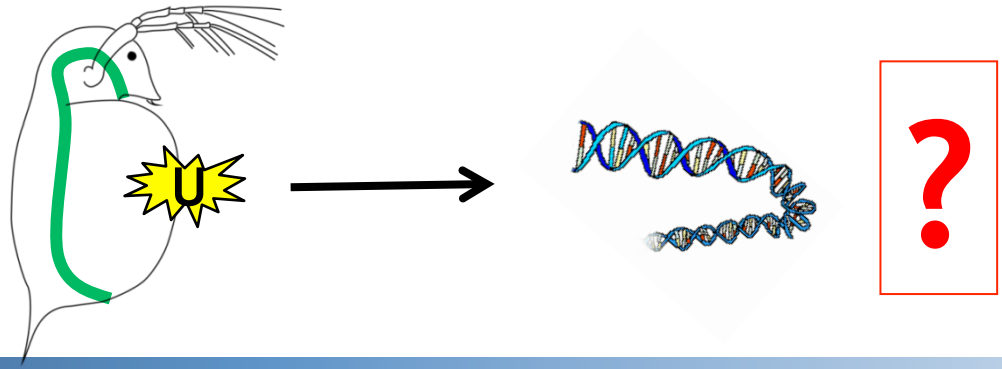
***Daphnia magna* as a biological model**

→ Sensitive to pollutant
→ Complex organism
→ Previous studies: correlations

- Freshwater microcrustacean used for standard ecotoxicological tests (OCDE, 2008)

✓ Short life cycle, small size
✓ Easy to reproduce and maintain under laboratory conditions
✓ Partenogenetic reproduction

- Multigenerational studies



Comet assay

- Comet assay on daphnids (den Bensten *et al.*, 2000)
  - Control cells are damaged: necessity to modify the cell dissociation method

Mechanical cell dissociation

- Egg number per sample: 5 to 40
- Filter diameter
- Time of lysis: 30 min

Comet assay

- Isolation des cellules : dissociation mécanique

- Rinçage des embryons dans 1,5mL de PBS + EDTA
- Ecraser avec une tige les œufs
- Filtrer la suspension cellulaire sur un filtre 100 µm
- Rincer à deux reprises le tube avec 500µL de PBS + EDTA
- Centrifugation à 110g, 8°C, 10 minutes
- Elimination du surnageant puis ajout de 1mL de L15 + HEPES
- Centrifugation à 110g, 8°C, 10 minutes
- Elimination du surnageant puis ajout de 100µL de L15 + HEPES

Comet assay

- Comet assay on daphnids (den Bensten *et al.*, 2000)
  - Control cells are damaged: necessity to modify the cell dissociation method

Mechanical cell dissociation

- Egg number per sample: 5 to 40
- Filter diameter
- Time of lysis: 30 min

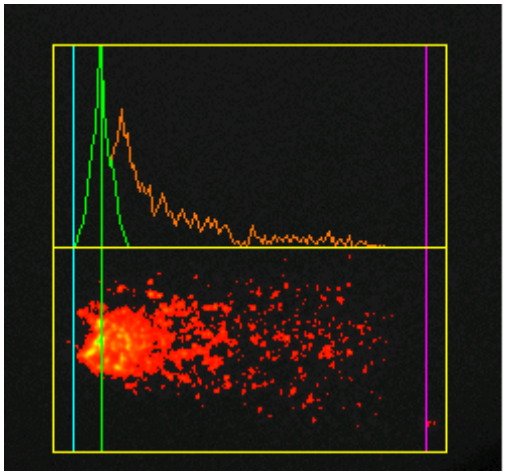
Enzymatic cell dissociation

- Collagenase IV: 15 and 30 min of incubation
- Pronase E

No cell dissociation

- Incision in eggs
- All eggs: no treatment

→ Control cells are still altered



Necessity to use another method to study genotoxicity on daphnids

RAPD-PCR

- RAPD-PCR (Random amplified polymorphic DNA) *(Atienzar et al., 1999; Cambier et al., 2010)*
  - Detection of a wide damage varieties and mutations in the whole genome

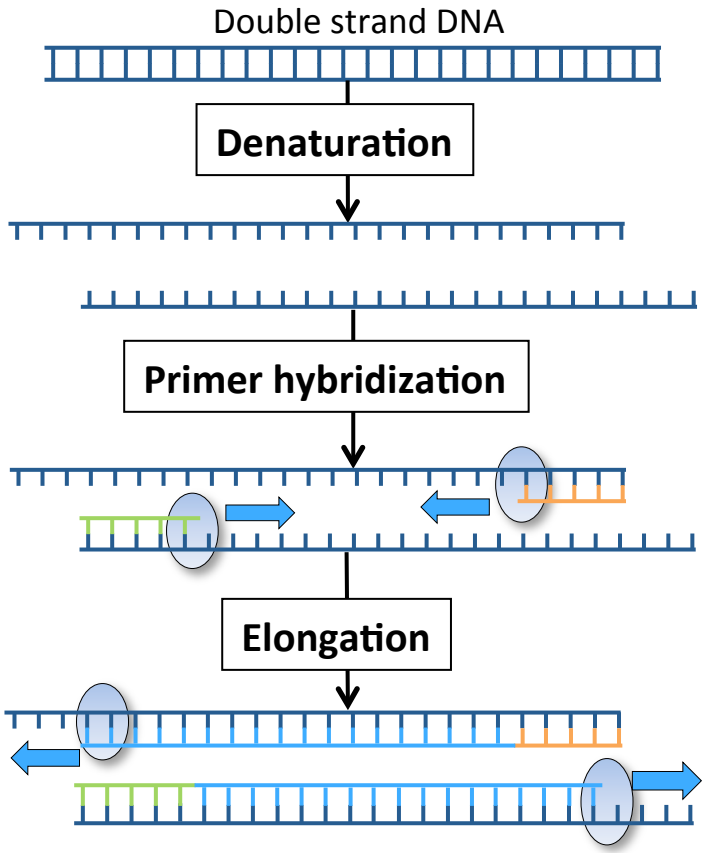
- Amplification of random DNA fractions

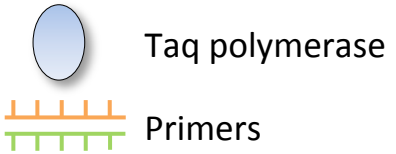
  - DNA extraction

• Ajouter 1% de SDS (solution stock 10%) et le 2% β-mercaptoéthanol dans le tampon de lyse
• Mettre 400 µL de ce tampon par échantillon et écraser les individus avec une tige
• Mettre 40 µg de RNAse par échantillon
• Incuber 1h à 37°C
• Mettre 400 µL de phénol-chloroforme par échantillon
• Passer les échantillons au vortex : 30 secondes chacun
• Centrifuger 5 min à 20°C à la vitesse maximale
• Prélever la phase aqueuse et ajouter 1/10 d'acétate de sodium par échantillon
• Mettre 2 volumes d'éthanol pur
• Mettre les échantillons au -80°C pendant 30 minutes
• Centrifuger 15 minutes à 4°C à la vitesse maximale
• Enlever le surnageant et ajouter 1 mL d'éthanol à 70% pour nettoyer
• Stocker les échantillons à -20°C

RAPD-PCR

- DNA Amplification

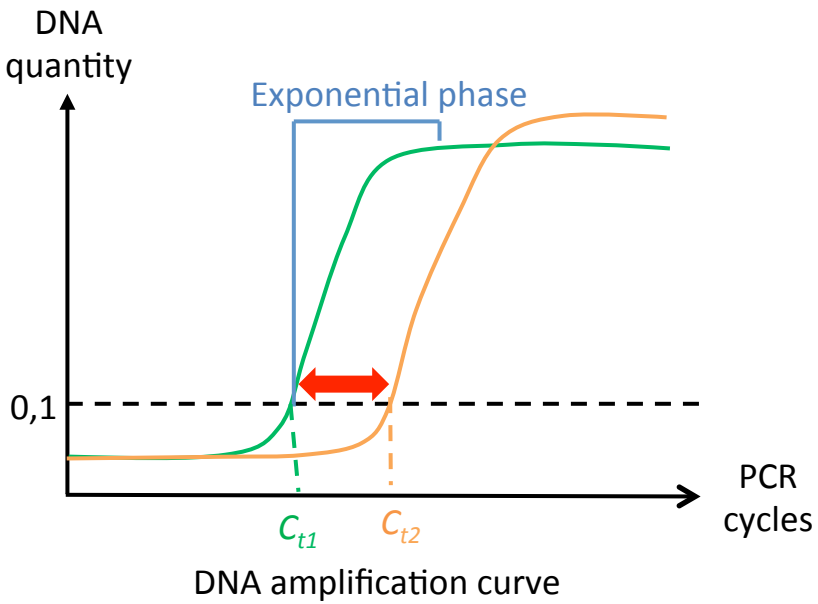




Using random primers in the case of RAPD

The RAPD-PCR

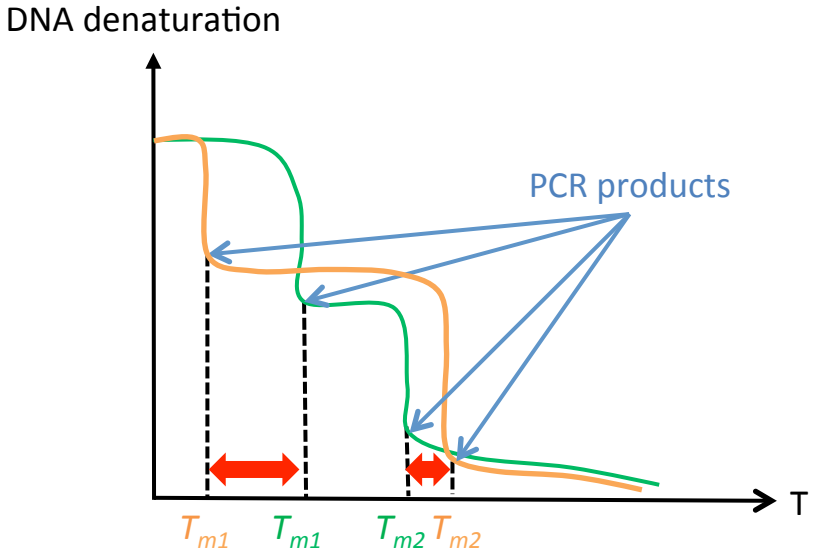
Monitoring DNA amplification



DNA amplification curve

- Calculation of the mean number of cycles (C_t) necessary to enter into exponential phase of DNA amplification
- Changes in C_t reflects a creation or loss of hybridization sites

Analysis of melting temperature



DNA denaturation curve

- PCR products distributed according to melting temperature
- Distribution of T_m characterized the composition of PCR products
- Changes in T_m reflects modifications in hybridization sites

Variations → ***DNA alterations***

Table 3
Number of hybridization sites with RAPD probe OPA9 on daphnid genomic DNA sampled from adults and neonates at release of broods 1 and 5 (B1 and B5) and at different uranium concentrations in two successive generations (F0 and F1) during experiments (A) continuous exposure, (B) post-hatching exposure and (C) embryo exposure only. Results are mean ± SEM (multiplied by 10^2 to facilitate reading). Statistics: $^*p < 0.05$, $n = 4$. P corresponds to parents of F0, exposed during egg incubation at the beginning of experiments.

A	Adults	P	F0		F1	
		B5	B1	B5	B1	B5
	0 µg L^{-1}	(9.59 ± 3.72)	(11.74 ± 2.86)	(5.05 ± 2.10)	(8.94 ± 2.48)	(21.99 ± 4.65)
	2 µg L^{-1}	(9.62 ± 1.97)	(9.13 ± 1.87)	(4.43 ± 0.96)	**(67.08 ± 3.74)** *	**(5.04 ± 3.38)** *
	9.9 µg L^{-1}	(21.25 ± 8.00)	(10.12 ± 4.67)	(10.33 ± 1.62)	(19.71 ± 0.72)	**(4.85 ± 2.64)** *
	22.2 µg L^{-1}	(8.80 ± 0.22)	(34.22 ± 18.64)	**(18.92 ± 5.56)** *	(10.46 ± 5.01	(8.32 ± 5.51)
	50 µg L^{-1}	(11.35 ± 2.06)	(35.10 ± 16.10)	(13.25 ± 4.61)	(1.94 ± 0.66)	**(7.83 ± 1.88)** *
	Neonates	**F0**	**F1**		**F2**	
	0 µg L^{-1}	(10.35 ± 1.77)	(8.12 ± 2.59)	(1.36 ± 0.21)	(16.02 ± 3.28)	(30.24 ± 21.61)
	2 µg L^{-1}	(7.41 ± 1.78)	(8.52 ± 2.21)	**(4.45 ± 1.35)** *	(10.94 ± 2.09)	**(11.64 ± 0.69)** *
	9.9 µg L^{-1}	(5.11 ± 0.37)	**(20.46 ± 8.46)** *	**(12.44 ± 4.10)** *	(5.81 ± 0.04)	**(4.11 ± 0.22)** *
	22.2 µg L^{-1}	(3.99 ± 1.17)	(2.34 ± 2.19)	(1.05 ± 2.10)	(4.02 ± 0.75)	**(1.29 ± 0.90)** *
	50 µg L^{-1}	(6.02 ± 0.38)	**(2.18 ± 0.42)** *	**(6.82 ± 3.13)** *	(7.87 ± 1.41)	(1.44 ± 1.15)
B	**Adults**	**P**	**F0**		**F1**	
		B5	**B1**	**B5**	**B1**	**B5**
	0 µg L^{-1}		(15.45 ± 2.93)	(18.68 ± 6.04)	(4.02 ± 1.12)	(1.45 ± 0.38)
	2 µg L^{-1}		(17.86 ± 3.87)	(6.74 ± 0.92)	(19.40 ± 4.96)	(2.98 ± 0.69)
	9.9 µg L^{-1}		(19.53 ± 4.32)	(7.99 ± 3.76)	**(0.82 ± 0.19)** *	(2.56 ± 0.70)
	22.2 µg L^{-1}		**(6.90 ± 0.61)** *	**(2.28 ± 1.73)** *	**(8.51 ± 0.96)** *	**(14.51 ± 2.46)** *
	50 µg L^{-1}		(18.86 ± 6.11)	**(3.93 ± 1.40)** *	(3.25 ± 2.27)	(40.00 ± 4.50)
	Neonates	**F0**	**F1**		**F2**	
	0 µg L^{-1}	(23.84 ± 9.63)	(18.70 ± 4.31)	(6.74 ± 0.92)	(8.06 ± 4.72)	(0.82 ± 0.42)
	2 µg L^{-1}		(8.17 ± 1.00)	(34.42 ± 14.76)	**(0.29 ± 0.09)** *	**(3.30 ± 0.75)** *
	9.9 µg L^{-1}		(4.88 ± 1.97)	(7.34 ± 3.07)	(1.11 ± 0.77)	(3.65 ± 1.76)
	22.2 µg L^{-1}		(2.96 ± 1.29)	**(2.49 ± 1.13)** *	(3.86 ± 1.05)	**(13.76 ± 2.69)** *
	50 µg L^{-1}		(2.97 ± 1.37)	**(1.71 ± 0.49)** *	(1.42 ± 0.65)	(0.16 ± 0.03)
C	**Adults**	**P**	**F0**		**F1**	
		B5	**B1**	**B5**	**B1**	**B5**
	0 µg L^{-1}	(23.79 ± 6.34)	(15.45 ± 2.93)	(18.68 ± 6.04)	(4.02 ± 1.12)	(1.45 ± 0.38)
	2 µg L^{-1}	**(0.14 ± 0.04)** *	(70.80 ± 26.25)	**(4.24 ± 0.41)** *	(8.42 ± 2.05)	(0.48 ± 0.19)
	9.9 µg L^{-1}	**(0.05 ± 0.01)** *	**(0.15 ± 0.08)** *	(7.95 ± 1.51)	(8.04 ± 2.64)	(1.22 ± 0.44)
	22.2 µg L^{-1}	**(0.10 ± 0.05)** *	**(1.09 ± 0.99)** *	(7.77 ± 2.15)	(2.18 ± 1.27)	(3.51 ± 1.32)
	50 µg L^{-1}	(16.00 ± 1.60)	**(5.00 ± 1.49)** *	**(0.75 ± 0.16)** *	(7.00 ± 2.92)	(2.13 ± 0.46)
	Neonates	**F0**	**F1**		**F2**	
	0 µg L^{-1}	(23.84 ± 9.63)	(18.70 ± 4.31)	(6.74 ± 0.92)	(1.37 ± 0.27)	(0.82 ± 0.42)
	2 µg L^{-1}	**(0.33 ± 0.16)** *	**(3.00 ± 2.94)** *	(6.18 ± 0.77)	(0.25 ± 0.17)	(1.60 ± 1.15)
	9.9 µg L^{-1}	**(0.03 ± 0.01)** *	**(0.05 ± 0.01)** *	**(2.95 ± 0.33)** *	**(0.39 ± 0.12)** *	(1.05 ± 0.37)
	22.2 µg L^{-1}	(7.40 ± 3.37)	**(0.67 ± 0.27)** *	(7.72 ± 1.91)	(3.96 ± 2.24)	(1.57 ± 0.28)
	50 µg L^{-1}	(63.12 ± 15.30)	**(2.93 ± 0.53)** *	**(0.34 ± 0.09)** *	(0.77 ± 0.31)	(6.44 ± 3.82)

Table 4A

Continuous exposure: Frequency of PCR products in relation to melting temperature (per temperature class) with RAPD probe OPA9 on daphnid genomic DNA from adults and neonates at release of broods 1 and 5 (B1 and B5) and at different uranium concentrations in two successive generations (F0 and F1). Statistics: $^{*}p < 0.05$, $n = 4$. P corresponds to parents of F0, exposed during egg incubation at the beginning of experiment.

Temperature class (°C)	P					F0										F1									
	B5					B1					B5					B1					B5				
	[U] µg L^{-1}					[U] µg L^{-1}					[U] µg L^{-1}					[U] µg L^{-1}					[U] µg L^{-1}				
	0	2	9.9	22.2	50	0	2	9.9	22.2	50	0	2	9.9	22.2	50	0	2	9.9	22.2	50	0	2	9.9	22.2	50
Adults																									
I1: [74–78[	0	0	0	0	0	0	0	0	0.5	0	0.25	0	0	0	0	0	0	0.25	0.25	0.5	0	0	0.25	0	0.25
I2: [78–80[	0	0	0	0	0	0	0.25	0.75	0	0.75	0.75	0.5	0.25	0	0.25	1	1	0.75	0.5	0.5	0	0.5	1	1	1
I3: [80–81[	0	0	0	0	0.25	0	0	0.25	0.5	0.25	0	0	0.5	0	0	0.25	0	0.25	0	0	1	**0***	**0***	**0***	**0***
I4: [81–82[	1	1	**0***	**0***	**0***	0.5	1	0.75	0.25	0	1	1	0.25	1	1	0.75	0.75	0.5	1	0.25	0	**1***	**1***	**1***	**1***
I5: [82–83[	0	0	0	0	0	0	0	0	0	0.25	0	0	0	0	0	0	0.25	0.25	0	0.5	0	0	0	0	0
I6: [83–84[	0	0	0	0	0	0	0	0	0.75	0.5	0	0	0	0	0.25	0	0	0	0	0	0	0	0	0	0
I7: [84–85[	0.25	0	0	0	0	0	0	0	0	0.25	1	0.75	**0***	**0***	**0***	0.75	0	0	0.75	0.75	0	0	0.75	0.5	0
I8: [85–86[	0	0	0	0	0.25	0	0	0	0	0	0	0	0	0	0	0	0	0	0.25	0.25	0	0.5	0.25	0	0.5
I9: [86–87[	1	1	0.75	1	**0***	0.75	1	1	1	1	1	0.5	0.75	0.75	0.5	0.75	1	1	0.75	0.75	1	0.5	0.5	0.75	**0***
I10: [87–88[	0	0	0.25	0	**1***	0.25	0	0	0	0	0	0.5	0.25	0.25	0.5	0.25	0	0	0.25	0	0	0.5	0.25	0.25	**1***
I11: [88–89[	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
I12: [89–92[	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Neonates	**F0**					**F1**										**F2**									
I1: [74–78[	0	0	0	0	0	0	0	0.5	0.25	0.33	0	0.25	0	0	0	0.5	0	1	0	0	0.25	0	0	0	0
I2: [78–80[	0	0	0	0	0	0	0.5	0.25	0	0	1	0.25	**0***	1	**0***	0.25	1	0	0.5	0	0.5	0.75	1	0.67	1
I3: [80–81[	0	0	0	0	0	0	0	0.75	0.75	0.67	0	0	0	0	0	0.25	0	0	0.25	0.5	0.75	0	0	0	0
I4: [81–82[	1	1	**0***	**0***	**0***	1	1	0.25	0.25	0.33	1	1	1	1	1	0.5	1	1	0.75	0.5	0.25	1	1	1	1
I5: [82–83[	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
I6: [83–84[	0	0	0	0	0	0	0	0.75	0.25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
I7: [84–85[	0	0	0	0	0	0.5	0	0	0.25	0	0.5	0.25	0	0	0	0.25	0	0	0.25	0	0	0.25	**1***	0.67	0
I8: [85–86[	0	0	0	0	0	0	0	0	0	0.67	0.25	0	0	0	0	0	0	0	0	0	0.5	0	0	0	0.67
I9: [86–87[	1	1	0.33	0.5	0.25	0.5	1	1	0.25	1	0.5	0	1	1	0.25	1	1	1	0.25	1	1	**0***	**0***	**0***	**0***
I10: [87–88[	0	0	0.67	0.5	0.75	0.5	0	0	0.75	0	0.5	1	0	0	0.75	0	0	0	0.5	0	0	**1***	**1***	**1***	**1***
I11: [88–89[	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
I12: [89–92[	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

The RAPD-PCR

Lowest concentration for significant DNA damage ([U] µg L^{-1})

F0			F1			F2
Néo	*B1*	*B5*	*Néo*	*B1*	*B5*	*Néo*
9.9	> 50	9.9	2	2	2	2

Time

- DNA damage are significant at hatching of F0

  Result of direct exposure of eegs in females

- DNA damage at a lower concentration at B5 than B1

  DNA damage appear to increase gradually in exposed females

- DNA damage at a lower concentration in F1 et F2 than in F0

  Accumulation and transmission of DNA damage across generation

  F1 et F2 : Resulting from both a direct exposure of embryos and a transmission of DNA alterations

Conclusion

- A lot of genotoxic compounds > a lot of DNA damage
- Comet assay : detection of single and double strand breaks
- RAPD PCR : quantitative method

Thanks for your attention

Publications

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